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Thin-filament regulation of force redevelopment kinetics in rabbit skeletal muscle fibres

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Thin-filament regulation of isometric force redevelopment (k_{tr}) was examined in rabbit psoas fibres by substituting native TnC with either cardiac TnC (cTnC), a site I-inactive skeletal TnC mutant (xsTnC), or mixtures of native purified skeletal TnC (sTnC) and a site I- and II-inactive skeletal TnC mutant (xxsTnC). Reconstituted maximal Ca^{2+}-activated force (rF_{max}) decreased as the fraction of sTnC in sTnC : xxsTnC mixtures was reduced, but maximal k_{tr} was unaffected until rF_{max} was <0.2 of pre-extracted F_{max}. In contrast, reconstitution with cTnC or xsTnC reduced maximal k_{tr} to 0.48 and 0.44 of control ($P < 0.01$), respectively, with corresponding rF_{max} of 0.68 ± 0.03 and 0.25 ± 0.02 F_{max}. The k_{tr}–pCa relation of fibres containing sTnC : xxsTnC mixtures ($rF_{max} > 0.2$ F_{max}) was little effected, though k_{tr} was slightly elevated at low Ca^{2+} activation. The magnitude of the Ca^{2+}-dependent increase in k_{tr} was greatly reduced following cTnC or xsTnC reconstitution because k_{tr} at low levels of Ca^{2+} was elevated and maximal k_{tr} was reduced. Solution Ca^{2+} dissociation rates (k_{off}) from whole Tn complexes containing sTnC ($26 \pm 0.1\ s^{-1}$), cTnC ($38 \pm 0.9\ s^{-1}$) and xsTnC ($50 \pm 1.2\ s^{-1}$) correlated with k_{tr} at low Ca^{2+} levels and were inversely related to rF_{max}. At low Ca^{2+} activation, k_{tr} was similarly elevated in cTnC-reconstituted fibres with ATP or when cross-bridge cycling rate was increased with 2-deoxy-ATP. Our results and model simulations indicate little or no requirement for cooperative interactions between thin-filament regulatory units in modulating k_{tr} at any [Ca^{2+}] and suggest Ca^{2+} activation properties of individual troponin complexes may influence the apparent rate constant of cross-bridge detachment.

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Calcium regulates the cyclic actomyosin interactions responsible for isometric tension (force) development in mammalian striated muscle. Contraction is initiated when Ca^{2+} binds to the N-terminal domain of the troponin C (TnC) subunit of troponin (Tn), leading to movement of tropomyosin (Tm) over the surface of actin to a position that allows strong cross-bridge formation, force generation or sarcomere shortening (reviewed in (Gordon *et al.* 2000)). In both skeletal and cardiac demembranated muscle cells, there is a Ca^{2+} dependence of steady-state force and the rate of tension redevelopment (k_{tr}) following a sudden release and restretch (Gordon *et al.* 2000). k_{tr} is thought to mainly report the rate of cross-bridge transitions between detached or weakly attached states and force-generating states (Brenner & Eisenberg, 1986). While the number of cycling cross-bridges is the primary determinant of steady-state force, and Ca^{2+} regulates the apparent rates of transitions within the actomyosin cross-bridge cycle (Chalovich *et al.* 1981; Brenner, 1988), the mechanism by which Ca^{2+} controls k_{tr} in muscle fibres remains a subject of debate with important implications for striated muscle function.

In rabbit psoas fibres, the relationship between Ca^{2+}-activated steady-state force and k_{tr} is highly curvilinear at 10–15°C, such that k_{tr} is slow ($1–2\ s^{-1}$) and essentially constant at force levels <50% of maximal force (F_{max}), while the magnitude increases 10–15-fold as force increases to F_{max}. In maximally activated fibres, i.e. at saturating levels of Ca^{2+}, the intrinsic rate of cross-bridge cycling is the primary determinant of k_{tr} (Gordon *et al.* 2000). In contrast, we and others have shown that a Ca^{2+}-dependent process is rate-limiting to force development during submaximal thin-filament activation in skeletal fibres (Brenner, 1986, 1988; Sweeney & Stull,

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1990; Chase *et al.* 1994; Regnier *et al.* 1996, 1998*b*). We have previously shown that Ca^{2+} modulates k_{tr} primarily through binding to TnC (Chase *et al.* 1994), leading to the hypothesis that the dynamics of thin-filament activation limits k_{tr} at subsaturating levels of Ca^{2+} (Chase *et al.* 1994; Regnier *et al.* 1996, 1999*b*; Morris *et al.* 2001). In addition, the magnitude of the Ca^{2+}-dependent increase in cardiac muscle k_{tr} is less (only 2–4-fold) than in skeletal muscle, and we have provided evidence that even maximal k_{tr} is limited by thin-filament activation kinetics (Regnier *et al.* 2004). This kinetic limitation (for skeletal and cardiac muscle) likely involves not only the Ca^{2+}-binding properties of TnC (Regnier *et al.* 1996, 1999*b*), but also kinetic interactions of the other thin-filament regulatory subunits of Tn (Brenner & Chalovich, 1999) or Tm.

Additional influence of thin-filament dynamics on k_{tr} could come from cooperative spread of activation between adjacent thin-filament regulatory units (RUs = 1 troponin, 1 tropomyosin, 7 actins) and/or allosteric modulation of strong cross-bridge formation during Ca^{2+} activation (Gordon *et al.* 2001; Regnier *et al.* 2002). The relationship between $[Ca^{2+}]$ and steady-state isometric force in skeletal muscle fibres is highly cooperative, and much of this cooperativity is due to interactions between neighbouring RUs (Regnier *et al.* 2002). The spread of activation and strong cross-bridge formation from activated RUs (those with Ca^{2+} bound to TnC) to neighbouring inactive RUs (those without Ca^{2+} bound to TnC) probably occurs via head-to-tail interactions of the neighbouring Tm molecules, or alternatively could be through actin itself in regulated thin filaments. There is no conclusive evidence that cooperative mechanisms between RUs are required to explain the Ca^{2+} activation dependence of k_{tr}. However, there is some evidence to suggest that k_{tr} may reflect events within individual RUs. Partial extraction of sTnC from skeletal fibres, that reduced maximal forces to as low as 0.13 F_{max} resulted in little or no change in submaximal or maximal k_{tr} as a function of $[Ca^{2+}]$ (Metzger & Moss, 1991). Chase *et al.* (1994) varied the degree of thin-filament activation in a Ca^{2+}-independent manner by reconstituting fibres with mixtures of cTnC and a constitutively activated TnC (aTnC), and also found that k_{tr} is not dependent on the level of force. Quantitative assessment of cooperative mechanisms in these earlier studies was limited because removing some of the native sTnCs or reconstituting the thin filaments with cardiac TnC may substantially alter the complex interactions that occur among TnC, TnI, TnT, Tm and actin (Piroddi *et al.* 2003; Clemmens *et al.* 2005). In addition, to determine the role of cooperative mechanisms in the Ca^{2+} activation of k_{tr}, fibres should be activated by Ca^{2+} as in the normal activation process as opposed to the permanent activation with aTnC (Chase *et al.* 1994) or extraction of whole Tn (Metzger & Moss, 1991).

Mathematical models have also been used to investigate the possible role of cooperative mechanisms between RUs in the Ca^{2+} activation dependence of k_{tr}. Campbell, (1997) and Razumova *et al.* (2000) have suggested that cooperative RU interactions can be used to explain both the Ca^{2+} dependence of force and k_{tr}. However, Hancock *et al.* (1997), using a four-state model (Landesberg & Sideman, 1994) that coupled thin-filament states with a two-state cross-bridge cycle, showed that a non-linear k_{tr}–force relationship could be simulated without including any cooperative mechanisms.

Here we investigated the contribution of TnC Ca^{2+} binding properties and the subsequent spread of activation along thin filaments in determining force redevelopment kinetics of rabbit psoas fibres. TnC Ca^{2+}-binding properties were altered by replacing native TnC with cardiac TnC or a mutant sTnC (D28A) that does not bind Ca^{2+} at site I (xsTnC) (Moreno-Gonzalez *et al.* 2005). Cooperative spread of activation along thin filaments into near-neighbour RUs was reduced by replacing native TnC with different mixtures of purified native sTnC and a mutant sTnC (D28A, D64A) that does not bind Ca^{2+} at either N-terminal site (xxsTnC) (Regnier *et al.* 2002). Our results clearly demonstrate that reducing near-neighbour RU interactions greatly reduced F_{max}, and had no effect on maximal k_{tr} and little influence on the magnitude of the Ca^{2+}-dependent increase of k_{tr}. In contrast, cTnC or xsTnC in fibres reduced or eliminated the Ca^{2+} dependence of k_{tr} and this reduction was correlated with increased Ca^{2+} dissociation kinetics of Tn (k_{off}). Further experiments and modelling simulations suggest the Ca^{2+}-binding properties of Tn influence the kinetics of both cross-bridge attachment and detachment. Thus our results strongly suggest that k_{tr} is regulated at the level of individual RUs and that cooperative interactions between near-neighbour RUs have little influence on the magnitude of the Ca^{2+}-dependent increase in k_{tr}. Instead this increase appears to depend on the properties of TnC and its interactions with TnI or other proteins in the activation pathway. Preliminary reports of this work have been published previously (Regnier *et al.* 1999*a*; Moreno-Gonzalez *et al.* 2003).

Methods

Fibre preparation

Chemically skinned segments of single fibres from rabbit psoas muscle were prepared as previously described (Regnier *et al.* 2002). 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. The animals were killed with pentobarbital (120 mg kg^{-1})

administered through the marginal ear vein. All protocols involving animals were approved by the UW Animal Care Committee.

For mechanical experiments, fibre segments were isolated from bundles immediately prior to use, and were further treated with 1% Triton X-100 (v/v) in relaxing solution to remove membranous residue. The fibre segment ends were chemically fixed by focal application of 1% glutaraldehyde in H_2O to form artificial tendons that minimize end compliance (Chase & Kushmerick, 1988).

Experimental solutions

Compositions of relaxing and activating solutions for fibre experiments were calculated and solutions were made as previously described (Martyn *et al.* 1994; Regnier *et al.* 2002). Solutions were maintained at 0.17 M ionic strength and pH 7.0 at 15°C (experimental temperature), and contained (mM): 5 MgATP or 5 dATP, 15 phosphocreatine (PCr), 15 EGTA, at least 40 MOPS, 1 free Mg^{2+}, 135 $Na^+ + K^+$, 1 dithiothreitol (DTT), at least 250 u ml^{-1} creatine kinase (CK), 4% w/v Dextran T-500. Ca^{2+} levels (given as $pCa = -\log[Ca^{2+}]$) were established by varying the amount of $Ca(propionate)_2$.

Proteins

Native rabbit skeletal TnC, TnI, TnT were purified from rabbit skeletal back and leg muscles and native rat cTnC from rat hearts according to the method of Potter (1982) and Dong *et al.* (1996), respectively. The rabbit *sTnC* gene was cloned, and wild-type (WT) and mutants were expressed in *E. coli* as previously described (Regnier *et al.* 2002; Liang *et al.* 2003). Mutations were introduced at the x position of the low-affinity, N-terminal Ca^{2+}-binding sites I and II as previously described (Regnier *et al.* 2002; Moreno-Gonzalez *et al.* 2005). Site I alone is inactive (D28A) in xsTnC, whereas both site I (D28A) and II (D64A) are inactive in xxsTnC. The purity of all native and recombinant proteins was assessed by SDS-PAGE and protein concentration was determined by UV-absorbance spectroscopy. Purified TnT and TnI were complexed with different TnCs, according to Potter (1982) for stopped-flow measurements.

Mechanical data acquisition and analysis

Mechanical measurements were performed in a previously described apparatus (Regnier *et al.* 2002; Moreno-Gonzalez *et al.* 2005). In all experiments, sarcomere length (SL) was set initially to 2.5 μm using HeNe laser diffraction and adjusted to between 2.4 and 2.6 μm during activations. To maintain structural and functional integrity, fibres were periodically (every 5 s) unloaded by rapid (10 L_F s^{-1}) 15% release of total fibre segment length (L_F) for ~40 ms, followed by rapid restretch to the initial L_F (Brenner, 1983). Force, L_F and SL signals were digitized and analysed as previously described using custom data acquisition software (Chase *et al.* 1994; Regnier *et al.* 2002).

Steady-state force. Isometric force was measured during the steady-state period just prior to the release/restretch of a digitized k_{tr} record (see protocol below) (Sweeney *et al.* 1987; Chase *et al.* 1994; Martyn *et al.* 1994). Passive force was determined at pCa 9.0 and was subtracted from the total force at the various pCa levels to obtain the Ca^{2+}-activated force that is reported. Passive force following reconstitution did not differ from pre-extracted values under any of the experimental conditions (data not shown). The Ca^{2+}-activated force was normalized to fibre cross-sectional area, calculated from the diameter (58.5 ± 1.3 μm) assuming cylindrical geometry. F_{max} ($n = 57$) was 286.0 ± 11.5 mN mm^{-2} prior to extraction of endogenous sTnC. A small number of fibres included in this study exhibited ~15% rundown of F_{max}, although the great majority exhibited <10%; all yielded similar results.

Kinetics of isometric tension (force) redevelopment (k_{tr}). The rate of isometric tension redevelopment was determined from the half-time of force recovery after a rapid release–restretch transient as previously described (Chase *et al.* 1994; Regnier *et al.* 1996, 1998*b*). In brief, after the development of steady-state force, the fibre was shortened by 15% L_F with a 4 or 10 L_F s^{-1} ramp, which reduced force to zero, followed by a rapid (300 μs) under-damped restretch to the initial L_F. Force was reduced to baseline during shortening, transiently spiked with rapid restretch to L_F, then the subsequent force redevelopment kinetics were characterized by an apparent rate constant derived from the half-time of force recovery (Eqn 1) (Chase *et al.* 1994). k_{tr} traces where the force redevelopment started above 50% of steady-state force were not included in this study, to avoid over-estimation of the rate.

$$k_{tr} = \tau^{-1} = -\ln 0.5\left(t_{1/2}^{-1}\right) \quad (1)$$

Individual fibre force was normalized to either F_{max} (pre-extracted) or rF_{max} (reconstituted). k_{tr} was normalized to maximal k_{tr} from the same fibre prior to the extraction–reconstitution procedure when indicated. Values in all graphs (except Fig. 1) are shown as mean ± S.E.M. with some of the S.E.M. error bars being smaller than the symbols. Comparisons were made using paired t tests and are considered significant for $P < 0.01$.

Troponin C extraction and replacement

TnC was selectively extracted from fibres as previously described using trifluoperazine (TFP) (Regnier *et al.* 1999*b*, 2002; Moreno-Gonzalez *et al.* 2005). Fibres were placed in extracting solution for 30 s, followed by 10–15 s in relaxing solution (pCa 9.0), and this procedure was repeated in sets of five for an average of 9 min total in the extracting solution. Additional extraction was performed if Ca^{2+}-activated force at pCa 4.5 (F_{max}) was $\geq 2\%$ of pre-extracted F_{max}. Extracted fibres were washed extensively in relaxing solution to remove residual TFP.

Reconstitution of Tn complexes in TnC-extracted fibres with cTnC, xsTnC or mixtures of sTnC : xxsTnC was achieved by 1–3 min incubations in 1 mg ml^{-1} (total) TnC in pCa 9.2 without CK or Dextran, as previously described (Regnier *et al.* 2002; Moreno-Gonzalez *et al.* 2005). Reconstitution was considered complete when force at pCa 4.5 no longer increased (rF_{max}) with subsequent incubations. We previously reported that additional incubation with sTnC for 1–3 min following cTnC or xsTnC reconstitution does not increase F_{max}. This incubation time would normally completely reconstitute Tn complexes (Moreno-Gonzalez *et al.* 2005), thus suggesting that all troponin complexes were reconsituted. We have also previously demonstrated relatively equal binding affinities for sTnC and xxsTnC in the absence of Ca^{2+}, suggesting the procedure for reconstitution with sTnC : xxsTnC mixtures should yield a random distribution of these two TnCs along individual thin filaments throughout the entire fibre diameter. (Regnier *et al.* 2002). Reconstitution of extracted fibres with 100% sTnC results in restoration of F_{max} to >90% (often >95%) of the pre-extracted level, and reconstitution with 100% xxsTnC provides no active force even at pCa 4.0 (Regnier *et al.* 2002; Moreno-Gonzalez *et al.* 2005).

Ca^{2+} dissociation rates from troponin

The rate of Ca^{2+} dissociation (k_{off}) from TnC was measured at 15°C in whole Tn complexes using an Applied Photophysics Ltd. (Leatherhead, UK) model SX.18 MV stopped-flow instrument with a dead time of 1.4 ms as previously described for TnC by Tikunova *et al.* (2002), and modified slightly for whole Tn. Three Tn complexes were created: sTnC–sTnI–sTnT, xsTnC–sTnI–sTnT, and cTnC–sTnI–sTnT. The Ca^{2+} k_{off} was determined in a buffer containing (mM): 20 MOPS, 250 KCl, 1 DTT, 5 MgCl at pH 7.0. Ca^{2+} dissociation from Tn was quantified from an increase in fluorescence of the Ca^{2+} chelator Quin-2 (150 μM). Quin-2 was excited at 330 nm and fluorescence was monitored through a 510 nm broad band-pass interference filter (Tikunova *et al.* 2002). For N-terminal Ca^{2+}-dissociation rates, each k_{off} represents the mean of at least 15 averaged traces and was well fitted by a single exponential (r^2 = variance less than 1.0×10^{-4}). Values are reported as mean ± S.E.M. For TnC C-terminal Ca^{2+}-dissociation rates, representative traces were obtained at a slower timescale and were well fitted by a double exponential. All TnC C-terminal Ca^{2+} k_{off} rates were = 0.2 s^{-1}.

Results

Maximal k_{tr}

For each muscle fibre, steady-state force and k_{tr} were measured at varying levels of activating Ca^{2+} prior to extraction of endogenous sTnC and following replacement with either cTnC, xsTnC or mixtures of sTnC : xxsTnC. In all cases passive force (pCa 9.0) following reconstitution did not differ from pre-extracted values, indicating that thin-filament regulation was complete. Maximal steady-state force (F_{max}) and rate of force redevelopment (maximal k_{tr}) were compared for pCa 4.5 activations just prior to the extraction protocol and then following reconstitution. Figure 1 shows example pCa 4.5 force traces for fibres reconstituted with 100% cTnC (Fig. 1*A*) or 100% xsTnC (Fig. 1*B*). In both cases, maximal k_{tr} was ~15 s^{-1} prior to extraction of native TnC (control) and was reduced to ~6 s^{-1} following reconstitution with cTnC or xsTnC. However, the reconstituted F_{max} (rF_{max}) differed greatly for the fibre containing 100% cTnC (0.79 F_{max}) *versus* the fibre containing 100% xsTnC (0.23 F_{max}). To determine how reduction of near-neighbour RU interactions influence maximal k_{tr}, fibres were reconstituted with mixtures of sTnC : xxsTnC that produced similar rF_{max} as that obtained in the cTnC- or xsTnC-reconstituted fibres. Reconstituted fibres with sTnC : xxsTnC mixtures ratios produced rF_{max} that was greater than proportionality between force and sTnC content, as we have previously reported (Regnier *et al.* 2002). Figure 1*C* shows a fibre reconstituted with 60% sTnC : 40% xxsTnC (rF_{max} = 0.80 F_{max}) and Fig. 1*D* shows a fibre reconstituted with 5% sTnC : 95% xxsTnC (rF_{max} = 0.25 F_{max}). In these fibres, control maximal k_{tr} was ~16 s^{-1} but, in contrast to fibres with cTnC and xsTnC, maximal k_{tr} was not decreased even though rF_{max} was reduced.

These example maximal k_{tr} traces were representative of the measurements obtained for all fibres reconstituted with the various TnCs and sTnC : xxsTnC mixtures. The data for all experiments are summarized in Fig. 2, with sTnC : xxsTnC fibres grouped by force increments (see legend). Maximal k_{tr} stayed nearly constant as rF_{max} progressively decreased to ~0.10 F_{max} (0.13 ± 0.03) when fibres were reconstituted with decreasing fractions of functional sTnC (●). In fact only fibres with

$rF_{max} < 0.20\ F_{max}$ had a significantly reduced maximal k_{tr} compared with control (✚) or fibres reconstituted with 100% sTnC (&). In contrast, maximal k_{tr} was reduced by >50% for fibres reconstituted with 100% cTnC (□) or 100% xsTnC (▽). Interestingly, as in the example fibres in Fig. 1, maximal k_{tr} for fibres reconstituted with cTnC or xsTnC was the same even though rF_{max} in xsTnC-reconstituted fibres (0.25 ± 0.02, $n = 13$) was only ~37% of that in cTnC-reconstituted fibres (0.68 ± 0.03, $n = 9$). Figure 2 illustrates two salient points. First, maximal Ca^{2+}-activated k_{tr} has little or no dependence on the fraction of functional RUs or the level of steady-state force, i.e. maximal k_{tr} does not appear to depend on interactions between near-neighbouring RUs (Regnier *et al.* 2002). Second, while maximal k_{tr} does not depend on the level of isometric force *per se*, it is greatly influenced by the Ca^{2+}-binding properties of TnC, which also affect steady-state force (Moreno-Gonzalez *et al.* 2005).

Ca^{2+} dependent increases in k_{tr}

The dependence of k_{tr} on $[Ca^{2+}]$ in the bathing solutions (k_{tr}–pCa relationship) is summarized in Fig. 3. The steady-state force–pCa relationship is shown as fitted

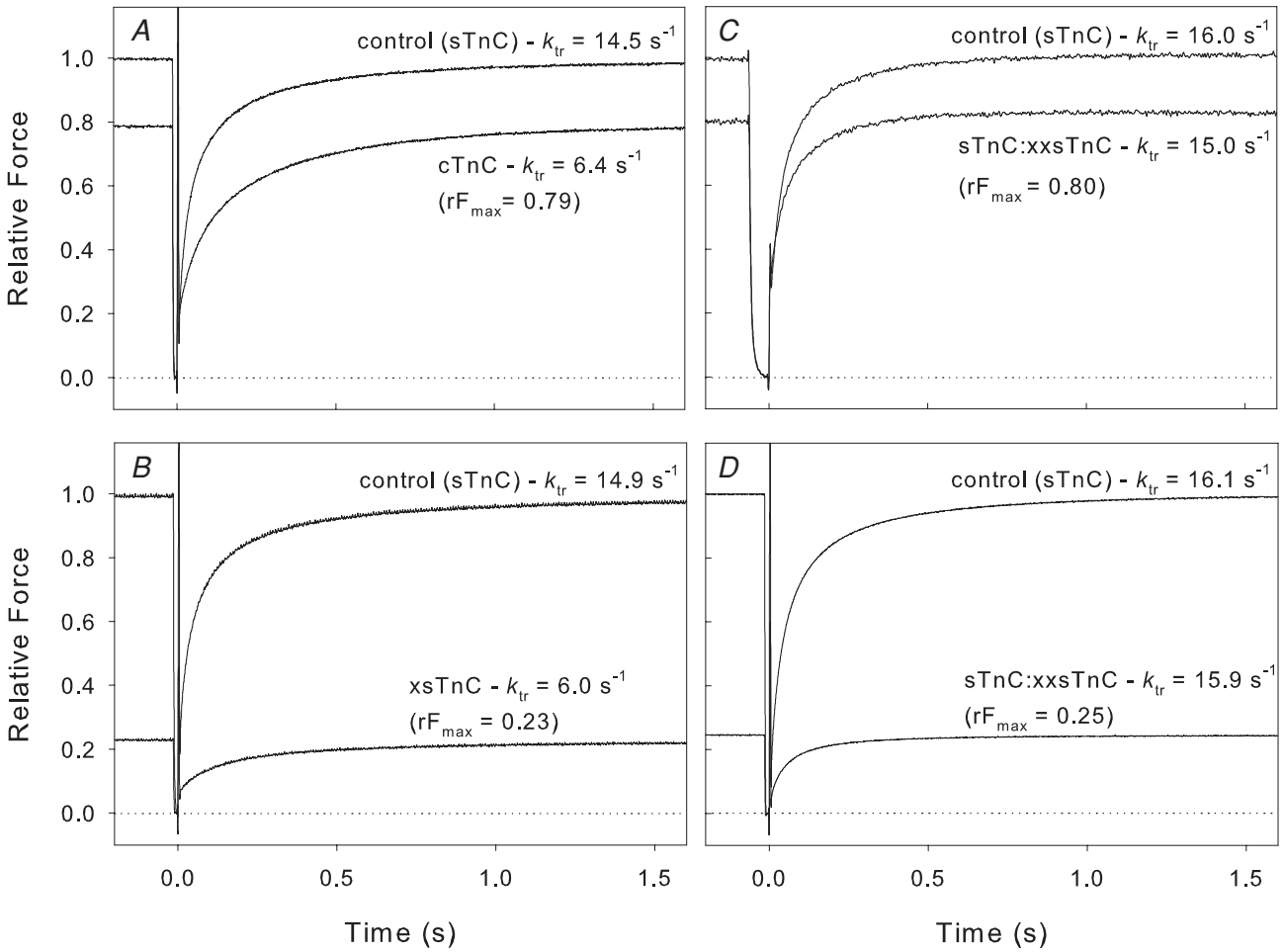


Figure 1. Kinetics of force redevelopment (k_{tr}) in single permeabilized rabbit psoas muscle fibres at saturating [Ca^{2+}] (pCa 4.5) before native sTnC extraction and after reconstitution with either cTnC (*A*) or mutant sTnCs (*B–D*)

Force records comparing k_{tr} in four different example fibres prior to extraction of endogenous TnC (control) and after reconstitution with 100% cTnC (*A*), 100% sTnC,D28A (xsTnC) (*B*), or mixtures of sTnC and sTnC,D28A,D64A (xxsTnC) (*C* and *D*). Force traces were normalized relative to maximal force under control conditions (F_{max}) for each fibre. Reconstituted F_{max} (rF_{max}) and k_{tr} for each trace are given next to the respective force record. Example fibres with similar reconstituted steady-state force levels were paired (*A–C* and *B–D*) to demonstrate that k_{tr} is very similar between control conditions and when fibres are reconstituted with varied mixtures of sTnC : xxsTnC, but not when they are reconstituted with either cTnC or xsTnC. Control force for each trace is as follows: 366.5 mN mm^{-2} (*A*), 429.6 mN mm^{-2} (*B*), 317.3 mN mm^{-2} (*C*), and 322.4 mN mm^{-2} (*D*).

lines for comparison (data reported in (Regnier *et al.* 2002 and Moreno-Gonzalez *et al.* 2005)). Figure 3*A* shows that prior to extraction of native TnC (control) the Ca^{2+} dependence of k_{tr}, defined as the magnitude increase in k_{tr} from the first measurable value to maximal Ca^{2+} activation, was ~10-fold as Ca^{2+} was varied. However, the Ca^{2+} dependence of k_{tr} varied only ~2-fold following reconstitution with 100% cTnC (Fig. 3*A*) and was eliminated in fibres reconstituted with 100% xsTnC (Fig. 3*B*). We report only the magnitude of Ca^{2+}-dependent increase in k_{tr} because it was so diminished in fibres containing cTnC or xsTnC that it was impossible to determine the Ca^{2+} sensitivity (pCa_{50}) of this rate. These reductions in the Ca^{2+} dependence of k_{tr} resulted from both a decreased maximal k_{tr} and an elevated k_{tr} at low levels of Ca^{2+}, demonstrating that the properties of Tn complexes can play a prominent role in determining k_{tr}.

To compare the magnitude of the Ca^{2+}-dependent increase in k_{tr} of fibres with cTnC or xsTnC *versus* those with reduced numbers of RUs, fibres were reconstituted with sTnC : xxsTnC mixtures that yielded

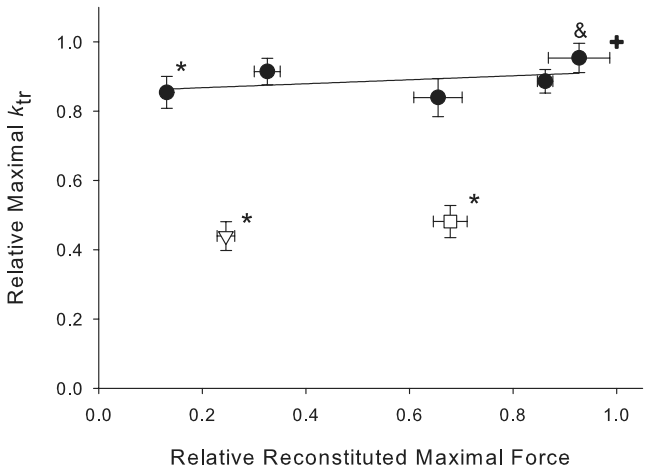


Figure 2. Relationship between maximal k_{tr} and reconstituted maximal isometric force (rF_{max}) (pCa 4.5) for fibres reconstituted with cTnC (□, 9 fibres), xsTnC (▽, 13 fibres) or sTnC : xxsTnC mixtures (●, 34 fibres)
Maximal k_{tr} and rF_{max} of TnC-reconstituted fibres were normalized to maximal k_{tr} and F_{max}, respectively, obtained in the same fibre prior to TnC extraction (control ✚, 56 fibres). Fibres reconstituted with various mixtures of sTnC and xxsTnC to give different rF_{max} levels were binned in 0.2 or 0.3 rF_{max} increments even when the proportions of sTnC (10–100%) and xxsTnC (0–90%) varied within some groups. Note that maximal k_{tr} does not depend on the level of reconstituted force but on the properties of TnC. Values are means ± S.E.M.; some error bars are smaller than the symbols. Data for sTnC : xxsTnC mixtures were fitted with a linear regression (solid line);. *$P < 0.01$ *versus* maximal k_{tr} under control conditions (✚). & Fibres reconstituted with 100% sTnC. Relative rF_{max} between any group (except for &) and control F_{max} (✚) is statistically significant ($P < 0.01$). Relative maximal k_{tr} values among sTnC : xxsTnC groups are not statistically significant. Relative maximal k_{tr} between cTnC and xsTnC is not statistically significant.

~0.70 F_{max} (Fig. 3*C*) and ~0.20 F_{max} (Fig. 3*D*). For these experiments, fibres reached F_{max} at ~pCa 5.5 under control conditions, even though k_{tr} was still rising. To minimize the time of Ca^{2+} activation and to maintain fibre integrity prior to extraction of native TnC, the preparation was then placed in a pCa 4.5 solution to obtain maximal k_{tr}. Following reconstitution, measurements were also made over the pCa range 5.5–5.0 because steady-state force continued to increase over this range of $[Ca^{2+}]$. Interestingly, the Ca^{2+}-dependent range of k_{tr} was minimally reduced for fibres with the different fractions of functional (sTnC-containing) *versus* non-functional (xxsTnC-containing) Tn. In fibres reconstituted with sTnC : xxsTnC mixtures that produced ~0.70 F_{max} (Fig. 3*C*; mixture ratios given in legend), the magnitude of the Ca^{2+}-dependent increase in k_{tr} was minimally reduced due to a small elevation at low levels of Ca^{2+}. This contrasts with the much larger reduction in cTnC-containing fibres (Fig. 3*A*) that produced a similar rF_{max}. For fibres reconstituted with sTnC : xxsTnC mixtures that produced ~0.20 F_{max} (Fig. 3*D*; mixture ratios given in legend), the Ca^{2+}-dependent range of k_{tr} values was reduced by ~40%, due to some elevation of k_{tr} at low levels of Ca^{2+}. This contrasts with the complete elimination of the Ca^{2+}-dependent range of k_{tr} values for fibres reconstituted with xsTnC (Fig. 3*B*), even though rF_{max} was similar. These results indicate that near-neighbour RU interactions are not the primary determinant of the Ca^{2+} dependence of k_{tr}. Instead the major determinant of both maximal k_{tr} and the Ca^{2+} dependence of k_{tr} appear to be the local thin-filament activation by Ca^{2+} and subsequent strong cross-bridge formation.

To determine if Ca^{2+}-dependent increases in k_{tr} resulted from differences in the number of cross-bridges available to participate in thin-filament activation and force generation, the data in Fig. 3 were replotted as k_{tr} *versus* steady-state force produced at each level of $[Ca^{2+}]$ (Fig. 4). This allows comparison of k_{tr} at similar levels of steady-state force, under different experimental conditions, independent of the $[Ca^{2+}]$. At force levels <0.20 F_{max}, fibres reconstituted with cTnC (Fig. 4*A*) or xsTnC (Fig. 4*B*) had similar elevated k_{tr}, which was constant within that range of forces. With cTnC-reconstituted fibres, k_{tr} increased with increasing force but remained elevated above control conditions over the range of forces from 0.20 to 0.70 F_{max}, as previously observed (Chase *et al.* 1994). In contrast, fibres with sTnC : xxsTnC mixtures that produced ~0.70 F_{max} (Fig. 4*C*) had similar or only slightly increased k_{tr} at steady-state force levels below 0.20 F_{max} compared with pre-extracted controls values, and k_{tr} was elevated at forces >0.20 F_{max}. In comparison, fibres with sTnC : xxsTnC mixtures that produced ~0.20 F_{max} (Fig. 4*D*) had slightly elevated k_{tr} at the lowest force, but there was a steep

increase towards maximal k_{tr} as force approached 0.20 F_{max} at maximal Ca^{2+} activation. However, k_{tr} remained constant over the range of forces <0.20 F_{max} under control conditions. This difference is probably due to the fact that obtaining similar forces required much more Ca^{2+} when fibres contained the sTnC : xxsTnC mixture. Combined, the data in Figs 3 and 4 suggest that k_{tr} at submaximal levels of Ca^{2+} depends much more on the Ca^{2+}-activation properties of Tn than on interactions between near-neighbouring RUs along the length of thin filaments.

N-terminal Ca^{2+} dissociation rates (k_{off}) of TnCs from whole Tn complexes

At submaximal levels of Ca^{2+} activation, k_{tr} is correlated with Ca^{2+} k_{off} measured from isolated TnCs (Johnson *et al.* 1994) for fibres reconstituted with TnC mutants (Regnier *et al.* 1999*b*) or under the effects of Ca^{2+}-sensitizing agents (Regnier *et al.* 1996). However, k_{off} of isolated TnCs (Johnson *et al.* 1994) is quite rapid and not indicative of the rates in whole Tn complexes (Table 1) or in skeletal muscle fibres. To more closely approximate fibre conditions,

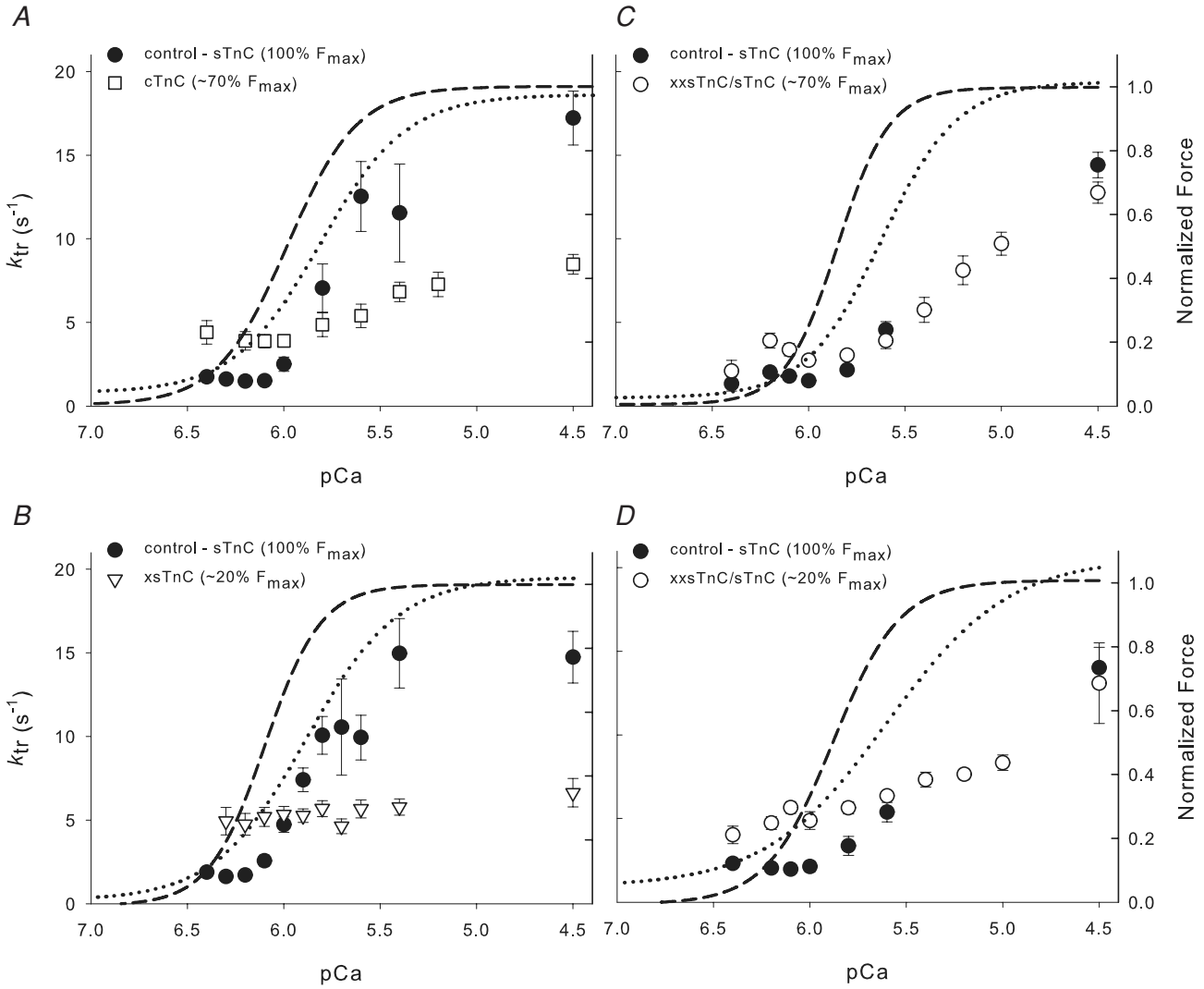


Figure 3. Ca^{2+} dependence of k_{tr}
Summary of k_{tr}–pCa data for fibres prior to native TnC extraction (●) and after reconstitution with cTnC (□) (*A*, 6 fibres), xsTnC (▽) (*B*, 10 fibres), or sTnC : xxsTnC mixtures (○) (*C*, 8 fibres and *D*, 13 fibres). Fibres reconstituted with mixtures of sTnC : xxsTnC were grouped according to rF_{max} (~0.70 F_{max} in panel *C* using 20 : 80 or 60 : 40 sTnC : xxsTnC mixtures, or ~0.20 F_{max} in *D* using 10–15% sTnC and 80–90% xxsTnC mixtures) to compare with fibres reconstituted with 100% cTnC or 100% xsTnC, respectively. Notice the substantial reduction in (*A*) or elimination of (*B*) the Ca^{2+} dependence of k_{tr} with cTnC or xsTnC. Values are means ± S.E.M.; some error bars are smaller than the symbols. Force–pCa curves previously reported (Regnier *et al.* 2002; Moreno-Gonzalez *et al.* 2005) are included for control (dashed lines) and experimental conditions (dotted lines) for visualization of the effect on pCa_{50} and Hill coefficient for steady-state isometric force.

we complexed recombinant cTnC, xsTnC, xxsTnC or native sTnC with purified native sTnI and sTnT for measurements of N-terminal Ca^{2+}-dissociation kinetics using stopped-flow spectrofluorimetry with Quin-2. Table 1 shows that Ca^{2+} k_{off} increased in the order sTnC–Tn < cTnC–Tn < xsTnC–Tn. As expected, we were unable to detect a signal associated with Ca^{2+} binding to N-terminal site I and II in Tn containing xxsTnC, suggesting Ca^{2+} binding was minimal or non-existent at these sites.

These results indicate an inverse correlation between fibre F_{max} and Tn Ca^{2+}-dissociation rates at the N-terminal (Fig. 2, Table 1), and suggest that increasing k_{off} may reduce the maximal attainable level of thin-filament activation. The faster k_{off} of xsTnC–Tn and cTnC–Tn was also associated with slower maximal k_{tr} and elevated k_{tr} at low [Ca^{2+}] (Fig. 3) and thus at low force levels (Fig. 4). Interestingly, there was an inverse correlation between Ca^{2+} k_{off} in solution (sTnC–Tn < cTnC–Tn < xsTnC–Tn) (Table 1), and the magnitude of Ca^{2+}-dependent increase in k_{tr} (Figs 3 and 4). If whole Tn solution kinetics mimic conditions found in fibres, this would suggest that the ~2-fold difference in Ca^{2+} k_{off} between sTnC–Tn and xsTnC–Tn may be enough to eliminate the Ca^{2+}-dependent increase of k_{tr}. Therefore, these data clearly suggest an important role for Ca^{2+}-dissociation kinetics of Tn in determining both steady-state force and the kinetics of force development.

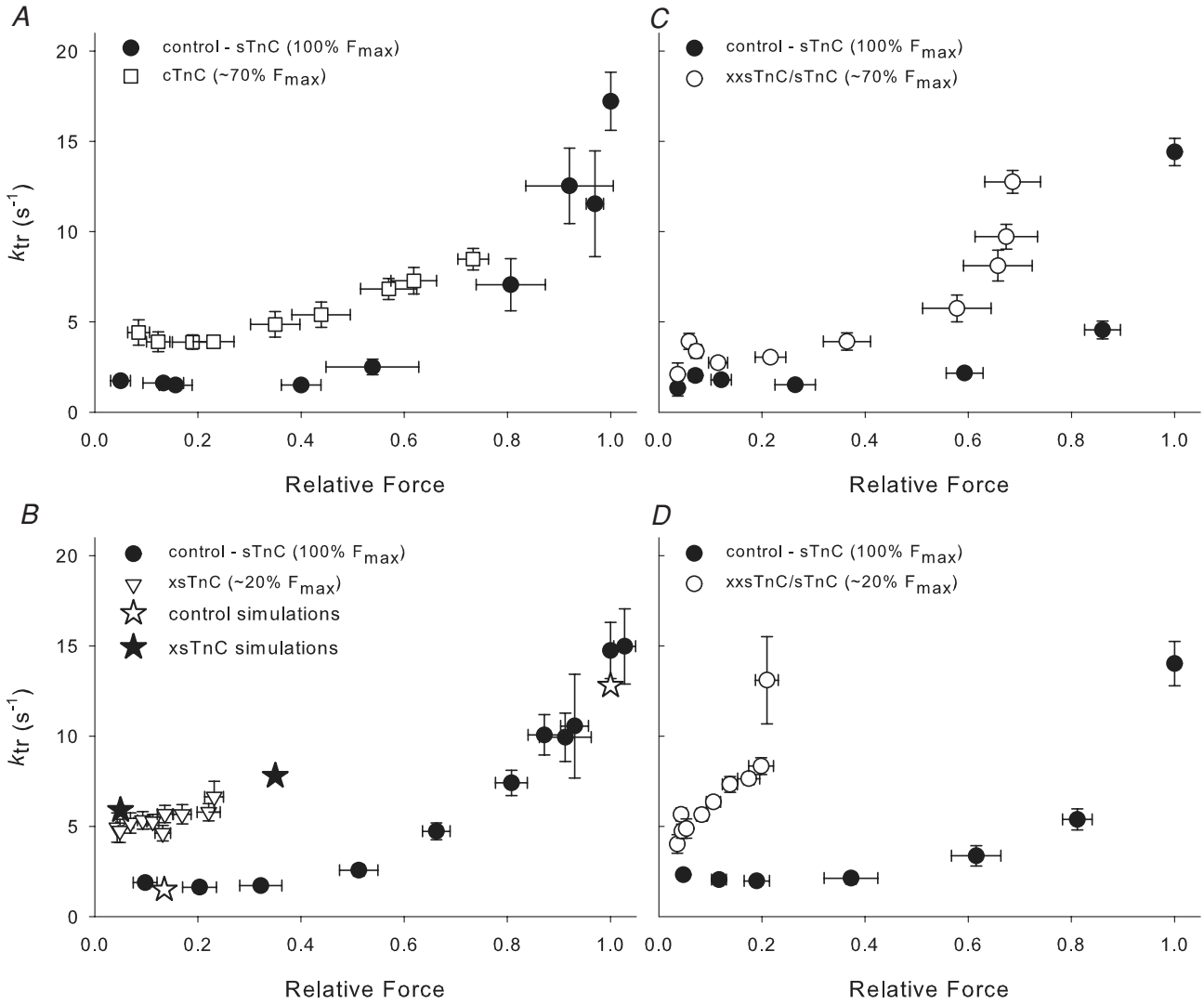


Figure 4. Relationship between k_{tr} and steady-state isometric force as pCa is varied
k_{tr} data from Fig. 3 were replotted against steady-state force, normalized relative to pre-extracted F_{max} (control ●), for fibres reconstituted with 100% cTnC (□) (*A*, $rF_{max} = 0.73 \pm 0.03$), 100% xsTnC (▽) (*B*, $rF_{max} = 0.23 \pm 0.02$), or sTnC : xxsTnC mixtures (○) (*C*, $rF_{max} = 0.69 \pm 0.05$ and *D*, $rF_{max} = 0.21 \pm 0.02$). Data were binned by pCa. Values are means ± S.E.M.; some error bars are smaller than the symbols. In *B*, k_{tr} simulation values (see Appendix) for control (☆) and xsTnC (★) conditions at low and high force are included for comparison with experimental data.

Table 1. N-terminal Ca^{2+} dissociation rates (k_{off}) of whole Tn complexes (sTnI–sTnT) with different TnCs

TnC protein	k_{off} (s^{-1})
sTnC	26 ± 0.1
cTnC	38 ± 0.9
xsTnC	50 ± 1.2
xxsTnC	Undetectable

Values from stopped-flow measurements using Quin-2 at 15°C. Each TnC was reconstituted with native sTnI and sTnT in solution. All values are given as mean ± S.E.M. All means are different from each other ($P < 0.05$).

Influence of increasing cross-bridge cycle rate on k_{tr}

In skeletal muscle fibres, k_{tr} is limited by the dynamics of thin-filament activation during submaximal levels of Ca^{2+} (Chase *et al.* 1994; Regnier *et al.* 1996, 1999*b*), while maximal k_{tr} is thought to be determined predominantly by the intrinsic rate of cross-bridge cycling (Brenner & Eisenberg, 1986). If reduction of maximal k_{tr} and elevation of k_{tr} at low levels of Ca^{2+} activation (force) result from the faster k_{off} for cTnC–Tn and xsTnC–Tn (Figs 3*A* and *B* and 4*A* and *B*, Table 1), increasing the rate of cross-bridge cycling should have little or no effect on the k_{tr}–force relationship except during maximal Ca^{2+} activation. To test this hypothesis we substituted 2-deoxy-ATP (dATP) for ATP. We have previously demonstrated that dATP increases (by 10–30%) the rate of actomyosin NTPase in solution, *in vitro* motility of actin filaments, and maximal k_{tr} and unloaded shortening (but not F_{max}) in skinned skeletal muscle fibres (Regnier & Homsher, 1998; Regnier *et al.* 1998*a*, 1997*b*; Clemmens & Regnier, 2004). Figure 5 summarizes the effect of dATP on maximal k_{tr}. As previously reported (Regnier *et al.* 1998*b*), dATP increased maximal k_{tr} by ~15% in control conditions. A similar increase was seen for fibres with cTnC but not for fibres with xsTnC, suggesting that the faster k_{off} of xsTnC–Tn (Table 1) may limit maximal k_{tr}.

In Fig. 6 the relation between k_{tr} and force as $[Ca^{2+}]$ was varied is plotted for control (●; pre-extraction) conditions and following reconstitution with cTnC in 5 mM ATP (□). The elevation of k_{tr} at low forces and reduction of the maximal value can be seen with cTnC, as in Fig. 4*A*. These fibres were then exposed to the same Ca^{2+} activating solutions, but with 5 mM dATP replacing ATP (△). Under this condition, there was an increase in the maximal k_{tr} as well as in the level of steady-state force attained at saturating $[Ca^{2+}]$ (compare left-most two *). In contrast, at submaximal Ca^{2+} activation, there was no change in k_{tr} at equivalent levels of steady-state force obtained with ATP. This implies that submaximal k_{tr} was not limited by cross-bridge kinetics because otherwise it should have been elevated by dATP. Combined, the results in Figs 5 and 6 support the hypothesis that elevation of k_{tr} at submaximal steady-state force levels results not from altered actomyosin cycling rates *per se*, but from rate-limiting Ca^{2+}-dependent interactions in thin-filament activation that may alter cross-bridge cycling.

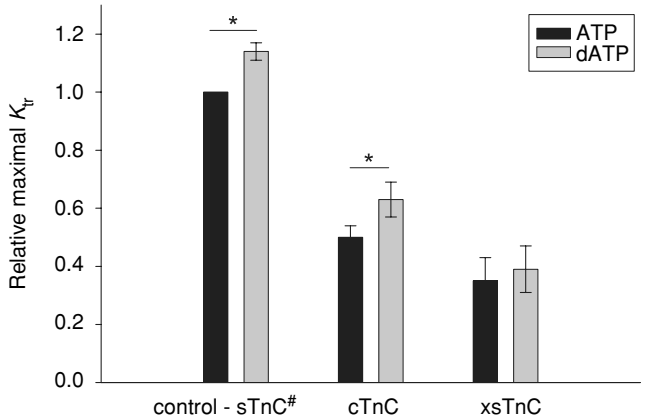


Figure 5. Effect of dATP on maximal k_{tr} for fibres reconstituted with cTnC (6 fibres) and xsTnC (3 fibres)
Maximal k_{tr} with 5 mM ATP (black bars) or 5 mM dATP (grey bars) as the contractile substrate was normalized to maximal k_{tr} obtained in the same fibre prior to TnC extraction (control – sTnC) with ATP. Values are means ± S.E.M. *$P < 0.01$ *versus* maximal k_{tr} with ATP. #Values from Regnier *et al.* (1998*b*). Note that dATP increases maximal k_{tr} under control conditions and in fibres reconstituted with 100% cTnC, but not with 100% xsTnC.

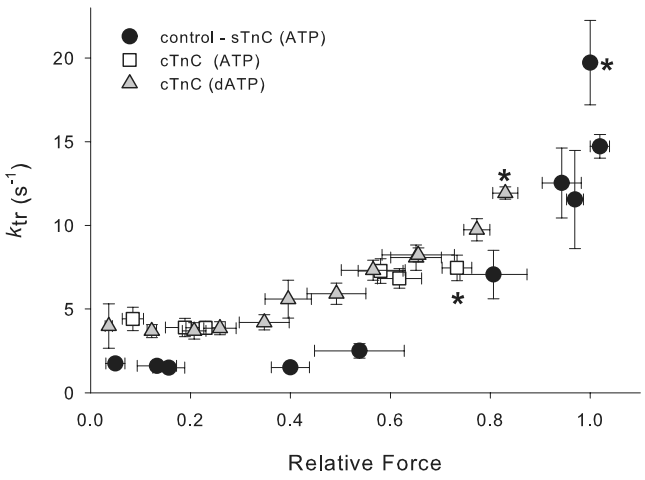


Figure 6. Effect of dATP on the relationship between k_{tr} and steady-state isometric force for fibres reconstituted with 100% cTnC (□, 6 fibres)
k_{tr} data from Fig. 4*A* were replotted for ATP conditions (control ● and cTnC □). In addition, the relationship between k_{tr} and force with dATP for those cTnC-reconstituted fibres (△) shows that dATP extends the curve beyond maximal values of force and k_{tr} with ATP at high levels of Ca^{2+} activation. *Maximal values under each condition. Data were binned by pCa. Values are means ± S.E.M.; some error bars are smaller than the symbols.

Discussion

The goal of this study was to investigate how Ca^{2+}-binding properties of individual Tn complexes and the interaction of RUs along thin filaments influence the rate of force redevelopment (k_{tr}) in rabbit psoas muscle fibres during isometric contraction. The main findings of this study are that maximal k_{tr} and the Ca^{2+} dependence of k_{tr}, defined as the magnitude increase in k_{tr} from the first measurable value to maximal Ca^{2+} activation: (1) do not depend on F_{max} or near-neighbour regulatory unit (RU) interactions; (2) are greatly influenced by the Ca^{2+}-activation properties of each Tn; and (3) are inversely correlated with the Ca^{2+}-dissociation kinetics (k_{off}) of whole Tn measured in solution. Below we discuss how these data may provide clues to the mechanism of thin-filament regulation of k_{tr} during Ca^{2+} activation in skeletal muscle.

Maximal k_{tr} and the Ca^{2+} dependence of k_{tr} are properties of individual RUs

We found that maximal k_{tr} was independent of F_{max} as the number of interacting RUs was progressively reduced (Fig. 2), and that the magnitude of Ca^{2+}-dependent increase in k_{tr} was minimally altered (Figs 3*C* and *D* and 4*C* and *D*). These observations indicate that k_{tr} is determined primarily by thin–thick-filament protein interactions within the confines of individual RUs, and that interactions between RUs have little influence on k_{tr} in skinned skeletal muscle fibres. This contrasts the strong influence of near-neighbour RU interactions on setting the level of Ca^{2+}-activated steady-state isometric force (Regnier *et al.* 2002). Comparable results have been obtained by others. Metzger & Moss (1991) found that partial extraction of sTnC from skinned rabbit psoas fibres had little effect on maximal k_{tr} and k_{tr} at low levels of Ca^{2+} activation. However, these studies were not definitive because regions of thin filaments with incomplete Tn complexes could have altered the interactions that occur between TnI, TnT, Tm and actin, potentially affecting the spread of activation along thin filaments. An alternative approach was used by Morris *et al.* (2001), who extracted native sTnC and reconstituted Tn complexes with mixtures of cTnC and a cTnC mutant that does not bind Ca^{2+} at N-terminal site II (CBMII). This approach is similar to our experiments in which fibres were reconstituted with mixtures of sTnC : xxsTnC (Figs 1*C* and *D*, 2, 3*C* and *D* and 4*C* and *D*), with an important difference being the type of functional TnC used, i.e. cardiac TnC *versus* skeletal TnC. In their study using cTnC : CBMII mixtures, maximal k_{tr} was not affected by the fractional content of cTnC. However, maximal k_{tr} and the Ca^{2+} dependence of k_{tr} in fibres reconstituted with 100% cTnC were diminished from pre-extracted values, similar to what we observed (Figs 2, 3*A* and 4*A*).

The length of thin filament activated by Ca^{2+} binding to each Tn may also be diminished by replacement of native sTnC with cTnC and xsTnC. In a previous study (Regnier *et al.* 2002), we estimated this length to be 10–12 actin monomers for skeletal fibres with sTnC. If this length is less in fibres with cTnC or xsTnC, the interaction between neighbouring RUs may be reduced because fewer actins are available for myosin binding when Ca^{2+} binds to one Tn. This is suggested by a large decrease in the Hill coefficient (n_H) and reduced Ca^{2+} sensitivity of the force–pCa relationship seen when these proteins replace native sTnC (dotted lines in Fig. 3*A* and *B*) (Moreno-Gonzalez *et al.* 2005). Additionally, we find that the length of thin filament activated by Ca^{2+} binding to Tn is smaller in cardiac muscle (containing cTnC) than in skeletal muscle (unpublished observations). Thus, our data and other studies support the hypothesis that maximal k_{tr} and the Ca^{2+} dependence of k_{tr} are primarily determined by the properties of the Tn complex at the level of individual functional RUs, with no observable dependence on F_{max} and little or no influence of near-neighbour RU interactions along thin filaments.

Factors that regulate k_{tr} within individual RUs

The rate that force develops is dependent on both myosin and thin-filament properties (Gordon *et al.* 2000). Maximal Ca^{2+}-activated k_{tr} in skinned skeletal fibres is primarily determined by the intrinsic rate of actomyosin cross-bridge cycling, when the contribution of thin-filament dynamics should be least limiting (Brenner, 1986; Metzger & Moss, 1990). Brenner & Eisenberg (1986) proposed that k_{tr} reflects the sum of the forward (f_{app}) and reverse (g_{app}) rates of cross-bridge transitions from weak-binding to strong-binding, force-generating states. They also proposed that the steep relationship between k_{tr} and Ca^{2+}-activated force (as seen in Fig. 4) reflected a Ca^{2+} dependence of f_{app}. However, we and others have shown that this model is too simplistic to predict results from experiments when either cross-bridge cycling rate or thin-filament activation are altered independently of [Ca^{2+}] (Chase *et al.* 1994; Regnier *et al.* 1998*b*, 1999*b*; Fitzsimons *et al.* 2001). These experiments led us to conclude that k_{tr} is controlled by a complex kinetic interaction between cross-bridge cycling and Ca^{2+}-dependent thin-filament dynamics. This idea is further supported by our present experiments showing that cTnC and xsTnC in skeletal fibres can reduce or eliminate the magnitude of Ca^{2+}-dependent increase in k_{tr} (Figs 3*A* and *B* and 4*A* and *B*).

The Ca^{2+} dependence of k_{tr} within RUs could occur via regulation of thin-filament activation kinetics and/or thin-filament effects on cross-bridge-binding and -cycling kinetics. The Ca^{2+}-binding kinetics of TnC are probably

not rate limiting *per se*, at least in skeletal muscle (Brenner & Chalovich, 1999). Ca^{2+} binding to TnC is rapid and close to diffusion limited in the Tn complex or in regulated thin filaments. However, the transmission of the Ca^{2+}-binding signal through the interaction of TnC with TnI, and the subsequent movement of Tm could limit thin-filament activation kinetics. This is supported by the much slower apparent kinetics of TnC Ca^{2+} binding when complexed in Tn, compared to isolated TnC (Dong *et al.* 1996, 1997*a*, 1997*b*). In the current study, when native skeletal TnC was replaced with either cTnC or xsTnC, the interaction between TnC and TnI may well have been affected. Both of these TnCs have a single N-terminal Ca^{2+}-binding site. Ca^{2+} binding to cTnC results in much less hydrophobic patch exposure for interaction with TnI than found for the skeletal isoform (Gagne *et al.* 1995; Li *et al.* 1999). In the presence of Ca^{2+}, weaker interaction between TnC and TnI could shift cTnI or sTnI binding towards increased interaction with actin, thereby decreasing thin-filament activation. It could also increase the probability of de-activation if cross-bridge binding is not sufficient to maintain the activated state. The faster Ca^{2+} k_{off} from cTnC–Tn and xsTnC–Tn in solution (Table 1) and the correlated decrease in rF_{max} and k_{tr} (Fig. 2) support this idea. The inverse correlation between rF_{max} and k_{off} suggests that the ability of Tn to activate thin filaments is in the order of fibres containing sTnC > cTnC > xsTnC. This provides an explanation for the lower maximal k_{tr} seen with cTnC- or xsTnC-reconstituted fibres (Fig. 2). It does not, however, explain why maximal Ca^{2+}-activated k_{tr} is similar for cTnC- *versus* xsTnC-reconstituted fibres, while rF_{max} with xsTnC-reconstituted fibres is only one-third of that with cTnC (Fig. 2). This cannot be explained by a difference in the extent of Tn complex reconstitution for xsTnC *versus* cTnC, as our control measurements demonstrated that most or all of the Tn complexes are complete (see Methods). Additionally, it is not readily apparent how increased k_{off} can explain the elevation of k_{tr} (compared with control) at low levels of $[Ca^{2+}]$ (Fig. 3) and at similar levels of Ca^{2+}-activated force (Fig. 4). The potential coupling between Ca^{2+} k_{off} and g_{app} as a possible explanation for these issues is discussed below and in the Appendix.

In addition to determining the dynamics of Ca^{2+}-induced changes in thin-filament protein interactions, thin-filament regulatory proteins may more directly affect cross-bridge cycling. We (Gordon *et al.* 1997; Kohler *et al.* 2003; Clemmens & Regnier, 2004) and others (Homsher *et al.* 1996; 2000) have demonstrated that regulatory proteins increase actomyosin kinetics in the *in vitro* motility assay, and this is best explained by thin-filament regulatory proteins increasing cross-bridge detachment rate (g_{app}). Microneedle force measurements in the *in vitro* motility assay suggest that regulatory proteins may also affect f_{app} (Clemmens & Regnier, 2004). In addition, there is growing evidence that alterations in the molecular structure of regulatory proteins influence cross-bridge cycling and force production in muscle fibres (Regnier *et al.* 1999*b*; Homsher *et al.* 2000; Morris *et al.* 2001; Piroddi *et al.* 2003; Chandra *et al.* 2005; Hernandez *et al.* 2005; Kruger *et al.* 2005; Moreno-Gonzalez *et al.* 2005). As such, our data lead us to hypothesize that a faster Ca^{2+} k_{off} from Tn may somehow result in an increased g_{app} in skeletal muscle fibres, especially at low levels of Ca^{2+} activation.

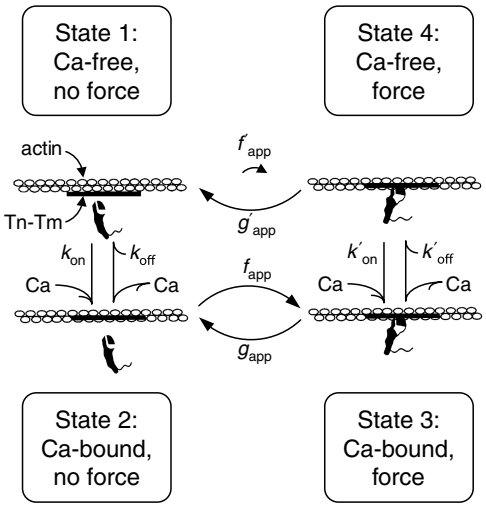
We (Regnier *et al.* 1996) previously reported that the Ca^{2+}-sensitizing compound calmidazolium (CDZ), which reduces Ca^{2+} k_{off} from isolated TnC (Johnson *et al.* 1994), increases k_{tr} at submaximum levels of Ca^{2+} activation. In contrast, here we show even greater elevation of sub-maximal k_{tr} in fibres reconstituted with either cTnC or xsTnC (Fig. 4*A* and *B*), both of which increased (rather than reduced) Ca^{2+} k_{off} when measured in whole Tn complexes containing sTnI and sTnT (Table 1). In the model by Hancock *et al.* (1997), slower k_{off} elevated k_{tr} at submaximal $[Ca^{2+}]$, as with CDZ. In the Hancock study, however, k_{off} was based on solution measurements using isolated TnC (Johnson *et al.* 1994). In contrast, here we measured k_{off} for Ca^{2+} bound to whole Tn. We propose that skeletal Tn containing cTnC or xsTnC has a lower apparent affinity for the switch region of TnI in the presence of Ca^{2+}, thereby favouring TnI binding to actin and reducing thin-filament activation. Since TnI and cross-bridges compete for binding sites on thin filaments, this competition may increase the apparent rate of cross-bridge detachment (g_{app}), thereby increasing k_{tr} ($f_{app} + g_{app}$) (Brenner, 1986; Brenner & Eisenberg, 1986). This is a reasonable explanation because in the absence of Ca^{2+}, the TnI–actin interaction is strong enough to prevent cross-bridge transition from weak to strong, force-generating states. In contrast, slowed k_{off} of sTnC by CDZ should shift TnI binding towards TnC within an RU, elevating thin-filament activation at submaximal $[Ca^{2+}]$ by prolonging exposure of cross-bridge-binding sites on actin and increasing submaximal k_{tr}. In this case, faster k_{tr} would result from increased f_{app}.

In conclusion, our experimental data demonstrate that loss of near-neighbour RU interactions does not greatly affect maximal k_{tr} or the magnitude of Ca^{2+}-dependent increase in k_{tr} (Figs 2, 3 and 4). Instead, k_{tr} is strongly influenced by the Ca^{2+}-binding properties of individual thin-filament RUs, without the involvement of cooperative mechanisms between RUs. In contrast, steady-state isometric force depends strongly on near-neighbour RU cooperative interactions (Regnier *et al.* 2002; Moreno-Gonzalez *et al.* 2005). Taken together our results indicate that in skinned skeletal fibres, k_{tr} is determined by a balance of actin-binding site availability, the kinetics of cycling cross-bridges, and the influence of

regulatory proteins on the apparent rate constants in the cross-bridge cycle.

Appendix

To help understand the influence of regulatory protein dynamics on the rate of force development, we have used a four-state model, originally proposed by Landesberg & Sideman (1994) and modified by Hancock *et al.* (1997). This model is based on the coupling of thin-filament activation and cross-bridge cycling kinetics. Scheme 1



depicts an individual thin-filament RU and the associated cross-bridges. Its features have been described in detail elsewhere (Hancock *et al.* 1997; Regnier *et al.* 1998*b*; 1999*b*) and therefore, will only be described briefly here. In this scheme the apparent rate constants (f_{app} and g_{app}) that control cross-bridge entry into and out of force-generating states (States 2 and 3) are coupled with a thin-filament activation component that is controlled by Ca^{2+} binding to (k_{on}) and dissociation from (k_{off}) Tn. State $1 \leftrightarrow 2$ is a rapid equilibrium between detached and weak cross-bridge attachment. Transitions between states $3 \leftrightarrow 4$ and $4 \leftrightarrow 1$ are necessary because Ca^{2+} can dissociate from Tn while cross-bridges are in attached, force-bearing conformations. While this model greatly simplifies cross-bridge chemo-mechanical transitions, it is sufficient to explain the k_{tr} *versus* force relationship for conditions where the kinetics of Ca^{2+} binding to TnC (Chase *et al.* 1994; Regnier *et al.* 1996; 1999*b*) or cross-bridge cycling (Regnier *et al.* 1998*b*) are varied independently.

To simulate control steady-state force and k_{tr} at 15°C, the apparent rate constants regulating cross-bridge cycling (f_{app}, f'_{app}, g_{app} and g'_{app}) were set to generate a maximal k_{tr} of $\sim 13\ s^{-1}$ (controlled by $f_{app} + g_{app}$) and a minimal k_{tr} of $1.5\ s^{-1}$ (controlled by g_{app}) (as in Fig. 4). To simulate varying $[Ca^{2+}]$ we initially assumed that Ca^{2+} affects only thin-filament activation kinetics, and values for k_{on} and k_{off} were selected in the following manner. A wide range of k_{off} values could be used to simulate the control k_{tr} *versus* force relationship, as k_{on} was varied to simulate changing $[Ca^{2+}]$. Even though the k_{off} value obtained for sTn in solution (Table 1) was within this range, we selected a somewhat larger value ($50–100\ s^{-1}$). This allowed us to simulate the k_{tr} *versus* force relationship of fibres reconstituted with cTnC or xsTnC by increasing k_{off} in a proportional amount as indicated from our stopped-flow measurements (Table 1). An apparent second-order binding constant of Tn for Ca^{2+} was calculated ($\sim 10^7\ M^{-1}s^{-1}$) that is within the range reported by Rosenfeld & Taylor (1985) for thin filaments in solution, and this allowed us to simulate a k_{tr} *versus* force relationship. Simulation values for k_{tr} at low and high force are shown in Fig. 4*B* (★ control, ☆ xsTnC).

To simulate results from skeletal muscle fibres containing cTnC and xsTnC, g_{app} was increased from $1.5\ s^{-1}$ to $3–4\ s^{-1}$ and Ca^{2+} k_{off} was increased 2-fold. Using these values, simulated F_{max} was reduced by ~30% and maximal k_{tr} was reduced by ~50%, similar to levels seen for cTnC-reconstituted fibres (Fig. 2). In addition, simulations of k_{tr} for low $[Ca^{2+}]$ were increased ~2-fold, as we also observed in cTnC-reconstituted fibres (Fig. 4*A*). Further increasing Ca^{2+} k_{off} (4-fold) and increasing g_{app} to $6\ s^{-1}$ reduced F_{max} down to 25% of control F_{max}. Interestingly, these simulations had little further effect on maximal k_{tr} (~10% reduction) but almost completely eliminated the Ca^{2+} dependence of k_{tr}, reproducing our results of xsTnC-reconstituted fibres (Fig. 4*B*). No combination of altering k_{on} and k_{off} (without increasing g_{app}) was able to simulate elevated k_{tr} at low force (Ca^{2+}) levels. Additionally, no combination of altering f_{app} and g_{app} (without changing Ca^{2+} k_{on} and/or k_{off}) was able to simulate a similar maximal Ca^{2+}-activated k_{tr} with a greatly different F_{max}, as observed in fibres reconstituted with cTnC *versus* xsTnC (Fig. 2). Thus, we conclude that the simplest explanation of our data is that faster Ca^{2+} k_{off} with cTnC and xsTnC, compared with sTnC (Table 1), is coupled to an increased rate of cross-bridge detachment (g_{app}). This apparent coupling reduces or eliminates the Ca^{2+} dependence of k_{tr}, as observed in Figs 3 and 4. Using this model we also tested whether, when Ca^{2+} k_{off} is increased (to simulate cTnC- or xsTnC-reconstituted fibres), increases in $f_{app} + g_{app}$ alter k_{tr} at low levels of Ca^{2+} activation. They did not, in agreement with our results when cross-bridge cycling rate was increased with dATP (Fig. 6).

As mentioned above, Scheme 1 describes a simple coupled relationship between activation of the thin filament and cross-bridge cycling. As such, we have not incorporated any cooperative mechanisms of Ca^{2+} or cross-bridge binding on the thin-filament activation steps.

Thus we cannot model the cooperativity observed in the relationship between steady-state force and pCa. Campbell *et al.* (1997) and Razumova *et al.* (2000) have suggested from modelling studies that cooperative interactions between near-neighbour RUs can be used to explain both the Ca^{2+} dependence of force and k_{tr}. However, using the simple model depicted in Scheme 1, we were able to predict the k_{tr} *versus* force relationship as $[Ca^{2+}]$ is varied (Fig. 4).

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Thin-filament regulation of force redevelopment kinetics in rabbit skeletal muscle fibres

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