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Influence of enhanced troponin C Ca^{2+}-binding affinity on cooperative thin filament activation in rabbit skeletal muscle

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We studied how enhanced skeletal troponin C (sTnC) Ca^{2+}-binding affinity affects cooperative thin filament activation and contraction in single demembranated rabbit psoas fibres. Three sTnC mutants were created and incorporated into skeletal troponin (sTn) for measurement of Ca^{2+} dissociation, resulting in the following order of rates: wild-type (WT) sTnC–sTn > $sTnC^{F27W}$–sTn > M80Q sTnC–sTn > M80Q $sTnC^{F27W}$–sTn. Reconstitution of sTnC-extracted fibres increased Ca^{2+} sensitivity of steady-state force (pCa_{50}) by 0.08 for M80Q sTnC, 0.15 for $sTnC^{F27W}$ and 0.32 for M80Q $sTnC^{F27W}$ with minimal loss of slope (n_H, degree of cooperativity). Near-neighbour thin filament regulatory unit (RU) interactions were reduced in fibres by incorporating mixtures of WT or mutant sTnC and D28A, D64A sTnC (xxsTnC) that does not bind Ca^{2+} at N-terminal sites. Reconstitution with sTnC: xxsTnC mixtures to 20% of pre-exchanged maximal force reduced pCa_{50} by 0.35 for sTnC: xxsTnC, 0.25 for M80Q sTnC: xxsTnC, and 0.10 for M80Q $sTnC^{F27W}$: xxsTnC. It is interesting that pCa_{50} increased by ∼0.1 for M80Q sTnC and ∼0.3 for M80Q $sTnC^{F27W}$ when near-neighbour RU interactions were reduced; these values are similar in magnitude to those for fibres reconstituted with 100% mutant sTnC. After reconstitution with sTnC: xxsTnC mixtures, n_H decreased to a similar value for all mutant sTnCs. Altered sTnC Ca^{2+}-binding properties (M80Q $sTnC^{F27W}$) did not affect strong crossbridge inhibition by 2,3-butanedione monoxime when near-neighbour thin filament RU interactions were reduced. Together these results suggest increased sTnC Ca^{2+} affinity strongly influences Ca^{2+} sensitivity of steady-state force without affecting near-neighbour thin filament RU cooperative activation or the relative contribution of crossbridges *versus* Ca^{2+} to thin filament activation.

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Contraction in skeletal muscle is initiated by Ca^{2+} binding to the N-terminus of troponin C (TnC), a subunit of the troponin (Tn) complex, which leads to thin filament activation and force generation (reviewed by Gordon *et al.* (2000)). Following Ca^{2+} binding to TnC, there is increased interaction between the N-terminus of TnC and the inhibitory subunit of Tn (TnI) and between TnC and the tropomyosin (Tm)-binding subunit (TnT). The increased interaction between these Tn subunits results in the release of TnI from actin and greater mobility of Tm. Tm mobility exposes myosin binding sites on actin and allows for cyclic actomyosin (crossbridge) interactions, which result in contractile force and shortening. Strong crossbridge binding increases and stabilizes Tm displacement (Xu *et al.* 1999; Lehman *et al.* 2000) allowing further cooperative crossbridge binding (Gordon *et al.* 2000). This positive crossbridge feedback to increase thin filament activation (defined as the availability of myosin binding sites on actin) occurs with little effect on Ca^{2+} binding to TnC in skeletal muscle (Fuchs & Wang, 1991; Wang & Fuchs, 1994; Martyn & Gordon, 2001). However, how Ca^{2+} binding properties affect cooperative activation of force and spread of activation along the thin filament in skeletal muscle remains unknown.

A number of cooperative mechanisms have been proposed for skeletal muscle to explain the steep force–Ca^{2+} relationship observed experimentally in demembranated muscle fibres (Gordon *et al.* 2000, 2001; Regnier *et al.* 2002). We and others have reported that reduction of nearest-neighbour structural regulatory

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unit (RU; i.e. 7 actins : 1 Tn : 1 Tm) interactions along the length of the thin filament considerably decreases cooperativity in single skinned skeletal fibres (Moss *et al.* 1985, 1986; Regnier *et al.* 2002). Completely extracting native skeletal TnC (sTnC) from demembranated rabbit psoas fibres and reconstituting Tn complexes with varying mixtures of sTnC and a non-Ca^{2+}-binding sTnC mutant (D28A, D64A; xxsTnC) allows for the study of how near-neighbour RU interactions influence maximal Ca^{2+}-activated force, Ca^{2+} sensitivity of force, and slope or Hill coefficient (used as a measure of cooperativity) of the force–log [Ca^{2+}] (pCa) relationship (Regnier *et al.* 2002). In this earlier study we concluded that (1) near-neighbour RU interactions are the dominant form of cooperativity in skeletal muscle (possibly occurring through end-to-end overlap of Tm), (2) these near-neighbour cooperative RU interactions play a major role in setting the maximal force and Ca^{2+} sensitivity of force, and (3) there remains some cooperativity in the system even with isolated RUs (Regnier *et al.* 2002). Cooperative mechanisms that may occur within these individual isolated RUs include cooperative Ca^{2+} binding between the two EF-hand Ca^{2+}-binding sites in the regulatory domain (N-terminus) of sTnC (Gordon *et al.* 2001) and/or crossbridge-induced increased crossbridge binding, perhaps via compliant realignment of myosin binding sites on actin (Daniel *et al.* 1998; Gordon *et al.* 2000; Tanner *et al.* 2007).

Ca^{2+} sensitivity and cooperativity of force generation may also be influenced by the Ca^{2+}-binding properties of sTnC. Engineering single amino acid mutations into the regulatory domain of sTnC alters Ca^{2+}-binding kinetics, as measured by changes in Ca^{2+} dissociation rate (k_{off}) and steady-state Ca^{2+} affinity in solution (Tikunova *et al.* 2002; Davis *et al.* 2004). Reconstitution of skinned rabbit psoas fibres with sTnC mutants alters Ca^{2+} sensitivity of steady-state force (pCa_{50}) such that increased pCa_{50} is correlated with increased Ca^{2+} affinity of sTnC in complex with the TnI peptide $TnI_{96\text{-}148}$ and vice versa for decreased Ca^{2+} affinity (Davis *et al.* 2004). Recent work from our group is in agreement with this. Reduced Ca^{2+} affinity of TnC reduced Ca^{2+} sensitivity of force, and comparison with fibres with mixtures of sTnC and xxsTnC suggested that changes in pCa_{50} were primarily due to the Ca^{2+}-binding properties of TnC, but changes in slope were primarily due to the spread of activation between RUs in the thin filament (Moreno-Gonzalez *et al.* 2005). This demonstrates that Ca^{2+}-binding properties of sTnC play a major role in determining the level of thin filament activation and force generation in skeletal muscle fibres. However, how Ca^{2+}-binding properties of sTnC are coupled to near-neighbour RU cooperativity remains to be determined.

In the current study, we sought to more fully understand the relationship between the Ca^{2+}-binding properties of sTnC, near-neighbour RU cooperativity, and crossbridge binding in activation of skeletal muscle contraction. sTnC proteins were produced with point mutations that progressively increased Ca^{2+}-binding affinity and were then reconstituted into demembranated rabbit psoas fibres. To study how these mutants influence the Ca^{2+}-activation properties of individual RUs and near-neighbour RU interactions along the thin filament, fibres were reconstituted with mixtures of sTnC (purified, wild-type (WT) or mutant) and xxsTnC (as used by us to reduce the number of Ca^{2+}-activatible RUs (Regnier *et al.* 2002)). Finally, 2,3-butanedione monoxime (BDM) was used to examine how strong crossbridge binding affects cooperative thin filament activation under conditions of altered Ca^{2+} binding (with sTnC mutants) and reduced RU interactions (with mixtures containing xxsTnC). The results of the present studies confirm that near-neighbour RU interactions are the dominant form of cooperativity in skeletal muscle and suggest that while Ca^{2+} affinity of sTnC is a primary determinant of activation within individual RUs, enhancing Ca^{2+} affinity of sTnC does not influence either the spread of activation along the thin filament or the contribution of crossbridges to activation. A preliminary report of this work has been previously published (Kreutziger *et al.* 2004).

Methods

Preparation of proteins

A phenylalanine (Phe) to tryptophan (Trp) mutation at residue 27 (F27W) and a methionine to glutamine mutation at residue 80 (M80Q) were incorporated into rabbit sTnC cDNA using oligonucleotide primers for site-directed mutagenesis using the QuikChange kit from Stratagene (La Jolla, CA, USA). WT or mutant rabbit sTnC protein was extracted and purified from *E. coli* according to the method of Dong *et al.* (1996). Native rabbit sTnC, skeletal TnI (sTnI) and skeletal TnT (sTnT) were purified from ether powder of rabbit skeletal back and leg muscles according to the method of Potter (1982). Whole Tn complexes were formed using recombinant WT or mutant rabbit sTnC and purified native rabbit sTnI and sTnT as previously described with modification (Szczesna *et al.* 2000). Tn subunits (C, I and T) were dialysed into 10 mM 3-(*N*-morpholino) propanesulphonic acid (MOPS), 4.6 M urea, 1 mM dithiothreitol (DTT) and 0.01% NaN_3; pH 7.0 at 4°C. TnC, TnI and TnT were mixed at a molar ratio of 1 : 1.5 : 1.5 and allowed to sit at room temperature (20°C)for 20 min. The concentrations of urea, KCl and $MgCl_2$ were reduced by serial dialysis against the following three buffers (a, b and c) for > 6 h (with changing concentrations indicated as a–b–c): 10 mM MOPS, 2–0–0 M urea, 3–1–1 mM $MgCl_2$, 1 mM DTT and 0.01% NaN_3, pH 7.0 at 4°C. Dialysis in the final buffer was repeated twice, followed by centrifugation of

precipitated excess TnI–TnT as confirmed by SDS-PAGE. Non-Ca^{2+}-binding rabbit mutant sTnC (D28A and D64A; xxsTnC) and TnI peptide (TnI_{96-148}) were prepared as previously described (Regnier *et al.* 2002). sTnC concentrations were calculated from peak absorbance at 280 nm with extinction coefficients of 2680 cm^{-1} M^{-1} for sTnC and M80Q sTnC, and 8370 cm^{-1} M^{-1} for $sTnC^{F27W}$ and M80Q $sTnC^{F27W.}$ Purity of native and recombinant Tn subunits was assessed by SDS-PAGE.

Ca^{2+} dissociation rates from sTnC or sTn

Ca^{2+} dissociation rates (k_{off}) from rabbit isolated sTnC and whole sTn (sTnC + sTnI + sTnT) were measured at 5.0 and 15.0 ± 0.1°C using an Applied Photophysics Ltd (Leatherhead, UK) model SX-18MV stopped-flow instrument with a dead time of 1.4 ms at 15°C and a 150 W xenon arc source as previously described (Tikunova *et al.* 2002; Gomes *et al.* 2004). Two methods were used to measure Ca^{2+} k_{off}: (1) Ca^{2+} was removed from all proteins, including isolated sTnC and whole sTn complexes, using Quin-2 (Calbiochem), a fluorescent Ca^{2+} chelator, that was excited at 330 nm; and (2) Ca^{2+} was removed from isolated sTnC proteins containing Phe to Trp mutations using excess EGTA by following decreases in Trp fluorescence with excitation at 275 nm. Quin-2 fluorescence reports Ca^{2+} binding to Quin-2 as it dissociates from sTnC, whereas Trp fluorescence reports a conformational change within the N-terminus when Ca^{2+} binds to sites I and II of sTnC. Either method – Quin-2 or Trp – can be used with $sTnC^{F27W}$ proteins, even with addition of TnI_{96-148} (which does not contain any Trp residues). However, only the Quin-2 method can be used with non-F27W sTnC proteins (because there is no Trp to report Ca^{2+} kinetics) or with whole sTn complexes (because Trp residues exist in sTnI and sTnT and confound the sTnC Trp signal). k_{off} was measured with addition of a peptide fragment of TnI, residues 96–148 (TnI_{96-148}; switch and inhibitory regions) to isolated sTnC using Trp fluorescence because TnI_{96-148} does not contain Trp residues. Reactions for Trp and Quin-2 fluorescence were monitored with the appropriate band-pass optical filters, as previously described (Tikunova *et al.* 2002). Raw data traces were collected and then two to four traces were averaged before being fitted with an exponential curve. Reported k_{off} values represent an average (± S.E.M.) of the rate reported by the fit from $n = 6$–8 traces. Whole sTn-containing M80Q $sTnC^{F27W}$ was fitted with a double exponential equation using fixed parameters for C-terminal rate and endpoint in order to accurately fit the longer time duration (5 s) of these traces that was required to capture the full N-terminal rate of M80Q $sTnC^{F27W}$–sTn (variance less than 2.5×10^{-4}). All other traces for both Trp and Quin-2 methods for isolated sTnC and whole sTn were well fitted by a single exponential. Variance was less than 3.5×10^{-3} at 15°C and 4.4×10^{-3} at 5°C for isolated sTnC and less than 4.3×10^{-4} at 15°C for whole sTn. Quin-2 also measures Ca^{2+} dissociation from the C-terminal domain of sTnC, requiring a longer time course (10 s for isolated sTnC and 200 s for whole sTn). Collecting traces of different time durations and fitting the data accordingly resulted in reproducibility of results for both isolated sTnC and whole sTn experiments to determine N- and C-terminal Ca^{2+} dissociation rates. Further, we were able to calculate the molar concentration of Ca^{2+} dissociating from sTnC using a calibration where 0, 5, 10 and 20 μM Ca^{2+} rapidly (~1 ms) mixed with 150 μM Quin-2, giving a linear relationship of $[Ca^{2+}]$ to change in voltage (related to change in fluorescence of Quin-2). This calibration indicated that we were detecting dissociation of ~65% of the Ca^{2+} bound to the N-terminal domain of sTnC (1.3 mol Ca^{2+} per mol protein) during Quin-2 measurements. The buffer used for isolated sTnC or sTnC + TnI_{96-148} stopped-flow experiments contained (mM): MOPS 10, KCl 90 and DTT 1; pH 7.0 at experimental temperature as previously described (Tikunova *et al.* 2002). 30 μM Ca^{2+} was added to 6 μM sTnC for experiments and was mixed with 150 μM Quin-2. The buffer used for whole sTn stopped-flow experiments contained (mM): MOPS 10, KCl 150, $MgCl_2$ 1 and DTT 1; pH 7.0 at 15°C. This buffer system for whole sTn was chosen so that the ionic strength would be comparable to that used for fibre experiments (0.17 M), which approximates physiological conditions. No additional Ca^{2+} was added to whole sTn as previously described (Gomes *et al.* 2004) because contaminating Ca^{2+} was sufficient for obtaining both N- and C-terminal rates with Quin-2 (150 μM).

Steady-state Ca^{2+} affinity of rabbit sTnC

The affinity of Ca^{2+} binding to WT and mutant sTnCs was determined using a model LS50B Perkin Elmer Luminescence Spectrometer (Wellesley, MA, USA) with a circulating refrigerated water bath (model 1160 A, PolyScience, Niles, IL, USA) to maintain cuvette temperature at 15.0 ± 0.1°C. Trp fluorescence was measured during Ca^{2+} titration of $sTnC^{F27W}$ or M80Q $sTnC^{F27W}$ by using an excitation wavelength of 276 nm and an emission wavelength of 330 nm, as previously described (Gillis *et al.* 2003). Slit widths were the same for excitation and emission and were set to 9 or 10 nm to maximize the fluorescence signal range. Each titration was normalized by first subtracting the fluorescence at the lowest $[Ca^{2+}]$ and then normalizing the resulting values to the maximum fluorescence value. Each data set of Ca^{2+} dependence of fluorescence was fitted with the Hill equation:

$$F = F_o/\left(1 + 10^{n_H(pCa - pCa_{50})}\right) \quad (1)$$

using non-linear least-squares regression analysis (SigmaPlot version 9.0, SPSS, Inc., Chicago, IL, USA). Parameters of slope (n_H), pCa_{50} (pCa at half-maximal fluorescence) and dissociation constant (K_d) were calculated for each trace, averaged and reported ± S.E.M. Ca^{2+} association rates (k_{on}) were calculated from $K_d = k_{off}/k_{on}$ using average values of K_d and k_{off} (Quin-2).

Muscle fibre preparation

Rabbits were housed in the Department of Comparative Medicine at the University of Washington (UW) and cared for in accordance with UW Institutional Animal Care and Use Committee procedures. All animal protocols were in accordance with the US National Institutes of Health Policy on Humane Care and Use of Laboratory Animals and were approved by the UW Animal Care Committee. Male New Zealand white rabbits were anaesthetized with an intravenous injection of pentobarbital (40 mg kg^{-1}) in the marginal ear vein and were exsanguinated when all reflexive response were absent. Small bundles of psoas fibres were excised, demembranated and stored at −20°C for up to 6 weeks as previously described (Regnier *et al.* 2002). Segments of single fibres dissected from fibre bundles were prepared and for most experiments fibre ends were chemically fixed with 1% gluteraldehyde in water (Chase & Kushmerick, 1988) and wrapped in aluminium foil T-clips for attachment to the mechanical apparatus. For all experiments, initial sarcomere length (L_o) was set to 2.5 μm and continuously monitored with helium–neon laser diffraction. Average unfixed L_o of gluteraldehyde-treated fibres was 1.27 ± 0.04 mm (mean ± S.E.M.; $n = 47$) and diameter was 55 ± 1 μm.

Ca^{2+} solutions for measurements of fibre mechanics

Experimental solutions contained (mM): phosphocreatine 15, EGTA 15, MOPS 80, free Mg^{2+} 1, ($Na^+ + K^+$) 135, ATP 5, DTT 1, and 250 units ml^{-1} creatine kinase (Sigma, St Louis, MO, USA) and 4% (w/v) Dextran T-500 (Pharmacia, Piscataway, NJ, USA); pH 7.0, 15°C and an ionic strength of 0.17 M. For activating solutions, the Ca^{2+} concentration (expressed as pCa) was varied between pCa 9.0 and 4.0 by adjusting calcium propionate concentration. Some solutions contained 1–50 mM BDM.

Measurements of fibre mechanics

Two experimental setups were used for collecting mechanical data on individual skinned fibre segments. For studies examining recombinant mutant sTnC and xxsTnC mixtures in fibres (Fig. 5) and inhibition of steady-state force by BDM (Fig. 7), fibre segment ends (unfixed) were wrapped around wire hooks to a force transducer and manual micromanipulator as previously described (Martyn *et al.* 1993) for measurement of steady-state force. All other mechanical data were collected with fixed and clipped fibre segments attached to an Aurora Scientific (Ontario, Canada) force transducer and a General Scanning model G120DT (Watertown, MA, USA) servo-motor (adjusted for 300 μs step time) by minutien pin hooks and mounted on a Nikon (Japan) inverted microscope as previously described (Regnier *et al.* 2002). At 5 s intervals, fibre segments were shortened by 15% L_o at 10 L_o s^{-1}, then rapidly restretched to initial L_o to maintain fibre integrity (Brenner, 1983; Sweeney *et al.* 1987; Chase & Kushmerick, 1988). Resulting force transients are visible as vertical lines in the chart records shown in Fig. 3. Measurement of steady-state isometric force was made prior to the release–restretch cycle as $[Ca^{2+}]$ in the activating solutions was varied. The fibre preparation was moved between pCa solutions that were held in individual temperature-controlled troughs as previously described (Regnier *et al.* 2002). Passive force was determined at pCa 9.0 with a release–restretch protocol and subtracted from total force measured in solutions of higher $[Ca^{2+}]$ to obtain the active force values reported. Maximal fibre force (F_{max}), measured at pCa 4.5 just prior to extraction, was 354 ± 11 mN mm^{-2} ($n = 47$; assuming circular cross-sectional area). Fibres with greater than 12% loss of F_{max} from initial value to just prior to extraction were discarded. sTnC extraction solution contained (mM) MOPS 10, EDTA 5 and trifluoperazine (TFP) 0.5; pH 6.6 at 15°C, and selective sTnC extraction was completed to 1.3 ± 0.1% F_{max} with repeated incubations in extraction solution (30 s) and pCa 9 solution (10 s) as previously described (Regnier *et al.* 1999; Moreno-Gonzalez *et al.* 2005). Reconstitution with 1 mg ml^{-1} of total protein in pCa 9 relaxing solution was completed with 1–2 min incubations (unless otherwise noted). Reported reconstituted F_{max} values (Tables 2–4) are from back-to-back measurements comparing just prior to extraction and immediately following reconstitution. Steady-state force–pCa relationships were fitted with the Hill equation (eqn (1)) to obtain pCa at half-maximal force (pCa_{50}; defined as the Ca^{2+} sensitivity of force) and slope (n_H; the apparent cooperativity of contractile activation). Reported pCa_{50} and n_H values represent the means of the values from the individual fits (± S.E.M). Plots show average (± S.E.M) of each point with curves fitted to the average data. Analysis of variance (ANOVA) was used to compare between groups and when differences between groups were significant, Student's paired or unpaired t tests were used with statistical significance set at $P < 0.05$.

Table 1. Solution Ca^{2+}-binding kinetics of sTnC and sTn at 15°C

	Isolated sTnC		Whole sTn*	
Rabbit sTnC Type	k_{off} (s^{-1})	K_d (μM)	$k_{on} \times 10^8$ ($M^{-1}\,s^{-1}$)	k_{off} (s^{-1})
WT sTnC	Too fast	—	—	5.57 ± 0.04
M80Q sTnC	888 ± 44‡†	—	—	3.25 ± 0.01§
$sTnC^{F27W}$	353 ± 5	2.19 ± 0.16	1.6	4.57 ± 0.02§
M80Q $sTnC^{F27W}$	70.6 ± 0.7‡	0.75 ± 0.01‡	0.9	2.16 ± 0.02§‡

*Whole sTn complexes contain purified native rabbit sTnI and sTnT. †A slower rate of $24.4 \pm 1.0\ s^{-1}$ was 27% of amplitude signal, and this double exponential signal appeared only with isolated M80Q sTnC. §$P < 0.01$ *versus* WT sTn; ‡$P < 0.01$ *versus* $sTnC^{F27W}$.

Results

Ca^{2+} kinetics of recombinant rabbit sTnC mutants

Ca^{2+} binding properties were determined for isolated sTnC and in complex with $TnI_{96\text{-}148}$ or sTnI + sTnT. Isolated sTnC measurements allowed us to determine steady-state Ca^{2+} affinity (K_d) and kinetics of Ca^{2+} dissociation (k_{off}), although determination of K_d was not possible using tryptophan fluorescence for whole sTn complexes. Table 1 summarizes Ca^{2+}-binding properties and shows that k_{off} progressively decreased, in the order of WT sTnC > M80Q sTnC > $sTnC^{F27W}$ > M80Q $sTnC^{F27W}$. k_{off} was also measured with Trp fluorescence for $sTnC^{F27W}$ ($350 \pm 2\ s^{-1}$) and M80Q $sTnC^{F27W}$ ($83.5 \pm 0.7\ s^{-1}$) as a control, and these values are comparable to those previously reported for the chicken isoform of sTnC (Davis *et al.* 2004). Measurements at 5°C were made for quantitative comparisons between isolated sTnC mutants because k_{off} for WT sTnC is too fast to measure at 15°C. Lower temperature decreased k_{off} for isolated sTnC proteins and was $725 \pm 2\ s^{-1}$ for WT sTnC, and decreased by 5-fold, 17-fold and 40-fold at 5°C for M80Q sTnC, $sTnC^{F27W}$ and M80Q $sTnC^{F27W}$, respectively. Additional measurements were made in the presence of an sTnI peptide (residues 96–148 containing switch and inhibitory regions; $TnI_{96\text{-}148}$) using Trp fluorescence. k_{off} decreased by 50-fold for $sTnC^{F27W}$–$TnI_{96\text{-}148}$ ($7.0 \pm 0.1\ s^{-1}$) and by 20-fold for M80Q $sTnC^{F27W}$–$TnI_{96\text{-}148}$ ($3.5 \pm 0.1\ s^{-1}$) *versus* isolated $sTnC^{F27W}$ and M80Q $sTnC^{F27W}$, respectively, at 15°C. This trend agrees with previous reports and suggests an important contribution of intermolecular interactions between TnI and TnC to slowing k_{off} (Davis *et al.* 2004).

Steady-state K_d was measured for isolated $sTnC^{F27W}$ and M80Q $sTnC^{F27W}$ at 15°C (Fig. 1) and used to determine Ca^{2+} association rate (k_{on}). K_d for $sTnC^{F27W}$ was ~2.2 μM and decreased for M80Q $sTnC^{F27W}$ (Table 1). Values of k_{on} ($k_{on} = k_{off}/K_d$) for both F27W mutants (Table 1) were similar to those previously reported (Johnson *et al.* 1994; Tikunova *et al.* 2002), approach diffusion limitations at ~$10^8\ M^{-1}\,s^{-1}$, and were somewhat greater for $sTnC^{F27W}$. This calculation suggests that the ~3-fold increase in steady-state K_d for M80Q $sTnC^{F27W}$ (compared to $sTnC^{F27W}$) is probably not due to differences in k_{on} but determined primarily by changes in k_{off}.

Whole sTn complexes contained WT or mutant sTnC and purified native rabbit sTnI and sTnT. All k_{off} values in whole sTn were slower than k_{off} values for isolated sTnC or sTnC–$TnI_{96\text{-}148}$, suggesting that interactions between all three subunits of troponin slow k_{off} to its greatest extent. Mutations in sTnC decreased k_{off} in whole sTn *versus* WT sTnC–sTn by 42% for M80Q sTnC–sTn, 18% for $sTnC^{F27W}$–sTn and 61% for M80Q $sTnC^{F27W}$–sTn (Fig. 2 and Table 1). While the order of decreasing k_{off} changed from isolated sTnC values (with M80Q sTnC–sTn having a slower k_{off} than $sTnC^{F27W}$–sTn), these k_{off} rates in whole sTn confirmed that these mutations reduced k_{off} and M80Q $sTnC^{F27W}$ had the slowest k_{off} of the three sTnC mutants. It is important to note that these rates at 15°C are 2–6 s^{-1} which could be slow enough to affect the kinetics of force development or relaxation, especially during submaximal Ca^{2+} activation.

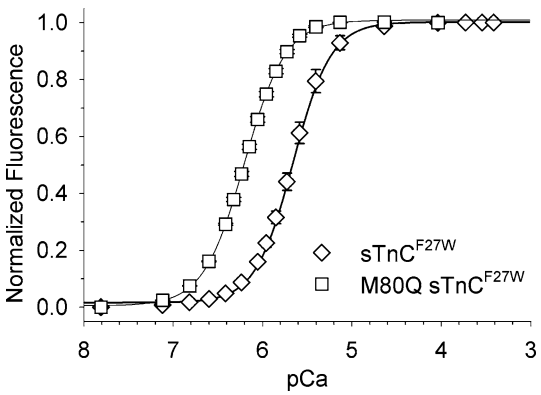

Figure 1. Steady-state Ca^{2+} affinity measurements for isolated $sTnC^{F27W}$ and M80Q $sTnC^{F27W}$ at 15°C
Ca^{2+} sensitivity of steady-state affinity (pK) was 5.67 ± 0.03 for $sTnC^{F27W}$ ($n = 11$) and increased for M80Q $sTnC^{F27W}$ to 6.13 ± 0.01 ($n = 10$). The calculated slopes of the binding curves were 2.0 ± 0.1 for $sTnC^{F27W}$ and 1.6 ± 0.1 for M80Q $sTnC^{F27W}$. Error bars (± S.E.M.) are present and often smaller than symbols.

Steady-state force–Ca^{2+} relationships of mutant sTnC-reconstituted fibres

Measurement of the steady-state force–pCa relationship for each skinned rabbit psoas fibre was completed before extraction of native sTnC (see Methods) and after reconstitution of Tn complexes with purified native or recombinant sTnC (Fig. 3). Reconstitution with native purified sTnC (control) resulted in an F_{max} that was $96 \pm 1\%$ of pre-extracted values, demonstrating the ability to completely re-occupy Tn complexes. Fibres reconstituted with 100% recombinant mutant sTnC showed some reduction in F_{max} compared with control (100% purified sTnC; Table 2). To determine whether all Tn complexes were occupied, recombinant sTnC-reconstituted fibres were incubated for two additional 1 min periods in 100% purified native sTnC. No further change in F_{max} confirmed that all Tn complexes had been occupied with recombinant sTnC. Possible reasons for lower F_{max} with recombinant mutant sTnC are discussed below.

Fully reconstituting fibres with the different sTnC mutants clearly altered Ca^{2+} sensitivity of force (pCa_{50}). There were no differences between fibre groups in pCa_{50} prior to sTnC extraction. Reconstitution with 100% purified sTnC did not change pCa_{50} or slope (Table 2), demonstrating that the extraction–reconstitution protocol had no effect on Ca^{2+}-dependent activation of steady-state force. All sTnC mutants had an increased pCa_{50} of the force–pCa relationship (Fig. 4). Paired comparisons were made within fibre groups for pre-extraction *versus* post-reconstitution conditions to determine magnitude changes in pCa_{50} (ΔpCa_{50}) with individual sTnC mutants. ΔpCa_{50} increased from 0.08 with M80Q sTnC, to 0.15 with $sTnC^{F27W}$ and to 0.32 with M80Q $sTnC^{F27W}$ (Fig. 4 and Table 2). These increases in pCa_{50} are inversely related to decreases in solution k_{off} and K_d, indicating that progressively greater Ca^{2+} affinity of sTnC may result in a progressively greater increase in the Ca^{2+} sensitivity of steady-state force. It is important to note that the F27W mutation, which has been used as a reporter of confirmation change with Ca^{2+} binding (Pearlstone *et al.* 1992; Chandra *et al.* 1994; Johnson *et al.* 1994; Tikunova *et al.* 2002; Davis *et al.* 2004), slows k_{off} (by ~20% at 15°C in whole sTn) and alters fibre mechanics (increases pCa_{50} by 0.15). It is worth noting that $sTnC^{F27W}$ is used in this study both as a reporter of sTnC conformational changes with Ca^{2+} (Table 1) and as a tool to study functional effects in fibres (Fig. 4*B* and Table 2).

Fibres fully reconstituted with the different sTnC mutants had slightly reduced n_H, a measure of the cooperativity of force generation. ANOVA between groups reconstituted with M80Q sTnC, $sTnC^{F27W}$ or M80Q $sTnC^{F27W}$ showed no statistical difference. However, in paired comparisons (pre-extraction *versus* post-reconstitution) within fibre groups, n_H was unchanged for purified sTnC (control) and reduced for each mutant sTnC ($P < 0.05$; Table 2). These data suggest that some loss of cooperative activation probably occurred as a result of the altered Ca^{2+}-binding properties or altered quaternary structure of the sTnC mutants. In spite of this slight reduction in n_H, these sTnC mutations had a dramatic Ca^{2+}-sensitizing effect on force in skeletal muscle fibres.

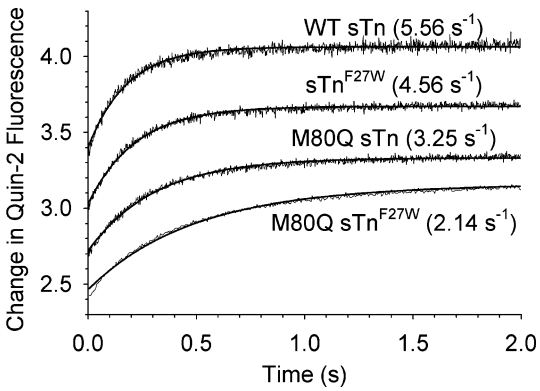

Figure 2. Example traces of Ca^{2+} dissociation from whole sTn with sTnC mutants
Stopped-flow spectroscopy traces show the time course of fluorescence increase in Quin-2 as it binds Ca^{2+} for WT sTnC, $sTnC^{F27W}$, M80Q sTnC and M80Q $sTnC^{F27W}$ in complex with purified native sTnI and sTnT at 15°C. Y-axis is displayed in volts. Traces are fitted with a single exponential curve from which Ca^{2+} dissociation rates (k_{off}) are determined except for M80Q $sTnC^{F27W}$–sTn where a double exponential curve with fixed slow C-terminal rate was used (see Methods). Traces are the average of three runs and are vertically spaced for clarity.

Mutant sTnC: xxsTnC mixtures in fibres

To determine whether sTnC Ca^{2+}-binding properties influence cooperative interaction between structural RUs along thin filaments during Ca^{2+} activation, fibres were reconstituted with mixtures of WT or mutant rabbit sTnC and xxsTnC. Steady-state force–pCa relationships for mixtures of M80Q $sTnC^{F27W}$ and xxsTnC (denoted as sTnC: xxsTnC) showed reduced cooperative activation and force generation (Fig. 5*A* and Table 3). Prior to extraction, pCa_{50} and n_H were not different between groups of fibres. Following reconstitution, F_{max} was reduced as the content of M80Q $sTnC^{F27W}$ in the reconstitution mixture was reduced (and correspondingly, xxsTnC content increased). For these experiments, pCa_{50} increased (leftward shift) by 0.25 for the 100 : 0 mixture (compared with 0.32 in Table 2) and this was progressively reduced for all other mixtures, with a maximum rightward shift of 0.51 for only 20% M80Q $sTnC^{F27W}$ (80% xxsTnC) in the reconstitution mixture (20 : 80; Table 3). n_H also decreased with diminishing M80Q $sTnC^{F27W}$ content

Table 2. Force–Ca^{2+} relationship parameters in skinned psoas fibres fully reconstituted with sTnC

	Pre-extracted		Reconstituted			
sTnC (*n*)	pCa_{50}	n_H	F_{max}‡	pCa_{50}	n_H	ΔpCa_{50}§
Purified sTnC (7)	5.98 ± 0.02	3.1 ± 0.2	1.0	5.96 ± 0.03	3.2 ± 0.4	−0.01 ± 0.02
M80Q sTnC (6)	6.08 ± 0.05	3.4 ± 0.2	0.89 ± 0.03*	6.15 ± 0.05*	2.8 ± 0.2	0.08 ± 0.02*†
$sTnC^{F27W}$ (9)	6.08 ± 0.05	2.9 ± 0.1	0.92 ± 0.02*	6.23 ± 0.04*	2.7 ± 0.1	0.15 ± 0.03*
M80Q $sTnC^{F27W}$ (9)	6.06 ± 0.01	3.4 ± 0.1	0.85 ± 0.01*#	6.38 ± 0.02*#	2.5 ± 0.1	0.32 ± 0.02*#

‡Fraction of reconstituted F_{max} compared to purified sTnC F_{max} where purified sTnC was 0.96 ± 0.01 of pre-extracted F_{max} ($P < 0.01$). §Change in pCa_{50} *versus* pre-extracted native sTnC value calculated for each fibre and reported as average ± S.E.M. within group of $n = 6$–9 fibres. *$P < 0.01$ *versus* purified sTnC; #$P < 0.01$ *versus* $sTnC^{F27W}$; †$P < 0.05$ *versus* $sTnC^{F27W}$. All reconstituted values are significantly different (for n_H, $P < 0.05$; for pCa_{50} and F_{max}, $P < 0.01$) by paired *t* test *versus* pre-extracted native sTnC within each group of $n = 6$–9 fibres, except for purified sTnC values for pCa_{50} and n_H ($P > 0.05$).

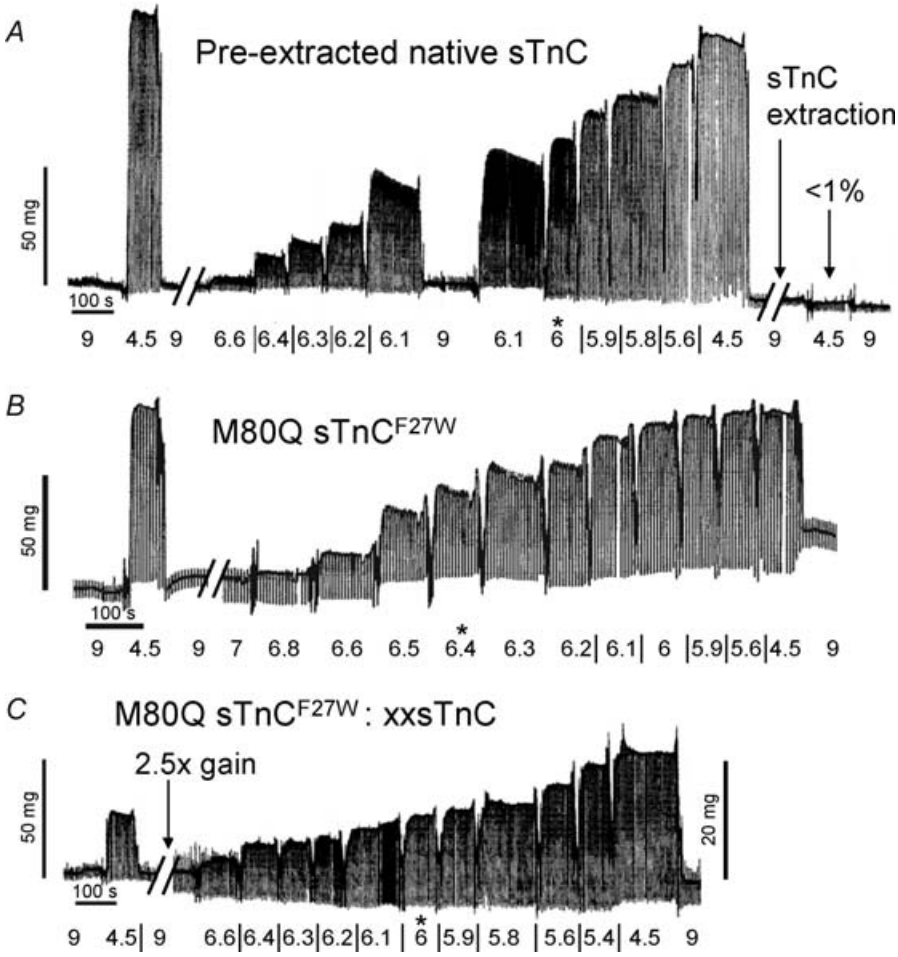

Figure 3. Examples of chart record traces from protocols to measure skinned rabbit psoas fibre mechanics

A, trace shows a maximal activation (pCa 4.5) followed by measurement of force–pCa curve ending with maximal activation, F_{max}, and then sTnC extraction. Force–pCa curve shows relaxation (pCa 9) after first pCa 6.1 activation for readjusting sarcomere length to 2.5 μm. Hill fit parameters were $pCa_{50} = 6.04$ and $n_H = 2.5$. All panels have pCa_{50} value marked by an asterisk to emphasize changes in Ca^{2+} sensitivity between panels. After sTnC extraction in *A* (first arrow; see Methods), 0.7% F_{max} remained at pCa 4.5 (second arrow). *B*, example trace from a separate experiment shows reconstitution of Tn complexes with 100% M80Q $sTnC^{F27W}$ where 84% F_{max} was recovered (see Methods), followed by a force–pCa curve where $pCa_{50} = 6.35$ and $n_H = 2.5$. The wandering baseline does not effect measurement of steady-state isometric force as true zero force is recorded with each trace recorded. *C*, example trace (same fibre as *A*) shows reconstitution of Tn complexes with a mixture of M80Q $sTnC^{F27W}$ and xxsTnC where 23% F_{max} was recovered. Signal gain on chart recorder was increased by 2.5 for improved resolution (arrow). $pCa_{50} = 5.98$ and $n_H = 1.4$. Fibre diameters were 78 μm (*A* and *C*) and 59 μm (*B*).

of the mixture by paired comparisons (although no difference was found between groups by ANOVA), similar to previous observations for sTnC: xxsTnC mixtures (Regnier *et al.* 2002). These data suggest a reduced ability for near-neighbour RU interactions to contribute to cooperative force generation. n_H is not reported for the 20 : 80 mixture because these fibres had a very low reconstituted F_{max} and often exhibited a step-like on/off response to submaximal Ca^{2+} concentrations. For each M80Q sTnC^{F27W}: xxsTnC mixture, F_{max} was lower than the percentage of M80Q sTnC^{F27W} in the protein incubation solution and was only 7% of pre-extracted F_{max} for 20 : 80 M80Q sTnC^{F27W}: xxsTnC (compared with 23% F_{max} for 20 : 80 sTnC: xxsTnC). Fibres reconstituted in 20 : 80 mixtures of M80Q sTnC: xxsTnC or sTnC^{F27W}: xxsTnC produced only 14% or 17% F_{max}, respectively (data not shown). Although this result was not expected for sTnC mutants with greater Ca^{2+}-binding affinity, it could indicate a reduced ability of the mutant proteins to activate thin filaments compared to native sTnC at maximal [Ca^{2+}] (in agreement with slightly reduced F_{max} in 100 : 0 mixtures).

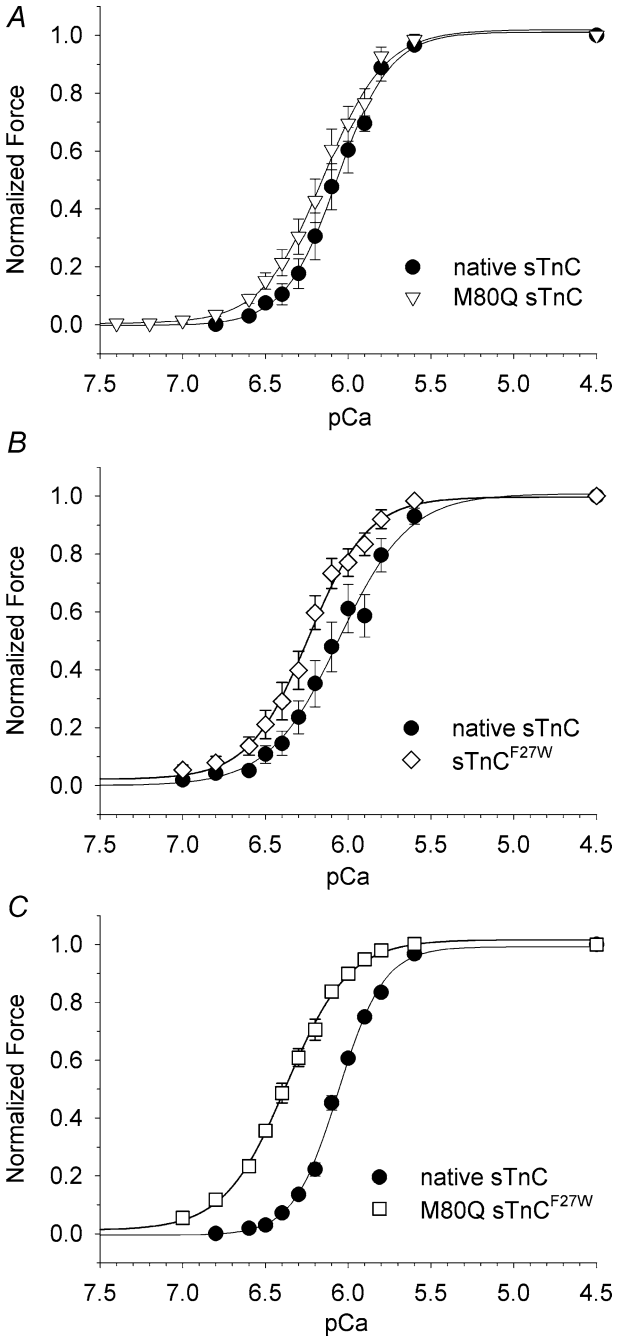

Figure 4. Force–pCa relationships for fibres reconstituted with mutant rabbit sTnC
Extraction of native sTnC was followed by reconstitution with M80Q sTnC (*A*), sTnC^{F27W} (*B*) or M80Q sTnC^{F27W} (*C*). Each panel shows normalized force as a function of pCa and clearly demonstrates that the Ca^{2+} sensitivity of force (pCa_{50}) gradually increases by 0.08 to 0.15 to 0.32 in *A*, *B* and *C*, respectively, as the Ca^{2+} dissociation rate (k_{off}) of the mutant sTnC decreased (see Table 1). Some error bars are smaller than symbols; see Table 2 for reconstituted F_{max}, pCa_{50} and n_H values.

An alternative explanation for unexpectedly low F_{max} values for mixtures containing mutant sTnC and xxsTnC is that different relative binding affinities between the mutants and xxsTnC result in a different ratio of proteins that bind to TnI–TnT complexes in fibres. We previously showed that purified sTnC and xxsTnC bind to TnI–TnT complexes with similar affinity in fibres (Regnier *et al.* 2002), but this may be altered if sTnC mutants bind with different affinities from sTnC and xxsTnC. To test the relative binding affinity of sTnC mutants for TnI–TnT complexes in fibres, native sTnC was extracted and Tn complexes were reconstituted by short interval incubations in solutions of low [sTnC] (0.1 mg ml^{-1}), each followed by a measurement of F_{max} (pCa 4; Fig. 5*B*). Reconstitution with each sTnC was considered complete when successive incubations no longer increased F_{max}. In a subset of these experiments, additional incubations in a high concentration of purified sTnC (1 mg ml^{-1}; 2 incubations for 2 min and 1 min) did not change F_{max}, confirming that all TnI-TnT complexes were occupied with M80Q sTnC^{F27W} (black stars, Fig. 5*B*). The force plateau occurred at ~0.8 F_{max} with M80Q sTnC^{F27W}, similar to previous measurements with 100% M80Q sTnC^{F27W} (Tables 2 and 3). Extraction of M80Q sTnC^{F27W} and reconstitution with 1 mg ml^{-1} purified sTnC (filled diamonds, Fig. 5*B*) showed that force recovered to ~0.8 F_{max}, suggesting that some rundown occurred because of the long protocol and a second extraction–reconstitution of sTnC. Force curves were well fit by a single exponential rising curve (see legend to Fig. 5). The time constant for force restoration ($1/b$) was 8.14 ± 1.59 min for M80Q sTnC^{F27W}, which was approximately three times slower than for purified sTnC (2.48 ± 0.11 min). This force assay demonstrated that M80Q sTnC^{F27W} had a lower affinity than sTnC for skeletal TnI–TnT complexes in muscle fibre thin filaments. We verified that the single mutant sTnC^{F27W} also had a reconstitution time constant different from that of purified sTnC (3.01 ± 0.55 min; data not shown). These

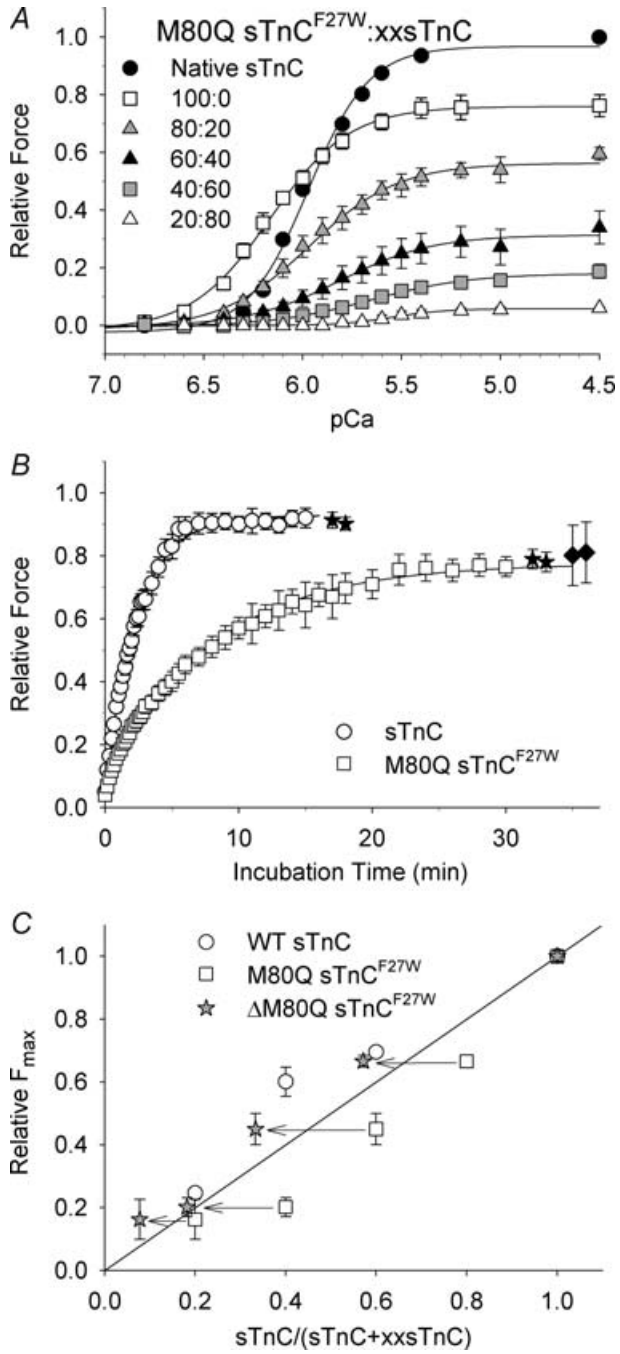

Figure 5. Reduction of thin filament near-neighbour cooperativity in fibres by increasing xxsTnC content in protein mixtures for reconstitution

A, force–pCa relationships of fibres reconstituted with mixtures of M80Q sTnC^{F27W} and xxsTnC are shown with pre-extracted native sTnC (●) as control. Mixture ratios of M80Q sTnC^{F27W}: xxsTnC are shown for each symbol. F_{max} values for mixtures are slightly less than the consecutive activation values reported in Table 3 due to fibre rundown that occurred with the force–pCa protocol. *B*, force assay to determine the relative binding affinity of M80Q sTnC^{F27W} *versus* sTnC in sTnC-extracted thin filaments of fibres shows F_{max} (pCa 4) measured after short interval incubations in 0.1 mg ml^{-1} sTnC (○) or M80Q sTnC^{F27W} (□). Black stars indicate F_{max} after incubations in 1 mg ml^{-1} purified sTnC. Black diamonds show F_{max} after fully extracting M80Q sTnC^{F27W} and reconstituting fibres in 1 mg ml^{-1} purified sTnC. Force curves were well fit by a single exponential rising curve: $f = y_0 + a(1-e^{-bx})$ with $r^2 > 0.995$ for all proteins. For sTnC, baseline $y_0 = 5\%$, amplitude $a = 88\%$, and the time constant for force rise (1/*b*) was 2.48 ± 0.11 min. For M80Q sTnC^{F27W}, $y_0 = 8\%$, $a = 69\%$ and 1/*b* was 8.14 ± 1.59 min. *C*, plot of F_{max} (relative to F_{max} of 100% protein) *versus* functional sTnC content of reconstitution mixture shows WT sTnC controls (○) and M80Q sTnC^{F27W} (□) at each mixture ratio (100 : 0, 80 : 20, 60 : 40, 40 : 60 and 20 : 80; $n = 2$–8). Transformation of data (ΔM80Q sTnC^{F27W}; grey stars) to account for unequal binding in the fibre of M80Q sTnC^{F27W} and xxsTnC (see Appendix) shifts mixture content of M80Q sTnC^{F27W} (arrows). Continuous line represents the unity line $y = x$. Some error bars are smaller than symbols.

data suggest that sTnC mutant content is lower in the fibre than in the protein incubation mixture.

Figure 5*C* shows how F_{max} varies with the fraction of functional sTnC in fibres. The unity line reflects expected F_{max} if each functional Tn complex activates a seven actin length of thin filament, equal to the structural RU size. As previously shown for purified sTnC (Regnier *et al.* 2002), mixtures of WT sTnC: xxsTnC resulted in a curve that was above the unity line, suggesting that more than seven actin monomers are activated by Ca^{2+} binding within an RU. It is odd that data for M80Q sTnC^{F27W} were below the unity line. This could indicate that the spread of activation along the thin filament with Ca^{2+} binding to individual Tn complexes was reduced. However, the results shown in Fig. 5*B* suggest the mixture ratio content did not accurately reflect the fibre content of functional sTnC. Thus, a second-order non-linear binding affinity calculation was used to estimate the protein composition in fibres assuming a 3-fold slower protein binding rate for M80Q sTnC^{F27W} *versus* xxsTnC across the range of protein ratio mixtures used for these studies (eqn (3) in Appendix). These calculations transform the F_{max} data for M80Q sTnC^{F27W}: xxsTnC mixtures (open squares, Fig. 5*C*) by adjusting the solution mixture content value (*x* axis) to reflect predicted content in fibres (grey stars, Fig. 5*C*). This transformation shifts all data points above the unity line (arrows, Fig. 5*C*), as we find for sTnC: xxsTnC mixtures. Therefore, the transformed data suggest that similar fractions of either WT or M80Q sTnC^{F27W} incorporated into thin filaments allow for Ca^{2+} to activate muscle fibre force production to a comparable extent.

To study the effect of sTnC mutants on the Ca^{2+} dependence of force development when near-neighbour RU interactions along thin filaments were greatly reduced, we reconstituted fibres with mixtures of sTnC: xxsTnC that produced ~0.2 of the pre-extracted F_{max}. To achieve 0.2 F_{max}, solution protein mixtures were approximately 20 : 80 for sTnC: xxsTnC, 25 : 75 for M80Q sTnC: xxsTnC and 40 : 60 for M80Q sTnC^{F27W}: xxsTnC. Force–pCa relations in fibres were determined (Fig. 6) and examination of pCa_{50} and n_H values between xxsTnC mixtures (Table 4)

Table 3. Force–Ca^{2+} parameters for fibres reconstituted with M80Q $sTnC^{F27W}$: xxsTnC mixtures

	Pre-extracted		Reconstituted			
M80Q $sTnC^{F27W}$: xxsTnC (*n*)	pCa_{50}	n_H	F_{max}*	pCa_{50}	n_H	ΔpCa_{50}†
100 : 0 (5)	5.92 ± 0.02	2.9 ± 0.3	0.85 ± 0.02	6.17 ± 0.02	2.2 ± 0.1	0.25 ± 0.02
80 : 20 (4)	5.97 ± 0.01	2.9 ± 0.1	0.57 ± 0.03	5.91 ± 0.09	1.9 ± 0.1	−0.07 ± 0.09
60 : 40 (5)	5.96 ± 0.01	2.8 ± 0.1	0.33 ± 0.06	5.75 ± 0.05	1.8 ± 0.9	−0.21 ± 0.05
40 : 60 (3)	6.00 ± 0.01	2.8 ± 0.1	0.19 ± 0.03	5.65 ± 0.03	1.7 ± 0.2	−0.35 ± 0.03
20 : 80 (3)	5.98 ± 0.01	3.0 ± 0.1	0.07 ± 0.01	5.47 ± 0.11	—	−0.51 ± 0.11

*Fraction of reconstituted F_{max} compared to pre-extracted F_{max} (normalized to 1.0). †Change in pCa_{50} *versus* pre-extracted native sTnC value calculated for each fibre and reported as average ± S.E.M. within group of $n = 3$–5 fibres. All reconstituted values are significantly different ($P = 0.01$) by paired *t* test *versus* pre-extracted native sTnC within each group of $n = 3$–5 fibres, except for pCa_{50} of 80 : 20 ratio. All reconstituted F_{max} and pCa_{50} values are different ($P < 0.05$) between groups, except pCa_{50} values of 80 : 20 *versus* 60 : 40 and 60 : 40 *versus* 40 : 60. n_H values do not significantly differ by ANOVA.

Table 4. Force–Ca^{2+} parameters for force-matched fibres reconstituted with sTnC: xxsTnC mixtures to ∼0.2 F_{max}

	Pre-extracted		Reconstituted			
sTnC Mixture (*n*)	pCa_{50}	n_H	F_{max}#	pCa_{50}	n_H	ΔpCa_{50}§
sTnC: xxsTnC (6)	5.97 ± 0.05	2.9 ± 0.1	0.24 ± 0.03*	5.62 ± 0.05**	2.1 ± 0.1**	−0.35 ± 0.03*
M80Q sTnC: xxsTnC (6)	5.98 ± 0.05	3.1 ± 0.3	0.21 ± 0.01*	5.73 ± 0.06*	2.0 ± 0.2*	−0.25 ± 0.04*†
M80Q $sTnC^{F27W}$: xxsTnC (7)	6.03 ± 0.03	3.1 ± 0.2	0.20 ± 0.01*	5.92 ± 0.05##	2.0 ± 0.2*	−0.10 ± 0.03**‡

Fraction of reconstituted F_{max} compared to 100% purified sTnC F_{max} (reported in Table 2). §Change in pCa_{50} *versus* pre-extracted native sTnC value calculated for each fibre and reported as average ± S.E.M. within group of $n = 6$–7 fibres. *$P < 0.01$ *versus* purified sTnC value (reported in Table 2); **$P < 0.05$ *versus* purified sTnC value (reported in Table 2); ##$P < 0.01$ *versus* sTnC: xxsTnC and $P < 0.05$ *versus* M80Q sTnC: xxsTnC; †$P < 0.05$ *versus* sTnC: xxsTnC; ‡$P < 0.01$ *versus* sTnC: xxsTnC and *versus* M80Q sTnC: xxsTnC. All reconstituted values are significantly different ($P < 0.05$) by paired *t* test *versus* pre-extracted native sTnC within each group of $n = 6$–7 fibres. All F_{max} and n_H values do not significantly differ between xxsTnC mixture groups by ANOVA.

suggests that sTnC mutants modulate Ca^{2+} sensitivity of force and not cooperativity (as indicated by n_H) at this low level of force. Reconstitution with purified native sTnC: xxsTnC decreased pCa_{50} by 0.35 and reduced n_H, similar to previous results (Regnier *et al.* 2002), suggesting that interactions between functional RUs along thin filaments were reduced. However, some interactions may persist, as n_H was not reduced to the value of 1.7 that we obtained previously using 15 : 85 sTnC: xxsTnC reconstituted fibres (Regnier *et al.* 2002). Matching the force level at 0.2 F_{max} using M80Q sTnC or M80Q $sTnC^{F27W}$ in mixtures with xxsTnC was important for creating similar thin filament activation levels. Under these conditions, ΔpCa_{50} was less for fibres reconstituted with M80Q sTnC: xxsTnC (−0.25) and M80Q $sTnC^{F27W}$: xxsTnC (−0.10) compared to sTnC: xxsTnC (Table 4). In other words, the force–pCa curve shifted to the left with M80Q sTnC: xxsTnC and M80Q $sTnC^{F27W}$: xxsTnC *versus* sTnC: xxsTnC by +0.13 and +0.28, respectively. This magnitude of increase in pCa_{50} in xxsTnC mixtures was similar to that of fibres fully reconstituted with each sTnC mutant (Table 2). It is interesting that for mutant sTnC: xxsTnC mixtures to 0.2 F_{max}, n_H was the same for sTnC and mutants, suggesting that altered Ca^{2+}-binding properties of sTnC do not affect cooperative activation within RUs in the absence of near-neighbour RU interactions. Together these data demonstrate that pCa_{50} is determined (at least partially) by the Ca^{2+}-binding properties of sTnC within individual RUs and by the extent of near-neighbour RU interactions, whereas n_H is determined primarily by the extent of

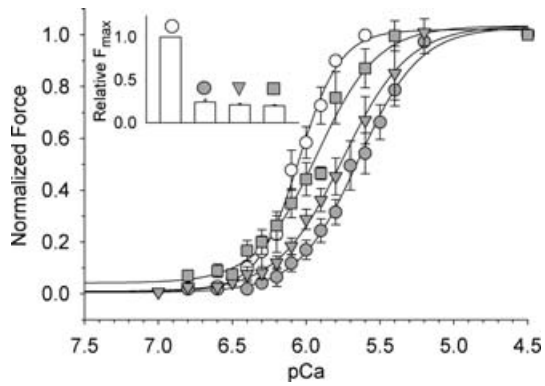

Figure 6. Force–pCa relationships for fibres reconstituted with mixtures of mutant sTnC and xxsTnC to 0.2 F_{max}
Normalized force–pCa relationships are shown for 100% sTnC (control; ○) and mixtures with xxsTnC resulting in 0.2 F_{max}: purified sTnC: xxsTnC (grey circles), M80Q sTnC: xxsTnC (grey triangles) and M80Q $sTnC^{F27W}$: xxsTnC (grey squares). Inset shows reconstituted F_{max} for 100% purified sTnC (○), sTnC: xxsTnC (grey circle), M80Q sTnC: xxsTnC (grey triangles) and M80Q $sTnC^{F27W}$: xxsTnC (grey squares). See Table 4 for values.

near-neighbour RU interactions and much less by the Ca^{2+}-binding properties of sTnC.

Inhibition of strong crossbridge formation with BDM

We next sought to determine how increasing Ca^{2+}-binding affinity under conditions of reduced near-neighbour RU interactions influences the crossbridge contribution to thin filament activation. We recently demonstrated that 10 mM BDM decreased F_{max} ~4-fold more in skeletal muscle fibres reconstituted with 20 : 80 sTnC: xxsTnC mixtures compared with native sTnC control (Gillis *et al.* 2007). The conclusion from these experiments was that when near-neighbour RU interactions were minimized, thin filament activation became more dependent on strong crossbridge binding. Figure 7 shows how inhibition by BDM differs between sTnC and M80Q $sTnC^{F27W}$ when near-neighbour RU interactions are reduced (with xxsTnC to 0.2 F_{max}). Plotting normalized F_{max} *versus* BDM concentration allows comparison of the extent of force inhibition under each thin filament condition. The sensitivity of F_{max} to increasing [BDM] prior to extraction (filled circles; inhibition constant (K_i), 12.3 ± 0.4 mM) was unaltered by reconstitution with 100% sTnC (open circles; K_i, 11.0 ± 1.0 mM) and slightly reduced for reconstitution with 100% M80Q $sTnC^{F27W}$ (K_i, 8.5 ± 2.2 mM; $P = 0.09$; data not shown). Force inhibition was more sensitive to BDM for fibres reconstituted with 20 : 80 sTnC: xxsTnC mixtures (grey circles; K_i, 2.6 ± 0.4 mM) compared with control (open circles), in agreement with previous work. To compare M80Q $sTnC^{F27W}$ at a similar level of thin filament activation, ~0.2 F_{max} was reached by reconstituting fibres with 40 : 60 M80Q $sTnC^{F27W}$: xxsTnC (inset, Fig. 7). It is surprising that M80Q $sTnC^{F27W}$: xxsTnC fibres showed no change in sensitivity to BDM inhibition (grey squares; $K_i = 2.0 \pm 0.2$ mM) compared with 20 : 80 sTnC: xxsTnC (grey circles). In comparison, for measurements with 40 : 60 sTnC: xxsTnC incubation mixtures, fibre F_{max} was ~0.4 (inset, Fig. 7) and sensitivity of force inhibition by BDM was reduced for 40 : 60 mixtures (open triangles; K_i, 4.2 ± 0.3 mM; $P < 0.02$ *versus* 0.2 F_{max} mixtures). Together these data indicate that the sensitivity of force inhibition to BDM was correlated with the number of functional RUs in the thin filament and suggest that enhancing the Ca^{2+} affinity of sTnC does not alter the strong crossbridge contribution to thin filament activation.

Discussion

This study was designed to investigate how enhancing the Ca^{2+}-binding affinity of sTn influences the myofilament protein interactions in cooperative contractile activation of skeletal muscle. Specifically we studied how near-neighbour RU interactions along the thin filament and strong crossbridge formation were altered when the Ca^{2+} affinity of sTnC was increased. By engineering specific point mutations into sTnC, we were able to increase Ca^{2+} affinity (reported as K_d), which was changed via slowed Ca^{2+} k_{off}. The sTnC mutants were then incorporated into sTn complexes in skinned fibres (following extraction of native sTnC) to examine their influence on the cooperativity of force generation. The main findings were: (1) complete reconstitution of fibres with sTnC containing a mutation that slowed k_{off} increased the Ca^{2+} sensitivity of steady-state force (pCa_{50}) but did not increase the slope of the force–pCa relationship (n_H); (2) when near-neighbour RU interactions were reduced (mutant sTnC: xxsTnC to produce 0.2 F_{max}), n_H was reduced and Ca^{2+} sensitivity of steady-state force decreased by 0.35–0.40 for each sTnC mutant from its own 100% reconstitution value; and (3) with reduced RU interactions, enhancing the Ca^{2+} affinity of sTnC did not alter the strong crossbridge contribution to thin filament activation. Below we discuss these findings and how they give insight into cooperative thin filament activation and force development in skeletal muscle.

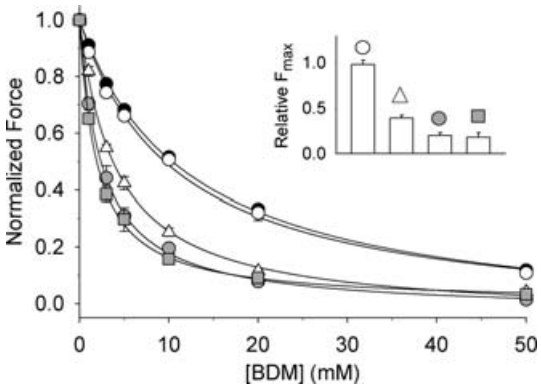

Figure 7. Inhibition of strong crossbridge formation with 2,3-butanedione monoxime (BDM)
Maximal force (pCa 4) was measured in the presence of increasing concentrations of BDM and with different reconstitution conditions of the thin filament to examine the effect of strongly bound crossbridges on thin filament activation level. F_{max} in the absence of BDM was normalized to 1.0 for each reconstitution condition for comparison. Thin filament conditions were pre-extracted native sTnC (●), 100% purified sTnC (control; ○), 40 : 60 sTnC: xxsTnC (△), 40 : 60 M80Q $sTnC^{F27W}$: xxsTnC (grey squares) and 20 : 80 sTnC: xxsTnC (grey circles). Inset shows reconstituted F_{max} for 100% purified sTnC (○; 0.98 ± 0.05; $n = 3$), 40 : 60 sTnC: xxsTnC (△; 0.39 ± 0.03; $n = 3$), 20 : 80 sTnC: xxsTnC (grey circles; 0.20 ± 0.03; $n = 3$) and 40 : 60 M80Q $sTnC^{F27W}$: xxsTnC (grey squares; 0.18 ± 0.05; $n = 3$). Curves were fit with the inhibition curve $F = F_{min} + (1 - F_{min}) \times (K_i/(K_i + [BDM]))$ to determine the asymptote of minimal force (F_{min}; where amplitude is $1 - F_{min}$) and the inhibition constant is K_i; (see text for values).

Ca^{2+}-binding properties of sTnC influence the Ca^{2+} sensitivity of force

The point mutations in sTnC were chosen based on previous work with chicken sTnC (Tikunova *et al.* 2002; Davis *et al.* 2004) but in this study species isoform (rabbit) was maintained between Tn subunits and other sarcomeric proteins in the fibre. The selected mutations at positions 80 and 27 created a graded decrease in Ca^{2+} k_{off} for isolated sTnC and these rates decreased in the presence of a TnI peptide ($TnI_{96\text{-}148}$), in agreement with previous work (Tikunova *et al.* 2002; Davis *et al.* 2004). Here we report k_{off} rates measured in whole sTn complex (Fig. 2 and Table 1) which agree well with those previously reported for sTnC in complex with either $TnI_{96\text{-}148}$ or whole TnI (Davis *et al.* 2004).

With incorporation of sTnC mutants into skinned rabbit psoas fibres via sTnC extraction and reconstitution, Ca^{2+} sensitivity of steady-state force (pCa_{50}) increased for each sTnC mutant (Fig. 4 and Table 2) demonstrating that reduction of k_{off} correlates with increased pCa_{50}. However, increasing the Ca^{2+}-binding properties of sTnC does not appear to enhance cooperative interactions of myofilament proteins in activation of the thin filament and force development. Indeed, cooperative mechanisms may actually be somewhat reduced for fibres containing these mutants with increased Ca^{2+}-binding affinity. One possibility is that communication between crossbridges and sTnC Ca^{2+} binding was disrupted, although this feedback has been shown to be minimal in skeletal muscle (Fuchs & Wang, 1991; Martyn & Gordon, 2001). Another possibility is that the structural change in the N-terminal domain of sTnC associated with decreased K_d (Table 1) results in a somewhat reduced affinity for sTnI in the presence of Ca^{2+}, as previously suggested for chicken M82Q $sTnC^{F29W}$ (Davis *et al.* 2004). This idea of altered TnC–TnI interaction is supported by a reduced F_{max} and n_H for fibres reconstituted with M80Q $sTnC^{F27W}$.

Ca^{2+}-binding properties of sTnC have little effect on near-neighbour RU interactions and the cooperativity of steady-state force generation

By reconstituting fibres with increasingly low fractional content of sTnC mutants (and high xxsTnC content) we were able to study the influence of sTnC mutants with altered Ca^{2+}-binding properties on the size and behaviour of increasingly isolated RUs in the sarcomere (Fig. 5). This procedure allows one to study the Ca^{2+}-activation properties in the absence of the dominant form of cooperativity in skeletal muscle (i.e. near-neighbour RU interactions). sTnC mutants could alter the Ca^{2+}-activation signalling process either by changing the sTnC–sTnI interaction (as stated above) or by reducing the functional unit (FU) size of regulatory units. Here the FU size is defined as the number of myosin binding sites on actin made available when Ca^{2+} binds to individual sTn complexes in the thin filament (also referred to as the spread of activation along the thin filament). We previously determined that the size of the FU is likely to be 10–12 actin monomers in skeletal muscle (Regnier *et al.* 2002). It is possible that mutant sTnCs could reduce this FU size, which would reduce n_H (the apparent cooperativity of force production). This was suggested previously by Moreno-Gonzalez *et al.* (2005) when skinned psoas fibres were reconstituted with either D28A sTnC (where Ca^{2+} does not bind to site I) or cardiac TnC to reduce the Ca^{2+} component of thin filament activation. In these fibres, n_H was significantly reduced. When these proteins were complexed with sTnI and sTnT to make whole Tn, k_{off} was significantly increased, suggesting a reduced Ca^{2+}-binding affinity (Moreno-Gonzalez *et al.* 2007).

In our current experiments, the manipulation of sTnC is essentially the opposite – an increase in the Ca^{2+} component of thin filament activation by increased Ca^{2+} affinity of sTnC (with decreased k_{off}). Because near-neighbour RU interactions play a dominant role in cooperative activation, we used our previous technique of reducing near-neighbour RU interactions (Regnier *et al.* 2002) to examine the effects of altered sTnC affinity within a local RU environment. We verified that reconstitution of thin filaments with progressively reduced mixture ratios of M80Q $sTnC^{F27W}$: xxsTnC shows a progressive decrease in n_H to a minimum ($n_H = 1.7$) at a force level of 0.2 F_{max} (Fig. 5*A* and Table 3). At maximal [Ca^{2+}], F_{max} data at different ratio mixtures (Fig. 5*C*) suggest that FU size may be maintained at more than seven actin monomers when values are corrected for unequal relative binding affinities of M80Q $sTnC^{F27W}$ *versus* xxsTnC (Fig. 5*B* and Appendix). These results suggest that (1) reduced n_H with mixtures of mutant M80Q $sTnC^{F27W}$: xxsTnC is caused primarily by loss of near-neighbour RU interactions and not reduction of FU size, and (2) comparison between mutants with reduced near-neighbour RU interactions should be made by matching F_{max} rather than matching protein mixture ratio with xxsTnC. Additionally, when 0.2 F_{max} was achieved for each mutant sTnC by reconstituting fibres with mixtures of xxsTnC and sTnC, M80Q sTnC or M80Q $sTnC^{F27W}$, RU interactions were reduced (low n_H), but there was no difference in n_H between mutants. This supports the idea that enhancing the Ca^{2+}-binding properties of sTnC in fibres with greatly reduced RU interactions does not alter n_H (Fig. 6 and Table 4) or increase FU size. Thus, we demonstrated that the Ca^{2+} sensitivity of force in skeletal muscle fibres can be increased by enhancing sTnC Ca^{2+} affinity, and large increases in Ca^{2+} affinity of sTnC can compromise the apparent cooperative activation of thin filaments.

This idea is further supported by our experiments where, during maximal Ca^{2+} activation, the relative influence of strong crossbridge binding in activating the thin filament was studied by force inhibition with increasing [BDM]. Minimizing near-neighbour RU interactions with sTnC: xxsTnC mixtures to 0.2 F_{max} allowed us to examine Ca^{2+}–crossbridge interactions within RUs along the thin filament. The sensitivity of F_{max} to BDM (i.e. $1/K_i$) was increased for both sTnC: xxsTnC and M80Q $sTnC^{F27W}$: xxsTnC mixtures to 0.2 F_{max}, but did not differ between them (Fig. 7). This suggests the enhanced Ca^{2+} binding of M80Q $sTnC^{F27W}$ did not help maintain thin filament activation as the number of strong crossbridges was reduced by BDM, and that native sTnC can fully activate individual RUs upon Ca^{2+} binding. Together these data suggest that (1) M80Q $sTnC^{F27W}$ does not alter the overall level of thin filament activation within individual RUs at saturating Ca^{2+}, and (2) the contribution of strong crossbridge binding to thin filament activation is not altered by enhanced Ca^{2+} binding with M80Q $sTnC^{F27W}$.

In summary, we have provided the first direct evidence that enhanced Ca^{2+} affinity of sTnC (via decreased k_{off}) activates the thin filament to set the Ca^{2+} sensitivity of steady-state force with no enhancement of the cooperativity of force generation. Therefore, the Ca^{2+}-binding properties of native sTnC may be optimal for providing near-neighbour RU interactions and subsequent strong crossbridge formation to cooperatively activate the thin filaments in skeletal muscle.

Appendix

Different reconstitution time constants ($1/b$) for the mutant sTnC proteins in a sTnC-extracted fibre (Fig. 5*B*) suggest different binding affinities to TnI–TnT in the fibre. Changes in steady-state affinity between sTnC with point mutations and the inhibitory fragment of TnI (residues 96–148) support this idea (Davis *et al.* 2004). These differences in affinity between Tn subunits may alter the ratio of proteins when reconstituted into thin filaments (compared with the protein solution mixture), as with a mixture of sTnC and xxsTnC. The following calculations characterize two proteins with different relative affinities binding into Tn complexes. Relative binding affinity (A_r) is defined as A_A/A_B, where A_A and A_B are the affinities of proteins A and B. Given a reconstitution mixture of protein A and protein B, the fraction of protein A (f_A) is [A]/[A] + [B]. This leads to a second-order binding rate to the thin filament for protein A (r_A) and B (r_B) as a function of f_A during reconstitution:

$$r_A(f_A) = A_A f_A, \text{ and } r_B(f_A) = A_B(1-f_A). \quad (2)$$

Normalizing for the ratio of these rates gives the reconstituted fraction of proteins A (p_A) or B (p_B) in the fibre:

$$p_A(f_A) = r_A(f_A)/(r_A(f_A) + r_B(f_A)) \quad (3)$$

with $p_A(f_A) + p_B(f_A) = 1$. We use the example of M80Q $sTnC^{F27W}$ (protein A) compared to xxsTnC (protein B), which was used for the data transformation in Fig. 5*C*. Data from Fig. 5*B* show a 3-fold slower binding of M80Q $sTnC^{F27W}$ into a sTnC-extracted fibre compared with sTnC. The similar binding affinity between sTnC and xxsTnC in a fibre (Regnier *et al.* 2002) suggests that M80Q $sTnC^{F27W}$ has a binding affinity one-third of that for xxsTnC, so that $A_r = 1/3$. The mixture ratio of A:B for $f_A = 0.20$, 0.40, 0.60 and 0.80 is rescaled to 0.08, 0.18, 0.33 and 0.57, respectively, for protein A incorporated into the thin filament. These values were used in the data transformation of Fig. 5*C*. Because this calculation was based on the probability of random binding events, it may better reflect what occurs as M80Q $sTnC^{F27W}$ and xxsTnC compete for binding sites in the thin filament.

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Influence of enhanced troponin C Ca2+-binding affinity on cooperative thin filament activation in rabbit skeletal muscle

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