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Contractile effects of the exchange of cardiac troponin for fast skeletal troponin in rabbit psoas single myofibrils

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The effects of the removal of fast skeletal troponin C (fsTnC) and its replacement by cardiac troponin C (cTnC) and the exchange of fast skeletal troponin (fsTn) for cardiac troponin (cTn) were measured in rabbit fast skeletal myofibrils. Electrophoretic analysis of myofibril suspensions indicated that replacement of fsTnC or exchange of fsTn with cTnC or cTn was about 90 % complete in the protocols used. Mechanical measurements in single myofibrils, which were maximally activated by fast solution switching, showed that replacement of fsTnC with cTnC reduced the isometric tension, the rate of tension rise following a step increase in Ca^{2+} (k_{ACT}), and the rate of tension redevelopment following a quick release and restretch (k_{TR}), but had no effect on the kinetics of the fall in tension when the concentration of inorganic phosphate (P_i) was abruptly increased ($k_{P_i(+)}$). These data suggest that the chimeric protein produced by cTnC replacement in fsTn alters those steps controlling the weak-to-strong crossbridge attachment transition. Inefficient signalling within the chimeric troponin may cause these changes. However, replacement of fsTn by cTn had no effect on maximal isometric tension, k_{ACT} or k_{TR}, suggesting that these mechanics are largely determined by the isoform of the myosin molecule. Replacement of fsTn by cTn, on the other hand, shifted the pCa_{50} of the pCa–tension relationship from 5.70 to 6.44 and reduced the Hill coefficient from 3.3 to 1.4, suggesting that regulatory protein isoforms primarily alter Ca^{2+} sensitivity and the cooperativity of the force-generating mechanism.

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Skinned muscle fibres have been an important tool for the elucidation of the mechanisms of contraction and its regulation. This preparation permits control of the fibre lattice solution, while maintaining the relationship between the thick and thin filaments, during measurement of muscle contraction in a mechanically coupled system. Thus, extrapolation of crossbridge mechanisms to the living system can be made with confidence. Finally, one can alter the protein structure of the fibre to probe the function of the specific proteins in the contractile process. Nevertheless, single fibres do have limitations. First, even with ATP regeneration systems, gradients exist within the fibre for [ATP], [ADP] and the concentration of inorganic phosphate ($[P_i]$) arising from diffusion distances of > 25 μm that can alter mechanical behaviour. Working at temperatures greater than 10–15 °C exacerbates these gradients so that studies performed at greater temperatures are problematic. Furthermore, the long diffusion distances militate against the application of chemical transients to evaluate the kinetic behaviour of the system. While the use of photolabile caged compounds may circumvent this latter problem, they have inherent limitations with respect to the input of large radiant energies and the production of reactive photolytic by-products. Finally, the exchange of contractile proteins in fibres with exogenous proteins requires long incubation times (> 6 h; Kraft *et al.* 1995; Brenner *et al.* 1999).

The isolated single myofibril has the potential to eliminate these problems because the diffusion distances are < 2 μm, and by flowing streams of solution over the myofibril, the myofilament lattice can be effectively clamped at any desired composition. Furthermore, by rapidly moving micropipettes carrying the flow of different solutions, complete changes in the lattice solution can be made in < 10 ms (Tesi *et al.* 1999, 2000, 2002*a*,*b*). Finally, the smaller diffusion distances allow the exchange of proteins to be completed within minutes instead of hours.

Recent experiments in which more than 90 % of fast skeletal muscle troponin C (fsTnC) was removed and replaced by bovine ventricular cardiac troponin C (cTnC) in psoas muscle fibres produced a number of mechanical changes (Morris *et al.* 2001, 2003). These changes included a reduction of the maximal isometric tension and the maximal rate of tension redevelopment (k_{TR}) to 65 % of the values achieved in the presence of fsTnC, and a

reduction of the pCa_{50} and Hill coefficient (n_H) by 0.1 pCa units and 0.9 units, respectively. However, the unloaded shortening velocity was unaffected by the TnC exchange (Morris *et al.* 2003). The isometric tension and k_{TR} changes were explained by assuming that the presence of cTnC reduced the rate of crossbridge transition from a weakly to a strongly bound (but non-force-generating) form. The results were consistent with no change in the power stroke or the rate of ADP release from the cycling crossbridges. It was hypothesized that the modification of strong crossbridge binding was a consequence of a less effective signalling of the cTnC Ca^{2+} binding to the fsTnI and fsTnT subunits and the subsequent positioning of tropomyosin on the thin filament (Morris *et al.* 2001, 2003).

The exchange of whole troponin (Tn) allows one to exchange whole cTn for fast skeletal troponin (fsTn) and test whether the slowed k_{TR} and reduced tension in the presence of cTnC is a property of the cTn molecule itself or it involves altered communication between Tn subunits. The quantitative exchange of Tn in skeletal muscle fibres can be made by incubation of the contractile lattice in a 4–20 μM Tn solution (Brenner *et al.* 1999). While the complete exchange in single fibres requires 6–15 h, exchange in myofibrils is complete within 60 min (She *et al.* 2000). Furthermore, force transients that occur following abrupt increases in $[P_i]$, which are thought to monitor the rate of the power stroke (Dantzig *et al.* 1992), can be measured directly in single myofibrils by fast solution switching (Tesi *et al.* 2000).

In the present study we monitored the mechanical behaviour of fast skeletal myofibrils in controls, in myofibrils in which endogenous fsTnC had been extensively extracted and replaced by cTnC, and in myofibrils in which endogenous fsTn had been almost completely exchanged for cTn. Extensive removal of fsTnC and its replacement with cTnC in myofibrils reduced both the maximal isometric tension and apparent rate of force generation, as observed previously in skinned fibres (Morris *et al.* 2001). Moreover, the rate of force decline following a step increase in P_i ($k_{P_i(+)}$) was not changed by cTnC replacement, implying that neither the power stroke nor P_i release was affected. These results support the hypothesis that cTnC substitution alters the rate of the weak-to-strong attachment step in the crossbridge cycle. On the other hand, replacement of the whole fsTn with cTn by exchange produced no change in either myofibril maximal isometric tension or rate of force generation, indicating that crossbridge mechanics at saturating Ca^{2+} concentrations are not altered by the presence of cTn. Thus, the changes in mechanical kinetics effected by cTnC replacement arise either from alterations in the signalling between cTnC, fsTnI and/or fsTnT or from incomplete reconstitution of the regulatory complex during cTnC replacement. However, the pCa–tension relationship of the myofibrils was markedly altered by the presence of cTn. This latter result supports the idea that regulatory proteins primarily alter Ca^{2+} sensitivity and the cooperativity of the force-generating mechanism, whereas the behaviour of crossbridges *per se* is largely determined by the isoform of the myosin molecule. Preliminary reports of these results have been published in abstract form (Piroddi *et al.* 2001, 2002).

METHODS

Myofibrils

Single myofibrils or bundles of two to three myofibrils were prepared from fast skeletal muscle by homogenization of glycerinated rabbit psoas muscles, as described previously (Tesi *et al.* 1999). Rabbits were killed by intravenous administration of pentobarbitone (120 mg kg^{-1}) through the marginal ear vein. All of the procedures were conducted in accordance with the official regulations of the European Community Council on use of laboratory animals (directive 86/609/EEC) and the study was approved by the Ethical Committee for Animal Experiments of the University of Florence.

All solutions to which the myofibrils were exposed contained a cocktail of protease inhibitors including leupeptin (10 μM), pepstatin (5 μM), phenylmethylsulphonyl fluoride (200 μM) and E64 (10 μM; all obtained from Sigma Chemical Co, St Louis, MO, USA), as well as NaN_3 (500 μM) and 1–10 mM DTT. Myofibril preparations, which were stored at 0–4 °C and were used for up to 5 days after preparation. For experiments, a small volume of myofibril suspension was transferred to a temperature-controlled chamber filled with a relaxing solution (pCa 8–9) mounted on an inverted microscope. Sarcomere lengths and myofibril diameters were measured from video images (× 1800, phase-contrast optics).

Apparatus for mechanical measurements and rapid solution changes

The system used to record force from single myofibrils and for rapid solution changes has been described earlier (Colomo *et al.* 1997, 1998; Tesi *et al.* 2000). Briefly, myofibrils selected for use were mounted horizontally between two glass micro-tools: a calibrated cantilevered force probe and a length-control motor. The myofibrils adhered strongly to the glass tools, which were positioned to maximize the attachment area using micromanipulators. The initial length of attached myofibrils between attachments averaged (mean ± S.E.M.) 51.4 ± 2.1 μm at a sarcomere length of 2.27 ± 0.02 μm (n = 94). Isometric force was measured from the deflection of the shadow of the force probe projected on a split photodiode (Cecchi *et al.* 1993).

Myofibrils were activated and relaxed by rapid translation between two continuous streams of relaxing (pCa 8–9) and activating (pCa 7.25–3.5) solutions flowing by gravity from a double-barrelled glass pipette placed at right angles to, and within 1 mm of, the preparation. Solution changes after the start of the paired-pipette movement (driven by a stepper-motor-controlled system) occurred with a time-constant of 2–4 ms and were complete within 10 ms (Colomo *et al.* 1998; Tesi *et al.* 2000).

Experiments were performed at 15 °C in a thermostatically controlled myofibril observation chamber and microscope enclosure. Release–restretch protocols were used to measure k_{TR}

(Brenner, 1988). Values of the rate of rise of tension following a step decrease in pCa by fast solution switching (k_{ACT}) were estimated from the time required to reach 50 % of the isometric force at a given pCa. In some experiments performed at 5 °C, sudden perturbations in the [P_i] of maximally activating solutions perfusing the myofibril were produced by fast solution switching. The rates of the myofibril tension responses following the stepwise increase and decrease in [P_i] ($k_{P_i(+)}$ and $k_{P_i(-)}$, respectively) were estimated from the time required to reach 50 % of the isometric force change.

Solutions

All activating and relaxing solutions, which were calculated as described previously (Tesi *et al.* 2000), were at pH 7.0. The solutions contained: 10 mM total EGTA (CaEGTA/EGTA ratio set to obtain different values of pCa in the range 9.0–3.5), 5 mM MgATP, 1 mM free Mg^{2+}, 10 mM Mops, propionate and sulphate to adjust the final solution to an ionic strength of 200 mM and a monovalent cation concentration of 155 mM. Although continuous solution flow minimizes alterations in the concentration of MgATP and its hydrolysis products in the myofibrillar space, the measurements were made in the presence of creatine phosphate (10 mM) and creatine kinase (200 units ml^{-1}) to prevent any ADP gradients. Contaminant [P_i] (around 170 μM in standard solutions) was reduced in some experiments to less than 5 μM (P_i-free solutions) by a P_i-scavenging enzyme system (purine-nucleoside-phosphorylase with substrate 7-methyl-guanosine) (Tesi *et al.* 2000, 2002*a*).

Proteins, TnC extraction/reconstitution and Tn exchange

Both bovine cTn and bovine cTnC were purified as described previously (Tobacman & Adelstein, 1986; Huynh *et al.* 1996). fsTn was purchased from Sigma. Dr F. Schachat generously supplied fsTnC.

For the removal of endogenous fsTnC and its replacement with cTnC or exogenous fsTnC, two different protocols were used, both of which are based on the low ionic strength TnC extraction method proposed by Brandt *et al.* (1984) and Moss *et al.* (1985). Results from the two protocols were not different. In the first protocol, a myofibril was attached to the glass micro-tools and its maximum ability to develop Ca^{2+}-activated force was measured. The endogenous fsTnC was then extracted by briefly (3 min) perfusing the myofibril with a low ionic strength rigor-EDTA solution stream at pH 7.8 (EDTA 5 mM, Tris 20 mM, and the protease inhibitor cocktail mentioned earlier). After measuring the residual ability of the preparation to produce maximal Ca^{2+}-activated isometric force, the myofibril was then briefly (2 min) perfused by a TnC-replacement solution stream at pH 7.0 (a rigor-EGTA solution, containing Tris 50 mM, KCl 100 mM, $MgCl_2$ 2 mM, EGTA 1 mM and the protease inhibitors, added with 0.05 mg ml^{-1} cTnC or fsTnC). When the flow of the replacement solution was halted and the myofibril returned to the MgATP-containing relaxing solution (pCa 8), it immediately relaxed. The ability of the myofibril to develop Ca^{2+}-activated force was then measured again. All of the steps in this extraction–reconstitution protocol were performed at 15 °C. Examples of the results obtained are shown in Fig. 2.

In the second protocol, or 'batch' TnC extraction and replacement, myofibril suspensions were first pelleted by centrifugation at 4000 *g* and 10 °C. To extract the fsTnC, the pellet was resuspended in a rigor-EDTA TnC-extraction solution containing 10 mM Mops, 5 mM EDTA and the protease inhibitors (pH 7.2). The myofibrils were incubated in this solution for 1 h at 30 °C and then centrifuged at 4000 *g* for 10 min at 10 °C. The pellet was washed by resuspension in the rigor-EGTA solution (composition given above) and immediately centrifuged as before. The pellet was then resuspended in a rigor-EGTA solution containing 0.3 mg ml^{-1} fsTnC or 0.3 mg ml^{-1} cTnC and incubated overnight at 0–4 °C to reconstitute the Tn. Myofibril batches, collected at different stages of this extraction–reconstitution protocol (untreated-control, TnC-extracted and cTnC- or fsTnC-reconstituted batches), were stored at 0–4 °C and used for mechanical experiments in the next 4–5 days after preparation. Force-generation parameters were measured in a number of myofibrils from each batch at 15 or 5 °C. Samples of myofibrils from the same batches as used for mechanical experiments were also employed for electrophoretic analysis (see Fig. 1*A*).

For the exchange of whole Tn, the method of She *et al.* (2000) was used. For this method, endogenous fsTn is exchanged for cTn in a rigor myofibril suspension with a 2 h exposure (at room temperature) to a high [Ca^{2+}] rigor solution containing 0.5 mg ml^{-1} (*ca* 6 μM) cTn or fsTn. The rigor solution for the exchange contained imidazole 10 mM (pH 7.0), NaCl 170 mM, $MgCl_2$ 5 mM, EGTA 2.5 mM, $CaCl_2$ 3 mM and the protease inhibitor cocktail. Myofibrils either remained in this solution until use (stored in ice) or were centrifuged at 4000 *g* for 10 min at 10 °C to form a myofibril pellet. The pellet was then resuspended in the usual rigor-EGTA solution (see above), in which it remained until use at 0–4 °C. Myofibrils from these solutions were attached to the glass micro-tools in a MgATP-containing relaxing solution (pCa 8–9) and were completely relaxed. Results from the two methods were the same.

SDS-PAGE

The extraction/reconstitution of the TnC in myofibrils was quantified using a 10–20 % gradient SDS-PAGE (Salviati *et al.* 1982), which is able to identify both cardiac and skeletal TnC isoforms (Fig. 1*A*). A 12 % SDS-PAGE (Giulian *et al.* 1983) was used to evaluate the exchange of cTn for the endogenous myofibril fsTn. The latter protocol was chosen because it is able to identify all isoforms of the cardiac and fast skeletal Tn complexes from each other and from all the other myofibril proteins, with the only exception of the cTnC isoform that co-migrates with one of the fast skeletal myosin light chain isoforms (LC_2). Myofibrils containing fsTnC, cTnC, fsTn or cTn were placed in a Laemmli sample buffer (Laemmli, 1970) and denatured to solubilize the muscle myofibrils. Following electrophoresis, the gels were stained using the silver stain technique (Giulian *et al.* 1983). Identification of TnC bands on gels was confirmed by co-migration with purified cTnC and fsTnC (Fig. 1*A*). Identification of Tn complex bands was confirmed by co-migration with purified cTn and fsTn (Fig. 1*B*). A scanning densitometer was used to normalize bands to the area under the actin peak or the sum of the areas of myosin light chains ($LC_1 + LC_2 + LC_3$). To confirm TnT isoform identification, Western blots were run of the control and cTn-exchanged rabbit psoas myofibrils using a monoclonal antibody for rabbit fsTnT. Proteins from unstained gels were transferred to nitrocellulose sheets by electrophoresis and a semi-dry transfer procedure (Towbin & Gordon, 1984). Nitrocellulose sheets were reacted first with a primary monoclonal antibody against TnT (Sigma, JLT-12) and then with a peroxidase-conjugated secondary antibody (P260, DAKO). TnT bands were visualized with the aid of an enhanced chemiluminescence method in which luminol was excited by peroxidase in the presence of H_2O_2 (Pollard-Knight *et al.* 1990).

RESULTS

Gel electrophoretic studies of TnC and Tn replacement and exchange in rabbit psoas myofibrils

To gauge the extent to which TnC replacement or Tn exchange was accomplished in these experiments, SDS gel electrophoresis measurements of the treated myofibrils were made, examples of which are shown in Fig. 1. Figure 1*A* shows representative 10–20 % SDS gradient gels of control unextracted myofibrils (ctrl), myofibrils from which the endogenous fsTnC had been extracted (TnC extr), those in which endogenous TnC had been replaced by exogenous fsTnC (fsTnC repl) and myofibrils in which the endogenous fsTnC had been replaced by cTnC (cTnC repl). Scans of the gel indicate that in the control myofibrils the ratio of the area under the fsTnC band to the sum of the areas of myosin light chain bands ($LC_1 + LC_2 + LC_3$) was 11.6 ± 0.5 % (mean ± S.E.M., $n = 3$), while that for the fsTnC and cTnC replacement averaged 15.3 ± 3.2 and 15.5 ± 3.5, respectively. Moreover, fsTnC was not observed in the gels of TnC-extracted or cTnC-replaced myofibrils. As shown in Table 1, the Ca^{2+}-activated tension of the TnC-extracted myofibrils fell to < 15 % of pre-extraction levels and replacement by exogenous fsTnC led to an almost complete tension recovery. The recovery of tension in TnC replacement by both fsTnC and cTnC (see below) is comparable to that reported earlier in single muscle fibres (Morris *et al.* 2001).

Figure 1*B* is a 12 % SDS gel of untreated control myofibrils (ctrl), cTn-exchanged myofibrils (cTn-exch) and lanes containing only cTn (cTn) or fsTn (fsTn). Insignificant amounts of fsTnT and fsTnI remain in the cTn-exchanged myofibrils as compared to the control myofibrils. Densitometric measurements of these gels indicate that 93.1 ± 1.0 % ($n = 3$) of the TnI in the myofibrils exchanged with cTn was cTnI, while 84.5 ± 1.0 % of the TnT was cTnT in such myofibrils. In 12 % gels, cTnC cannot be identified separately because it co-migrates with LC_2. However, scans of control myofibrils gave values of 24.6 ± 2.2 % ($n = 3$) for the ratio of the area under the LC_2 band to that under the actin band, while the same ratio in myofibrils in which cTn was exchanged was about twice as great (53.9 ± 0.2 %, $n = 2$), very close to the ratio of (LC_2 + fsTnC)/actin in control myofibrils (47.7 ± 3 % $n = 3$). This result plus the absence of fsTnC in the cTn-exchanged myofibrils suggest an almost complete exchange of the cTn for fsTn. This conclusion is strengthened by the Western blot in Fig. 1*C*, which shows that the amount of fsTnT remaining in the exchanged myofibrils is < 5 % of the TnT present. Thus the modified She *et al.* (2000) protocol results in an essentially complete exchange of cTn for fsTn.

Mechanical effects of TnC extraction–replacement

Figure 2 illustrates the mechanical behaviour of representative myofibrils in which the endogenous TnC was extracted from the preparation and then replaced with either fsTnC (*A* and *B*) or cTnC (*C* and *D*). Figure 2*A* and *C*

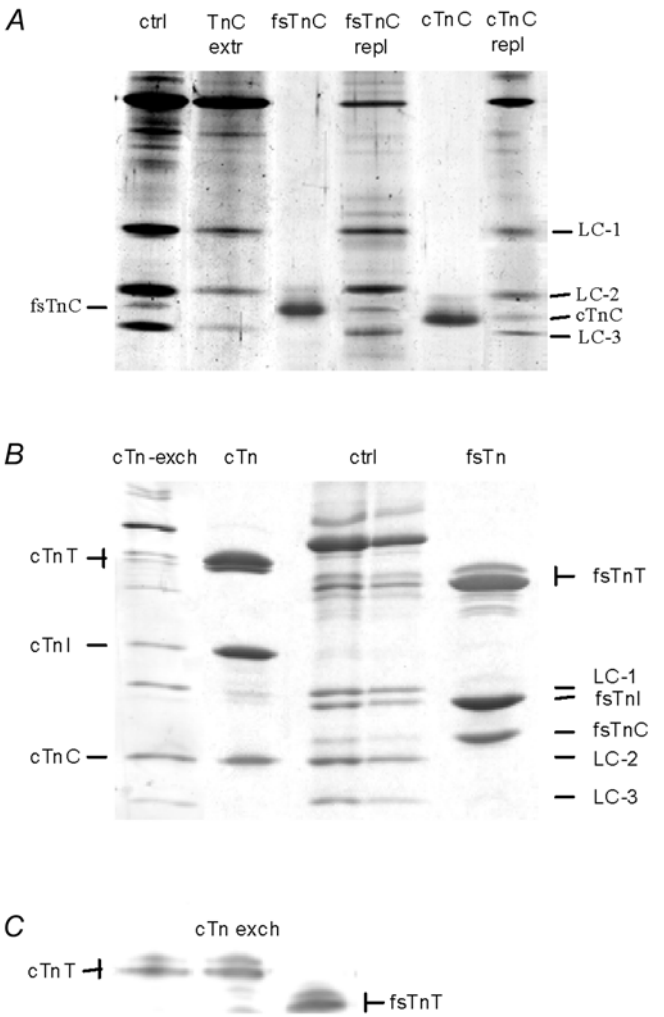

Figure 1. TnC and Tn replacement in rabbit psoas myofibril suspensions

In each gel the myofibrils loaded into the lane were washed of the solutions in which they had been centrifuged to form a pellet, the supernatant was removed and the pellet washed and then resuspended in rigor-EGTA solution. *A*, 10–20 % gradient SDS gel of untreated control myofibrils (ctrl), myofibrils that had been extracted to remove the endogenous TnC (TnC extr) and myofibrils from which endogenous TnC had been extracted and then replaced by either exogenous fsTnC (fsTnC repl) or cTnC (cTnC repl). *B*, 12 % SDS gel of untreated control myofibrils (ctrl), myofibrils in which the endogenous whole Tn complex had been exchanged with cardiac Tn (cTn-exch), and lanes containing only cardiac Tn (cTn) or fsTn (fsTn). *C*, Western blot of a gel containing cTn-exchanged myofibrils (cTn exch) shows a large amount of cardiac TnT (cTnT) in the myofibrils but less than 5 % of the native fsTnT. Thus, an almost complete replacement of endogenous fsTn has taken place. The left-most lane contains cTnT and the right-most, fsTnT.

Table 1. Effects of TnC and whole Tn replacement on tension generation in rabbit psoas myofibrils at 15°C

Myofibril batch	P_0 (mN mm^{-2})	k_{ACT} (s^{-1})	k_{TR} (s^{-1})
TnC extraction and replacement			
Control	312 ± 25 (11)	7.9 ± 0.4 (11)	8.0 ± 0.4 (11)
TnC-extracted	40 ± 7 (5)*	—	—
fsTnC-replaced	280 ± 23 (7)	7.8 ± 0.7 (7)	7.5 ± 0.5 (7)
cTnC-replaced	166 ± 22 (7)*	3.5 ± 0.3 (7)*	3.6 ± 0.3 (7)*
Whole Tn replacement			
Control	329 ± 20 (41)	8.2 ± 0.6 (16)	7.7 ± 0.3 (27)
cTn-exchanged	330 ± 23 (34)	8.0 ± 0.5 (15)	7.5 ± 0.3 (29)

Each group of data were obtained from different myofibril batches. All values are given as mean ± S.E.M.; the number in parentheses is number of myofibrils. P_0, maximum isometric tension; k_{ACT}, rate constant of tension rise following stepwise pCa decrease (8.0→4.5) by fast solution switching; k_{TR}, rate constant of tension redevelopment following release–restretch of maximally activated myofibrils. *$P < 0.01$ (Student's t test) *versus* the same parameter measured in control myofibrils.

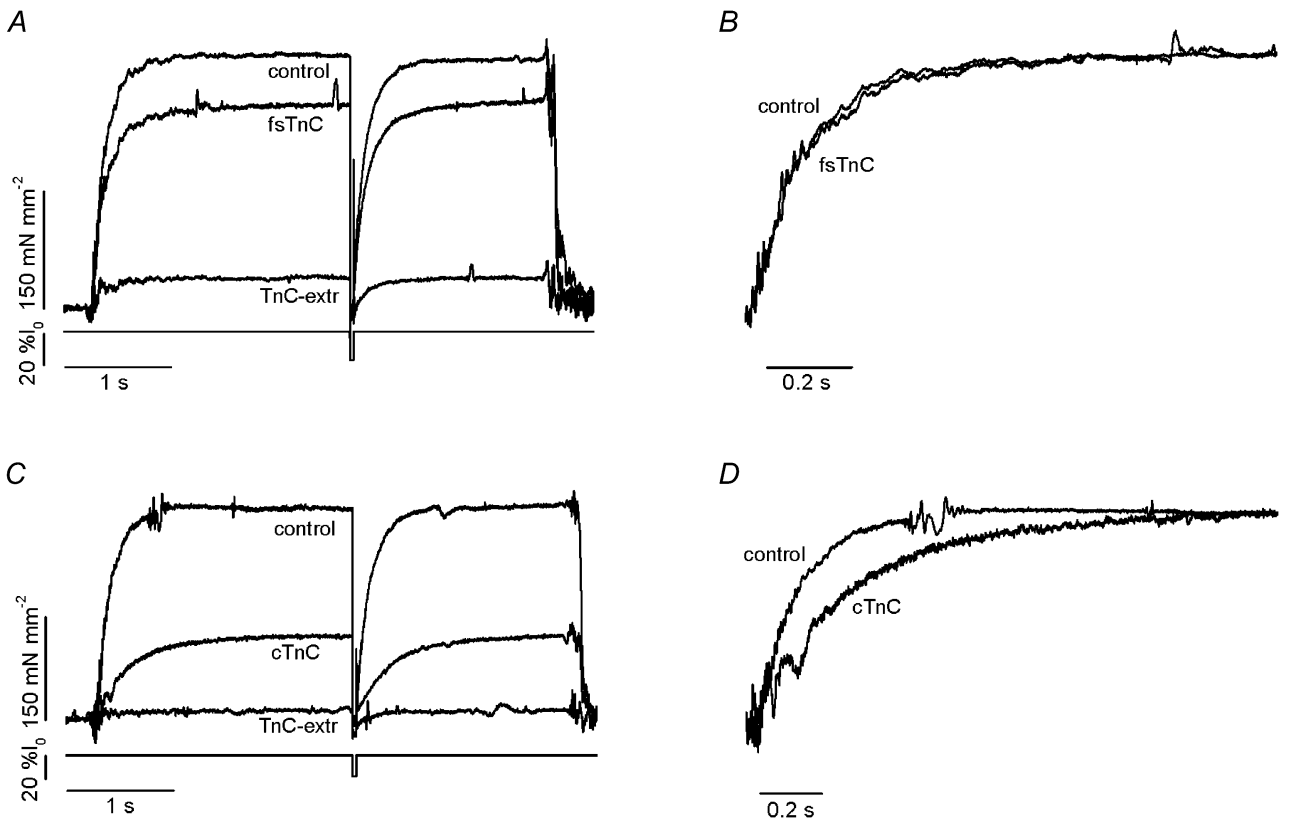

Figure 2. Tension generation in rabbit psoas myofibrils following TnC extraction and replacement with exogenous fsTnC or cTnC at 15 °C

Representative preparations treated with the single myofibril TnC extraction–replacement protocol. *A* and *C*, slow time base recordings of myofibril tension (upper traces) and length (lower traces) during full activation–relaxation cycles induced by fast solution switching between pCa 8.0 and 4.5. In both myofibrils, tension was recorded before (control) and after exhaustive extraction of endogenous TnC (TnC-extr). Finally, tension was recorded after reconstitution with either fsTnC (fsTnC, *A*) or cTnC (cTnC, *C*). In each contraction cycle a large release–restretch was applied to the preparation under steady-state conditions of activation to measure k_{TR}. k_{TR} values were: 8.2 and 7.9 s^{-1} in control and fsTnC, respectively (*A*), and 7.6 and 3.2 s^{-1} in control and cTnC, respectively (*C*). *B* and *D*, same traces as in *A* and *C*, respectively, on an expanded time base and normalized to the amplitude of the force change to illustrate the effects of TnC replacement on the kinetics of tension activation; *B*, k_{ACT} 8 s^{-1} in both control and fsTnC; D, k_{ACT} 7.6 and 3.3 s^{-1} in control and cTnC, respectively.

shows control recordings of tension production in response to maximal Ca^{2+} activation by fast solution switching, followed by a quick release–restretch applied to the myofibrils under conditions of steady activation. As reported earlier (Tesi *et al.* 2002*b*), the rate of rise of tension following Ca^{2+} activation, k_{ACT}, and the rate of tension redevelopment following a release–restretch protocol, k_{TR}, are the same. Next, endogenous TnC was extracted (3 min perfusion with the extraction solution) and upon activation, < 15 % of the control isometric tension was observed. Following TnC replacement with either fsTnC or cTnC, the myofibrils were again activated and recovered the isometric tension to 80 % or 40 % of the control values, respectively. The k_{ACT} and k_{TR} for the fsTnC-reconstituted myofibril were not different from the values measured in the control contraction, while those for the cTnC-replaced myofibril were 43 % of their control values. The effects of fsTnC and cTnC replacement on k_{ACT} are better seen in Fig. 2*B* and *D*, where the tension records are normalized and shown on a faster time base. On average, in 14 cTnC-replaced myofibrils, k_{ACT} and k_{TR} decreased to 46.6 ± 3.1 and 46.3 ± 2.6 % of their pre-extraction values, respectively, while no significant change was observed in 10 fsTnC-replaced myofibrils (99.8 ± 4.5 and 97.5 ± 3.3 % of the pre-extraction values for k_{ACT} and k_{TR}, respectively).

The observed reduction in kinetics following cTnC replacement is not a consequence of incomplete reincorporation of cTnC into the Tn complex of fast skeletal myofibrils. Partial TnC extraction from myofibrils, performed for 1 min and without subsequent TnC replacement reduced the maximal tension (Fig. 3*A* and *D*) to an extent that is comparable to the effect of cTnC replacement (Fig. 2). However, unlike cTnC replacement, partial TnC extraction did not significantly affect k_{ACT} and k_{TR}. This indicates that the reduction of the apparent kinetics of force generation is specific to cTnC replacement of fsTnC.

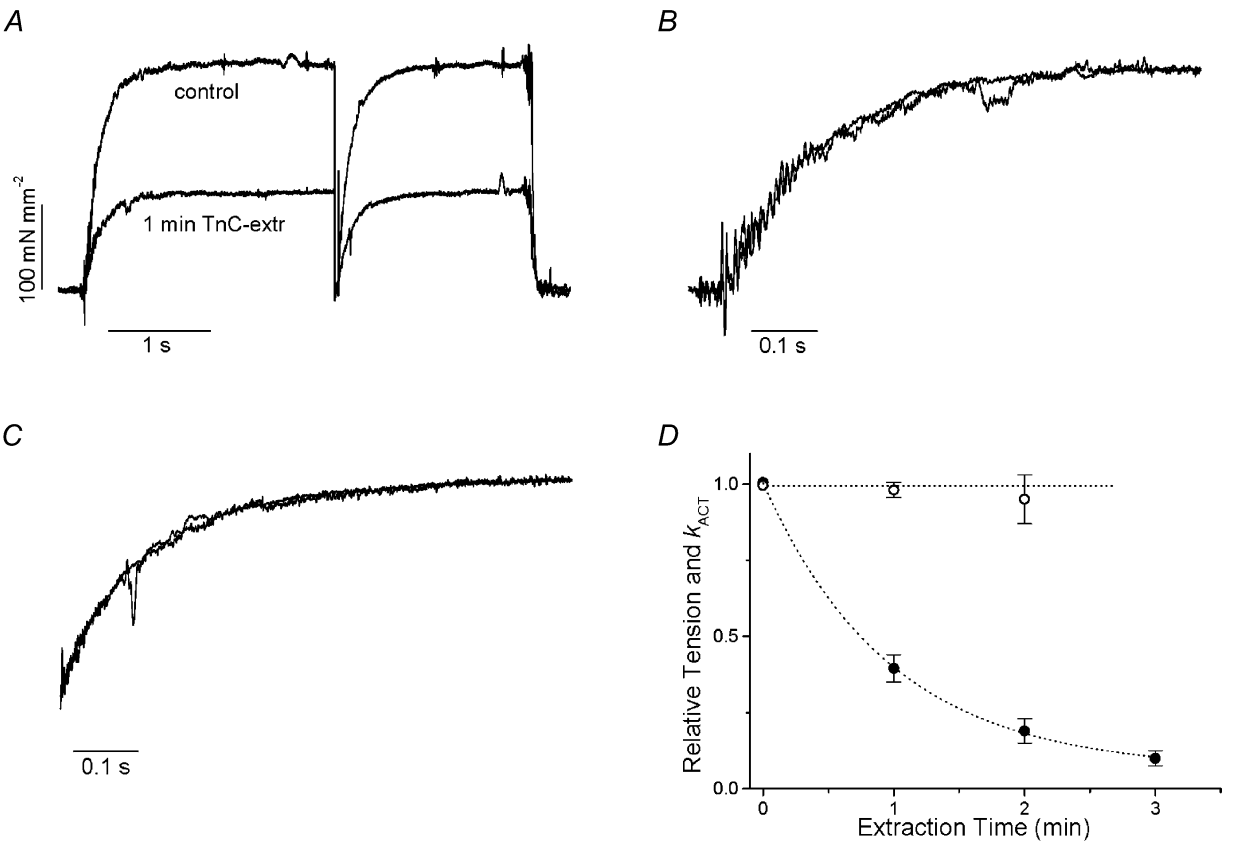

Figure 3. Effects of partial TnC extraction without TnC replacement on tension and apparent rates of tension generation in rabbit psoas myofibrils at 15 °C

A, full activation–relaxation cycles recorded from a myofibril under control conditions and after 1 min perfusion with the TnC extraction solution. In both cases, pCa was switched between 8.0 and 4.5.The drop in maximal tension induced by TnC extraction (P_0 after TnC extraction was 42 % of the control value) was not accompanied by any significant change in k_{ACT} (7.2 *vs.* 7.1 s^{-1} in control *vs.* TnC extraction) and k_{TR} (7.4 *vs.* 7.3 s^{-1}), as better shown in *B* (for k_{ACT}) and *C* (for k_{TR}) on a faster time base and after normalization of the traces for the maximal tension. *D*, average behaviour of maximal tension (filled circles) and k_{ACT} (open circles) following progressively longer exposure of myofibrils to the extraction solution. Data points are means ± S.E.M. of six to nine myofibrils.

Essentially similar results as found with the 'single myofibril protocol' of TnC extraction and replacement were obtained from the 'batch protocol' (see Methods), which has also been used for the electrophoretic analysis. Table 1 summarizes the results from these experiments. The average maximal tension in fsTnC-replaced myofibrils was 90 % of that of the controls (the difference, however, was not statistically significant), and in cTnC-replaced myofibrils it was 53 % of that of the controls (59 % of the mean value found in fsTnC-replaced myofibrils). The mean values of k_{ACT} and k_{TR} at saturating Ca^{2+} concentration were not different in controls and fsTnC-replaced myofibrils, while in cTnC-replaced myofibrils they were 44 and 45 % of the control values, respectively. The results of these experiments show that myofibrils behave in a manner practically identical to skinned muscle fibres (Morris *et al.* 2001, 2003).

One possible explanation for the reduced force, k_{TR} and k_{ACT} seen on cTnC replacement is that it reduces crossbridge cycling by reducing the rate of the power stroke. Measurement of the fsTnC- and cTnC-containing myofibril response to a step increase in $[P_i]$ tested this possibility. This manoeuvre is thought to detect the rate of the power stroke (Dantzig *et al.* 1992). These experiments were performed at 5 °C to better resolve the time course of the fast tension transient following a sudden increase in $[P_i]$. Figure 4 illustrates the effects of abrupt changes in $[P_i]$ on the tension of representative myofibrils in which the endogenous TnC was extracted and then replaced with either fsTnC (*A* and *B*) or cTnC (*C* and *D*). Figure 4*A* and *C* shows slow time base recordings of exposure of the myofibrils to maximally activating pCa 4.5 solution, a sudden increase in $[P_i]$ from 5 μM to 2 mM and a reduction in $[P_i]$ from 2 mM to 5 μM, followed by Ca^{2+} removal and

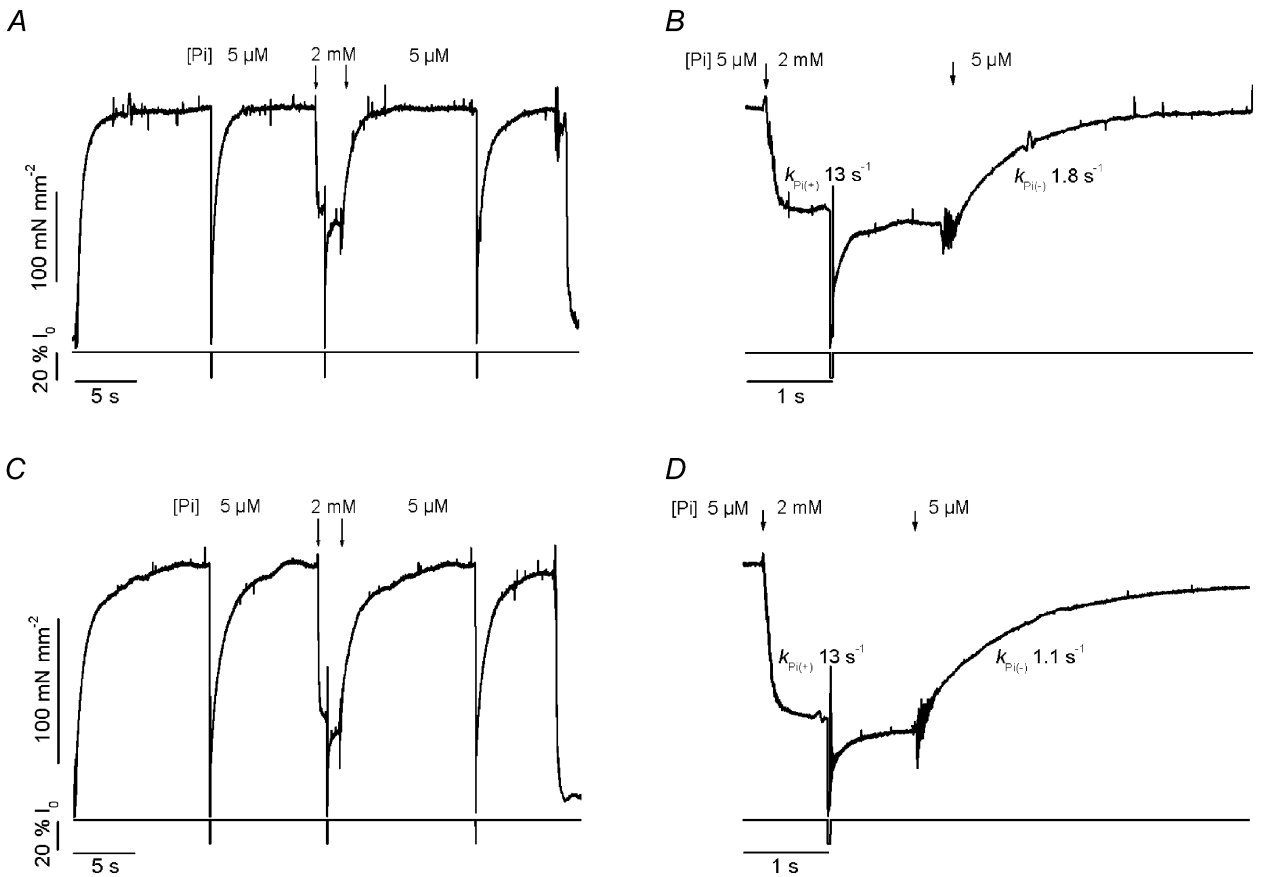

Figure 4. Tension transients of fsTnC- and cTnC-replaced myofibrils in response to sudden changes in $[P_i]$ at 5 °C

Representative examples from batches of myofibrils from which endogenous TnC had been extracted and then replaced by either fsTnC (*A* and *B*) or cTnC (*C* and *D*). *A* and *C*, slow time base recordings of myofibril tension (upper traces) and length (lower traces). Both myofibril types were initially fully relaxed in a P_i-free solution (pCa 8.0, $[P_i]$ 5 μM) and then maximally activated by switching to a pCa 4.5 P_i-free solution. After a quick release–restretch was applied to the myofibrils to measure k_{TR}, a sudden increase in $[P_i]$ was produced by switching to a 2 mM P_i maximally activating solution. A new release–restretch was applied to the myofibrils, after which $[P_i]$ was suddenly reduced back to 5 μM. After a final release–restretch, the myofibrils were fully relaxed (pCa 8.0). *B* and *D*, fast time base recordings of the myofibril tension responses to sudden changes in $[P_i]$. $k_{P_i(+)}$ and $k_{P_i(-)}$ are the rate constants of the tension changes in response to sudden $[P_i]$ increase and decrease, respectively.

Table 2. Effects of P_i on tension generation in fsTnC- and cTnC-replaced myofibrils at 5°C.

Myofibril batch	P_0 5 μM P_i (mN mm^{-2})	k_{ACT} 5 μM P_i (s^{-1})	k_{TR} 5 μM P_i (s^{-1})	$k_{Pi(+)}$ 5 μM → 2 mM P_i (s^{-1})	$k_{Pi(-)}$ 2 mM →5μM P_i (s^{-1})	P/P_0 2 mM P_i	n
fsTnC-replaced	249 ± 21	2.2 ± 0.2	2.1 ± 0.1	12.5 ± 0.8	2.1 ± 0.3	0.54 ± 0.03	8
cTnC-replaced	126 ± 17*	1.3 ± 0.1*	1.2 ± 0.1*	12.6 ± 1.1	1.1 ± 0.1*	0.40 ± 0.03*	8

Data from experiments as shown in Fig. 3. All values are given as mean ± S.E.M.; n, number of myofibrils. $k_{Pi(+)}$ and $k_{Pi(-)}$ are rate constants of the myofibril tension responses to sudden [P_i] increase and decrease, respectively; P/P_0, maximum Ca^{2+}-activated tension measured at 2 mM P_i relative to that measured in P_i-free solution (5 μM P_i). *$P < 0.01$ (Student's t test) *versus* the same parameter measured in fsTnC-replaced myofibrils.

full relaxation of the myofibrils. Release–restretches were applied to the myofibrils to measure k_{TR} at all stages of this protocol. In P_i-free solutions (5 μM P_i), the slowed k_{ACT} and k_{TR} in the myofibril containing cTnC (1.2 s^{-1} and 1.1 s^{-1}, respectively) compared to that containing fsTnC (2.1 s^{-1} and 2 s^{-1}, respectively) is obvious even on the slow time base. In Fig. 4*B* and *D*, on a faster time base, the time course of the $k_{P_i(+)}$ and $k_{P_i(-)}$ bracketing the release–restretch at 2 mM P_i is shown. As reported earlier (Tesi *et al.* 2000), $k_{P_i(+)}$ is significantly faster than k_{TR}, and $k_{P_i(-)}$ is essentially the same as the k_{TR} at a given [P_i]. The value of k_{TR} at 2 mM P_i (5 °C) for the fsTnC myofibril in this case was 5.6 s^{-1}, while that for the cTnC-containing myofibril was 3.5 s^{-1}. The increased k_{TR} at higher concentrations of P_i was the same as has been reported previously (Millar & Homsher, 1990; Tesi *et al.* 2000). A data summary for the P_i transient studies is given in Table 2. The constancy of $k_{P_i(+)}$ in the presence of both fsTnC and cTnC indicates that the power stroke *per se* is not affected by isoforms of TnC. The slowed rate of $k_{P_i(-)}$ in the presence of cTnC compared to fsTnC agrees with previous suggestion that $k_{P_i(-)}$, unlike $k_{P_i(+)}$, reflects the same processes as k_{ACT} and k_{TR} (Tesi *et al.* 2000). The results are consistent with the hypothesis that the cTnC slows the rate of strong crossbridge formation (Morris *et al.* 2001).

Mechanical effects of whole Tn exchange

Next the mechanical sequelae of cTn (whole Tn complex) replacement of the endogenous fsTn were measured to learn whether the results already described here were a consequence of alterations in signalling between heterologous Tn subunits, or the specific Tn isoform. Figure 5 shows the effects of exchange of cTn for fsTn in single myofibrils (15 °C). Figure 5*A* shows force traces from a fsTn-containing myofibril, and Fig. 5*B* shows those from a cTn-exchanged myofibril. In these experiments the myofibrils were first activated at pCa 6.25 (and k_{TR} measured if the tension was greater than 20 % of the maximal tension) and then exposed to a pCa of 3.5 followed by a k_{TR} measurement. The data given in the figure show that the rate of force development following activation at pCa 3.5 and the k_{TR} are the same for the cTn- and fsTn-containing myofibrils. This conclusion is

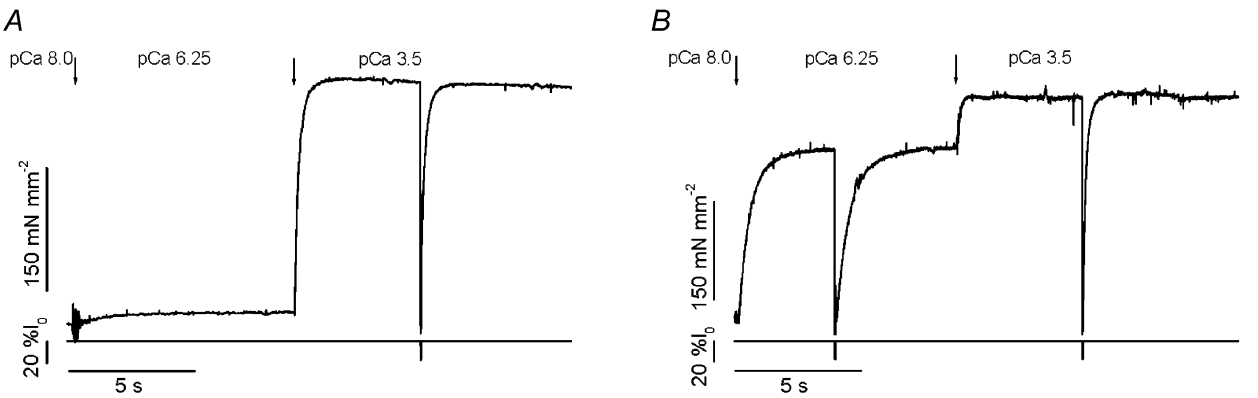

Figure 5. Tension generation in rabbit psoas myofibrils at 15 °C following whole Tn complex replacement by cTn

Representative recordings of tension (upper traces) and length (lower traces) from batches of control, unexchanged myofibrils (*A*) and myofibrils whose native fsTn had been exchanged with cTn (*B*). Both myofibril types were initially activated by switching the pCa from 8.0 to 6.25 and then maximally activated by a further stepwise pCa decrease to 3.5. Large release–restretches were applied to the preparations under steady-state conditions of activation to measure k_{TR}. Tension, k_{ACT} and k_{TR} measured at maximal activation appear to be the same for the cTn- and fsTn-containing myofibrils, while the larger submaximal tension developed by the cTn-containing myofibril at pCa 6.25 indicates that the Ca^{2+} sensitivity of tension is greater in the cTn-exchanged myofibril.

confirmed in Table 1, which shows that there is no difference in the maximal isometric tension, k_{ACT} or k_{TR} between myofibrils containing cTn or fsTn. Thus, for the case of the whole Tn complex exchange, crossbridge mechanics at saturating Ca^{2+} concentrations is independent of the Tn source.

The data shown in Fig. 5 do suggest, however, that the Ca^{2+} sensitivity of the cTn-containing myofibrils is significantly greater than that for fsTn-containing myofibrils. The relative submaximal tension developed by cTn-containing myofibrils at pCa 6.25 was much larger (*ca* 70 % of maximal isometric tension, P_0) than that developed by control fsTn-containing myofibrils (< 10 % P_0). The Ca^{2+}-sensitizing effect of cTn exchange was not a consequence of the exchange procedure itself because the same very small tension levels (8.4 ± 4.0 % P_0) were measured at pCa 6.25 in four myofibrils exchanged with homologous fsTn.

To better describe the Ca^{2+}-sensitizing effect of cTn exchange in fast skeletal myofibrils, the relationships between tension and pCa measured at 15 °C in myofibrils containing cTn and fsTn are shown in Fig. 6. The pCa–tension plots reveal that the pCa_{50} for cTn-containing myofibrils is 0.74 pCa units greater than that for the fsTn-containing myofibrils. cTn-containing myofibrils also exhibit a significantly reduced Hill coefficient (1.42 compared to 3.30). These results are consistent with those of Brenner *et al.* (1999) obtained from skinned muscle fibres.

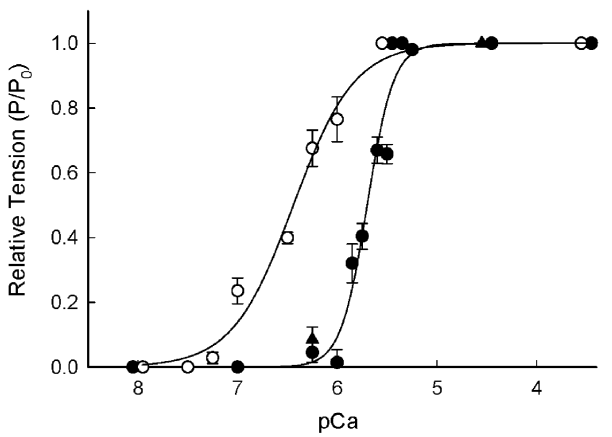

Figure 6. Effect of cTn replacement for fsTn on the Ca^{2+} sensitivity of tension in rabbit psoas myofibrils

pCa–tension relationships of control, fsTn-containing, myofibrils (filled circles) and cTn-replaced myofibrils (open circles). Data points are means ± S.E.M. of five to 13 myofibrils at 15 °C. The continuous lines are drawn according to the parameters developed by fitting the data to the Hill equation: $P/P_0 = 1/(1 + 10^{(-n_H(pCa50 - pCa))})$; pCa_{50} = 5.70 ± 0.02 and 6.44 ± 0.04; n_H = 3.30 ± 0.49 and 1.42 ± 0.15 for the control and cTn-replaced myofibrils, respectively. Filled triangles are average results from four control experiments in which myofibril endogenous fsTn was replaced by exchange with an exogenous fsTn complex.

Given the differences in the pCa–tension curves, we next examined the relationship between tension and k_{TR} in myofibrils containing cTn to learn whether it behaves in a fashion different from that in myofibrils containing fsTn. Figure 7 plots the observed k_{TR} against the relative tension exerted by cTn- and fsTn-containing myofibrils at 15 °C. Such plots normalize the differences in pCa used to obtain the specific values (e.g. the k_{TR} values corresponding to the lowest tension levels shown in this figure were measured at pCa 7 for the cTn-containing myofibrils and pCa 6 for the fsTn-containing myofibrils). The superimposition of the relative tension–k_{TR} relationships is consistent with the conclusion given above, that the kinetics of crossbridge formation are similar in the presence of cTn and fsTn. The data given by the open triangles are the results from the experiments in which the relative force and k_{TR} for cTnC-replaced and fsTnC-containing myofibrils were measured. In this case, the maximal relative force was that obtained with the fsTnC-containing myofibrils. When referenced to the control force, the data from cTnC-containing myofibrils are almost superimposable to the control set of data (fsTn-containing myofibrils). This superimposition suggests that the creation of the heterologous complex (fsTnI/fsTnT/cTnC) reduces myofibril force and kinetics primarily because it compromises thin-filament activation. When myofibrils containing fsTnI/fsTnT/cTnC and fsTn are compared at the same probability of strong crossbridge attachment, the kinetics of the crossbridge formation is the same.

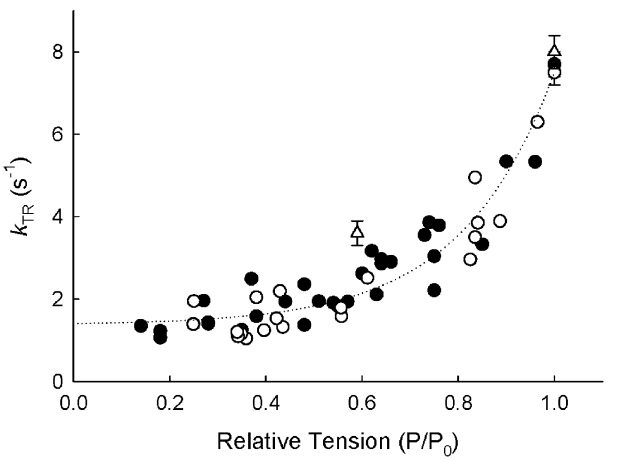

Figure 7. Dependence of the kinetics of tension development on the level of Ca^{2+} activation in fsTn- and cTn-containing myofibrils

Scattered k_{TR} values of control, fsTn-containing, myofibrils (filled circles) and cTn-replaced myofibrils (open circles) are plotted *versus* steady-state isometric tension at different values of [Ca^{2+}] (15 °C). Data points at maximal activation are means ± S.E.M. of 27–29 myofibrils (see Table 1). The dotted line is a single exponential function fitted to the data: $k_{TR} = 1.37 + 0.0345 \exp(P/P_0/0.193)$. Open triangles are mean k_{TR} values measured at maximal Ca^{2+} activation in seven cTnC-replaced myofibrils (relative tension 0.59) and in 11 control, TnC-unextracted myofibrils (relative tension = 1; see Table 1).

DISCUSSION

Summary

The results of the present study have several implications, regarding both the mechanism of regulation by Tn and the significance of cardiac *vs.* skeletal muscle Tn and Tn subunits. First, the effects of replacement of endogenous TnC in fast skeletal myofibrils by cTnC extend previous work in rabbit psoas skinned fibres (Morris *et al.* 2001, 2003). Maximal isometric tension and the apparent rate of force generation (as measured by k_{ACT} and k_{TR}) were significantly reduced after cTnC replaced the endogenous fsTnC isoform. The hypothesis that the chimeric protein produced by cTnC replacement in fsTn compromises thin-filament activation and reduces the probability of crossbridge transition from a weakly to a strongly bound state preceding the power stroke (Morris *et al.* 2001) is strengthened by the finding that cTnC replacement had no effect on the rate constant of the tension fall initiated by rapid elevation of $[P_i]$ ($k_{P_i(+)}$, an indicator of power stroke kinetics; Dantzig *et al.* 1992). Secondly, the mechanical consequences of whole cTn exchange for the endogenous fsTn have been described in isolated myofibrils for the first time. The Ca^{2+} sensitivity of tension was much higher in cTn- than in fsTn-containing psoas myofibrils. However, maximal tension, k_{TR} and k_{ACT} were the same in the two groups of myofibrils, suggesting that these mechanics are determined largely by the isoform of the myosin molecule. Thirdly, the lack of effect of whole Tn exchange on maximal tension and crossbridge kinetics demonstrates that the activation failure occurring in fast skeletal myofibrils after cTnC replacement is more a problem of mismatching proteins in the chimeric Tn complex than real differences between TnC isoforms. Finally, there is a large and unexplained increase in the Ca^{2+} sensitivity of tension following whole cTn exchange into skeletal muscle. These findings are further discussed below.

fsTnC replacement by cTnC: comparison with previous work and implications

Replacement of fsTnC by cTnC has been used to examine the regulation of contraction, but most studies have used partial extraction of fsTnC because exhaustive extraction reduces the maximal isometric force (Moss *et al.* 1986, 1991; Metzger, 1996). In partial extractions (< 70 % TnC removed), replacement with cTnC produced a variable reduction of maximal isometric force, a reduction of cooperativity (n_H fell by 1 unit) and a slight shift in the pCa_{50} (−0.1 pCa unit; Moss *et al.* 1986, 1991; Babu *et al.* 1987). That cTnC replacement produces a reduced cooperativity is paralleled by experiments in transgenic mice expressing fsTnC in their cardiac muscle. Compared to control cardiac myocytes, myocytes from transgenic mice expressing fsTnC/cTnI/cTnT exhibited an increased cooperativity (McDonald *et al.* 1995).

Recent work in skinned skeletal muscle fibres (Morris *et al.* 2001) from which > 90 % of the fsTnC was extracted and replaced with cTnC, confirmed these specific observations and showed that both isometric force and k_{TR} at saturating $[Ca^{2+}]$ fell to 65 and 60 %, respectively, of their control values (see also Chase *et al.* 1994). Identical extraction of fsTnC and replacement with exogenous fsTnC restored isometric force to ~ 90 % of the control (unextracted) value and k_{TR} to 100 % percent of the control values. Extensive extraction of fsTnC (> 90 %) and its replacement with exogenous fsTnC or cTnC in single myofibrils in the current work produced results similar to those obtained in fibres (Morris *et al.* 2001). These TnC-extracted myofibrils exhibited no fsTnC (as assessed by SDS-PAGE; see Fig. 1*A*). After cTnC replacement, maximal isometric tension fell to 55–60 % of the control values, while k_{TR} and k_{ACT} were both reduced to 45 % (see Table 1). Replacement of myofibrils with the homologous fsTnC restored all parameters to their pre-extraction values. These results imply that the heterologous Tn created by extraction and reconstitution of TnC (mixing of the cardiac and fast skeletal Tn subunits) compromises myofibril activation.

These results appear to be inconsistent with those of Moss *et al.* (1991) and Metzger (1996), who found that less extensive removal of fsTnC (30–70 % removal) and replacement with cTnC produced no change in maximal isometric force. The loss of function in the more extensive extraction procedures described here is not a consequence of the extraction procedure itself because homologous replacement of TnC restores control values of force, k_{TR} and k_{ACT}. It could be hypothesized that the significant loss of function in cTnC replacement seen here and in extensively extracted fibres (Chase *et al.* 1994; Morris *et al.* 2001, 2003) is a consequence of a failure of the exogenous cTnC to bind to the sites lacking TnC in the extracted fibres. The present results and several observations argue against this hypothesis. First, quantitative SDS-PAGE analysis, although unable to identify the location of specific TnC isoforms in myofibrils, indicates a small surfeit of TnC after reconstitution, not a deficit, regardless of whether cTnC or fsTnC was used (see the first section of Results and Fig. 1*A*). Second, and more significantly, the diminished k_{ACT} and k_{TR} observed after cTnC exchange cannot be explained by the presence of regulatory units that are inhibited because they lack TnC. As shown previously in fibres (Metzger & Moss, 1990, 1991), when fsTnC is partially removed from the myofibril, the isometric force at saturating pCa is reduced, but k_{ACT} and k_{TR} are unchanged (see Fig. 3). In the experiments reported here, isometric force at saturating pCa was reduced from control values when cTnC replaced the missing fsTnC, but the k_{TR} was also reduced (not unchanged as in the case of fsTnC removal). Similarly, in the presence of saturating

levels of Ca^{2+}, Morris *et al.* (2001) have shown in fibres that k_{TR} remains maximal despite 60 % replacement of fsTnC with an inhibitory, mutant cTnC that does not bind Ca^{2+} at site II. Third, Moss (1986) has shown that partial removal of fsTnC (to a point at which the maximal force is only 50 % of control values) produces two phases of shortening (a high velocity and low velocity phase) at saturating pCa in slack tests. However, when 90 % of the fsTnC has been replaced by cTnC, the unloaded shortening velocity at saturating pCa exhibits only one phase of shortening, which is unchanged from that of controls (Morris *et al.* 2003). Fourth, using similar fsTnC extraction and cTnC replacement as reported here, it was found that removal of > 90 % of the fsTnC from rabbit psoas fibres and its subsequent replacement with rat cTnC at maximal activation produces only 60–65 % of the control isometric force (Moreno Gonzales *et al.* 2003 and M. Regnier, personal communication). When these muscle fibres are subsequently incubated in a solution containing fsTnC (1 mg ml^{-1}) to saturate any Tn sites not containing cTnC, maximal isometric force is unchanged from that obtained prior to the exposure to fsTnC. This result argues directly against the presence of sites on the thin filament TnT/TnI binding sites lacking a cTnC molecule. Thus it is unlikely that the reductions of isometric force, k_{ACT} and k_{TR} seen on cTnC replacement of fsTnC stem from lack of TnC on the Tn sites.

The question then is why, in previous work by Moss *et al.* (1991) and Metzger (1996), partial replacement of fsTnC by cTnC had so little effect on isometric force. In those studies, fsTnC extraction and cTnC replacement occurred at sarcomere lengths of > 2.5–2.6 μm, the TnC removal from the region of the thin filament lacking overlap was much more extensive than that from the region of overlap (Yates *et al.* 1993; Swartz *et al.* 1996). In muscle fibres at a sarcomere length of 2.5 μm, partial TnC extraction preferentially removes TnC from a 0.4 μm length of the 1.1-μm-long thin filament region adjacent to the Z-line and lacking overlap (i.e. 37 % of the total sarcomeric TnC is removed and would have little impact on isometric force). If a total of 70 % of the TnC is removed from the fibre (Metzger, 1996), 37 % would come from the non-overlap region and 33 % from the overlap region, which accounts for only 52 % of the TnC in the overlap region. Thus only 50 % of the fsTnC is removed from the overlap region, producing the Ca^{2+}-sensitive force. With low extraction of fsTnC in the overlap region, the cooperative effects of adjacent active regions may obscure a reduction of communication between cTnC and fsTnI/fsTnT.

The effects of cTnC replacement on force transients following abrupt changes in $[P_i]$ have not been previously measured. Values of $k_{P_i(+)}$ were the same in cTnC- and fsTnC-replaced myofibrils (12.6 and 12.5 s^{-1} respectively at 5 °C and 2 mM final $[P_i]$, see Table 2). These values are consistent with those we reported previously for untreated rabbit psoas myofibrils (7.4 and 18.2 s^{-1} at 1 and 5 mM final $[P_i]$, respectively; Tesi *et al.* 2000). They are also in reasonable agreement with results from caged P_i experiments in skinned fibres (Millar & Homsher, 1990; Dantzig *et al.* 1992; Walker *et al.* 1992; Regnier & Homsher, 1998) when differences in experimental temperature are taken into account. As reported previously (Tesi *et al.* 2000), $k_{P_i(+)}$ values were two to four times faster (see Fig. 4) than the apparent rate of force generation measured at the same $[P_i]$ by k_{TR} (as well as by k_{ACT}, data not shown). $k_{P_i(+)}$ is thought to probe the crossbridge transitions associated with force generation and, unlike k_{ACT} and k_{TR}, it is either unaffected (Millar & Homsher, 1990; Tesi *et al.* 2000) or only marginally affected (Walker *et al.* 1992) by thin filament activation level. The facts that activation-independent indicators of power stroke kinetics (such as $k_{P_i(+)}$ (present data) and unloaded shortening velocity; Morris *et al.* 2003) are unchanged by cTnC replacement, while k_{ACT} and k_{TR} are reduced support the hypothesis that cTnC substitution compromises myofibril activation. Thus cTnC replacement alters a step associated with a weakly to strongly bound crossbridge transition preceding the power stroke, while subsequent crossbridge steps are unaffected (Morris *et al.* 2001, 2003).

The rate constant of the rise in force initiated by a sudden decrease in $[P_i]$ ($k_{P_i(-)}$) was not significantly different from k_{ACT} and k_{TR} and, like k_{ACT} and k_{TR}, was significantly reduced following cTnC replacement in fast skeletal myofibrils (see Table 2 and Fig. 4). It has been found that $k_{P_i(-)}$ shares the same activation dependence shown by k_{ACT} and k_{TR} (Tesi *et al.* 2000). Although the striking differences between $k_{P_i(-)}$ and $k_{P_i(+)}$ are inconsistent with the predictions of simple one- or two-step crossbridge kinetic models of force generation (e.g. Pate & Cooke, 1989; Millar & Homsher, 1990; Kawai & Halvorson, 1991; Dantzig *et al.* 1992; Regnier & Homsher, 1998), the reduction of $k_{P_i(-)}$ observed after cTnC replacement confirms the failure of the chimeric Tn complex to maximally activate crossbridge interactions even in the presence of saturating concentrations of Ca^{2+}.

An important question raised by the cTnC replacement studies (present study and Morris *et al.* 2001, 2003) is why cTnC interaction with fsTnI/fsTnT produces a small reduction in pCa_{50} and cooperativity, no significant change in unloaded shortening velocity and $k_{P_i(+)}$, but does produce significant reductions in isometric force, k_{TR}, k_{ACT} and $k_{P_i(-)}$. Comparison of the structures of fsTnC and cTnC yields some clues. First, cTnC lacks a Ca^{2+}-binding region in the N-terminal domain of cTnC (in the loop linking helix A and helix B) because negatively charged residues chelating Ca^{2+} in fsTnC (residues 28, 30 and 34) are uncharged in cTnC (Tobacman, 1996). This change alone

should reduce the cooperativity. Second, when Ca^{2+} binds to the N-terminal domain of fsTnC, helices B and C move away from a structural unit composed of helices N, A and D in transition from a 'closed' to an 'open' structure. This motion exposes a 'hydrophobic' patch to which a regulatory region of fsTnI (fsTnI residues 115–131 or cTnI residues 147–163) binds (Herzberg & James, 1988; Gagne *et al.* 1995; Vassylyev *et al.* 1998). This binding is thought to detach the inhibitory peptide of fsTnI from a binding site on actin and allows movement of Tn and tropomyosin structures to expose S-1 binding sites on actin (Tobacman, 1996; Gagne *et al.* 1995; Herzberg *et al.* 1986). In cardiac muscle, Ca^{2+} binding to the N-terminal regions does not create an 'open' structure (Spyracopoulos *et al.* 1998). Rather, residues $cTnI_{150–158}$ of the regulatory region of $cTnI_{147–163}$ bind to hydrophobic side chains (particularly the alanine residues 22 and 23 of cTnC) in helices A, B and D, which detach the cTnI inhibitory peptide from its binding site on actin resulting in thin filament activation (Vassylyev *et al.* 1998; Li *et al.* 1999). The binding of $cTnI_{147–163}$ to the cTnC N-terminal domain is only about 15 % as strong as the binding of the analogous regions of fsTnI ($fsTnI_{115–131}$) to fsTnC (Li *et al.* 1999). If the strength of this interaction affects the distribution among thin filament states in the presence of Ca^{2+} and the absence of bound crossbridges, then the rate of activation of the thin filament (and therefore k_{ACT}, k_{TR} and $k_{P_i(-)}$) might be correspondingly slowed by substitution of cTnC for fsTnC. More generally, any of the structural differences between the cardiac and fast skeletal Tn subunits could compromise the efficiency of the signalling between the cTnC and fsTnI.

Regardless of which set of interactions within Tn are perturbed, we suggest that the mechanical consequence is a slowing of the rate of the weak-to-strong crossbridge binding, which limits the rate of force development and the steady-state number of strongly bound crossbridges. Earlier we showed that such a kinetic interpretation is consistent with the reduction in isometric force and k_{TR} seen with cTnC substitution in single skinned skeletal muscle fibres (Morris *et al.* 2001). The Geeves model (McKillop & Geeves, 1993) and the structural interpretations of Lehman and co-workers (Xu *et al.* 1999) of thin filament regulation suggest an explanation of these results. We suggest that the rate of the weak-to-strong (non-force-bearing) crossbridge transition is determined by the equilibrium between two of the structural states of the thin filament: the B state, and the C state (Lehman *et al.* 2000; Vibert *et al.* 1997). In this model of regulation, strong crossbridge formation, even though non-force-bearing, requires that the local tropomyosin position be that of the C (or M state), but not that of the B state. In thin filaments containing chimeric Tn, weak TnC–TnI interactions cause one-third of the thin filament to remain in the B state in the presence of saturating Ca^{2+}. Such an altered distribution might decrease k_{ACT}, k_{TR} and $k_{P_i(-)}$, as well as reducing maximal isometric tension.

Tn complex exchange and Ca^{2+} regulation

The failure of the replacement of fsTn by cTn to alter crossbridge mechanics (isometric force, k_{TR}, k_{ACT}) *per se* suggests that the crossbridges themselves determine these effects (providing that regulatory proteins do not limit the rate of crossbridge attachment). These findings agree with the effects on nucleotide-free myosin S1–thin filament binding resulting from substitution of cardiac for skeletal muscle Tn (Maytum *et al.* 2003). In the presence of Ca^{2+}, neither the kinetics nor the equilibrium of skeletal myosin S1–actin binding was altered by the Tn source. The current data show that this is also true in the sarcomere for cycling crossbridges in the presence of ATP, as assessed for a broad panel of crossbridge kinetic parameters. In the presence of Ca^{2+}, myosin–thin filament interactions are unaffected by Tn isoform.

On the other hand, significant changes in the pCa_{50} and Hill coefficient seen in pCa–tension experiments (see Fig. 6) indicate that Tn isoforms cause important changes in Ca^{2+} regulation. The observed effects in myofibrils are thoroughly consistent with those reported previously for cTn-exchange in rabbit psoas muscle fibres (Brenner *et al.* 1999; see their Fig. 2). As shown here by control experiments in which homologous fsTn was used in the exchange protocol, the large Ca^{2+}-sensitizing effect of cTn-replacement in rabbit psoas myofibrils was not a consequence of the exchange procedure itself.

It is unclear how exchanging fsTn for cTn produces the combination of increased Ca^{2+} sensitivity and decreased cooperativity. The most straightforward explanation involves two separate mechanisms. After cTn for fsTn exchange, the thin filament regulatory sites have: (1) a decreased cooperativity due to the presence of one rather than two Ca^{2+} binding sites in each cTnC regulatory domain and (2) a higher intrinsic Ca^{2+} affinity. A reduction of the number of regulatory Ca^{2+}-binding sites could reduce the cooperativity by about 1 Hill unit (Grabarek & Gergely, 1983). However, an increased intrinsic Ca^{2+} affinity is not supported by comparisons among various chimeric and non-chimeric thin filaments (Rosenfeld & Taylor, 1985; Walsh *et al.* 1985; Tobacman, 1987; Tobacman & Sawyer, 1990; Korman & Tobacman, 1999). An alternative explanation for the increased pCa_{50} following exchange is a greater myosin-induced activation of the thin filament. A weakness of this idea is that this same mechanism should promote cooperativity in the pCa–tension curve, compensating for the decrease in cooperativity resulting from one Ca^{2+} binding site per cTn. Indeed, substitution of cTn for fsTn in the presence of EGTA does cause the myosin S1–thin filament binding

curve to become more cooperative (Maytum *et al.* 2003). However, in the present experiments the Hill coefficient after cTn exchange drops to 1.4, not much greater than 1. It is not clear how myosin-mediated effects might greatly shift the pCa_{50} and yet not preserve or increase cooperativity. Despite this uncertainty, the most likely explanation for the increased pCa_{50} after Tn exchange is enhanced thin-filament activation by crossbridges, and the decreased cooperativity presumably reflects the lone regulatory site of cTnC.

To account in more detail for the Ca^{2+}-sensitizing effect of cTn in fast skeletal myofibrils, we suggest that cTn is less effective than fsTn in suppressing thin-filament activation. Several observations support this view. First it is known that the addition of cardiac regulatory proteins does not alter the V_{max} of the actin activated S1 ATPase (Tobacman & Adelstein, 1986; Williams *et al.* 1988; Tobacman *et al.* 2002), but addition of skeletal muscle Tn–tropomyosin decreases the V_{max} (Williams *et al.* 1988). In addition, the same data show that the cardiac regulatory proteins decrease the thin filament K_m, but the skeletal muscle proteins do not. In filaments regulated by fsTn, addition of Ca^{2+} does not produce a fully active ATPase unless strongly bound crossbridges (e.g. NEMS-1) are present or the ratio of actin to S1 is less than 2–4 (Williams *et al.* 1988). However, thin filaments controlled by cTn are almost fully activated in the presence of Ca^{2+}, independent of the ratio of actin to S1 (Butters *et al.* 1997; Tobacman *et al.* 2002). Furthermore, unlike the equal results found in the presence of Ca^{2+}, in the presence of EGTA myosin S1–thin filament binding is more inhibited by skeletal than by cardiac Tn (Maytum *et al.* 2003). Maytum and co-workers suggested this is due to a larger occupancy of the fully active or M state of the thin filament when cardiac rather than skeletal Tn is present, in EGTA. However, other interpretations are possible because their analysis used a model that accounts for some but not all major aspects of thin filament behaviour (Tobacman & Butters, 2000) and fails to explain the profound inhibition of actin-S1 phosphate release in the absence of Ca^{2+} (Heeley *et al.* 2002). Finally, in the case of skeletal muscle myofibrils containing exchanged cTn, there is also the added complication that the regulation by cTn occurs on thin filaments controlled by fast tropomyosin (fsTm). In that case, the effectiveness of the cooperativity may be altered by differences in the structures of fsTm and cardiac tropomyosin (i.e. different ratios of alpha and beta tropomyosins). It is possible that the cooperativity in the presence of fsTm/fsTn is therefore greater than that of fsTm/cTn present in the exchange experiments described above.

The ease with which Tn exchange occurs in myofibrils means that many of these hypotheses can be tested by using chimeras of Tn in which those areas hypothesized to control the behaviour of the muscle fibre have been altered. The relative ease of exchange of whole Tn also means that such exchanges can be made using hypertrophic cardiomyopathy mutant Tn (TnI and TnT forms) to test for effects of these mutations on Ca^{2+} sensitivity and cooperativity. However, additional work is needed using cardiac skinned myocytes or myofibrils to learn whether whole Tn exchange in these preparations is as effective as it is in skeletal muscle myofibrils. The ease with which such exchange can be made in skeletal muscle preparations opens the way to perform tests of cooperativity using different ratios of fsTn or cTn and permanently inhibited fsTn or cTn (Morris *et al.* 2001; Regnier *et al.* 2002).

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