Coupled expression of troponin T and troponin I isoforms in single skeletal muscle fibers correlates with contractility

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Brotto, Marco A., Brandon J. Biesiadecki, Leticia S. Brotto, Thomas M. Nosek, and Jian-Ping Jin. Coupled expression of troponin T and troponin I isoforms in single skeletal muscle fibers correlates with contractility. Am J Physiol Cell Physiol 290: C567–C576, 2006.—Striated muscle contraction is powered by actin-activated myosin ATPase. This process is regulated by Ca$^{2+}$ via the troponin complex. Slow- and fast-twitch fibers of vertebrate skeletal muscle express type I and type II myosin, respectively, and these myosin isoenzymes confer different ATPase activities, contractile velocities, and force. Skeletal muscle troponin has also diverged into fast and slow isoforms, but their functional significance is not fully understood. To investigate the expression of troponin isoforms in mammalian skeletal muscle and their functional relationship to that of the myosin isoforms, we concomitantly studied myosin, troponin T (TnT), and troponin I (TnI) isoform contents and isometric contractile properties in a large number of Triton X-100-skinned rat skeletal muscle single fibers. We characterized a large number of Triton X-100-skinned single fibers from soleus, diaphragm, gastrocnemius, and extensor digitorum longus muscles and selected fibers with combinations of a single myosin isoform and a single class (slow or fast) of the TnT and TnI isoforms to investigate their role in determining contractility. Types IIA, IIX, and IIB myosin fibers produced higher isometric force than that of type I fibers. Despite the polyploidy of adult skeletal muscle fibers, the expression of fast or slow isoforms of TnT and TnI is tightly coupled. Fibers containing slow troponin had higher Ca$^{2+}$ sensitivity than that of the fast troponin fibers, whereas fibers containing fast troponin showed a higher cooperativity of Ca$^{2+}$ activation than that of the slow troponin fibers. These results demonstrate distinct but coordinated regulation of troponin and myosin isoform expression in skeletal muscle and their contribution to the contractile properties of muscle.

Muscle contraction is powered by actin-activated myosin ATPase (11). In cardiac and skeletal muscles, the contractile process is regulated by Ca$^{2+}$ through the troponin complex in the thin filament (14). The binding of Ca$^{2+}$ to troponin induces a series of allosteric changes in the thin filament, allowing the myosin head to form a strong cross bridge with filamentous actin to activate myosin ATPase and initiate contraction (32). Vertebrate skeletal muscle contains slow- and fast-twitch fibers (12, 47). Both myosin and troponin have evolved into slow and fast fiber type-specific isoforms. Slow and fast skeletal muscle fibers express type I and type II myosin, respectively, and these myosin isoenzymes have different ATPase activity (2). Previous studies from multiple investigators have demonstrated the contribution of four skeletal muscle myosin heavy chain (MHC) isoforms (types I, IIA, IIX, and IIB) to the magnitude and velocity of contraction of different types of muscle fibers (29, 45).

The troponin complex is at the center of Ca$^{2+}$ regulation of muscle contraction (32). Troponin consists of three subunits: the Ca$^{2+}$-binding subunit, troponin C (TnC); the inhibitory subunit, troponin I (TnI); and the tropomyosin-binding subunit, troponin T (TnT). TnC belongs to a family of Ca$^{2+}$-signaling proteins that includes CaM and myosin light chains (10). A fast isoform of TnC is found in fast-twitch fibers, and slow-twitch and cardiac muscles share another isoform of TnC (10, 38). In contrast, TnI and TnT are striated muscle-specific proteins, and both have diverged into three homologous isoforms corresponding to the cardiac, slow, and fast skeletal muscle fiber types (22, 39). The three TnI and three TnT isoform genes are closely linked in three pairs in the vertebrate genome. The fast TnI and fast TnT genes are linked in one pair (3). However, the cardiac TnI gene is linked to the slow TnT gene (24), and the slow TnI gene is linked to the cardiac TnT gene (49), although these genes have distinct expression patterns. These scrambled links of TnI and TnT isoform genes suggest that TnT and TnI isoform gene expression is regulated by the cellular environment rather than by genomic organization.

In contrast to the extensive studies of the functional role of skeletal muscle myosin isoforms (40, 41), the physiological significance of the fiber type-specific TnT and TnI isoforms is not well understood. The fast and slow cardiac TnC isoforms differ in that they contain two and one regulatory Ca$^{2+}$-binding sites, respectively (10). In contrast, the fast and slow TnI and TnT isoforms are highly conserved homologous proteins and have only minor structural differences (22, 39). Nonetheless, the finding that the loss of only slow skeletal TnI causes a lethal nemaline myopathy (30) provides strong evidence for the importance of the fiber type-specific TnT and TnI isoforms in the function of skeletal muscle. Therefore, the hypothesis that the functional difference between slow and fast TnI and TnT isoforms may have a critical role in the function of corresponding types of muscle fibers deserves detailed investigation.

Most skeletal muscles contain mixed slow and fast fibers (20). Therefore, it is necessary to study the contractility of individual fibers with clearly defined myofilament protein isoform content to understand the functional contribution of the fiber type-specific myosin, TnT, and TnI isoforms. In the present study, we investigated the myosin, TnI, and TnT isoform contents and isometric contractile properties in a large number of Triton X-100-skinned rat skeletal muscle single fibers.
fibers. Characterization of fibers containing representative combinations of a single myosin isoform and a single class of TnT and Tnl isoforms showed that the expression of fast or slow Tnl and TnT isoforms is tightly coupled in each fiber, despite the polyploid nature of skeletal muscle cells. Fibers containing slow Tnl and TnT showed higher Ca\(^{2+}\) sensitivity and lower cooperativity of Ca\(^{2+}\)-activated contraction than that of fast troponin fibers. The results reveal distinctive but concerted contributions of troponin and myosin isoforms to the contractility of skeletal muscle.

MATERIALS AND METHODS

Muscle tissues. Rats (10- to 12- wk-old males weighing ~250 g; Sprague-Dawley) were used in this study to provide skeletal muscle samples. They were euthanized by CO\(_2\) inhalation. A total of 12 diaphragm, 24 extensor digitorum longus (EDL), 24 gastrocnemius, and 24 soleus muscles from 12 rats were used to obtain single skinned muscle fibers.

All animal procedures were performed with the approval of the Case Western Reserve University Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals approved by the National Institutes of Health.

Specific MAbs. We previously developed a mouse MAb using rat cardiac muscle protein extract as an immunogen (27). This MAb (FA2) recognizes the cardiac β-MHC that is equivalent to MHC type I (MHC-I) in skeletal muscle and has no cross reaction to MHC type II (MHC-II). FA2 was therefore used to identify MHC-I skeletal muscle fibers.

We previously developed another MAb (CT3) that recognizes cardiac and slow skeletal muscle TnT, but not fast skeletal muscle TnT (26). The clear size difference between cardiac TnT and slow TnT allows convenient identification of slow TnT by performing Western blot analysis of muscle protein extracts.

An MAb T12 raised against rabbit fast skeletal muscle TnT (33) (a gift from Prof. Jim Lin, University of Iowa, Iowa City, IA) was used to identify fast TnT. Although MAB T12 binds weakly to cardiac TnT and slow TnT at high concentrations, we established a Western blot working concentration at which T12 specifically recognized only fast skeletal muscle TnT.

A mouse MAB Tnl-1 was used in Western blot analysis to identify fast and slow Tnl isoforms that showed clear difference in SDS gel mobility (28). The specificities of these MAbs are summarized in Fig. 1.

Separation of MHC isoforms by SDS-PAGE. Muscle tissue or single fibers were examined by performing SDS-PAGE for MHC contents. The gel formula and running conditions were slightly modified from the method of Talmadge et al. (48). Briefly, total protein was extracted from the tissue with SDS-PAGE sample buffer (2% SDS, 0.1% bromophenol blue, 10% glycerol, 50 mM Tris-HCl, pH 6.8) using a high-speed mechanical tissue homogenizer. Each single skinned muscle fiber was dissolved in 10 μL of SDS-PAGE sample buffer to extract myofibril proteins. The samples were heated at 80°C for 5 min, followed by brief vortexing and centrifugation at top speed in a microcentrifuge at room temperature for 5 min to remove insoluble materials. MHC isoforms were resolved using 8% polyacrylamide gel with an acrylamide-to-bisacrylamide ratio of 50:1 and containing 30% glycerol prepared in 200 mM Tris base, 100 mM glycine (pH 6.8), and 0.4% SDS. The stacking gel contained 4% acrylamide with an acrylamide-to-bisacrylamide ratio of 29:1, 70 mM Tris-HCl (pH 6.7), 4 mM EDTA, and 0.4% SDS. Aliquots of the muscle protein samples were loaded onto 0.75-mm-thick Mini-PRO-TEAN II gels (Bio-Rad Laboratories, Hercules, CA) and electrophoresed in an icebox for 18 h at 72 V, followed by another 6 h at 83 V. Resolved protein bands were visualized using silver staining as previously described (25).

Western blot analysis. Muscle tissue homogenates and protein extracted from Trition X-100-skinned muscle fibers used for contractility experiments were also analyzed by performing Western blot analysis for MHC, TnC, and TnT contents. The total protein extracts were resolved by performing SDS-PAGE on 14% Laemmlli gels with an acrylamide-to-bisacrylamide ratio of 180:1 and Coomassie Brilliant Blue R250 staining. B: dilutions of the same samples were analyzed using 8% SDS-PAGE gel with an acrylamide-to-bisacrylamide ratio of 50:1 and 30% glycerol followed by silver staining to reveal the 4 MHC isoforms. The samples were also examined using Western blot analysis with anti-MHC-I MAB FA2 (C), anti-slow skeletal muscle TnT MAB CT3 (D), anti-fast skeletal muscle TnT MAB T12 (E), and anti-Tnl MAB Tnl-1 (F). The results demonstrate effective identification of MHC, TnT, and Tnl isoforms using our experimental procedures. Similar to previously reported findings in mouse soleus muscle (26), 3 alternatively spliced slow TnT bands were found in the rat soleus on the CT3 Western blot. The two high molecular weight and one low molecular weight rat slow TnT shown correspond to the recently sequenced rat slow TnT isoforms 1, 2, and 4 (31).

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Fig. 1. Identification of troponin T (TnT), troponin I (Tnl), and myosin heavy chain (MHC) isoforms in muscle homogenate. A: total protein extracts of adult rat extensor digitorum longus (EDL), soleus (SOL), and diaphragm (DPH) muscles were resolved using 14% SDS-PAGE with an acrylamide-to-bisacrylamide ratio of 180:1 and Coomassie Brilliant Blue R250 staining. B: dilutions of the same samples were analyzed using 8% SDS-PAGE gel with an acrylamide-to-bisacrylamide ratio of 50:1 and 30% glycerol followed by silver staining to reveal the 4 MHC isoforms. The samples were also examined using Western blot analysis with anti-MHC-I MAb FA2 (C), anti-slow skeletal muscle TnT MAB CT3 (D), anti-fast skeletal muscle TnT MAB T12 (E), and anti-Tnl MAB Tnl-1 (F). The results demonstrate effective identification of MHC, TnT, and Tnl isoforms using our experimental procedures. Similar to previously reported findings in mouse soleus muscle (26), 3 alternatively spliced slow TnT bands were found in the rat soleus on the CT3 Western blot. The two high molecular weight and one low molecular weight rat slow TnT shown correspond to the recently sequenced rat slow TnT isoforms 1, 2, and 4 (31).
containing 0.5% Triton X-100 and 0.05% SDS, the membrane was incubated with alkaline phosphatase-labeled goat anti-mouse IgG secondary antibody (Sigma) in TBS-BSA. After being washed again as described above, the blots were developed in 5-bromo-4-chloro-3-indolylphosphate nitro blue tetrazolium substrate solution to reveal the specific protein bands recognized by the antibodies.

To obtain multiple Western blots of the limited amount of proteins extracted from a single rat muscle fiber, two strategies were applied. Because TnT and TnI are represented at significantly different sizes on Western blots, the anti-slow TnT and anti-TnI MAbs could be used as mixture in the first blot. After color development, the first blots were scanned to record the data before being dried completely. The semiwet blots were reprocessed using the Western blot analysis procedure for additional probing with the anti-fast TnT MAb T12. By subtracting the first blot information, the expression of fast TnI in the muscle fibers could be evaluated.

Examination of myofilament protein isoform expression in different parts of the rat diaphragm. The entire diaphragm was dissected from the rib cage and dissected free of thoracic abdominal wall muscles. The diaphragm was dissected into twelve 30° sectors (Fig. 2A). The central tendon was carefully removed. Each sector was homogenized in 40 volumes (vol/wt) of SDS-PAGE sample buffer, heated, and centrifuged, and then the samples were examined by performing SDS-PAGE and Western blot analysis for TnT and TnI isoform expression as described above. The diaphragm muscle extracts were diluted 1,000-fold (vol/vol) and analyzed using glycerol SDS-PAGE as described above to examine the MHC isoform contents.

Skinned single muscle fibers. In all experiments, we followed the protocols described previously by Brotto and Nosek (8) as recently modified (25). Isometric contractile properties were investigated in single rat muscle fibers of diaphragm, EDL, gastrocnemius, and soleus muscles. Intact muscles were carefully removed from the animals and then immediately transferred and dissected on ice in a solution containing (in mM) 1.0 MgCl₂, 1.0 MgATP, 15 phosphocreatine, 140.0 potassium methanesulfonate, 50.0 imidazole, 20.0 EGTA, 170 ionic strength, pH 7.0, at 4°C and pCa >8.5. The skinnning solution also contained a cocktail of protease inhibitors (0.1 mM PMSF, 0.1 mM leupeptin, 1.0 mM benzamidine, and 10 μM aprotinin) to protect the fibers from proteolytic damage. After single muscle fibers were dissected, they were exposed for 30 min to the above-described solution containing 0.5% wt/vol Triton X-100 (a nonionic detergent that permeabilizes the sarcolemmal membrane and all membrane organelles). Triton X-100 skinnning of muscle fibers allows for accurate control of Ca²⁺ and other ionic concentrations surrounding the myofilaments for the study of contractile protein function (8, 25, 34).

Mechanical measurement. Single muscle fibers were mounted between an optical electric force transducer (Scientific Instruments, Heidelberg, Germany) and a movable arm by wrapping the fibers three times around small stainless steel wires. After being mounted, only a small portion of the fibers (∼300–500 μm) remained free between the mounting wires, which allowed the avoidance of excitation of mechanical artifacts. The muscle fibers were then briefly exposed (∼30 s) to pCa 6.0 to induce a contraction to ensure the secure mounting of the single fibers to the wires. This initial contraction was induced to tighten the wrapping to minimize artificial compliance due to loose mounting of the fiber. As demonstrated previously (8, 25, 34), the central part of muscle fibers mounted in this way is undamaged and generates highly reproducible data regarding the force versus pCa relationship. In addition, mounting without the use of adhesives allows for easier use of muscle fibers for biochemical analysis after completion of the force versus pCa curves, because fibers can be removed without having to be sectioned, allowing for the recovery of more tissue for protein analysis.

To examine the isometric properties of the wrapping method directly with the commonly used adhesive method of fiber mounting, we conducted a series of experiments to compare the Ca²⁺ sensitivity and maximal Ca²⁺-activated force of rat EDL muscle fibers before and after fixation of the extremities of the wrapped fibers with adhesives. As shown in Fig. 3 and Table 1, this comparison confirmed that the wrapping method used in the present study produced reliable recording of Ca²⁺-activated force development for single muscle fibers.

Muscle fibers were bathed in room temperature (24 ± 1°C) solutions contained in 2.5-ml troughs milled in a spring-loaded Plexiglas plate. The composition of all solutions was calculated using a customized computer software program (Turbo-Pascal 87, version 3.0; Borland International, Scotts Valley, CA) with equilibrium constants (13) routinely used in our laboratory (25). To measure the isometric contractile force, the fibers were optically stretched (∼20%) from their slack length to control the sarcromere length at 2.5 ± 0.1 μm as measured by its laser diffraction.
The MHC, Tnl, and TnT isofrom contents of each single muscle fiber were used to classify the muscle fiber type. Fibers that had clear single MHC and a single class (slow or fast) of TnT and Tnl isoforms were selected to investigate the role of myosin and TnT and Tnl isoforms in determining contractility features. Only the muscle fibers with a complete set of data (i.e., force versus pCa relationships, SDS-PAGE, and immunoblot analysis performed on the same fiber) are summarized in this report.

**Analysis of Fmax versus sarcomere lengths.** Because diaphragm muscles may have optimal sarcomere lengths that are significantly longer than those of the other muscles analyzed (42), we compared isometric Fmax and Ca50 for diaphragm fibers at a sarcomere length of 3.0 ± 0.1 μm compared with that at 2.5 ± 0.1 μm. This procedure was performed as described above. To extend the evaluation to other muscles, we also compared isometric properties for soleus fibers at a sarcomere length of 3.0 ± 0.1 μm compared with that at 2.5 ± 0.1 μm.

**Statistical analysis.** SigmaStat 3.0 software (SPSS, Chicago, IL) was used for statistical analysis. The criterion for statistical significance was P < 0.05. Statistical analysis of parametric sets of data was performed using one-way ANOVA with SigmaStat software followed by Tukey’s post hoc test. For nonparametric sets of data, we used the Kruskal-Wallis one-way ANOVA on ranks test. Sets of data comprising the force versus pCa relationships were tested using the Kruskal-Wallis one-way ANOVA on ranks test followed by Dunn’s test for multiple comparisons.

### RESULTS

**Effective identification of TnT, Tnl, and MHC isoforms in isolated single skeletal muscle fibers.** To define the contribution of TnT and Tnl isoforms to skeletal muscle contractility and their correlation to myosin isoforms, it is essential to identify single fibers containing simple combinations of TnT, Tnl, and MHC isoforms. Therefore, we developed biochemical and immunohistochemical analysis methods sufficiently sensitive to determine the MHC, TnT, and Tnl isoform contents in the proteins extracted from a single skinned rat skeletal muscle fiber, which allowed us to focus the present study on the fibers that contained a single MHC isoform and a single class of TnT and Tnl isoforms.

Figure 1B shows that the glycerol SDS-PAGE gel used in our study was able to resolve the four MHC isoforms (types I, IIa, IIx, and IIb) clearly in rat skeletal muscle in agreement with the gel mobility and specific antibody identification performed in previous studies (4, 48). The Western blot shown in Fig. 1C further demonstrates that MAb FA2 raised against rat cardiac myosin (27) recognized MHC-I (equivalent to cardiac β-MHC) but not MHC-IIa, MHC-IIx, or MHC-IIb (Fig. 1B).

Figure 1D shows that the cardiac and slow TnT-specific MAb

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Fmax, kN/m²</th>
<th>Ca50, μM</th>
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<tr>
<td>EDL</td>
<td></td>
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<tr>
<td>Before glue, n = 10</td>
<td>305.00 ± 29.58</td>
<td>2.31 ± 0.05</td>
</tr>
<tr>
<td>After glue, n = 10</td>
<td>270.00 ± 35.00</td>
<td>2.48 ± 0.1</td>
</tr>
<tr>
<td>Soleus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before glue, n = 6</td>
<td>166.00 ± 22.78</td>
<td>1.81 ± 0.08</td>
</tr>
<tr>
<td>After glue, n = 6</td>
<td>155.00 ± 35.00</td>
<td>1.86 ± 0.1</td>
</tr>
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</table>

Data are averages ± SE. No statistical significance was present in EDL (P = 0.189) and in soleus muscle fibers (P = 0.236). Fmax, maximum Ca2⁺-activated isometric force; Ca50, Ca2⁺ concentration producing half-maximal force; EDL, extensor digitorum longus.
Table 2 summarizes the results showing that more than one MHC isoform, we found 80 fibers that contained a single MHC isoform together with slow or fast TnI/TnT isoforms in several representative combinations (Table 2). The representative SDS-PAGE and Western blot analysis results in Fig. 4 show that the following groups of fibers were found in rat muscles at significant frequencies: 1) MHC-I with slow TnI and slow TnT, 2) MHC-Iib with fast TnI and fast TnT, 3) MHC-Iix with fast TnI and fast TnT, and 4) MHC-IIa with fast TnI and fast TnT. Notably, although MHC-IIa has been reported in slow fibers (1, 4), in the present study it was found only in fibers expressing fast TnI and fast TnT, whereas slow TnI and slow TnT were found only in fibers expressing MHC-I. Those fibers were investigated for functional correlations.

Ca\(^{2+}\) sensitivity and cooperativity of force production in Triton X-100-skinned single fibers of rat skeletal muscle. Figure 5 shows the normalized isometric force versus pCa relationships in rat diaphragm muscle fibers containing 1) MHC-I and slow TnI and slow TnT; 2) MHC-IiA, fast TnI, and fast TnT; 3) MHC-IIX, fast TnI, and fast TnT; and 4) MHC-Iib, fast TnI, and fast TnT. Table 2 summarizes the results showing that in the fibers containing MHC-I and slow TnI/TnT, a higher level of MHC-Iib compared with that in other regions. Western blot analysis of TnT and TnI isoforms in different regions of diaphragm muscle showed similar ratios of slow- to fast-twitch skeletal muscle TnT and slow- to fast-twitch skeletal muscle TnI (Fig. 2B). These ratios were similar to those detected in the total diaphragm muscle homogenate (Fig. 1). Although the functional significance of the lower MHC-Iib content in the dorsal region of the rat diaphragm remains to be investigated, the results reported herein indicate that picking single fibers from most areas of the rat diaphragm would have yielded similar results. This observation was confirmed by the results of our random selection of skinned single fibers, which showed no bias between fibers from various parts of the diaphragm.

Expression of fast or slow TnI and TnT isoforms is coupled in all single muscle fibers examined and the presence of fibers containing simple combinations of MHC and TnT and/or TnI isoforms. Using sensitive detection methods with small amounts of myofilament protein extract, we determined MHC, TnI, and TnT isoform contents in all single skinned fibers of rat diaphragm, soleus, EDL, or gastrocnemius muscles after functional analysis. Not all representative combinations of MHC, TnT, and TnI isoforms were found in the rat diaphragm. Therefore, we analyzed single fibers from representative slow (soleus) and fast (EDL) fiber muscles as well as from a mixed fiber muscle (gastrocnemius) to include the most possible combinations of MHC, TnT, and TnI isoforms in the functional characterization. We studied a total of 390 single fibers and were able to collect complete sets of data from 310 fibers. In fibers containing a single class (slow or fast) of the TnI or TnT isoform, we found that the expression of slow or fast isoforms of TnT and TnI are 100% coupled; slow TnI is always accompanied by slow TnT, and the same is true for fast TnI and fast TnT in individual muscle fibers, despite their muscle origin or the expression of one or more MHC isoforms.

Although a significant portion of the single fibers examined expressed more than one MHC isoform, we found 80 fibers that contained a single MHC isoform together with slow or fast TnI/TnT isoforms in several different regions of the rat diaphragm muscle (Fig. 2B). These results demonstrate effective identification of all skeletal muscle MHC, TnI, and TnT isoforms and form a foundation for our experimental system.

Similar expression patterns of TnI, TnT, and MHC isoforms in different regions of the rat diaphragm. To determine whether a random selection of single fibers from the whole rat diaphragm can yield representative fibers for characterization, we examined different regions of the rat diaphragm for overall protein contents and patterns of MHC, TnI, and TnT isoform expression. SDS-PAGE demonstrated that the twelve 30° sectors of rat diaphragm muscle (Fig. 2A) had no apparent significant differences in protein contents (Fig. 2B). The 12 sectors of diaphragm muscle were further examined for MHC isoforms by performing glycerol SDS-PAGE. The results shown in Fig. 2B demonstrate that nearly all areas of the diaphragm muscle had similar patterns of MHC isoform expression, except in the dorsal region (sectors 1 and 12, Fig. 2A), which had a lower level of MHC-Iib compared with that in other regions. Western blot analysis of TnT and TnI isoforms in the different regions of diaphragm muscle showed similar ratios of slow- to fast-twitch skeletal muscle TnT and slow- to fast-twitch skeletal muscle TnI (Fig. 2B). These ratios were similar to those detected in the total diaphragm muscle homogenate (Fig. 1). Although the functional significance of the lower MHC-Iib content in the dorsal region of the rat diaphragm remains to be investigated, the results reported herein indicate that picking single fibers from most areas of the rat diaphragm would have yielded similar results. This observation was confirmed by the results of our random selection of skinned single fibers, which showed no bias between fibers from various parts of the diaphragm.

Expression of fast or slow TnI and TnT isoforms is coupled in all single muscle fibers examined and the presence of fibers containing simple combinations of MHC and TnT and/or TnI isoforms. Using sensitive detection methods with small amounts of myofilament protein extract, we determined MHC, TnI, and TnT isoform contents in all single skinned fibers of rat
fibers were more sensitive to Ca²⁺ produced higher levels of force than slow diaphragm fibers (P < 0.05). No significant differences were found between the contractile parameters of slow troponin and EDL muscles also had no significant differences in contractility. These features are summarized in Table 2.

The isometric contractility of skinned single fibers from different muscles demonstrated that the Fₘₐₓ for the diaphragm fibers containing MHC-II + fast TnI and fast TnT was slightly lower than that of the EDL and gastrocnemius fibers containing the same combination of myosin and TnI and TnT isoforms (Table 2). Because muscle isometric force development is dependent on the optimal length of the sarcomere (15), we further measured isometric contraction of rat diaphragm fibers at a longer sarcomere length (3.0 μm) and compared it with that at the 2.5-μm sarcomere length used in all other measurements. The results listed in Table 3 show that the diaphragm fibers had increased Fₘₐₓ at the longer sarcomere length, reaching a level comparable to that of the fibers from other muscles at 2.5-μm sarcomere length. In contrast, an increase in sarcomere length from 2.5 to 3.0 μm did not produce significant changes in Fₘₐₓ and Ca₅₀ values in soleus (174.7 ± 25 vs. 160.8 ± 17 kN/m² and 1.89 ± 0.01 vs. 1.96 ± 0.02 μM, respectively; n = 9) and gastrocnemius fibers (306.00 ± 29 vs. 290.7 ± 19 kN/m² and 2.23 ± 0.01 vs. 2.21 ± 0.03 μM, respectively; n = 9).

These data suggest the presence of other myofilament elements that may determine the optimal sarcomere length for contractile force in fibers from different skeletal muscles. This factor is apparently different between diaphragm and other muscles and remains to be investigated.

### DISCUSSION

A polyploid skeletal muscle fiber can express a single isoform of MHC and/or a single class of TnT and TnI. We previously reported that in Amish nemaline myopathy, the loss of slow TnT results in lethal neuromuscular abnormalities (25). The absence of only one isoform of TnT causing severe myopathy is evidence of a critical role for the fiber type-specific troponin isoforms in skeletal muscle development and function. Most skeletal muscles contain both fast- and slow-twitch fibers at various ratios. To investigate the contribution

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### Table 2. Isometric contractile parameters of Ca²⁺-activated force of single fibers at sarcomere length of 2.5 μm for four distinct fiber types from four different rat muscles

<table>
<thead>
<tr>
<th>Muscle Fiber Types</th>
<th>Fₘₐₓ, kN/m²</th>
<th>Ca₅₀, μM</th>
<th>Hill Coefficient</th>
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<tbody>
<tr>
<td>Diaphragm</td>
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<tr>
<td>Slow TnI/TnT + MHC-I (n = 8)</td>
<td>155.23±34.49</td>
<td>1.83±0.02</td>
<td>2.00±0.2</td>
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<tr>
<td>Fast TnI/TnT + MHC-IIa (n = 15)</td>
<td>214.03±27.83</td>
<td>2.51±0.002</td>
<td>4.75±0.31</td>
</tr>
<tr>
<td>Fast TnI/TnT + MHC-IIx (n = 17)</td>
<td>214.82±33.71</td>
<td>2.61±0.002</td>
<td>5.04±0.60</td>
</tr>
<tr>
<td>Gastrocnemius</td>
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<tr>
<td>Fast TnI/TnT + MHC-IIb (n = 7)</td>
<td>321.04±38.04</td>
<td>2.32±0.005</td>
<td>4.70±0.59</td>
</tr>
<tr>
<td>Fast TnI/TnT + MHC-IIx (n = 9)</td>
<td>291.65±44.30</td>
<td>2.20±0.02</td>
<td>4.72±0.43</td>
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<tr>
<td>Soleus</td>
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<td></td>
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<tr>
<td>Slow TnI/TnT + MHC-I (n = 10)</td>
<td>169.74±34.50</td>
<td>1.92±0.01</td>
<td>1.95±0.25</td>
</tr>
<tr>
<td>EDL</td>
<td></td>
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<tr>
<td>Fast TnI/TnT + MHC-IIb (n = 14)</td>
<td>297.92±39.98</td>
<td>2.30±0.003</td>
<td>4.41±0.24</td>
</tr>
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</table>

Data are means ± SE. Numbers in parentheses are the number of fibers analyzed. Statistical analyses revealed no significant differences among slow troponin fibers from diaphragm and soleus muscles or among fast troponin fibers from diaphragm, EDL, and gastrocnemius muscles. In contrast, diaphragm fast fibers produced higher levels of force than slow diaphragm fibers (P < 0.001), and all fast fibers from diaphragm, EDL, and gastrocnemius muscles produced higher levels of force than slow diaphragm and soleus muscle fibers (P < 0.01). Hill coefficients were greater in fast troponin fibers (P < 0.02), whereas slow troponin fibers were more sensitive to Ca²⁺ (P < 0.05). No significant differences were found between the contractile parameters of slow troponin + myosin heavy chain (MHC)-I fibers from diaphragm and soleus muscles. TnI, troponin I; TnT, troponin T.
of troponin isoforms to muscle contractility, one needs to take into consideration the function of myosin isoforms. Therefore, it is necessary to characterize isolated single fibers with definitive troponin and myosin isoform contents. We have demonstrated in the present study the feasibility of analyzing large numbers of single muscle fibers for contractility and determination of myofilament protein isoform contents. The present results reveal that polyploid adult vertebrate skeletal muscle fiber cells can express a single class of TnI, TnT, and/or MHC isoforms. This finding indicates a cellular environment-determined regulation of myofilament protein isoforms that may coordinate the multiple nuclei inside a muscle cell (fiber). This finding justifies the use of a muscle fiber as the functional unit with which to investigate the differentiation and functional adaptation of muscle during development and under physiological and pathological stress conditions. The present results lay a foundation for investigating the functional significance of fast- and slow-twitch muscle fibers as well as the fast and slow TnT and TnI isoforms.

Fig. 5. Isometric force vs. pCa relationship of representative diaphragm and gastrocnemius muscle fibers. A: diaphragm muscle fibers containing MHC-I and slow TnI/TnT (slow troponin), MHC-IIa and fast TnI/TnT (fast troponin), or MHC-IIx and fast troponin were compared. The normalized force-pCa curves show that the cooperativity of activation was higher in the MHC-IIa and fast troponin groups than in the fibers expressing MHC-I and slow troponin. However, the MHC-I and slow troponin fibers were more sensitive to Ca\(^{2+}\) (Table 2). No significant differences were detected between the MHC-IIa and MHC-IIx groups. B: gastrocnemius muscle fibers containing MHC-IIa and fast troponin or MHC-IIx and fast troponin also showed no significant differences in contractile properties.

Fig. 6. Isometric force vs. pCa relationship of slow and fast fibers from different muscles. A: slow fibers [containing MHC-I and slow TnI/TnT (slow troponin)] from diaphragm or soleus muscles showed similar [Ca\(^{2+}\) \(_{50}\)] producing half-maximal force (Ca\(_{50}\)) and cooperativity. B: fibers containing MHC-IIx and fast TnI/TnT (fast troponin) from diaphragm or gastrocnemius muscles demonstrated similar isometric contractile features. C: fibers containing MHC-IIb and fast troponin from gastrocnemius or EDL muscle also showed similar isometric contractile properties.

TnT and TnI isoforms in muscle differentiation, adaptation, and fatigue as well as in a number of pathological conditions. Coupled expression and function of fast or slow TnT and TnI isoforms. In agreement with previous observations in rabbit and chicken skeletal muscle fibers (17, 44), the well-matched slow or fast TnT and TnI isoform contents in single fibers isolated from different rat muscles demonstrated a tightly
coupled expression of the two troponin subunit isoforms. In contrast to TnC isoforms that are members of the CaM gene family (10), TnT and TnI are encoded by striated, muscle-specific genes with a coevolutionary relationship (24). Together with the slow and fast fiber type-specific expression of TnC isoforms (38), the coupled expression of fast or slow TnT and TnI in all fibers analyzed indicates that the troponin subunit gene is uniformly regulated in a polyploid muscle fiber. It is important to note that the slow Tnl and slow TnC genes are not physically paired in the vertebrate genome (24, 49). Therefore, their strictly coupled expression in each muscle type and all single fibers examined indicates a determinative role of cellular trans-regulatory factors other than genomic linkage. This observation supports the functional importance of slow and fast TnT and TnI isoforms to muscle contractile features. This finding also indicates the presence of a coordinated regulatory mechanism for the TnI and TnT isoforms, which may play roles in muscle fiber differentiation and functional adaptation.

TnT and TnI isoforms are useful markers for the classification of skeletal muscle fibers. MHC isoforms have classically been used as markers for skeletal muscle fiber typing (4, 5, 7, 29). However, the complex pattern of MHC isoform contents in most muscle fibers makes fiber typing complicated. It is commonly understood that MHC-I is specific to the slow fibers and that MHC-IIx and IIb are specific to fast fibers. The typical slow-twitch soleus muscle is known to express both MHC-I and MHC-IIa (Fig. 4), whereas the classic fast-twitch EDL muscle expresses both MHC-IIa and MHC-IIb. Although MHC-IIa is expressed in soleus muscle, our isoformic force results have shown that MHC-IIa fibers behave similarly to MHC-IIb and MHC-IIx fibers, but with higher isometric force than that of MHC-I fibers (Table 2). In contrast, we have shown that the expression of TnT and TnI isoforms is highly specific to the muscle fiber type and is related to the contractile features of the fiber type. Unlike MHC isoforms, most muscle fibers express only fast or slow TnT and TnI. For example, although both slow and fast TnT are detected in the homogencate of whole soleus muscle, most soleus fibers express either slow or fast TnT with matched TnI isoforms. Thus slow and fast TnT and TnI isoforms are useful markers for the identification of the functional characteristics of fast and slow muscle fiber types. The expression and functional relationships between troponin and MHC isoforms revealed in the present study provide a novel approach to the functional classification of skeletal muscle fiber types.

Table 3. Fmax and Ca50 for fast fibers from diaphragm muscles at sarcomeric lengths of 2.5 and 3.0 μm

<table>
<thead>
<tr>
<th>Fiber Types</th>
<th>Fmax, kN/m²</th>
<th>Ca50, μM</th>
<th>Fmax, kN/m²</th>
<th>Ca50, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5-μm SL</td>
<td></td>
<td></td>
<td>3.0-μm SL</td>
<td></td>
</tr>
<tr>
<td>Fast TnT/TnT + MHC-IIa, n = 3</td>
<td>204.83±17.67</td>
<td>2.48±0.06</td>
<td>293.22±21.56</td>
<td>2.55±0.07</td>
</tr>
<tr>
<td>Fast TnT/TnT + MHC-IIx, n = 3</td>
<td>209.12±23.71</td>
<td>2.59±0.05</td>
<td>289.30±13.72</td>
<td>2.63±0.08</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. Average Fmax was higher when sarcomere length (SL) was set at 3.0 μm compared with 2.5 μm. The 2.5-μm SL Fmax values in this set of experiments were comparable to those obtained for fast fibers of other muscles studied (see Table 2). *P < 0.001.

Slow and fast TnT isoforms determine the Ca²⁺ sensitivity and cooperativity of myofilaments. The expression of TnT and TnI isoforms is regulated during muscle development and fiber type differentiation. Most adult skeletal muscles of vertebrate animals, including the representative slow soleus muscle, contain mixed fast and slow fibers (4, 24, 41). The slow fibers are known to be important in sustained muscle contraction (21, 46). The results shown in Fig. 5A demonstrate that the rat muscle single fibers containing slow troponin had higher Ca²⁺ sensitivity than those that contained fast troponin. In addition, the fibers expressing fast troponin showed higher cooperativity during Ca²⁺ activation of contraction than did fibers that expressed slow troponin (Table 2). Although the slow troponin fibers analyzed in the present study were all MHC-I fibers and all fast troponin fibers contained MHC-II, we previously identified soleus fibers expressing slow myosin (MHC-I) but fast TnT and TnI (25). In those fibers, the Fmax is lower than that found in MHC-II fibers and Ca²⁺ sensitivity is lower than that of slow troponin fibers. Therefore, the present data suggest that the TnT and TnI isoforms rather than the MHC isoform may be determining factors for Ca²⁺ regulation of slow and fast muscle fibers.

In support of this observation, we previously demonstrated that cardiac muscle from transgenic mice overexpressing fast skeletal muscle TnT demonstrated increased cooperativity during Ca²⁺ activation of contraction (23). We also showed in chicken skeletal muscles expressing identical isoforms of TnI and TnC that acidic or basic isoforms of TnT had different Ca²⁺ sensitivities (36). Together with the observation that TnT-tropomyosin interaction is cooperative (37), these results indicate TnT isoforms may be a key element for the role of slow and fast troponin in the modulation of Ca²⁺ sensitivity and cooperativity of myofilaments. It is worth noting that the central and COOH-terminal domains of TnT are conserved in all isoforms and are the determinants for acidic and basic TnT isoforms; therefore, the observed functional differences are the amount of their NH₂-terminal negative charge (23, 36, 50).

Moss et al. (35) showed that the Hill coefficient was markedly different between 0 and 50% of maximum tension compared with that between 50 and 100% of maximum tension, thus giving rise to two different nH values. This observation supports the idea that the property of the troponin complex can affect muscle cooperativity. Our force versus pCa data points were properly fitted using a single Hill coefficient. The difference may be due to the fact that we were analyzing fibers with one class (fast or slow) of TnT and TnI isoform and one myosin isoform instead of comparing fibers with undefined TnT and TnI isoforms. Although TnC deficiency might alter cooperativity features, we found in a previous study (9) that when TnC was partially lost, the force versus pCa parameters would be significantly different from those reported in the present study. The integrity of the troponin complex in the fibers analyzed in the present study is further supported by the high absolute force levels, as a decrease in TnC content would have decreased the maximal Ca²⁺-activated force.
Similar isometric contractility of muscle fibers expressing MHC-IIa, MHC-IIx, and MHC-Ib. The single-fiber contractility analysis suggests that the myosin isoform content determines the maximum force of isometric contraction. The data in Table 2 show that maximal force produced by fibers expressing MHC-II was higher than that produced by fibers containing MHC-I. These results are consistent with the fact that slow myosin has lower ATPase activity than that of the fast myosin isoenzyme (2), which may contribute to the different \( F_{\text{max}} \) that developed. Previous studies have shown that skeletal muscle containing MHC-IIa, MHC-IIx, and MHC-Ib have different contractile velocities (6). On the other hand, our results demonstrate similar maximum steady-state force produced by the rat muscle fibers containing MHC-IIa, MHC-IIx, and MHC-Ib (Table 2). It is interesting to note that the moderately different \( F_{\text{max}} \) values of diaphragm and EDL type II muscle fibers were dependent on the resting length of the muscle, suggesting different optimal lengths for the generation of maximum steady-state force that may be determined by other myofilament components.

It is also plausible to speculate that such differences may be related to functional differences between muscles; for example, diaphragm muscle is the only rhythmic skeletal muscle among the muscles studied herein that possesses a remarkably different geometry compared with other skeletal muscles (42, 43). In diaphragm muscles, it has been demonstrated that the transmission of force occurs not only longitudinally but also transversely or diagonally because of the special architecture of this muscle. Furthermore, there is evidence suggesting that elastic components may play an important role in contributing to force development, maintenance, and relaxation in diaphragm muscles (18). It is also important to point out that our study was focused on investigation of isometric contractile properties and not on providing information regarding contractile velocity or isotonic and lengthening properties of contractile function. In addition, because the present study focused on the function of TnT and TnI isoforms by analysis of the relationships under isometric contractile conditions, the relationship of isometric force production and contractile velocity in the presence of different myosin isoforms remains to be investigated.

In summary, the present study demonstrates that TnT and TnI isoform expression is tightly coupled in individual fibers of skeletal muscle and contributes to myofilament Ca\(^{2+}\) regulation. The separated gene regulation of troponin and myosin isoforms is coordinated in muscle fibers, providing a molecular basis for the highly plastic adaptability of adult skeletal muscle.

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