Functional consequence of mutation in rat cardiac troponin T is affected differently by myosin heavy chain isoforms

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Cardiac troponin T (cTnT) is an essential component of the thin filament regulatory unit (RU) that regulates Ca^{2+} activation of tension in the heart muscle. Because there is coupling between the RU and myosin crossbridges, the functional outcome of cardiomyopathy-related mutations in cTnT may be modified by the type of myosin heavy chain (MHC) isoform. Ca^{2+} activation of tension and ATPase activity were measured in muscle fibres from normal rat hearts containing α-MHC isoform and propylthiouracil (PTU)-treated rat hearts containing β-MHC isoform. Muscle fibres from normal and PTU-treated rat hearts were reconstituted with two different mutations in rat cTnT; the deletion of Glu162 ($cTnT_{E162DEL}$) and the deletion of Lys211 ($cTnT_{K211DEL}$). α-MHC and β-MHC isoforms had contrasting impact on tension-dependent ATP consumption (tension cost) in $cTnT_{E162DEL}$ and $cTnT_{K211DEL}$ reconstituted muscle fibres. Significant increases in tension cost in α-MHC-containing muscle fibres corresponded to 17% ($P < 0.01$) and 23% ($P < 0.001$) when reconstituted with $cTnT_{E162DEL}$ and $cTnT_{K211DEL}$, respectively. In contrast, tension cost decreased when these two cTnT mutants were reconstituted in muscle fibres containing β-MHC; by approximately 24% ($P < 0.05$) when reconstituted with $cTnT_{E162DEL}$ and by approximately 17% ($P = 0.09$) when reconstituted with $cTnT_{K211DEL}$. Such differences in tension cost were substantiated by the mechano-dynamic analysis of cTnT mutant reconstituted muscle fibres from normal and PTU-treated rat hearts. Our observation demonstrates that qualitative changes in MHC isoform alters the nature of cardiac myofilament dysfunction induced by mutations in cTnT.

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Cardiac troponin T (cTnT) is a subunit of the troponin (Tn) complex, which binds Ca^{2+} in the heart muscle. cTnT plays an important role in regulating Ca^{2+}-activated tension by interacting with tropomyosin (Tm) and other thin filament regulatory proteins (Gordon *et al.* 2000). Several mutations in human cTnT (hcTnT) are known to be causal in familial hypertrophic cardiomyopathy (FHC) (Gomes & Potter, 2004; Tardiff, 2005). Two of these mutations in hcTnT are the deletion of Glu160 ($hcTnT_{E160DEL}$) and the deletion of Lys210 ($hcTnT_{K210DEL}$). $hcTnT_{E160DEL}$ mutation leads to ventricular hypertrophy and incidences of sudden death (Watkins *et al.* 1995) and the $cTnT_{K210DEL}$ mutation causes an early onset of ventricular dilatation and diminished contractile function, and frequently causes heart failure (Kamisago *et al.* 2001). The impact of mutations on the sequence of events that eventually lead to heart failure is not well understood. This issue takes on new significance in view of our recent findings that cTnT participates in regulating the dynamics of crossbridge (XB) cycling kinetics (Chandra *et al.* 2006), which suggests that mutations may interfere with important functions of cTnT.

A complication in the interpretation of some of the previous mutation studies is that such studies were undertaken with the use of the transgenic mouse (TG) that expressed a specific mutated sarcomeric gene in the heart. Although TG mouse models of FHC will continue to play an important role in the study of heart failure, some inherent limitations in the use of the mouse must always be noted (Kass *et al.* 1998). Many of the determinants of myocardial contractility in the rapidly contracting small ventricles of mouse hearts are significantly different from those of larger mammals (Li *et al.* 1997; Bers, 2000; Rice *et al.* 2000; Georgakopoulos & Kass, 2001; Stull *et al.* 2002). At the myofilament level, one of the major differences between the hearts of smaller and larger mammals is in

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the type of force generator, myosin heavy chain (MHC), present in the thick filament. Hearts of smaller animals contain predominantly the fast cycling α-MHC isoform, whereas the hearts of larger animals express the slow cycling β-MHC isoform (McNally *et al.* 1989). Given that the kinetic properties of MHC isoforms are the major determinants of the dynamic properties of left ventricular function, it is not surprising that the heart fails to adapt in certain forms of heart disease when the ratio of these two functionally diverse MHC isoforms is altered (Dillmann, 1980; Swynghhedauw, 1986; Miyata *et al.* 2000).

Coupling between the mechanical cycle (heart rate) and biochemical processes controlled by MHC and thin filament regulatory proteins (Rouslin & Broge, 1996; Campbell *et al.* 2004; Chandra *et al.* 2006) suggests an important link between myocardial contractility and heart muscle adaptation. This is consistent with the experimental observation that the spontaneous heart rate decreased significantly when the slower cycling β-MHC isoform was expressed in the mouse heart (Tardiff *et al.* 2000). A small increase in the level of β-MHC in the TG mouse hearts led to maladaptation of the heart as indicated by a significant systolic dysfunction (Tardiff *et al.* 2000), whereas a small increase in α-MHC in rat cardiac myocytes augmented power output (Herron & McDonald, 2002). Expression of nearly 40% of fast cycling α-MHC in rabbit hearts conferred protection against experimentally induced tachycardia (James *et al.* 2005).

Functional coupling between the thin filament regulatory unit (RU; Tm–Tn), and force-bearing cross-bridges (XBs) suggest that the left ventricular function may be modulated by the RU through an impact on XB kinetics (Razumova *et al.* 2000). Thus, the manner in which the heart adapts to changes in contractility depends not only on MHC isoform, but also on changes in the composition of the RU. Whether or not the mutation-dependent triggers in the thin filament are affected by differences in MHC isoforms is the focus of this study. Our study demonstrates that the consequence of a mutation in cTnT is dependent on the type of MHC isoform.

Methods

PCR mutagenesis

Full-length cDNA clones for adult rat cardiac troponin I (cTnI) and rat cardiac troponin C (cTnC) were isolated, as previously described (Chandra *et al.* 2006). Adult rat cTnT DNA (Jin & Lin, 1989) was a gift from Dr J. J. Lin (University of Iowa, IA, USA). All rat cTnT DNA constructs used in this study were tagged with the human *c-myc* epitope (Tardiff *et al.* 1998; Chandra *et al.* 2001). Amino acids Glu162 (E162) and Lys211 (K211) in our rat cTnT sequence correspond to E160 and K210 in the human cTnT sequence, respectively. To delete the codons for E162 and K211 in the rat cTnT DNA clone, we used a standard PCR mutagenesis protocol (Hi-fidelity, Roche). Two separate PCR reactions were carried out. Reaction 1 in each case was designed to amplify the DNA fragment from the 5′-end of the cTnT sequence to the E162 or the K211 region of cTnT and reaction 2 in each case was designed to amplify the DNA fragment from the E162 or the K211 region of cTnT to the 3′-end of the cTnT sequence. The 3′-end of PCR product from reaction 1 had a sequence overlap of 30 nucleotides at the 5′-end of the PCR product from reaction 2. Oilgonucleotide primers for the first set of $cTnT_{E162DEL}$ mutagenesis are as follows. Primer 1, 5′-GCAGAATTCAGGCATATGGAGCAGAAGCTGATCT-CCGAGGAGGACCTGTCTGACGCCGAGGAAGAGGT-G-3′; primer 2, 5′-CCTGTTCTCCTCCTCACGCCGGGC-CCTCTC-3′. Oilgonucleotide primers for the second set of $cTnT_{E162DEL}$ mutagenesis are as follows. Primer 1, 5′-GAGAGGGCCCGGCGTGAGGAGGAGAACAGG-3′; primer 2, 5′-TGCTGGAATTCAGGATCCCTATTTCCAA-CGCCCGGTGACTTT-3′. Oilgonucleotide primers for the first set of $cTnT_{K211DEL}$ mutagenesis are as follows. Primer 1, 5′-GCAGAATTCAGGCATATGGAGCAGAA-GCTGATCTCCGAGGAGGACCTGTCTGACGCCGAGG-AAGAGGTG-3′; primer 2, 5′-CCTCTCTGCCAGAATCT-TCTTCTTCTCTCG-3′. Oilgonucleotide primers for the second set of $cTnT_{K211DEL}$ mutagenesis are as follows. Primer 1, 5′-CGAGAGAAGAAGAAGATTCTGGCAGAG-AGG-3′; primer 2, 5′-TGCTGGAATTCAGGATCCCTAT-TTCCAACGCCCGGTGACTTT-3′. PCR-amplified DNA fragments from reactions 1 and 2 were gel-purified, mixed in equal proportions and the full-length cTnT products were amplified in the final PCR using appropriate 3′ and 5′ oligonucleotide primers. Final PCR products were digested with Nde I-BamH I restriction enzymes and subcloned into the pSBETa expression vector. Clones containing proper DNA inserts were sequenced.

Expression and purification of recombinant rat cardiac troponin subunits

Recombinant rat cTnT, mutant rat cTnT, rat cTnC and rat cTnI (all in pSBETa plasmid DNA) were expressed in BL21 (DE3) cells (Novagen) and purified as previously described (Chandra *et al.* 2006). All pure protein fractions were extensively dialysed against deionized water containing 15 mM β-mercaptoethanol, lyophilized and stored at -80°C.

Reconstitution of recombinant rat cTnT into detergent-skinned rat cardiac muscle fibre bundles

All procedures were performed in accordance with the guidelines laid down by the Washington State University Institutional Animal Care and Use Committee. Sprague-Dawley rats were anaesthetized with sodium

pentobarbital (50 mg kg^{-1} body weight), the hearts were rapidly excised and placed into ice-cold, high-relaxing solution containing (mM): MOPS 20 (pH 7.0), KCl 53, EGTA 10, $MgCl_2$ 6.81, Na_2ATP 5.35 and DTT 1.0. The total ionic strength was 150 mM. A cocktail of protease inhibitors containing 4 μM benzamidine-HCl, 5 μM bestatin, 2 μM E-64, 10 μM leupeptin, 1 μM pepstatin and 200 μM phenylmethylsulfonyl fluoride were included in the buffer. Young adult Sprague-Dawley rats were treated with propylthiouracil (PTU) in the drinking water (0.6 g l^{-1}) for 4–5 weeks. Shift from α- to β-MHC isoform expression in PTU-treated rat heart is well documented (Pope *et al.* 1980; Metzger *et al.* 1999; Herron *et al.* 2001; Rundell *et al.* 2004). Left ventricular papillary muscle fibre bundles from normal and PTU-treated rat hearts were isolated, dissected and detergent-skinned as previously described (Chandra *et al.* 2001). Exchange of rat muscle endogenous Tn complex with rat recombinant Tn complex containing mutant cTnT was based on the method previously described (Chandra *et al.* 2006). In brief, detergent-skinned muscle fibres were treated with the extraction solution containing cTnT and cTnI for approximately 3–4 h at room temperature (22°C) with stirring. The extraction buffer contained (mM): BES 50 (pH 7.0 at 20°C), KCl 180, 2,3-Butanedione monoxime (BDM) 10, EGTA 5, $MgCl_2$ 6.27, DTT 1.0 and $MgATP^{2-}$ 5, as well as 0.01% NaN_3 and a cocktail of protease inhibitors. After washing muscle fibres with the extraction buffer, Ca^{2+}-activated maximal tension was measured in solution at −log of free Ca^{2+} concentration (pCa) 4.3 to determine the residual tension. After reconstitution with cTnC (3 mg ml^{-1}), Ca^{2+}-activated tension and ATPase activity were measured at various pCa levels. All other details of reconstitution procedure are as previously described (Chandra *et al.* 2006). The composition of solutions of different pCa was calculated using the methods described by Fabiato & Fabiato (1979).

Measurement of tension-dependent ATP consumption in reconstituted rat cardiac muscle fibre bundles

Tension and ATPase activity were measured simultaneously at 20°C using the system previously described by Stienen *et al.* (1995) and de Tombe & Stienen (1995). Maximum activation buffer (pCa 4.3) contained (mM): potassium propionate 31, Na_2ATP 5.95, $MgCl_2$ 6.61, EGTA 10, $CaCl_2$ 10.11, BES 50 (pH 7.0), NaN_3 10, NADH 0.9 and phosphoenol pyruvate 10, with 4 mg ml^{-1} pyruvate kinase (500 U mg^{-1}), 0.24 mg ml^{-1} lactate dehydrogenase (870 U mg^{-1}) and 20 μM Diadenosine-5′ pentaphosphate (A_2P_5) as well as a cocktail of protease inhibitors. The ionic strength of the buffer was 180 mM. Detergent-skinned muscle fibre was attached to a motor and a force transducer using aluminium clips. Sarcomere length was measured, as previously described (de Tombe & Stienen, 1995). The resting sarcomere length was readjusted to 2.2 μm (after 2–3 cycles of full activation and relaxation) and the resting sarcomere length monitored using a He-Ne laser diffraction system. For ATPase measurements, near UV light (340 nm) was projected through the muscle chamber just below the muscle fibre, then split via a beam splitter (50/50) and detected at 340 nm (sensitive to change in [NADH]) and 400 nm (insensitive to [NADH]). The light intensity at 400 nm served as a reference signal. An analog divider and log amplifier produced a signal proportional to the amount of ATP consumed in the muscle chamber solution. ATP regeneration from ADP was coupled by enzymatic reactions as previously described (de Tombe & Stienen, 1995). These measurements allowed us to determine the tension cost in reconstituted muscle fibres (Campbell *et al.* 2004; Chandra *et al.* 2006).

Measurement of the rate constant of XB distortion in reconstituted rat cardiac muscle fibre bundles

XB distortion dynamics were determined in reconstituted muscle fibres as previously described (Campbell *et al.* 2004; Chandra *et al.* 2006). Previously, we have demonstrated a strong correlation between the rate constant of XB distortion (c) and the experimentally measured values of tension cost (Campbell *et al.* 2004; Chandra *et al.* 2006). The rate constant of XB distortion was determined (pCa 4.3) using buffer conditions as described for tension/ATPase measurements. In brief, muscle length-perturbation episodes of continuously varying sinusoidal frequencies (chirps) were delivered over time a period of 5 s to provide force and length information at frequencies between 1.0 and 40 Hz. Muscle fibre length (L_M) was commanded to change at constant amplitude (0.5% of L_M) during chirp perturbations and the resultant changes in force (ΔF) were measured. Experimentally measured change in force was fitted to a recruitment–distortion model to derive the rate constant of XB distortion. The differential equations and all other description of the model are as previously described (Campbell *et al.* 2004). In previous studies (Campbell *et al.* 2004; Chandra *et al.* 2006), the model was shown to fit the data well leaving very little residual error ($R^2 > 0.98$) and the values of c were estimated with less than 1% error.

Polyacrylamide gel electrophoresis

Protein samples for gel electrophoresis and Western blot analysis were prepared and run on 12.5% SDS-polyacrylamide gels, as previously described (Chandra *et al.* 1999*b*). For Western blot analysis, proteins were transferred onto the Polyvinylidene fluoride (PVDF) membrane and probed using an anti-mouse primary antibody against the human *c-myc* epitope or an antibody against hcTnT as previously described (Tardiff *et al.*

1998; Montgomery *et al.* 2001). MHC from normal and PTU-treated rat heart muscle preparations were separated on 8% SDS-polyacrylamide gels, as previously described (Rundell *et al.* 2004).

Data analysis

Data from the normalized pCa–tension measurements were fitted to the Hill equation using a non-linear least-square regression procedure to obtain the pCa_{50} ($-\log$ of free Ca^{2+} concentration required for half maximal activation) and the Hill coefficient (n). pCa_{50} and n were determined separately from each muscle fibre experiment and the values averaged. pCa_{50} values were converted to $[Ca^{2+}]$ to calculate the percentage increase or decrease in Ca^{2+} sensitivity of mutant cTnT reconstituted muscle fibres. Data were analysed using two-way ANOVA, with one factor being mutation ($cTnT_{E162DEL}$, $cTnT_{K211DEL}$ or cTnT) and the other factor being PTU treatment (control or PTU-treated). The interaction effect was evaluated to test the hypothesis that PTU treatment (i.e. change in MHC composition) altered the effect of mutation in cTnT. Subsequent *post hoc* multiple comparisons using a Holm-Bonferroni corrected t test (Glantz, 2002) were then conducted as indicated, to compare each mutation with wild-type cTnT. Statistical significance was assumed for $P < 0.05$. Data are expressed as means ± S.E.M.

Results

Shift from α- to β-MHC isoform expression in PTU-treated rat heart muscle fibres

PTU treatment to induce changes in MHC isoform expression has been well studied in the past (Pope *et al.* 1980; Metzger *et al.* 1999; Herron *et al.* 2001; Rundell *et al.* 2004). Such previous studies have shown that the only demonstrable change in myofilament proteins is that of MHC isoform, which shifts from predominantly α- to β-MHC in rat hearts after 4–5 weeks of treatment with PTU. To ascertain the shift in MHC isoform expression, we used a recently described method for the separation of MHC isoforms on SDS-PAGE (Rundell *et al.* 2004). Figure 1 demonstrates that there is a complete shift from α- to β-MHC isoform in the PTU-treated rat heart muscle preparations. The predominant MHC isoform present in normal rat heart preparations is α-MHC (lane 1 in Fig. 1), which shifts to nearly 100% β-MHC in rat hearts treated with PTU (lanes 2 and 3 in Fig. 1).

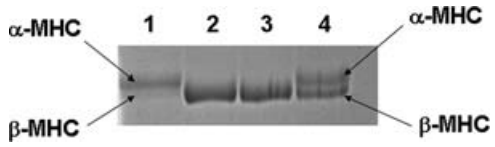

Figure 1. SDS-PAGE (8%) analysis of rat cardiac muscle fibre proteins from normal and PTU-treated rat hearts
Rats were made hypothyroid by feeding with tap water containing 0.6 g l^{-1} PTU for approximately 4–5 weeks. SDS-PAGE was performed as previously described (Rundell *et al.* 2004). Lane 1, muscle protein preparation from normal rat heart; lanes 2 and 3, muscle protein preparations from two different PTU-treated rat hearts; lane 4, mixture of normal and PTU-treated rat heart muscle preparations to highlight the size differences between α- and β-MHC isoforms. Lanes 2 and 3 demonstrates a complete shift to β-MHC isoform in PTU-treated rat heart muscle preparations. SDS gels were run at least once and stained with Coomassie blue as described before (Rundell *et al.* 2004).

Exchange of mutant rat cardiac Tn into detergent-skinned rat cardiac muscle fibre bundles with different MHC isoforms

All cTnT constructs used in this study were tagged with the human *c-myc* epitope (Tardiff *et al.* 1998; Chandra *et al.* 2001). Tn exchange in rat cardiac muscle fibre bundles was performed as previously described (Chandra *et al.* 2006). We have previously demonstrated that the endogenous Tn complex is removed as a whole (Chandra *et al.* 1999*a*, 2006) by the exogenously added cTnT. To demonstrate the incorporation of wild-type cTnT (WT-cTnT) and mutant cTnT constructs in

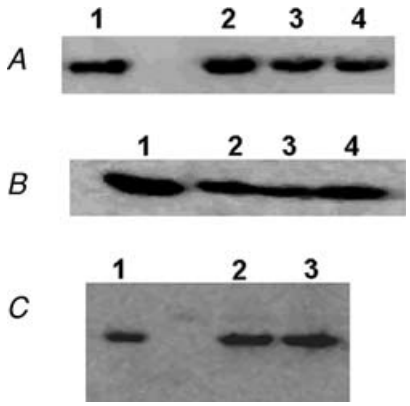

Figure 2. Western blot analysis of rat cardiac muscle fibres reconstituted with recombinant *c-myc*-tagged cTnT constructs
An antibody against either human *c-myc* epitope or human cTnT was used. Western blot analysis of reconstituted muscle fibres from normal rat hearts with antibody against human *c-myc* epitope (*A*), from PTU-treated rat hearts with antibody against human *c-myc* epitope (*B*) and from normal and PTU-treated rat hearts with antibody against hcTnT (*C*). *A* and *B*, lane identifications are as follows: lane 1, pure recombinant *c-myc* WT-cTnT; lane 2, muscle fibres reconstituted with *c-myc* WT-cTnT + cTnI + cTnC; lane 3, muscle fibres reconstituted with *c-myc* $cTnT_{E162DEL}$ + cTnI + cTnC; lane 4, muscle fibres reconstituted with *c-myc* $cTnT_{K211DEL}$ + cTnI + cTnC. No immunoreactivity was evident (data not shown) for the endogenous cTnT in reconstituted muscle fibres from both normal and PTU-treated rat cardiac muscle fibres. *C*, lane 1, pure recombinant cTnT; lane 2, muscle fibres from normal rat heart; lane 3, muscle fibres from PTU-treated rat heart. Lanes 2 and 3 demonstrate that there is only one adult cTnT isoform in these adult rat heart muscle fibres. Typically, Western blot was performed once or twice to detect different forms of cTnT.

muscle fibres from normal and PTU-treated rat hearts, Western blot (Fig. 2*A* and *B*) was performed with the antibody against human *c-myc* epitope. In Fig. 2*A* and *B*, immunoreactivity is evident in lanes 2, 3 and 4, which correspond to muscle fibres reconstituted with WT-cTnT, $cTnT_{E162DEL}$ and $cTnT_{K211DEL}$, respectively. As we have demonstrated previously, little or no immunoreactivity was evident (data not shown) when these reconstituted muscle fibres were probed with the antibody against cTnT, which demonstrated that the endogenous cTnT was replaced by *c-myc*-tagged cTnT. Furthermore, when these WT-cTnT/mutants + cTnI reconstituted muscle fibres were tested in pCa 4.3 solution, Ca^{2+}-activated residual tension was minimal ($\sim$1.0 mN mm^{-2}). We have previously demonstrated that the Tn exchange protocol had no major impact on myofilament Ca^{2+} sensitivity, cooperativity, Ca^{2+}-activated maximal tension and ATPase activity (Chandra *et al.* 2006). As previously observed by other investigators (Saggin *et al.* 1988; Fitzsimons *et al.* 1998; Metzger *et al.* 1999), Western blot analysis of heart muscle fibre preparations from normal and PTU-treated rat hearts (Fig. 2*C*) showed no shift in cTnT isoform expression.

Table 1. Normalized pCa–tension relationship in detergent-skinned rat heart muscle fibre bundles reconstituted with recombinant *c-myc*-tagged rat cTnT constructs

	WT-cTnT	E162DEL	K211DEL
Normal rat hearts			
pCa_{50}	5.55 ± 0.01	5.64 ± 0.02*	5.46 ± 0.02*
n	3.9 ± 0.2	2.8 ± 0.1*	4.2 ± 0.2
PTU-treated rat hearts			
pCa_{50}	5.54 ± 0.02	5.66 ± 0.03*	5.47 ± 0.03*
n	4.1 ± 0.2	3.1 ± 0.1*	4.5 ± 0.3

Values are means ± S.E.M. Data from normalized pCa–tension measurements were fitted to the Hill equation by using a non-linear least square regression procedure to derive pCa_{50} and the Hill coefficient (*n*). pCa_{50} and *n* were determined separately from each muscle fibre experiment and the values averaged. WT-cTnT represents WT-cTnT + cTnI + cTnC reconstituted muscle fibres; E162DEL represents $cTnT_{E162DEL}$ + cTnI + cTnC reconstituted muscle fibres and K211DEL represents $cTnT_{K211DEL}$ + cTnI + cTnC reconstituted muscle fibres. Number of determinations is at least eight for each. Statistical differences were analysed using two-way ANOVA as described in the Methods. *$P < 0.05$.

Impact of $cTnT_{E162DEL}$ and $cTnT_{K211DEL}$ mutants on muscle fibre bundles from normal and PTU-treated rat hearts

In reconstitution experiments, we used *c-myc*-tagged WT-cTnT as the control. In previous studies, we and others have demonstrated that the presence of *c-myc* epitope in cTnT has no significant effect on Ca^{2+} activation of tension and ATPase activity in detergent-skinned muscle preparations (Chandra *et al.* 2001; Montgomery *et al.* 2001) or intact heart function (Tardiff *et al.* 1998; Ertz-Berger *et al.* 2005). In the first line of experiments, the effects of WT-cTnT, $cTnT_{E162DEL}$ and $cTnT_{K211DEL}$ on Ca^{2+} activation of myofilaments were tested in cardiac muscle fibres from normal rat hearts, which contained predominantly the α-MHC isoform. When compared to WT-cTnT + cTnI + cTnC reconstituted muscle fibres, myofilament Ca^{2+} sensitivity, as measured by pCa_{50} values, increased significantly (23%) in $cTnT_{E162DEL}$ + cTnI + cTnC reconstituted muscle fibres and decreased significantly (23%) in $cTnT_{K211DEL}$ + cTnI + cTnC reconstituted muscle fibres (Fig. 3 and Table 1).

Next, we tested the effects of $cTnT_{E162DEL}$ and $cTnT_{K211DEL}$ on Ca^{2+} activation of cardiac muscle fibres from PTU-treated rat hearts, which contained nearly 100% β-MHC isoform. The impact of mutant cTnTs on Ca^{2+} sensitivity of muscle fibres containing β-MHC isoform (Fig. 4) was similar to that of muscle fibres from normal rat hearts which contained α-MHC isoform (Fig. 3). Ca^{2+} sensitivity of $cTnT_{E162DEL}$ + cTnI + cTnC reconstituted muscle fibres increased significantly (32%) and that of $cTnT_{K211DEL}$ + cTnI + cTnC reconstituted muscle fibres decreased significantly (17%). pCa_{50} values and the Hill coefficient values from the Hill fits are listed in Table 1. Data presented in Table 1 demonstrate that rat cardiac myofilament Ca^{2+} sensitivity is not MHC isoform dependent. $cTnT_{E162DEL}$ mutant decreased the Hill coefficient values in both α-MHC- and β-MHC-containing cardiac muscle fibres.

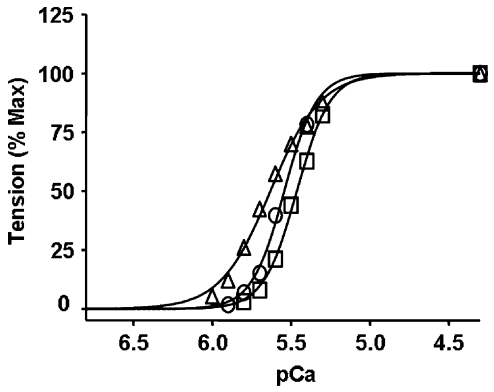

Figure 3. Normalized pCa–tension relations in reconstituted muscle fibres from normal rat hearts
Ca^{2+}-activated tension was measured at different pCa as described in the Methods for WT-cTnT + cTnI + cTnC (○), $cTnT_{E162DEL}$ + cTnI + cTnC (△) and $cTnT_{K211DEL}$ + cTnI + cTnC (□). pCa_{50} and the Hill coefficient values (*n*) are listed in Table 1. Standard error bars are smaller than symbols. Number of determinations is at least eight for each.

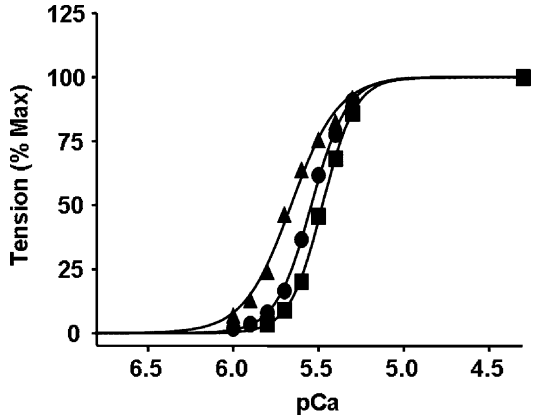

Figure 4. Normalized pCa–tension relations in reconstituted muscle fibres from PTU-treated rat hearts
Ca^{2+}-activated tension was measured at different pCa as described in the Methods for WT-cTnT + cTnI + cTnC (●), $cTnT_{E162DEL}$ + cTnI cTnC (▲) and $cTnT_{K211DEL}$ cTnI cTnC (■). pCa_{50} and the Hill coefficient values (*n*) are listed in Table 1. Standard error bars are smaller than symbols. Number of determinations is at least eight for each.

Compared to muscle fibres from normal rat hearts, Ca^{2+}-activated maximal tension was not affected significantly in muscle fibres from PTU-treated rat hearts. In this study, both the interaction effect ($P = 0.23$) and the main effect for muscle fibres from normal *versus* PTU-treated groups ($P = 0.33$) were not significant (Fig. 5*A*). Our observation is substantiated by two independent previous studies which demonstrated that a shift from α- to β-MHC in rat cardiac muscle had no significant impact on Ca^{2+}-activated maximal tension (Metzger *et al.* 1999; Rundell *et al.* 2004). However, the effect of $cTnT_{E162DEL}$ mutation on maximal tension was significant ($P < 0.001$). Ca^{2+}-activated maximal tension increased significantly when muscle fibres containing either α-MHC or β-MHC were reconstituted with the $cTnT_{E162DEL}$ mutant (Fig. 5*A*). On the other hand, $cTnT_{K211DEL}$ mutant had no significant effect on Ca^{2+}-activated maximal tension in muscle fibres containing either α-MHC or β-MHC (Fig. 5*A*).

In addition to the effect of cTnT mutants on myofilament Ca^{2+} sensitivity, another notable observation was the impact of $cTnT_{E162DEL}$ and $cTnT_{K211DEL}$ on Ca^{2+}-activated maximal ATPase activity in muscle fibres containing α-MHC and β-MHC (Fig. 5*B*). The interaction effect for Ca^{2+}-activated maximal ATPase activity was significant ($P = 0.007$), which indicated that β-MHC altered the effect of mutation in cTnT. ATPase activity increased when $cTnT_{E162DEL}$ and $cTnT_{K211DEL}$ were reconstituted into muscle fibres containing α-MHC (Fig. 5*B*). ATPase activity was approximately 23% higher ($P < 0.001$) in $cTnT_{E162DEL}$ reconstituted fibres and 11% higher ($P = 0.2$) in $cTnT_{K211DEL}$ reconstituted fibres. In contrast, there was a slight trend towards a decrease (non-significant) in Ca^{2+}-activated maximal ATPase activity when these two cTnT mutants were reconstituted into muscle fibres containing β-MHC isoform (Fig. 5*B*). ATPase activity was approximately 7% lower ($P = 0.2$) for $cTnT_{E162DEL}$ reconstituted fibres and 18% lower ($P = 0.5$) for $cTnT_{K211DEL}$ reconstituted fibres.

Because β-MHC altered the effect of mutation in cTnT on ATPase activity, we wanted to test whether

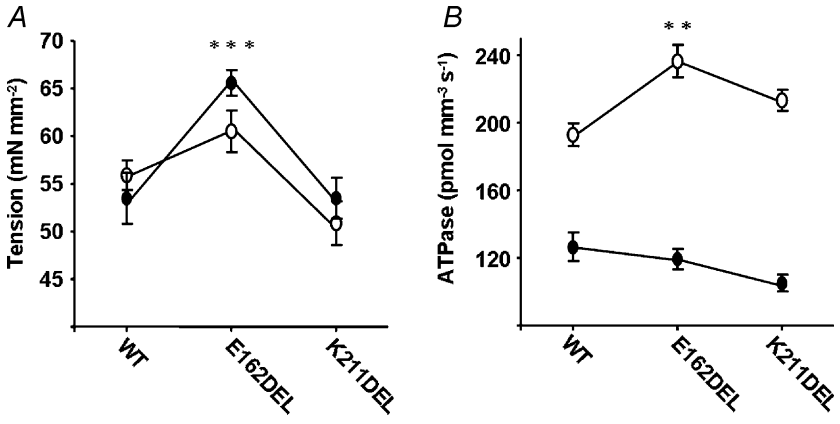

Figure 5. Ca^{2+}-activated maximal tension and ATPase activity in reconstituted muscle fibres from normal and PTU-treated rat hearts
Ca^{2+}-activated maximal tension (*A*) and ATPase activity (*B*) were measured in maximal activation buffer (pCa 4.3). For both A and B (○) represents experiments in muscle fibres from normal rat hearts and (●) represents experiments in muscle fibres from PTU-treated rat hearts. For both *A* and *B*, WT represents WT-cTnT + cTnI + cTnC reconstituted muscle fibres; E162DEL represents $cTnT_{E162DEL}$ + cTnI + cTnC reconstituted muscle fibres and K211DEL represents $cTnT_{K211DEL}$ + cTnI + cTnC reconstituted muscle fibres. Number of determinations is at least eight for each. Data were analysed using two-way ANOVA as described in the Methods. Subsequent *post hoc* multiple comparisons were made using a Holm-Bonferroni corrected *t* test (Glantz, 2002) to compare each mutation *versus* WT within PTU-treated and normal groups. $^{**}P < 0.01$, $^{***}P < 0.001$.

α- and β-MHC isoforms have different effects on the mutation-induced impact on tension-dependent ATP consumption. Previous TG mouse studies have suggested a causative link between cTnT mutation-induced impact on altered cardiac muscle energetics and cardiac phenotypes (Montgomery *et al.* 2001; Javadpour *et al.* 2003; Chandra *et al.* 2005). We simultaneously measured steady-state isometric tension and ATPase activity at different pCa in reconstituted muscle fibres from normal and PTU-treated groups. Data were fitted using a linear regression analysis as shown in Figs 6 and 7. The slope of the tension–ATPase relationship is a measure of the amount of ATP hydrolysed for a given amount of tension produced (tension cost). Mean slope values from linear fits of several muscle fibre bundles reconstituted with WT-cTnT + cTnI + cTnC, $cTnT_{E162DEL}$ + cTnI + cTnC and $cTnT_{K211DEL}$ + cTnI + cTnC are summarized in Fig. 8*A*. The interaction effect for tension cost was significant ($P = 0.0001$), which demonstrated that β-MHC altered the effect of mutation in cTnT. Tension cost increased significantly by approximately 17% ($P < 0.01$) when α-MHC-containing muscle fibres were reconstituted with $cTnT_{E162DEL}$ and by approximately 23% ($P < 0.001$) when reconstituted with $cTnT_{K211DEL}$. In contrast, tension cost decreased when these two cTnT mutants were reconstituted in muscle fibres containing β-MHC (Fig. 8*A*); by approximately 24% ($P < 0.05$) when reconstituted with $cTnT_{E162DEL}$ and by approximately 17% ($P = 0.09$, non-significant) when reconstituted with $cTnT_{K211DEL}$.

Impact of $cTnT_{E162DEL}$ and $cTnT_{K211DEL}$ mutants on the rate constant of XB distortion in muscle fibre bundles from normal and PTU-treated rat hearts

Results from the XB distortion dynamic measurements allowed us to validate the changes in tension cost as described above. XB distortion rate constant (c), estimated from model fitting, has a strong dependence on the kinetics of XB detachment. In previous studies (Campbell *et al.* 2004; Chandra *et al.* 2006), we have demonstrated that there is a strong correlation between c and the experimentally measured values of tension cost (ATPase/tension). Because the ratio of ATPase/tension is proportional to the rate constant of XB detachment (Brenner & Eisenberg, 1986), changes in the model-estimated c and tension cost can be correlated with changes in XB detachment kinetics.

It is interesting to note that model-estimated values of c varied in a manner similar to the experimentally derived values of tension cost (Fig. 8). The interaction effect for c was significant ($P = 0.017$), which demonstrated that β-MHC altered the effect of mutation in cTnT. For example, c increased significantly in α-MHC-containing normal muscle fibres by approximately 29% ($P < 0.02$) when reconstituted with $cTnT_{E162DEL}$ and by approximately 43% ($P < 0.001$) when reconstituted with $cTnT_{K211DEL}$ (Fig. 8*A*). In contrast, there was a small but non-significant decrease in c when these two cTnT mutants were reconstituted into muscle fibres containing β-MHC. For example, c in β-MHC-containing muscle fibres decreased by

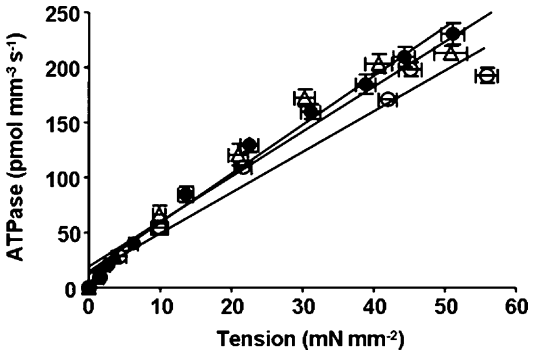

Figure 6. Relationship between tension and ATPase activity in reconstituted rat cardiac muscle fibres from normal rat hearts
Simultaneous measurement of tension and ATPase activity at different pCa are as described in the Methods. Averaged data from several experiments were fitted using a linear regression analysis. Departure from linearity was found to be non-significant in all cases. WT-cTnT + cTnI + cTnC reconstituted muscle fibres (○), $R^2 = 0.98$; $cTnT_{E162DEL}$ + cTnI + cTnC reconstituted muscle fibres (●), $R^2 = 0.97$; and $cTnT_{K211DEL}$ + cTnI + cTnC reconstituted muscle fibres (△), $R^2 = 0.97$. Comparison of mean slope values (tension cost) between reconstituted muscle fibres from normal and PTU-treated hearts are shown in Fig. 8A. Number of determinations is at least eight for each.

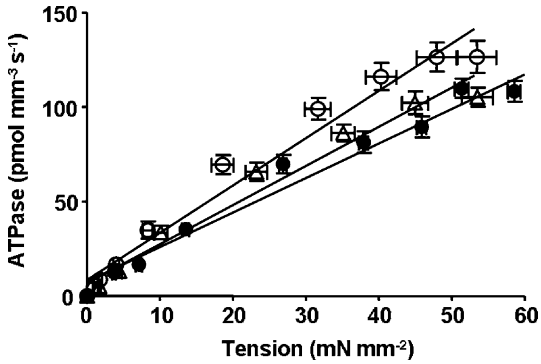

Figure 7. Relationship between tension and ATPase activity in reconstituted rat cardiac muscle fibres from PTU-treated rat hearts
Simultaneous measurement of tension and ATPase activity at different pCa are as described in the Methods. Averaged data from several experiments were fitted using a linear regression analysis. Departure from linearity was found to be non-significant in all cases. WT-cTnT + cTnI + cTnC reconstituted muscle fibres (○), $R^2 = 0.95$; $cTnT_{E162DEL}$ + cTnI + cTnC reconstituted muscle fibres (●), $R^2 = 0.96$; and $cTnT_{K211DEL}$ + cTnI + cTnC reconstituted muscle fibres (△), $R^2 = 0.97$. Comparison of mean slope values (tension cost) between reconstituted muscle fibres from normal and PTU-treated hearts are shown in Fig. 8A. Number of determinations is at least eight for each.

approximately 15% ($P = 0.9$) when reconstituted with $cTnT_{E162DEL}$ and by approximately 11% ($P = 0.5$) when reconstituted with $cTnT_{K211DEL}$ (Fig. 8*B*).

Discussion

The existence of multiple cTnT mutations and various cardiac phenotypes in FHC suggests that the primary molecular trigger in the sequence of events, which eventually leads to heart complications, varies with the nature of the mutation. Because there is functional coupling between the RU and XBs, it is expected that qualitative changes in MHC isoform will alter the nature of primary myofilament dysfunction induced by the mutation in cTnT. In view of our recent observation that cTnT participates in regulating the dynamics of XB cycling kinetics (Chandra *et al.* 2006), we wanted to test whether the effects of cTnT mutants on myofilament activation are modulated by changes in MHC isoform. To test our hypothesis, Ca^{2+} activation of tension, ATPase activity and myofilament dynamics were measured in mutant cTnT reconstituted muscle fibres from normal rat hearts (containing α-MHC) and PTU-treated rat hearts (containing β-MHC).

Effect of mutations in cTnT and MHC isoforms on rat cardiac myofilament Ca^{2+} sensitivity

The directionality of changes in myofilament Ca^{2+} sensitivity induced by the mutations in cTnT was not significantly affected by the type of MHC isoform. For example, $cTnT_{E162DEL}$ induced a significant increase in myofilament Ca^{2+} sensitivity and $cTnT_{K211DEL}$ induced a significant decrease in Ca^{2+} sensitivity, irrespective of the type of MHC isoform present in the myofilament. These observations demonstrate that the myofilament Ca^{2+} sensitivity is not MHC isoform-dependent and that the Ca^{2+}-sensitive step that regulates the transition of the RU from the non-permissive to permissive states (McKillop & Geeves, 1993) is not affected by the differences between α- and β-MHC isoforms. Because the extent of subsequent non-permissive to permissive RU transition depends on strong XB binding, a step in this process is differentially affected by α- and β-MHC isoforms to bring about differences in tension cost. Previous transgenic and reconstitution studies have also shown that $cTnT_{E162DEL}$ increases myofilament Ca^{2+} sensitivity (Chandra *et al.* 2005) and $cTnT_{K211DEL}$ decreases myofilament Ca^{2+} sensitivity (Morimoto *et al.* 2002). However, in contrast to our previous TG mouse studies (Chandra *et al.* 2005), we observed a significant decrease in the Hill coefficient values when $cTnT_{E162DEL}$ was reconstituted into either α-MHC- or β-MHC-containing rat cardiac muscle fibres. A possible explanation is that our previous study used a TG mouse model that expressed approximately 50% of the mutant cTnT in the myofilament, whereas in this study we used a reconstitution protocol to incorporate mutant cTnT protein in the thin filament. The presence of both WT-cTnT and mutant cTnT in the thin filament of heterozygous mouse may be responsible for

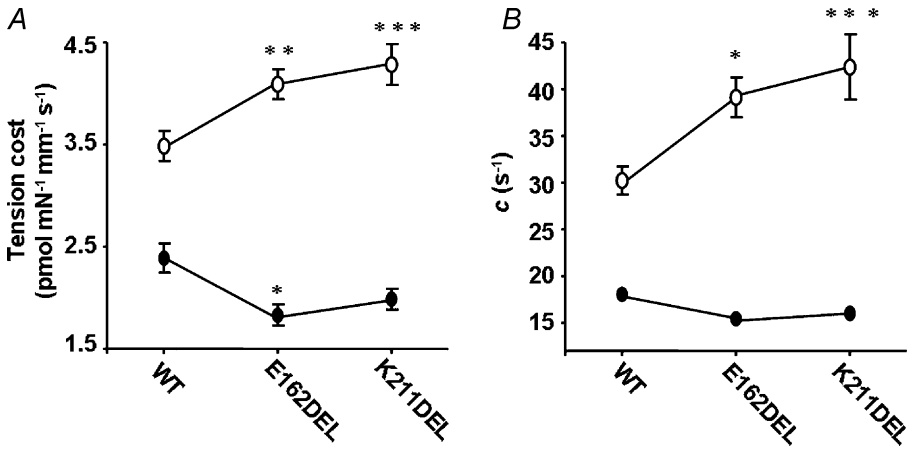

Figure 8. α- and β-MHC-induced differences in tension cost and XB distortion rate constant (*c*)
Tension cost was determined (*A*) from the slopes of tension–ATPase relationships as shown in Figs 6 and 7. The rate constant of distortion (c) was determined (*B*) as described in the Methods. For A and B (○) represents muscle fibres from normal rat hearts and (●) represents muscle fibres from PTU-treated rat hearts. For both *A* and *B*, WT represents WT-cTnT + cTnI + cTnC reconstituted muscle fibres; E162DEL represents $cTnT_{E162DEL}$ + cTnI + cTnC reconstituted muscle fibres and K211DEL represents $cTnT_{K211DEL}$ + cTnI + cTnC reconstituted muscle fibres. Number of determinations is at least eight for each. Standard error bars are smaller than symbols in some cases. Data were analysed using two-way ANOVA as described in the Methods. Subsequent *post hoc* multiple comparisons were made using a Holm-Bonferroni corrected *t* test (Glantz, 2002) to compare each mutation *versus* WT within PTU-treated and normal groups. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

differences in myofilament cooperativity that we observed in our earlier study (Chandra *et al.* 2005). Although $cTnT_{E162DEL}$ reconstituted muscle fibres showed decreased myofilament cooperativity as assessed by the changes in Hill coefficient values, myofilament Ca^{2+} sensitivity increased significantly. The Hill coefficient is a composite index of myofilament cooperativity that includes cooperativity in Ca^{2+} binding to the RU and near-neighbour interactions, including RU–RU, XB–RU and XB–XB interactions (Razumova *et al.* 2000). Such near neighbour interactions may also influence Ca^{2+} binding to the RU in ways that are not well understood.

Effect of MHC isoform on cTnT mutation-induced changes in tension cost

$cTnT_{E162DEL}$ and $cTnT_{K211DEL}$ significantly increased tension cost in α-MHC-containing muscle fibres (Fig. 8*A*). In our previous study, TG mouse cardiac muscle fibres expressing $cTnT_{E162DEL}$ showed no significant increase in Ca^{2+}-activated maximal tension, but demonstrated a significant increase in Ca^{2+}-activated maximal ATPase activity. Because the slope of tension–ATPase relationship (tension cost) has been proposed as a measure of the rate of XB detachment (Brenner & Eisenberg, 1986), we concluded in our previous TG mouse study that $cTnT_{E162DEL}$-induced increase in tension cost was due to an increase the rate of XB detachment (g). One way to validate this observation is to compare changes in tension cost with changes in rate constant of XB distortion (c), which is independently estimated by fitting the force response of small amplitude muscle length changes to the recruitment–distortion model (Campbell *et al.* 2004; Chandra *et al.* 2006). As demonstrated previously (Campbell *et al.* 2004; Chandra *et al.* 2006), model-estimated c has a strong dependence on g (tension cost), because XB distortion dynamics are principally determined by XB detachment kinetics (Campbell *et al.* 2004). The directionality of changes we observed in tension cost coincided with changes in c (Fig. 8). Therefore, our data suggest that the functional outcome of mutations in TnT varies depending on the type of MHC isoform present. Moreover, our observations also suggest that XB detachment kinetics are modulated by changes in cTnT structure (Chandra *et al.* 2005, 2006).

Structural changes in the RU have an impact on XB cycling kinetics

Differences in the composition of the RU have been linked to more prominent XB-dependent activation of force development in cardiac myofilaments (Fitzsimons *et al.* 2001; Chandra *et al.* 2006). Mutation in cTnT has been shown to increase XB detachment rate in mouse cardiac muscle fibres (Chandra *et al.* 2005). The pivotal location of cTnT in the thin filament (Gordon *et al.* 2000) may provide some clues as to how cTnT may modulate XB cycling kinetics. cTnT may modify the state of actin monomers in such a way that the kinetics of XB binding to actin is altered. cTnT may alter the strength of XB binding to actin in an indirect manner via its effect on TnI and Tm (Dahiya *et al.* 1994) or in a direct manner by its interaction with actin (Heeley & Smillie, 1988; Dahiya *et al.* 1994).

Because the strengths of cooperative interactions between the RU and XBs are influenced by multiple interactions within the myofilament (Greene & Eisenberg, 1980; Razumova *et al.* 2000), the dynamics of XB kinetic processes are likely to be affected by changes occurring within MHC as well as the RU. Just as Tn influences Tm binding to actin filaments, strongly bound XBs also modulate actin–RU interactions (Tobacman & Butters, 2000; Smith *et al.* 2003). Therefore, multiple amino acid sequence differences that exist in the head region of α- and β-MHC isoforms (McNally *et al.* 1989) are likely to have different effects on how XBs interact with actin and, importantly, how such interactions impact the feedback effect of XBs on the RU. In conclusion, our data demonstrate that the functional consequence of a mutation in cTnT is affected differently by the type of MHC isoform present in the thin filament. Therefore, our study suggests that some caution may be necessary when interpreting data from TG mouse studies that use mutant myofilament proteins. A limitation of this study is that we used a reconstitution method to incorporate mutant cTnT into cardiac myofilaments. The level of mutant protein expressed in human cardiomyopathy is one of the major 'unknowns' in the field. It is generally believed that the relative amount of mutant sarcomeric protein in human hearts may vary from very low to high levels. Therefore, conclusions drawn from our observations may not be applicable in some cases.

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