Cardiac troponin T isoforms demonstrate similar effects on mechanical performance in a regulated contractile system

PETER VANBUREN,1,2 SHARI L. ALIX,1 JOSEPH A. GORGA,1 KELLY J. BEGIN,1 MARTIN M. LEWINTER,1 AND NORMAN R. ALPERT2

1Cardiology Unit, Department of Medicine, and 2Department of Molecular Physiology and Biophysics, University of Vermont, Burlington, Vermont 05405

Received 29 October 2001; accepted in final form 24 December 2001

VanBuren, Peter, Shari L. Alix, Joseph A. Gorga, Kelly J. Begin, Martin M. LeWinter, and Norman R. Alpert. Cardiac troponin T isoforms demonstrate similar effects on mechanical performance in a regulated contractile system. Am J Physiol Heart Circ Physiol 282: H1665–H1671, 2002. First published January 3, 2002; 10.1152/ajpheart.00938.2001.—Alteration of troponin T (TnT) isoform expression has been reported in human and animal models of myocardial failure. The two adult beef cardiac TnT isoforms (TnT3 and TnT4) were isolated for comparative functional analysis. Thin filaments were reconstituted containing pure populations of the isoforms. The in vitro motility assay was used to directly compare the effect of the two TnT isoforms on force and unloaded shortening as a function of free calcium. We found no significant differences between the two isoforms in terms of calcium sensitivity, cooperativity, or maximal activation (velocity and force) as assessed in a fully calcium-regulated system. Activation by myosin strong binding was similar for thin filaments containing either of the two TnT isoforms. Whereas maximally activated velocity and cooperativity was depressed at pH 6.5, no difference between thin filaments containing the two isoforms was detected. From the small magnitude of the TnT isoform shifts detected in myocardial failure and the lack of significant mechanical effect detected in the motility assay, variable TnT isoform expression is unlikely to be any functional significance in heart failure.

thin filament; myocardial failure; in vitro motility

TROPNIN T (TnT) isoform shifts are observed in left ventricular hypertrophy and myocardial failure in mammalian models and humans (2, 7, 18, 39). These isoform shifts have been correlated with alterations in myocardial function as illustrated in human cardiac failure where expression of the fetal isoform (TnT4) is increased from 4% to 12% relative to TnT3, the predominant isoform in adult human myocardium (2). Specific isoform expression is the result of variable exon exclusion in the NH2-terminal segment of the protein with TnT3 and differing from TnT4 by the inclusion of one exon (5 amino acids). To date, there has been no proof of a cause and effect relationship between TnT isoform shifts and altered contractile function. The two actin-associated proteins troponin and tropomyosin regulate the activation of muscle. TnT, the tropomyosin binding subunit of the troponin complex, contains a globular COOH-terminus and a long NH2-terminal tail. The globular portion of TnT binds to troponin C (TnC; the calcium binding subunit), troponin I (TnI; the inhibitory subunit), and tropomyosin in a calcium-dependent manner. The NH2-terminus of TnT is highly charged and binds to tropomyosin in a calcium-independent manner, thus providing a tether for the entire troponin complex to the thin filament during muscle activation. The NH2-terminal segment of TnT binds at the NH2-terminal/COOH-terminal overlap of two adjacent tropomyosin molecules. The overlap of adjacent tropomyosins is felt to be a critical component of thin filament-mediated cooperative activation. Excimer fluorescence studies have demonstrated enhanced cooperativity of the thin filament in the presence of troponin compared with actin and tropomyosin alone (8). This enhanced cooperativity is felt to be largely an effect of the NH2-terminal segment of TnT, which enhances the binding of the tropomyosin to actin (12) and thus likely facilitates the communication of movement between adjacent tropomyosins with thin filament activation. The fact that the NH2-terminal segment of TnT is 1) highly charged, 2) overlies the overlap of adjacent tropomyosins, and 3) may impact the cooperativity of the thin filament, raises the possibility that even a small change in isoform content could significantly alter the cooperative activation of the thin filament.

To delineate the consequence of variable TnT isoform expression, we isolated the two adult beef TnT isoforms and characterized their effect on thin filament function. The sequence differences between the adult beef isoforms are nearly identical to those found between the two human TnT isoforms expressed in myocardial failure. To directly test the effect of different TnT isoforms on thin filament function, TnT isoforms were isolated and subsequently reconstituted with troponin C and troponin I. Functional differences between TnT isoforms were investigated by using calcium-regulated thin filaments in the in vitro motility assay.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
METHODS

Contractile Proteins

Myosin was isolated from chicken pectoralis skeletal muscle as previously reported (22). Skeletal myosin was used because it has proven to be more functionally stable than cardiac myosin once isolated and thus allows for greater consistency between experiments (35). Actin was isolated from chicken pectoralis skeletal muscle by standard techniques (24) and stored in filamentous form on ice. Troponin was isolated from rabbit skeletal muscle by the methods of Smillie (28), with further purification by hydroxyapatite chromatography. Isolation of the troponin subunits and separation of the TnT isoforms was as follows. Isolation from beef cardiac ether powder was performed as per the method of Potter (26) with minor modifications. In brief, crude cardiac troponin extract was initially run over an Uno-S cation exchange column (Bio-Rad) after equilibration [6 M urea, 50 mM citrate (pH 6.0), 1 mM EDTA and 0.1 mM dithiothreitol (DTT)]. Protein elution was achieved using a linear NaCl gradient. This step resulted in partial purification of TnC, TnI, and TnT. Further purification of TnC and TnI was achieved with the use of an anion exchange column (Uno-Q, Bio-Rad).

Final purification and separation of the two adult bovine TnT isoforms were achieved using the final TnT purification protocol (26) with the exception that the Uno-Q anion exchange column (Bio-Rad) was used. This protocol allowed nearly complete separation of the two TnT isoforms with TnT3 eluting at ~160 mM KCl and TnT4 eluting at ~190 mM KCl. Reconstitution of Tn from its subunits was as per Guo et al. (9). Troponin subunit reconstitution was followed by gel filtration chromatography. The purification and reconstitution resulted in troponins that demonstrate stoichiometric representation of the three subunits (Fig. 1). Proteins were snap-frozen and stored at ~80°C in a solution composed of (in mM) 100 KCl, 10 3-(N-morpholino)propanesulfonic acid (pH 7.0), 0.5 CaCl2, and 1 DTT until use. Protein concentrations were determined from their molecular weights and extinction coefficients (33). Thin filaments were reconstituted as previously described (13). Thin filaments were then labeled with rhodamine-phalloidin at a 1:1 actin-to-phalloidin ratio in low-salt buffer (in mM: 25 KCl, 25 imidazole, 5 MgCl2, 10 DTT, and 2 EGTA, pH 7.4) and stored overnight a 4°C before use in the in vitro motility assay. The actin binding protein α-actinin (Sigma; St. Louis, MO) was dialyzed into the above low-salt buffer before use.

To determine whether functional differences existed between reconstituted thin filament and isolated native thin filaments, native thin filaments were isolated from frozen bovine left ventricles as per the method of Lehman et al. (19) with the exception that the purification protocol was terminated after the 150-min centrifugation step. The final pellet was raised in low-salt buffer. Native thin filament protein concentration was determined with a protein assay (Bio-Rad) and microplate reader using actin as the standard. Native thin filaments were labeled with rhodamine-phalloidin as above.

In Vitro Assays

Motility. The in vitro motility assay was employed to assess the contribution of the two adult beef cardiac TnT isoforms on calcium-sensitive unloaded shortening and isometric force. The in vitro motility assay has been previously described in detail (13, 36). Free calcium was varied in the final motility solution (i.e., pCa 10–4.5) through the use of the public domain software called Bound and Determined (6). All experiments were performed at 30°C. As variable TnT expression occurs in perinatal development, thin filament calcium-sensitive motility was assessed under slightly acidic conditions to mimic a potential physiological condition of the perinatal period. In this set of experiments the final motility solutions were adjusted to pH 6.5 by using the above software. To determine the effect of myosin strong binding on thin filament activation, velocity as a function of myosin concentration on the motility surface was determined. In these experiments the thin filaments were calcium activated with the final motility solution containing 10 μM free calcium (i.e., pCa 5).

To assess whether α-actinin binding to actin affected either thin filament regulatory function or actomyosin interactions, control experiments were designed in which α-actinin was incubated overnight with reconstituted thin filaments (molar ratios of 1:4 and 1:2 α-actinin to actin, respectively). The pCa-velocity relation of these thin filaments containing α-actinin was determined under conditions in which no α-actinin was bound to the motility surface (i.e., unloaded conditions). The presence of α-actinin on the thin filament did not affect calcium-sensitive regulation nor maximal unloaded shortening (data not shown), demonstrating that the binding α-actinin to actin does not affect thin filament function. Thus α-actinin appears to be a suitable agent to assess isometric force in the motility assay (discussed in Isometric Force) because it does not artifactualy affect the mechanical properties of the contractile proteins.

In the in vitro motility assay, individual thin filaments were observed moving across the myosin-coated surface. Thin filament velocity was determined using the Motion Analysis System (Santa Rosa, CA) as previously described (14). Velocity of thin filaments as a function of pCa was assessed for thin filaments containing either the TnT3 or TnT4 isoform. Typically >250 individual filament velocities were averaged to determine the mean velocity-pCa data point for each TnT isoform. A nonlinear least-squares regression was fit to the data (SigmaPlot, Jandel Scientific) by

![Fig. 1. SDS-PAGE of reconstituted troponin (TnT) complexes. First lane is a mixture of troponins containing TnT3 or TnT4 isoforms. Second and third lanes demonstrate the purity of TnT3 and TnT4, respectively, in the reconstituted troponin complex.](http://ajpheart.physiology.org/)

AJP-Heart Circ Physiol • VOL 282 • MAY 2002 • www.ajpheart.org
using a four-parameter Hill equation (15). Statistical comparison was performed from the parameters of the fit by use of an unpaired t-test. All values are expressed as means ± SE.

**Isometric Force**

Relative isometric force was determined for thin filaments containing the two cardiac TnT isoforms and bovine cardiac native thin filaments by using α-actinin as an internal load. In brief, myosin was adhered to the nitrocellulose-coated coverslip as described above. α-Actinin was then attached to the coverslip surface (15–100 μg/ml in low-salt buffer), followed by a bovine serum albumin wash (0.5 mg/ml in low-salt buffer). Reconstituted thin filaments (10–20 nM) were then added to the motility surface. Motility was initiated with the addition of the motility buffer as described above. As α-actinin avidly binds to actin, the motion of the thin filament is impeded by the α-actinin adhered to the surface. The load placed on a thin filament is a function of the relative concentrations of the force generator (myosin) and the motion inhibitor (α-actinin). The amount of α-actinin adhered to the motility surface was gradually increased until motility was completely arrested, thus indicating an isometric state. As reported by Haeberle (10), image processing with background subtraction was employed to aid in this determination, because this technique is exquisitely sensitive to the slightest thin filament movement. Whereas thin filaments arrested on the motility surface may demonstrate a small amount of repletion (i.e., Brownian motion) under isometric conditions, no net movement of thin filaments was observed over time. Relative isometric force was then defined as the minimum amount of α-actinin needed to completely arrest thin filament motility. Others (4) have recently used this approach with consistent results. To further test the validity of this technique, force as a function of skeletal myosin concentration on the motility surface (12.5 to 100 μg/ml) was determined for actin. In previous experiments using the microneedle technique, we have demonstrated that for actin alone, force increases as a linear function of the myosin concentration on the surface (37). Force as a function of calcium activation was determined for thin filaments containing TnT3 and TnT4 isoforms, as well as bovine cardiac native thin filaments. Force data were fit and statistical significance was determined as described above for the velocity data.

**RESULTS**

Complete isolation of the two adult beef cardiac TnT isoforms and reconstitution with TnI and TnC was achieved as demonstrated by SDS-PAGE (Fig. 1). Isolation and reconstitution of the thin filament proteins had no apparent effect on either thin filament regulation or function. Full calcium regulation of the reconstituted thin filaments was demonstrated by the pCa-velocity relation (Fig. 2A), in which reconstituted thin filaments were nonmotile at pCa 10, cooperatively activated at transitional calcium concentrations, and fully activated at high calcium concentrations. Furthermore, the velocity thin filaments that underwent subunit isolation and reconstitution was similar to native thin filaments isolated intact from the sarcomere (Fig. 2B; Table 1). Thin filaments containing TnT3 demonstrated no difference in calcium sensitivity (pCa50), cooperativity (Hill coefficient), or maximal activation (Vmax) compared with thin filaments containing TnT4 (Fig. 2A; Table 1).

To assess thin filament activation via myosin strong binding, the myosin concentration on the motility surface was varied by altering the myosin concentration in the labeling solution (37). Under maximally calcium-activated conditions (pCa 5), thin filament velocity as a function of surface myosin concentration was determined (Fig. 3). Velocity of thin filaments was markedly slowed at low myosin concentrations and increased as a cooperative function of surface myosin concentration (i.e., the number of myosin cross bridges interacting with the thin filament). No difference in myosin strong binding activation was seen for thin filaments containing either of the TnT isoforms. To explore the possibility that myosin activation of the thin filament is more pronounced at submaximal calcium concentrations, the above experiments were repeated at pCa 6.25. No difference was found for the two beef TnT isoforms.
EFFECTS OF TROPONIN T ISOFORMS ON CONTRACTILITY

Table 1. Troponin T isoforms effects on calcium-sensitive activation of thin filament

<table>
<thead>
<tr>
<th></th>
<th>Velocity</th>
<th>Force</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max(^a)</td>
<td>pCa(^b)</td>
<td>Max(^c)</td>
</tr>
<tr>
<td>TnT(_3)</td>
<td>6.3 ± 0.3</td>
<td>6.33 ± 0.03</td>
<td>0.96 ± 0.12</td>
</tr>
<tr>
<td>TnT(_4)</td>
<td>5.9 ± 0.4</td>
<td>6.35 ± 0.04</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td>bNTF</td>
<td>6.6 ± 0.6</td>
<td>6.31 ± 0.08</td>
<td>0.99 ± 0.12</td>
</tr>
</tbody>
</table>

Values are expressed as least-square regression of the fit to the Hill equation ± SE. TnT, troponin T; bNTF, bovine cardiac native thin filaments; \(^a\) Maximal calcium-activated thin filament velocity (\(\mu\)m/s); \(^b\) negative log of the calcium concentration at half-maximal thin filament velocity; \(^c\) maximal calcium-activated thin filament force normalized to TnT\(_4\); \(^d\) negative log of the calcium concentration at half-maximal thin filament velocity; \(^e\) thin filament cooperative activation as estimated by the Hill Coefficient.

(data not shown), indicating that myosin activation of the thin filament is not differentially affected.

TnT isoforms are differentially expressed in perinatal development, raising the possibility that the TnT isoforms could function differently in an acidic environment. Velocity-pCa experiments were performed at pH 6.5 (Fig. 4). At pH 6.5 (Fig. 4) there was a reduction in maximal velocity (3.2 ± 0.3 and 3.1 ± 0.4 \(\mu\)m/s for TnT\(_3\) and TnT\(_4\), respectively; \(P < 0.001\)) compared with velocities at pH 7.4 (Table 1). There was also a reduction in the Hill coefficient for thin filaments at the lower pH (1.3 ± 0.3 and 1.1 ± 0.3 for TnT\(_3\) and TnT\(_4\), respectively; \(P < 0.01\)). No change in calcium sensitivity was observed (6.45 ± 0.08 and 6.42 ± 0.12 for TnT\(_3\) and TnT\(_4\), respectively; \(P = \text{not significant}\)). Irrespective of the difference in maximal activation and cooperativity at pH 6.5, compared with pH 7.4, no functional differences between the two TnT isoforms were detected at pH 6.5.

To determine whether the \(\alpha\)-actinin method is capable of detecting the changes in force generation that result from small changes in the number of myosin molecules on the surface, force as a function of myosin concentration on the surface was determined. Force increased as a linear function of the myosin concentration on the surface (Fig. 5; \(r^2 = 0.99\)) similar to previous force data using the microneedle technique under identical experimental conditions (37). These results indicate that this method is comparable to the microneedle technique in measuring force.

Force as a function of calcium activation was determined for myosins interacting with thin filaments containing either TnT\(_3\) or TnT\(_4\) (Fig. 6A; Table 1). Force increased as a cooperative function of the ambient calcium concentration and is similar to isolated intact native thin filaments (Fig. 6B; Table 1). The Hill coefficient was not different for the two TnT isoforms and is similar to that reported in skinned cardiac fiber studies (15). Thin filament calcium sensitivity for force was similar for thin filaments containing TnT\(_3\) and TnT\(_4\), and no difference in maximally activated force was observed.

DISCUSSION

Alterations in TnT isoform expression occur during perinatal development and in myocardial hypertrophy and failure. The transition to myocardial failure is associated with reexpression of a fetal gene program. Associated with this program is increased expression of the fetal isoform of TnT. One of the hallmarks of myocardial failure is a reduction of mehanical performance as measured by a decrease in ventricular ejection fraction and cardiac output. Skinned fiber studies...
have demonstrated that a substantial proportion of the deficit found in failing myocardium can be directly linked to an alteration in sarcomere function. Which specific changes at the contractile protein level are responsible for the functional alterations in heart failure are not well understood. A shift in TnT isoform expression has been proposed as a contributor to myofilament dysfunction and has been correlated with depression in ventricular and myocyte function in heart failure (2, 3, 18). This correlation has not been consistent, however, because shifts in TnT isoform expression are not always found in heart failure (17) and are reported in ventricular hypertrophy with no change in contractile function (7). Therefore, a causal relation has not been established between TnT isoform expression and contractility in myocardial failure, because a direct functional assessment of the effect of cardiac TnT isoform variation on contractile performance heretofore has not been performed.

The two adult beef TnT isoforms differ by the inclusion of a single exon (exon 4; encoding amino acids EAAEE) in TnT3 but not in TnT4 (20). The beef cardiac TnT isoforms have distinct parallels with the two TnT isoforms expressed in human myocardial failure. Like the beef TnT isoforms, human TnT isoforms differ by the inclusion of exon 4 (encoding amino acids EAAVE) in TnT3 but not TnT4 (1). The process of extraction and isolation of the TnT isoforms yielded pure populations of the two isoforms (Fig. 1). Reconstitution of the isoforms with the full complement of thin filament protein resulted in restoration of thin filament function, as was evident by complete inhibition of movement at low calcium concentrations and an activational plateau at higher calcium concentrations.

The NH2-terminal binding overlap of TnT with two adjacent tropomyosins is felt to be integral in the cooperative activation of the thin filament. With the "hypervariable" region of TnT being in the NH2-terminal segment, it is conceivable that TnT isoforms may differentially affect thin filament cooperative activation. This could occur through affecting the "communication" of movement between two adjacent tropo-myosins. Specifically, Geeves and Lehrer (8) have demonstrated that the number of myosin binding sites exposed (i.e., cooperative unit size) with myosin binding increases twofold with the addition of troponin to actin-tropomyosin. Presumably, this increase in unit size is predominantly the result of the calcium independent binding of the NH2-terminus of TnT binding to adjacent tropomyosins. Overexpression of fast skeletal TnT in the mouse heart resulted in a modest change in the cooperative activation of the thin filament, whereas no change in calcium sensitivity or maximally activated force was demonstrated (15). In contrast, we found no difference in cooperativity between the two beef cardiac isoforms as determined by the Hill coefficient (Table 1). Similarly, no difference between the two isoforms was discernable at submaximal (pCa50) or maximal calcium activation for both unloaded shortening and isometric force. Tobacman and Lee (34) investigated the effect of the two adult beef TnT isoforms on calcium-sensitive ATPase and demonstrated a small
increase in calcium sensitivity (0.1 pCa units) for TnT4 relative to TnT3. Our results reveal a small shift in the calcium sensitivity of force (Table 1), but this did not achieve statistical significance (P > 0.2).

Because the thin filament is activated by both calcium and by myosin strong binding, TnT isoform variation could affect thin filament activation through the cooperative effects of myosin strong binding. Previous estimates indicate in that in the in vitro motility assay at 100 μg/ml myosin concentration ~54 myosin cross bridges are available per micron of actin filament (11). Assuming 5.5 nm per actin monomer would yield an estimate of ~4 myosin cross bridges per the 12 actin cooperative unit size, as suggested by Lehrer and Geeves (8). Thus by altering the myosin concentration on the motility surface, myosin strong-binding activation of the filament can be investigated. Myosin binding affinity to the thin filament in both solution (38) and in muscle fibers (5) is increased at low ionic strength, and therefore the effect thin filament activation by myosin strong binding is most likely enhanced in the motility assay compared with studies at physiological ionic strength (30). Consistent with these concepts, thin filament motility increased as a cooperative function of the myosin concentration on the motility surface at maximal and submaximal calcium activation. However, no difference in the activation of the thin filament by myosin strong binding was seen for the two TnT isoforms.

Fetal cardiac fibers are less affected by acidic condition compared with adult cardiac fibers (29), raising the possibility that TnT isoforms may respond differently to acidic conditions. Whereas unloaded shortening and cooperativity was depressed under mildly acidic conditions, no discernable difference was detected for thin filaments containing the two TnT isoforms. The above results notwithstanding, variable TnT isoform expression is most extensive in the fetal heart (2, 16, 27), suggesting a role for the TnT isoforms in the perinatal period. Furthermore, nonisoform modification of TnT is known to have significant effects on muscle contraction. There are several phosphorylation sites on TnT that are known to affect myofilament function (23, 25), and studies of TnT mutations identified in familial hypertrophic cardiomyopathy reveal significant effects on contractile function (21, 31, 32, 35).

Whereas no difference in contractile performance was delineated for the two TnT isoforms, the motility assay is not without limitations. The motility assay is performed in the absence of normal sarcomeric structure and is performed at lower-than-physiological ionic strength both of which could affect the results reported using this technique. Here we have used pure populations of troponin T isoforms; however, using a mixture of the two isoforms to mimic TnT isoform composition in myocardial failure and using all cardiac contractile proteins could possibly reveal subtle differences in TnT isoform performance not discovered in this study. Additional studies in myofibers and transgenically manipulated hearts where TnT isoform content is modified might provide additional evidence regarding the effects of the cardiac TnT isoforms on contractility.

In summary, a small TnT isoform shift has been reported in human and animal models of myocardial hypertrophy and failure. We did not detect any functional difference between the two beef TnT isoforms with respect to in vitro motility or isometric force. From the small magnitude of the shift in human myocardium and the lack of a demonstrable functional mechanical effect in vitro using pure populations of TnT isoforms, it is unlikely that the TnT isoform shift reported with the transition to failure is of any functional significance.

This work was supported by the National Heart, Lung, and Blood Institute Grants HL-52087, HL-66157, HL-65586, and HL-59408.

REFERENCES


AJP-Heart Circ Physiol • VOL. 282 • MAY 2002 • www.ajpheart.org