Cardiac Troponin T Isoforms Affect the Ca$^{2+}$ Sensitivity and Inhibition of Force Development

INSIGHTS INTO THE ROLE OF TROPONIN T ISOFORMS IN THE HEART*

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Aldrin V. Gomes‡, Georgianna Guzman, Jiaju Zhao, and James D. Potter§
From the Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, Florida 33101

At least four isoforms of troponin T (TnT) exist in the human heart, and they are expressed in a developmentally regulated manner. To determine whether the different N-terminal isoforms are functionally distinct with respect to structure, Ca$^{2+}$ sensitivity, and inhibition of force development, the four known human cardiac troponin T isoforms, TnT1 (all exons present), TnT2 (missing exon 4), TnT3 (missing exon 5), and TnT4 (missing exons 4 and 5), were expressed, purified, and utilized in skinned fiber studies and in reconstituted actomyosin ATPase assays. TnT3, the adult isoform, had a slightly higher $\alpha$-helical content than the other three isoforms. The variable region in the N terminus of cardiac TnT was found to contribute to the determination of the Ca$^{2+}$ sensitivity of force development in a charge-dependent manner; the greater the charge the higher the Ca$^{2+}$ sensitivity, and this was primarily because of exon 5. These studies also demonstrated that removal of either exon 4 or exon 5 from TnT increased the cooperativity of the $p$Ca force relationship. Troponin complexes reconstituted with the four TnT isoforms all yielded the same maximal actin-tropomyosin-activated myosin ATPase activity. However, troponin complexes containing either TnT1 or TnT2 (both containing exon 5) had a reduced ability to inhibit this ATPase activity when compared with wild type troponin (which contains TnT3). Interestingly, fibers containing these isoforms also showed less relaxation suggesting that exon 5 of cardiac TnT affects the ability of Tn to inhibit force development and ATPase activity. These results suggest that the different N-terminal TnT isoforms would produce different functional properties in the heart that would directly affect myocardial contraction.

Several troponin T (TnT)$^{3}$ isoforms are encoded by cardiac muscle TnT genes through developmentally regulated alternative mRNA splicing (1, 2). Four isoforms of cardiac TnT (CtTnT) are expressed in human cardiac muscle through combinatorial alternative splicing of two 5′ exons (Fig. 1, exons 4 and 5) in a developmentally regulated manner (1, 2). CtTnT and fast skeletal TnT differ significantly in their N-terminal regions. First, the skeletal isoforms are shorter by about 30 amino acid residues compared with the cardiac isoforms, and the number of acidic residues (mainly glutamic acid) is much higher in the cardiac isoforms than in the skeletal isoforms. A comparison of CtTnTs from different species (human, bovine, rabbit, and chicken, see Fig. 2) shows near identity of the N-terminal 13 amino acid residues (exons 2 and 3), with most of the differences occurring in the next 60 residues (exons 4–8), whereas the next 220 residues (exons 9–18) show high homology. These differences suggest that the N-terminal region of CtTnT has possible tissue-specific and developmentally specific role(s).

Various studies (1, 3) have demonstrated different levels of HCTnT isoform expression in normal and diseased hearts. The HCTnT1 and HCTnT2 isoforms are expressed in the fetal heart, with HCTnT2 being expressed at a very low level. During perinatal heart development, the expression level of HCTnT1 decreases, and the expression of HCTnT3 increases and becomes the only HCTnT isoform in the normal adult heart. HCTnT4 is also expressed in the fetal heart and is re-expressed in the failing adult heart (1, 2, 4). The effects of disease and development on HCTnT isoform expression strongly suggest that these isoforms are important contributors to the vascular function of the heart, helping the heart to adapt to changes in workload. Whether these changes in CtTnT isoform expression are an adaptive compensatory response to heart failure is not known.

Because TnT is one of the major regulatory proteins in the thin filament (5), isoform switching of TnT during heart development may be important in the Ca$^{2+}$ regulation of myocardial contraction. However, the physiological relevance of HCTnT isoforms in human heart is still poorly understood partially due to the fact that TnT isoform expression is different between vertebrates. Extensive studies with human cardiac tissue have not been possible because of the difficulty of timely processing of the tissue and the limited availability of normal heart tissue.

Altered TnT isoform expression has been linked to cardiac hypertrophy, diabetes, and congenital heart disease (6, 7). It has been suggested that the changes in TnT isoform expression that occur in heart failure are important in maintaining contractility in the diseased heart. Failed hearts from patients with human idiopathic cardiomyopathy also show HCTnT alterations (1). In guinea pigs subjected to chronic pressure overload with aortic banding to induce ventricular hypertrophy, four distinct TnT isoforms were detected that underwent changes in the relative proportions of the different isoforms during hypertrophy (7). Tobacman and Lee (8, 9) investigated...
the two known bovine CTnT isoforms, BCTnT1 and BCTnT2, that differ from each other by the presence or absence of residues 15–19 (Fig. 2). Differences in the Ca\(^{2+}\) regulation of acto-S1 ATPase were found for these isoforms with BCTnT2 having the highest Ca\(^{2+}\) sensitivity. These results suggest that the N terminus of TnT affects the interaction of Tn with Ca\(^{2+}\).

However, some studies (10) were unable to find a correlation between the myofibrillar Ca\(^{2+}\) sensitivity of force in failing hearts and fetal TnT content.

The main goal of the present study was to gain insight into the role of the N-terminal hypervariable region in CTnT in cardiac muscle contraction. To address this question, we have characterized four HCTnT isoforms (Fig. 1) using two in vitro functional assays to determine whether any differences exist between the wild type (adult isoform) and other isoforms of TnT. In order to better understand the structure-function relationship of the alternatively spliced N-terminal region of HCTnT, we have also determined the secondary structure of the HCTnT isoforms. This is the first report of purified HCTnT isoforms being used in reconstituted systems and skinned fiber studies. Our results suggest that this alternatively spliced N-terminal region is an important modulator of the Ca\(^{2+}\) sensitivity of force development and also affects the overall secondary structure of the different HCTnT isoforms. Actomyosin ATPase assays also show that the alternatively spliced regions in HCTnT are important for regulating inhibitory ATPase activity.

**MATERIALS AND METHODS**

**Mutation, Expression, and Purification of HCTnT Isoforms—** Human CTnT isoforms and mutants were made using a sequential overlapping PCR method (11). The adult isoform of HCTnT (HCTnT3), which was already cloned in this laboratory (12), was used as a template for PCR with primers designed to introduce or delete the 15- or 30-bp regions which in various combinations results in isoforms 1, 2, and 4. HCTnT1 was created by adding a 30-bp region (exon 5) using the following primers: 3'/H11032 CTCCTCCTGCTCGTCTTCGTCCTCTCCAGTCCTCCTCTTCTTCAACAGCTGCTTCTTCCTGCTC; 5'/H11032 ACTAGTTCTAGAGGAGGACTGGAGAGAGGACGAAGACGAGCAGGAGGAGGCAGCGGGAAGAG. HCTnT1 was then used to create HCTnT2 by deleting the 15-bp region (exon 4) using the following primers: 3'/H11032 GTCCTCCTCTCCTTCCTCCTCGTACTC; 5'/H11032 GAGGAGCAGGAAGAAGAAGGAGGACTGGAGAGAGGAC. HCTnT3 was used as the template to create HCTNT4 (the 15-bp exon 4 was deleted) using the following oligonucleotides: 3'/H11032 CTCCTCCTGCTCTTCTGCTCCTCCTCCTCCTCGTC; 5'/H11032 CTAGTTCTAGAGGAGGACTGGAGAGAGGACGAAGACGAGCAGGAGGAGGCAGCGGGAAGAG. All subcloned DNA sequences were inserted into the expression plasmid pET3d and sequenced to verify the correct sequences prior to expression and purification. Standard methods previously used in this lab were utilized for expression and purification of the different CTnT mutants (13). Briefly, bacterially expressed and extracted HCTnTs were purified on a Fast Flow Q-Sepharose column. HCTnT was eluted...
from this column with a NaCl gradient (0–0.5 M). The semi-purified HCTnT was then further purified on a human troponin C (HCTnC) affinity column. HCTnT was eluted from this column with a double gradient of urea and EDTA (0–3 M, respectively). After each step, the ATPase activity was measured to determine the purity of the isolated HCTnT complex. The purity of the isolated HCTnT complex was determined by SDS-PAGE.

**Formation of the Troponin Complex**—Formation of the cardiac troponin complexes containing human recombinant HCTnT, HCTnC, and human cardiac troponin I (HCTnI) was carried out as described recently by Szczezna et al. (12). Proper stoichiometry was verified by SDS-PAGE. Although not done routinely, gel filtration of these Tn complexes showed that this reconstitution method resulted in each single species.

**Actin-TM-activated Myosin-ATPase Assay**—Porcine cardiac myosin, rabbit skeletal F-actin, porcine cardiac TM, and recombinant human cardiac TnC were prepared as described previously (12, 13). The ATPase inhibitory assay was performed in a 1-ml reaction mixture of 100 mM KCl, 4 mM MgCl₂, 1.0 mM EGTA, 2.5 mM ATP, 0.1 mM dithiothreitol, 10 mM MOPS, pH 7. The ATPase activation assay was carried out in the same 1 mL reaction mixture with 1 mM EGTA replaced with 0.5 mM CaCl₂, F-actin (3.5 μm), myosin (0.6 μm), and TM (1 μM) were homogenized and added to the reaction tube after the addition of buffer and either wild type (adult) human cardiac Tn (HCTn), HCTn containing HCTnT1 (HCTnT1), HCTn containing HCTnT2 (HCTnT2), HCTn containing HCTnT3 (HCTnT3), and HCTn containing HCTnT4 (HCTnT4) to the same extent. The ATPase reaction was initiated with the addition of ATP and stopped after 20 min with trichloroacetic acid (4% final concentration). After sedimenting the precipitate, the inorganic phosphate concentration was determined using a cell path length of 0.1 cm at ambient temperature (22 °C) in a 10 mM sodium phosphate, pH 7.0, 500 mM KCl solution. In some cases NaF was used instead of KCl to avoid the strong absorption of chloride ions in the far-ultraviolet region (17). Spectra were recorded at 190–250 nm with a bandwidth of 1 nm at a speed of 50 nm/min and a resolution of 0.2 nm. The concentration of the HCTnT isoforms was determined using a standard curve obtained with HCTnT3 as the standard. The protein concentration of the HCTnT3 was accurately determined by nitrogen analysis. Analysis and processing of data were carried out using the Jasco system software (Windows Standard Analysis, version 1.20). Ten scans were averaged, base lines subtracted, and no numerical smoothing applied.

Mean residue ellipticity (θ̃, in degrees cm² dmol⁻¹) for the spectra were calculated (utilizing the Jasco system software) using Equation 1.

\[
\theta_{e\text{mean}} = \theta/10 \times (c \times l) \quad (\text{Eq. 1})
\]

where \(\theta\) is the measured ellipticity in millidegrees, \(c\) is the mean residue molar concentration, and \(l\) is the path length in cm. The \(\alpha\)-helical content for each protein was calculated using the standard Equation 2 for \(\theta\) at 222 nm (18).

\[
\theta_{222} = -30,300 \rho \times 2340 \quad (\text{Eq. 2})
\]

\(\rho\) is the fraction of \(\alpha\)-helical content (\(\rho\) × 100, expressed in %). Spectra are presented as the mean residue ellipticity. The standard deviation of the mean was calculated from Student’s t test.

**RESULTS**

Four isoforms of HCTnT, which are known to be expressed in the human heart, have been generated, purified (Fig. 3), and functionally characterized. The regions of TnI involved in critical interactions with TnI and TnC in the Tn complex and with TM in the thin filament have been characterized by various biochemical and biological methods and are summarized in Fig. 1. Most of the known binding regions for TM, TnI, and TnC are shown in residues 70–278 of HCTnT3. Fig. 3 shows an SDS-PAGE of purified recombinant HCTnT isoforms. All samples were separated on a 12% SDS-polyacrylamide gel, and stained with Coomassie Brilliant Blue R-250. Lane 1, HCTnT1; lane 2, HCTnT2; lane 3, HCTnT3; and lane 4, HCTnT4.

**Circular Dichroism (Far UV) of HCTnT Isosforms**—CD spectra of the HCTnT isoforms were recorded on a Jasco J-720 spectropolarimeter using a cell path length of 0.1 cm at ambient temperature (22 °C) in a 10 mM sodium phosphate, pH 7.0, 500 mM KCl solution. In some cases NaF was used instead of KCl to avoid the strong absorption of chloride ions in the far-ultraviolet region (17). Spectra were recorded at 190–250 nm with a bandwidth of 1 nm at a speed of 50 nm/min and a resolution of 0.2 nm. The concentration of the HCTnT isoforms was determined using a standard curve obtained with HCTnT3 as the standard. The protein concentration of the HCTnT3 was accurately determined by nitrogen analysis. Analysis and processing of data were carried out using the Jasco system software (Windows Standard Analysis, version 1.20). Ten scans were averaged, base lines subtracted, and no numerical smoothing applied.

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**Actomyosin ATPase Activities on Reconstituted Troponin Complexes Containing Different HCTnT Isoforms**—The maximum ATPase activity for Tn complexes containing the different HCTnT isoforms in the presence of Ca²⁺ was similar for all the isoforms (Fig. 4). At Tn concentrations greater than 1 μM, the maximum ATPase activity only increased slightly (<10%), and as such the ATPase activity results are presented as a bar chart so that the different HCTnT isoforms could be directly compared. In these experiments the amount of Tn required for maximal ATPase activation was between 1 and 1.5 μM, which is...
consistent with a ratio of Tn:TM of 1:1. The human cardiac Tn complex containing HCTnT1 (HCTnT1) and HCTnT3 (HCTnT3) showed slightly lower maximal ATPase activity than the other complexes (Tn complex containing HCTnT2, HCTnI, and Tn complex containing HCTnT4, HCTnI) (Fig. 4). However, this difference in maximal ATPase activity was not found to be significant according to the Student’s t test. The ability of HCTnT isoforms to inhibit actin-TM-activated myosin ATPase activity in reconstituted thin filaments was also examined to determine whether the inhibitory activity of the HCTnT was affected by the deletions in the N-terminal region of HCTnT. The myosin ATPase activity in the absence of HCTnT was considered to be 100% activity (0% inhibition). Whereas HCTnT was able to inhibit ATPase activity nearly fully in the presence of EGTA (~92% at 3 μM HCTn concentration), HCTnI and HCTnC were both less effective at inhibiting actomyosin ATPase activity (~67% at 3 μM HCTn concentration, Fig. 5). Under the same conditions used for the HCTnI, HCTnC inhibited the actin-TM-activated myosin ATPase activity by 84 ± 2%. Although the Tn complexes do not inhibit the myosin ATPase activity fully at physiological concentrations, we are still able to observe a difference between HCTnI and HCTnC at physiological Tn concentrations (Fig. 5).

**Force Development and the Ca²⁺ Dependence of Force Development**—When the adult isoform of HCTnT (HCTnT3) was used to displace the native porcine cardiac troponin complex and the skinned fibers then reconstituted with a preformed HCTnT-HCTnC complex, the reconstituted fibers were less sensitive to Ca²⁺ (ΔpCa₅₀ about −0.14) than intact fibers (Fig. 6A). This difference is presumably due to the different Ca²⁺ response to the endogenous porcine and the human Tn complexes reconstituted into skinned muscle fibers and has been reported previously (19). Porcine skinned fiber studies using the TnT displacement of the endogenous Tn complex and HCTnT-HCTnC reconstitution is now a well-established method in this laboratory (19). Because HCTnT3 is the adult isoform, the dependence of all the other isoforms was compared with the Ca²⁺ dependence of HCTnT3-treated and HCTnI-HCTnC-reconstituted fibers. Table I summarizes the pCa₅₀ values of the force-pCa relationship and the Hill coefficients (nH) for the fibers treated with different HCTnT isoforms.

In these experiments, after displacing the endogenous porcine Tn complex with the different HCTnT isoforms (0.8 mg/ml), HCTnI-HCTnC was used to reconstitute the HCTnT-replaced skinned fibers. The thick and the thin filament proteins exhibit a strict stoichiometry with each other having specific interrelated role(s) in controlling muscle contraction and relaxation. No significant change in any of the other porcine fiber proteins was detected after HCTnT displacement. Both HCTnI and HCTnC levels were restored upon reconstitution as judged by SDS-polyacrylamide gel analysis (data not shown). After HCTnI-HCTnC was added to the skinned fibers, all the HCTnT isoforms investigated were able to restore the Ca²⁺ dependence of force development. The Ca²⁺ dependence of force development was measured before treating each fiber with HCTnT.

The level of Ca²⁺-unregulated force following the HCTnT treatment was equal to ~60% of the force developed by the untreated skinned fiber preparations. For clarity this level of Ca²⁺-unregulated force was set to 100% to determine the percentage of force activation and inhibition following the HCTnT treatment. In the absence of Ca²⁺, the level of force remaining after reconstituting the fibers with the HCTnT-HCTnC complex is equivalent to the percentage of Ca²⁺-unregulated force that was not inhibited by the Tn complex (Fig. 7). The fibers reconstituted with HCTnT1 and HCTnT-HCTnC (HCTnI) had a force of 9.1 ± 4% of unregulated force compared with 3 ± 1% force of fibers reconstituted with HCTnT3 and HCTnI-HCTnC (HCTnC). The level of force after reconstitution of porcine fibers with HCTnT at pCa 8.0 (3 ± 1%) is consistent with previously published values (11). This suggests that Tn containing HCTnT1 is unable to properly inhibit force development at low Ca²⁺ concentrations. The fibers reconstituted with HCTnT2 and HCTnI-HCTnC (HCTnI) had a force of 6.4 ± 2.7% of unregulated force. HCTnT-reconstituted fibers had a slightly lower force that HCTnI-reconstituted fibers at low Ca²⁺ concentrations (Fig. 7).

The maximum Ca²⁺-dependent force recovered is equivalent to the level of force developed in fibers after reconstituting the fibers with the appropriate Tn complex and treating the fiber with pCa 4 solution. The maximal level of force recovered with the different HCTnT isoforms was not significantly different from each other. As seen in Fig. 6, a significant change in the

![Graph](http://www.jbc.org/content/35344/Supplemental/figure4.png)  
**Fig. 4.** Effect of the HCTnT isoforms on the activation (+Ca²⁺) of the actin-TM-activated myosin-ATPase activity. The protein concentrations used in this assay are as follows: 3.5 μM actin, 1 μM TM, 1 μM Tn, and 0.6 μM myosin. Each data point represents the average of 4–5 separate experiments each performed in duplicate and is expressed as mean ± S.D. The myosin ATPase activity that occurs in the absence of Ca²⁺ is considered to be 100% ATPase activity.

![Graph](http://www.jbc.org/content/35344/Supplemental/figure5.png)  
**Fig. 5.** Effect of the HCTnT isoforms on the inhibition (−Ca²⁺) of the actin-TM-activated myosin-ATPase activity. The protein concentrations used in this assay are as follows: 3.5 μM actin, 1 μM TM, 0–3 μM Tn, and 0.6 μM myosin. Each data point represents the average of 4–5 separate experiments each performed in duplicate and is expressed as mean ± S.D. The minimal ATPase activity values for the HCTnI and HCTnC are significantly different from the HCTnT and HCTnC in the absence of Ca²⁺ (p < 0.05).
dependence of force was observed between the fibers displaced with the different HCTnT isoforms after reconstitution with HCTnI/HCTnC complex. Fig. 6 presents the Ca\(^{2+}\) response of force of HCTnT1-treated fibers. HCTnT1-treated fibers had an increased Ca\(^{2+}\) sensitivity when compared with HCTnT3. HCTnT2-treated fibers also had an increased Ca\(^{2+}\) sensitivity, which was statistically significant when compared with HCTnT3-treated fibers (Fig. 6 C). HCTnT4-treated fibers showed a slight decrease in Ca\(^{2+}\) sensitivity when compared with HCTnT3 (Fig. 6 D). These results are summarized in Fig. 8, which illustrates the effect of the net protein charge, at the pH that we carried out these fiber studies (pH 7.0), on the \(p_{Ca_{50}}\) of the different HCTnT isoforms.

Circular Dichroism of HCTnT Isoforms—CD spectroscopy was carried out to determine the secondary structure of the four HCTnT isoforms (Fig. 9 and Table II). The average mean residue molar ellipticity \((\theta)\) for HCTnT3 at 222 nm was \(-14,009\) degrees cm\(^2\) dmol\(^{-1}\) (corresponding to an \(\alpha\)-helical content of 38.5\%). HCTnT1 had \(\theta\) at 222 nm of \(-11,462\) degrees cm\(^2\) dmol\(^{-1}\) (corresponding to an \(\alpha\)-helical content of 30.1\%), HCTnT2 had \(\theta\) at 222 nm of \(-11072\) (28.8\% \(\alpha\)-helical), whereas HCTnT4 had \(\theta\) at 222 nm of \(-12,165\) degrees cm\(^2\) dmol\(^{-1}\) (corresponding to an \(\alpha\)-helical content of 31.0\%).

<table>
<thead>
<tr>
<th>HCTnT Isoform</th>
<th>(p_{Ca_{50}})</th>
<th>Hill coefficient, (n_H)</th>
<th>No. experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCTnT1</td>
<td>5.66 ± 0.03(^a)</td>
<td>1.76 ± 0.09(^b)</td>
<td>4</td>
</tr>
<tr>
<td>HCTnT2</td>
<td>5.63 ± 0.03(^a)</td>
<td>1.92 ± 0.10</td>
<td>3</td>
</tr>
<tr>
<td>HCTnT3</td>
<td>5.53 ± 0.01</td>
<td>2.01 ± 0.08</td>
<td>4</td>
</tr>
<tr>
<td>HCTnT4</td>
<td>5.48 ± 0.02</td>
<td>2.13 ± 0.10</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\) Indicates that the \(p_{Ca_{50}}\) values for the respective TnT isoforms are significantly different from HCTnT3 and HCTnT4 (\(p < 0.05\)).

\(^b\) Indicates that the \(n_H\) value for HCTnT1 isoform is significantly different from HCTnT4 (\(p < 0.05\)).

Fig. 6. The Ca\(^{2+}\) sensitivity of force development in skinned porcine cardiac muscle fibers treated with HCTnT isoforms. A, comparison of porcine fibers before and after treatment with HCTnT3 (adult HCTnT). B, comparison of HCTnT1 with HCTnT3. C, comparison of HCTnT2 with HCTnT3. D, comparison of HCTnT4 with HCTnT3. Each skinned muscle preparation was treated with an HCTnT isoform (0.8 mg/ml) to displace the endogenous Tn complex. The Ca\(^{2+}\) dependence of force was measured in each preparation after reconstituting whole HCTn. Each point is the average of 3–4 experiments and represents the mean ± S.D.
TnT Isoforms Affect Ca\(^{2+}\) Sensitivity and Inhibition

**DISCUSSION**

HCTnT is expressed in the human heart at the protein level as four isoforms, HCTnT1 through HCTnT4, numbered in the order of decreasing molecular size (Figs. 1–5). Previous results suggested that the first 45 residues of skeletal TnT are not essential for anchoring the Tn complex to the thin filament and do not play a crucial role in the cooperative response of actomyosin ATPase to Ca\(^{2+}\). Others have also shown (20, 21) that removal of the N-terminal region of TnT does not affect the Ca\(^{2+}\) sensitivity of actomyosin ATPase activity. However, we now know that TnT is more complex than previously thought, and changes in the N-terminal region have recently been shown to affect the structure of the C-terminal region of TnT (22, 23).

Our investigations demonstrate that removal of exon 5 (10 amino acids) decreases the Ca\(^{2+}\) sensitivity of force development in both HCTnT3 (\(\Delta pC_{50} = -0.13\)) and HCTnT4 (\(\Delta pC_{50} = -0.15\)) relative to HCTnT1 and HCTnT2 (Fig. 6, B–D). This decrease in Ca\(^{2+}\) sensitivity observed with exon 5 removal is consistent with the previous avian and rabbit cardiac studies (24–27). McAuliffe et al. (26) found that developmental changes in TnT isoforms in rabbit myocardium correlated with changes in myofilament Ca\(^{2+}\) sensitivity. Rabbit TnT2 has 10 amino acid residues EEEDREDEDE in the N-terminal region that contributed to the determination of the Ca\(^{2+}\) sensitivity of force development in a charge-dependent manner: the greater the net negative charge the higher the Ca\(^{2+}\) sensitivity (Fig. 8). This latter result is primarily due to exon 5. Tobacman and Lee (8, 9) investigated two bovine cardiac isoforms of TnT, BCTnT1 and BCTnT2, which differ by the presence or absence of 5 amino acids (Fig. 2), and they found that the smaller isoform, BCTnT2, had a higher Ca\(^{2+}\) sensitivity than BCTnT1 in actomyosin S1 ATPase assays. Because both HCTnT1 and HCTnT3 contain a 5-residue...
region (exon 4) similar to the 5 residues found in bovine heart TnT1 (EAAEE in BCTnT and EAAVE in HCTnT, Fig. 2), in contrast to what we found, it was expected that removal of these 5 residues (exon 4) in HCTnT would have increased the Ca$^{2+}$ sensitivity of force development.

It is most likely that the differences observed between these human and bovine TnTs are due to differences in the N-terminal regions of bovine and human CTnT. The BCTnT2 isoform is generated by alternative splicing of 5 amino acids resulting in a deletion of residues 15–19 (EAAEE) (Fig. 2). It should be noted that the sequence for BCTnT2 in the Swiss-Prot or NCBI databases (P13789) is different from that reported by Leszyk et al. (28). In the Swiss Protein or NCBI databases BCTnT2 also has a single amino acid change (H21 in BCTnT1 to N16 in BCTnT2). Two residues that are very close to this alternatively spliced region and are present in human, rabbit, and chicken TnT isoforms are missing in both BCTnT isoforms (Fig. 2). Single amino acid changes (as found in familial hypertrophic cardiomyopathy patients) in other regions of HCTnT have already been shown to cause changes in the Ca$^{2+}$ sensitivity of force development (12).

The fetal distribution of BCTnT isoforms in bovine heart is not known, and the adult isoform distribution is different from other vertebrates including chicken, rat, rabbit, and human (4) in which the adult isoform(s) is the smallest of the isoforms present in the adult heart. VanBuren et al. (29) recently investigated the two adult recombinant CTnT isoforms for functional differences using an in vitro motility assay. They found no differences in the Ca$^{2+}$ sensitivity, cooperativity, or maximal force between the two BCTnT isoforms. However, the small differences that may exist between these isoforms is unlikely to be detectable via the in vitro motility assay used by this group due to the relatively large standard error they obtained for these experiments.

A recent report by Ogut et al. (30) suggests that differences in Ca$^{2+}$ sensitivity between pectoralis (which expresses high molecular weight, acidic TnTs) and levator (which expresses low molecular weight basic TnTs) fast twitch skeletal muscle fibers in chicken muscles are likely due to TnT isoform expression, with higher Ca$^{2+}$ sensitivity in the muscles expressing acidic TnTs. These latter results are consistent with results presented here where the most acidic HCTnT isoform (HCTnT4, estimated pI = 4.94) showed the highest Ca$^{2+}$ sensitivity, and the most basic HCTnT (HCTnT4, estimated pI = 5.2) showed the lowest Ca$^{2+}$ sensitivity.

Heart failure associated changes in TnT isoform expression have been correlated with a change in Ca$^{2+}$ sensitivity of force development and a decreased myofibrillar ATPase activity (1). Left ventricular myofibrillar ATPase activity in patients with end-stage heart failure was also found to be lower than in normal patients by other investigators (31). In other studies of failing hearts the maximal myofibrillar ATPase activity was found to be lower, and the Ca$^{2+}$ sensitivity of force development was greater than in the normal heart (32). However, we were unable to find any significant difference between HCTnT3 and HCTnT4 with respect to maximal ATPase activity (Fig. 4).

Tobacman and Lee (8, 9) also found that the maximal ATPase rate was similar for BCTnT1 and BCTnT2. These results suggest that the fall in ATPase activity in some heart failure patients may not be due to a change in the expression of HCTnT isoforms as suggested previously. The effects seen in failing hearts could possibly be accounted for by different CTnI phosphorylation levels (32–35). TnI phosphorylation decreases the myofilament sensitivity to Ca$^{2+}$, increases the myofibrillar relaxation rate, and decreases the maximal myofibrillar ATPase activity (32–35). In failing hearts the level of TnI phosphorylation is decreased which would result in an increase in the Ca$^{2+}$ sensitivity in the myocardium (36).

Following treatment of fibers with different HCTnT isoforms and reconstitution with HCTnI-HCTnC complexes, we found a decrease in the inhibition of force at low Ca$^{2+}$ when either HCTnT1 or HCTnT2 was used, with HCTnT1 showing the highest residual force at pCa 8.0 (Fig. 7). These skinned fiber studies suggest that both HCTn1 and HCTn$_{ab}$ impair the inhibition of force (Fig. 7). This latter result together with the reduced inhibition of actomyosin ATPase activity by Tn complexes containing HCTnT1 and HCTnT2 (Fig. 5) suggests that the HCTnT1 and HCTnT2 (both which contain exon 5) may somehow hinder the release of myosin head binding to the actin filament in the skinned cardiac muscle system. These results suggest that exon 5 of cardiac TnT affects the ability of Tn to inhibit force development and ATPase activity. If present in the adult myocardium, these HCTnT isoforms may contribute to incomplete diastolic relaxation. Exon 4 also seems to be influencing the inhibition of force because Tn complexes with HCTnT isoforms containing this exon (HCTnT1 and HCTnT3) show lower inhibition of force than Tn complexes with HCTnT isoforms lacking this exon (HCTnT3 and HCTnT4) (Fig. 7). HCTnT4 exons 4 and 5 may cause their effect either directly via their interaction with TM or indirectly via their interaction with TnI and/or TnC. A possible inhibitory role for the N-terminal region of skeletal TnT (TnT1, residues 1–158) was shown by Onoyama and Ohtsuki (37). They found that TnT1 was able to inhibit actomyosin ATPase activity over a range of Ca$^{2+}$ concentrations. Changes in the tertiary structure of the N-terminal region of HCTnT by alternative splicing may affect its interaction with TM resulting in changes in the positioning of TM on the actin filament. If the position of TM on the actin filament is affected, the interaction between myosin and actin would be affected leading to changes in the inhibition of force and ATPase activity. TnT also binds with both TnI and TnC forming the regulatory complex Tn. Changes in the capability of TnT to interact with either TnI and/or TnC would result in structural changes in TnI that could affect the ability of TnI to bind actin, which in turn would affect the inhibition of force and ATPase activity.

Our results also suggest that the N-terminal alternatively spliced region affects the cooperativity of force development. Removal of either exon 4 or 5 increases cooperativity, whereas removal of both exons increased the cooperativity of force development even further (Table I). The rank order in Fig. 7 corresponds to the rank order in both force pCa$_{50}$ and the Hill coefficient (n$_H$). Two possibilities that could account for these results are that all these comparisons agree because they reflect the ability of the various TnTs to inhibit contraction. Another possibility is that the Tn exchange/reconstitution procedure had slightly different exchange levels that are not detected by SDS-PAGE. SDS-PAGE is unlikely to detect a small percentage of the Tns in the fibers that do not have a full complement of TnI and TnC. It is unclear how small differences in Tn reconstitution would affect Ca$^{2+}$ phosphorylation and inhibition. Following treatment of fibers with different HCTnT isoforms and reconstitution with HCTnI-HCTnC complexes, we found a decrease in the inhibition of force at low Ca$^{2+}$ when either HCTnT1 or HCTnT2 was used, with HCTnT1 showing the highest residual force at pCa 8.0 (Fig. 7). These skinned fiber studies suggest that both HCTn1 and HCTn$_{ab}$ impair the inhibition of force (Fig. 7). This latter result together with the reduced inhibition of actomyosin ATPase activity by Tn complexes containing HCTnT1 and HCTnT2 (Fig. 5) suggests that the HCTnT1 and HCTnT2 (both which contain exon 5) may somehow hinder the release of myosin head binding to the actin filament in the skinned cardiac muscle system. These results suggest that exon 5 of cardiac TnT affects the ability of Tn to inhibit force development and ATPase activity. If present in the adult myocardium, these HCTnT isoforms may contribute to incomplete diastolic relaxation. Exon 4 also seems to be influencing the inhibition of force because Tn complexes with HCTnT isoforms containing this exon (HCTnT1 and HCTnT3) show lower inhibition of force than Tn complexes with HCTnT isoforms lacking this exon (HCTnT3 and HCTnT4) (Fig. 7). HCTnT4 exons 4 and 5 may cause their effect either directly via their interaction with TM or indirectly via their interaction with TnI and/or TnC. A possible inhibitory role for the N-terminal region of skeletal TnT (TnT1, residues 1–158) was shown by Onoyama and Ohtsuki (37). They found that TnT1 was able to inhibit actomyosin ATPase activity over a range of Ca$^{2+}$ concentrations. Changes in the capability of TnT to interact with either TnI and/or TnC would result in structural changes in TnI that could affect the ability of TnI to bind actin, which in turn would affect the inhibition of force and ATPase activity.

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Although the molecular mechanism of cooperativity is still not clearly understood, the Ca$^{2+}$ dependence of muscle contraction is known to be highly cooperative with Hill coefficients ranging from 1 to 4 depending on the system used (12, 38–40). Biochemical studies on skeletal TnT peptides suggest that part
of their N-terminal region (residues 70–159, corresponding to residues 102–189 of HCTnT3) promotes cooperative interactions between functional units (41, 42). In the absence of actin, the binding of TnT to TM was found to be cooperative (43) suggesting that TnT can induce a conformational change in adjacent TM dimers promoting TnT interaction with them. Differences in the N-terminal region of TnT may affect the binding of TM to TnT resulting in subtle changes in the cooperative binding seen for the different TnT isoforms. This latter suggestion is not unlikely because studies on avian skeletal TnT isoforms showed binding of these avian isoforms to TM increased as they became more basic in nature (43).

It is possible that embryonic-specific exons 4 and 5 of TnT may change the conformation of other regions of TnT affecting the interaction between TnT and TM and/or TnI and TnC. For this reason, circular dichroism was carried out to determine whether any significant changes in secondary structure of the different HCTnT isoforms occurred (Fig. 9 and Table II). These results show that HCTnT3 has a higher α-helical content than the other HCTnTs, suggesting that the overall secondary structures of the different HCTnTs are slightly different. Which region(s) of HCTnT are structurally affected by these N-terminal deletions is not known. However, differences in secondary structure monitored by circular dichroism may be functionally important as changes in the N-terminal region of TnT have been shown recently to affect the structure of the C-terminal region (22, 23).

From our results and other published studies we suggest that in failing hearts, depending on the cause of the cardiac dysfunction, either no expression or increased expression of HCTnT4 would probably occur. It is unlikely that HCTnT1 would be re-expressed at the same time because the reduced inhibition of ATPase activity and force development would adversely affect the heart and be counter-productive. This would be consistent with a recent report by Mesnard-Rouiller et al. (44) who found that HCTnT3 was the major mRNA isoform in adult human heart and that HCTnT4 was a minor isoform, whereas HCTnT1 and HCTnT2 mRNAs were present but barely detectable in left ventricular specimens taken at the time of heart transplantation. Higher levels of HCTnT4 mRNA and protein (compared with controls) were detected in only half the failing ventricles independently of the cause of failure, suggesting that this increase in HCTnT4 may not be a general characteristic of left ventricular failure but instead could be related to stress. They also found a decrease in HCTnT1 protein expression in all failing ventricular samples studied (compared with controls). Chen et al. (45) found that shifts in TnT isoforms in rabbit cardiac muscle toward a more “fetal” pattern occurred during experimentally induced mild left ventricular hypertrophy and was suggested to be a general feature of the response to hemodynamic stress, rather than caused by end-stage disease.

Cooper and Or Dahl (46) hypothesized that the embryonic TnT isoform may play a role during sarcomere assembly. However, this seems unlikely because the fetal isoform of TnT has distinct functional effects, which are different from the adult isoform, and the fetal isoform is present in late fetal and early neonatal hearts where contractile function is already well developed.

Fetal human heart contains two isoforms of TnI, HCTnI and slow skeletal muscle TnI (SSTnI). Structural and physiological considerations indicate that these TnI isoforms confer differing contractile properties on the myocardium, particularly on the phosphorylation-mediated regulation of contractility (47). Human SSTnI (HSSTnI) is the predominant TnI isoform throughout fetal life and gradually decreases during the first few months of postnatal development (48). Because of this, the reduced inhibition of ATPase activity at low Ca2+ levels observed for HCTnT1 and HCTnT2 may be because these isoforms do not bind HCTnI as well as HSSTnI. The 10-residue peptide present in HCTnT1 and HCTnT2 may be important for conformational changes in these isoforms that are important for interaction with HSSTnI.

Maintenance of normal myocardial contractility depends on both the proper homeostasis of Ca2+ in the muscle cells and on the contractile responsiveness of the myofilaments to Ca2+. Changes in the Ca2+ sensitivity of force development seen with these HCTnT isoforms are therefore likely to affect myocardial contractility. Overall, these results suggest that the major fetal isoform (HCTnT1) is functionally distinct from the adult isoform (HCTnT3) and is likely to have a significant role in modulating cardiac muscle contraction.

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Cardiac Troponin T Isoforms Affect the Ca\(^{2+}\) Sensitivity and Inhibition of Force Development: INSIGHTS INTO THE ROLE OF TROPONIN T ISOFORMS IN THE HEART

Aldrin V. Gomes, Georgianna Guzman, Jiaju Zhao and James D. Potter

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