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

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(Summary)

Brotto, Marco A., Brandon J. Biesiadecki, Leticia S. Brotto, Thomas M. Nosek, and J.-P. Jin. 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 and troponin T (TnT) and troponin I (TnI) isoform contents and isometric contractile properties in single fibers of rat skeletal muscle. We characterized a large number of Triton 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 TnT and TnI isoform to investigate their role in determining contractility. Type 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, while fibers containing fast troponin showed a higher cooperativity of Ca^{2+} activation than that of the slow troponin fibers. The results demonstrate distinctive, but coordinated, regulation of troponin and myosin isoform expression in skeletal muscle and their contribution to the contractile properties.

Keywords

Skeletal muscle; skinned single fiber; troponin; myosin; isoform; contractility; diaphragm; soleus; gastrocnemius; extensor digitorum longus.

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 F-actin to activate myosin ATPase and initiate contraction (**32**). Vertebrate skeletal muscle contains slow and fast twitch

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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 (type 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 the Ca^{2+}-regulation of muscle contraction (**32**). Troponin consists of three subunits: the Ca^{2+}-binding subunit, TnC, the inhibitory subunit, TnI and the tropomyosin-binding subunit, TnT. TnC belongs to a family of Ca^{2+} signaling proteins including calmodulin and myosin light chains (**10**). A fast isoform of TnC is found in fast twitch fibers and slow 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 the TnT and TnI isoform gene expression is regulated by cellular environment rather than by genomic organization.

In contrast to the extensive studies on 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 differ by containing two or one regulatory Ca^{2+} binding sites, respectively (**10**). In contrast, the fast and slow TnT and TnI isoforms are highly conserved homologous proteins and have only minor structural differences (**22;39**). Nonetheless, the finding that the loss of only slow skeletal TnT causes a lethal nemaline myopathy (**30**) provided 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 contents in order to understand the functional contribution of the fiber type-specific myosin and 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-skinned rat skeletal muscle single fibers. Characterization of fibers containing representative combinations of single myosin and single class of TnT and TnI isoforms showed that the expressions of fast or slow TnI and TnT isoforms were tightly coupled in each fiber despite the polyploidy nature of skeletal muscle cells. Fibers containing slow TnI 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 (Sprague-Dawley, males, 6 months old, weighing ~250g) were used in this study to provide skeletal muscle samples. Rats were euthanized by CO_2 inhalation. A total of 9 diaphragm, 18 extensor digitorum longus (EDL), 18 gastrocnemius and 18 soleus muscles from 9 rats were used for obtaining single skinned muscle fibers in this study.

All animal procedures were carried out with approval by the Case Western Reserve University Institutional Animal Care and Use Committee and were conducted in accordance with the "Guiding Principles in the Care and Use of Animals" as approved by the National Institutes of Health, United States Public Health Service.

Specific Monoclonal Antibodies. We have previously developed a mouse monoclonal antibody (mAb) using rat cardiac muscle protein extract as immunogen (**27**). This mAb (FA2) recognizes the cardiac***b***-MHC that is equivalent to type I MHC in skeletal muscle and has no cross reaction to MHC-II. FA2 was, therefore, used to identify type I skeletal muscle fibers.

We have 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 on Western blot of muscle protein extracts.

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

A mouse mAb TnI-1 was used in Western blot to identify fast and slow TnI isoforms that show clear difference in SDS-gel mobility (**28**).

The specificities of these mAbs are summarized in Fig. 1

Separation of MHC Isoforms by SDS-Polyacrylamide Gel Electrophoresis. Muscle tissue or single fibers were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for the myosin heavy chain (MHC) contents. The gel formula and running conditions were slightly modified from the method of Talmadge et al. (**48**). Briefly, total protein was extracted 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 ***m***L SDS-PAGE sample buffer to extract myofibril proteins. The samples were heated at 80°C for 5 min followed by a brief vortexing and centrifugation at top speed in a microcentrifuge at room temperature for 5 min to remove insoluble materials. MHC isoforms were resolved by 8% polyacrylamide gel with an acrylamide:bisacrylamide ratio of 50:1 containing 30% glycerol prepared in 200 mM Tris base, 100 mM glycine (pH 8.8) and 0.4 % SDS. The stacking gel contained 4% polyacrylamide with an acrylamide: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 BioRad mini-Protean II gels and electrophoresed in an ice box for 18 hrs at 72 V, followed by 83 V for 6 hrs. Resolved protein bands were visualized by silver stain as previously described (**25**).

Western Blot Analysis. Muscle tissue homogenates and protein extracted from Triton-skinned muscle fibers used for contractility experiments were also analyzed by Western blotting for their MHC, TnI and TnT contents. The total protein extracts were resolved by SDS-PAGE on 14% Laemmli gels with an acrylamide-to-bisacrylamide ratio of 180:1 and visualized by silver stain or transferred to nitrocellulose using a BioRad semi-dry electrotransfer apparatus for Western blot analysis using the anti-MHC, TnI and TnT isoform mAbs. As described previously (**25**), individual or mixtures of the mAbs were diluted in Tris-buffered saline (TBS) containing 0.1% bovine serum albumin (BSA) and incubated with the nitrocellulose membrane. After high stringency washes with TBS containing 0.1% Triton X-100 and 0.05% SDS, the membrane was incubated with alkaline phosphatase-labeled goat anti-mouse IgG second antibody (Sigma) in TBS-BSA. Washed again as 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 on the limited amount of proteins extracted from a single rat muscle fiber, two strategies are applied. Since TnT and TnI have a significant size difference on the Western blots, the anti-slow TnT and anti-TnI mAbs can be used as mixture in the first blot. After color development, the first blots were scanned to record the data before completely dried. The semi-wet blots were reprocessed by the Western procedure for additional probing using the anti-fast TnT mAb T12. By subtracting the first blot information, the expression of fast TnT in the muscle fibers can be evaluated.

Examination of Myofilament Protein Isoform Expression in Different Parts of Rat Diaphragm. The entire diaphragm was removed 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 (v/w) of SDS-PAGE sample buffer, heated and centrifuged, the samples were examined by SDS-PAGE and Western blotting for TnT and TnI isoform expression as described above. The diaphragm muscle extracts were then diluted for 1,000 folds (v/v) and analyzed by the glycerol SDS-PAGE as above to examine the MHC isoform contents.

Skinned Single Muscle Fibers. All experiments followed the protocols as described in Brotto & 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, immediately transferred and dissected on ice in a solution containing (in mM): 1.0 $MgCl_2$; 1.0 MgATP; 15 phosphocreatine; 140.0 potassium methanesulfonate (KMS); 50.0 imidazole, 20.0 EGTA, 170 ionic strength, pH 7.0 at 4°C, pCa > 8.5. The skinning solution also contained a cocktail of protease inhibitors to protect the fibers from proteolytic damage (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 1.0 mM benzamidine, 10 μM aprotinin). After single muscle fibers were dissected, they were exposed for 30 min to the above solution containing 0.5% w/w Triton X-100 (a non-ionic detergent that permeabilizes the sarcolemmal membrane and all membrane organelles). Triton X-100 skinning of muscle fibers allows an accurate control of calcium and other ion concentrations surrounding the myofilaments for the study of contractile protein functions (**8;25;34**).

Mechanical Measurement. Single muscle fibers were mounted between an optical-electric force transducer (Scientific Instruments GMBH, Heidelberg, Germany) and a movable arm by wrapping the fibers 3 times around small stainless steel wires. After mounting, only a small portion of the fibers (~300-500 μm) remains free in between the mounting wires, which avoids the exacerbation of mechanical artifacts. The muscle fibers were then briefly exposed (~30 sec) to pCa 6.0 to induce a contraction for ensuring the secure mounting of the single fibers to the wires. This initial contraction is expected to tighten the wrapping to minimize the artificial compliance due to a 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 for the force versus pCa relationship. In addition, the mounting without use of adhesives allows for an easier utilization of muscle fibers for biochemical analyses after completion of the force versus pCa curves as fibers can be more easily removed, without having to be sectioned, allowing the recovery of more tissue for protein analysis.

To directly compare the isometric properties of wrapping method with the commonly used adhesives method for fiber mounting, we conducted a series of experiments where we compared the Ca^{2+} sensitivity and maximal Ca^{2+}-activated force of rat EDL muscle fibers before and after fixation of their extremities of the wrapped fibers with adhesives. As shown

in Fig. 3 and Table 1, the comparison confirmed that the wrapping method used in the present study produces reliable recording of Ca^{2+}-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 by using a customized computer program (Turbo-Pascal 87 Version 3.0, Borland International, Scotts Valley, CA) employing the equilibrium constants (**13**) that are routinely used in our laboratory (**25**).

To measure the isometric contractile force, the fibers were optimally stretched (~20%) from their slack length to control the sarcomere length at 2.5 ± 0.1 μm as measured by its laser diffraction pattern (**34**), at the beginning of each force versus pCa measurement. The diameter of the fiber was measured as the flat plan of horizontal width at 80X magnification by using a calibrated micrometer inside the eyepiece of the dissecting microscope. The fiber diameter was used to calculate the cross-sectional area. We have previously used a prism system where both the width and depth of the fibers were simultaneously measured but found no differences between the single and the dual measurement approaches. The secured single muscle fibers were relaxed in pCa 8.5 and then exposed to solutions of varying Ca^{2+} concentrations (pCa 8.5 to 4.0) to determine the force versus pCa relationship. At the end of each experiment, the sarcomere length and the diameter of each fiber were measured again. In the majority of fibers (> 90%), no significant changes were found. If significant differences were found, the fiber was discarded. The force versus pCa relationship of each fiber was analyzed as described previously (8; 25). Maximum calcium-activated isometric force (F_{max}) was recorded and normalized to the cross sectional area of each fiber (in kN/m^2) or to the maximal force produced by each fiber at pCa 4.0 (100%, F_{max}). Computer programs (Sigma Plot 5.0 and Origin 6.1 Professional, Jandel Scientific) were used to fit the force versus pCa curve for each fiber to the Hill equation:

$$\%F_{max} = 100\left[Ca^{2+}\right]^n / \left[\left(Ca_{50}\right)^n + \left[Ca^{2+}\right]^n\right]$$

Ca^{2+} sensitivity was evaluated from Ca_{50} (the Ca concentration producing half-maximal force). The steepness of the curve was evaluated from n, the Hill coefficient. After each parameter was obtained for individual fibers, average force versus pCa was calculated for each group of fibers. Each fiber used in the studies was carefully unwrapped and removed from the anchors, measured for the diameter and length and transferred to microcentrifuge tubes containing 10 μl of SDS-PAGE sample buffer described above and saved at -80°C for the analysis of myofilament protein contents.

The MHC, TnI and TnT isoform contents of each single muscle fiber were used to classify the muscle fiber type. Fibers that had clear single MHC and single class (slow or fast) of TnT and TnI isoforms were selected to investigate the role of myosin and TnT and TnI isoforms in determining contractility features. Only the muscle fibers with a complete set of data (i.e., force versus pCa relationships, SDS-PAGE and immunoblots were performed on the same fiber) were summarized in this report.

Analysis of F_{max} versus Sarcomere Lengths. Because diaphragm muscles may have significantly longer optimal sarcomere lengths than the other muscles analyzed (**42**), we compared isometric F_{max} and Ca_{50} for diaphragm fibers at sarcomere length of 3.0 ± 0.1 μm compared with that at 2.5 ± 0.1 μm. The procedure was carried out as described above. To extend the evaluation to other muscles, we also compared isometric properties for soleus fibers at 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 analyses. The criterion for statistical significance was $P < 0.05$. For statistical analysis of

parametric sets of data, One-Way Analysis of Variance (ANOVA, SigmaStat) followed by Tukey's post hoc test was employed. For non-parametric sets of data, Kruskal-Wallis One Way Analysis of Variance on Ranks was used. Sets of data comprising the force versus pCa relationships were tested with Kruskal-Wallis One-Way Analysis of Variance on Ranks followed by Dunn's method for multiple comparisons.

RESULTS

Effective identification of TnT, TnI and MHC isoforms in isolated single skeletal muscle fibers. To define the contribution of TnT and TnI isoforms to skeletal muscle contractility and their correlation to myosin isoforms, it is essential to identify single fibers containing simple combinations of TnT, TnI and MHC isoforms. Therefore, we developed biochemical and immunochemical analysis that are sufficiently sensitive to determine the MHC, TnT and TnI isoform contents in the protein 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 single class of TnI and TnT isoforms.

Fig. 1B shows that the glycerol SDS-PAGE used in our study is able to clearly resolve the four MHC (I, IIa, IIx, and IIb) isoforms in rat skeletal muscle, in agreement with the gel mobility and specific antibody identifications in previous studies (**4;48**). The Western blots in Fig. 1C further demonstrate that mAb FA2 raised against rat cardiac myosin (**27**) recognizes MHC-I (equivalent to cardiac***b***-MHC) but not MHC-IIa, IIx or IIb (Fig. 1B). Fig. 1D shows that the cardiac and slow TnT-specific mAb CT3 (**26**) recognizes slow TnT in diaphragm and soleus muscles, but only had very weak stain in the EDL muscle sample, in agreement with the predominant fast fiber feature of rat EDL observed by previous studies (**25**). Fig. 1E shows mAb T12 recognizes multiple alternatively spliced fast TnT bands (**50**) in diaphragm, soleus and EDL muscles. Western blot using mAb TnI-1 (Fig. 1F) shows that the EDL muscle only expresses the faster migrating fast skeletal muscle TnI while the soleus muscle expresses predominantly the slower migrating slow skeletal muscle TnI. Both fast and slow isoforms of skeletal muscle TnI are expressed in the diaphragm muscle. This is consistent with the fact that both slow and fast TnT is detected at a high level in the diaphragm muscle, confirming its mixed fiber nature. The results that rat soleus muscle expresses both slow and fast skeletal muscle TnTs are in agreement with data previously obtained from mouse muscles (**26**). It is worth noting that although the MHC gel did not show any MHC-I in EDL, the FA2 blots on 1,000 time more protein loaded showed a trace amount of MHC-I. The presence of slow Type I fiber in EDL is in agreement with the trace amount of slow TnT detected by the CT3 blot. On the other hand, the failed detection of such amount of slow TnI in EDL by the TnI-1 blot may be due to the lower sensitivity of this mAb (raised against chicken fast TnI) in detecting rat TnI. These results demonstrate an effective identification of all skeletal muscle MHC, TnT and TnI isoforms and formed a foundation of our experimental system.

Similar expression patterns of TnT, TnI and MHC isoforms in different regions of 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 for MHC, TnI and TnT isoform expression. SDS-PAGE demonstrated that the twelve 30° sectors of rat diaphragm muscle (Fig. 2A) had no significant apparent difference in protein contents (Fig. 2B). The 12 sectors of diaphragm muscle were further examined for MHC isoforms by SDS-glycerol-PAGE. The results in Fig. 2B demonstrate that nearly all areas of the diaphragm muscle had similar patterns of MHC isoform expression except for the dorsal region (sectors 1 and 12, Fig. 2A) that has a lower level of MHC-IIb as compared with that in other regions. Western blot examination of TnT and TnI isoforms in the different regions of diaphragm showed similar ratios of slow/fast skeletal muscle TnT and slow/fast skeletal muscle TnI (Fig. 2B). These ratios were also similar

to that detected in the total diaphragm muscle homogenate (Fig. 1). While the functional significance of the lower MHC-IIb content in the dorsal region of mouse diaphragm remains to be investigated, the results indicate that picking single fibers from most areas of rat diaphragm would have similar yields. This observation was then confirmed by the results of our random picking of skinned single fibers, which showed no bias among fibers from various parts of the diaphragm.

The 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/TnI isoforms. By the sensitive detection methods using very 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 and 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 a mixed fiber muscle (gastrocnemius) to include most possible combinations of MHC, and 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 TnI/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 so for the fast TnI and fast TnT, in individual muscle fibers, despite their muscle origin or the expression of one or more MHC isoforms (Table 2).

Although a significant portion of the single fibers examined expresses more than one MHC isoform, we found 86 fibers containing a single MHC isoform together with slow or fast TnI/TnT isoforms in several representative combinations. As shown by the representative SDS-gels and Western blots in Fig. 4, the following groups of fibers were found in the 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. It is worth noting that although MHC-IIa is reported in slow fibers (**1;4**), it was only found in fibers expressing fast TnT and fast TnI, whereas slow TnT and slow TnI are only found in fibers expressing MHC-I. Those fibers were investigated for functional correlations.

Calcium sensitivity and cooperativity of force production in Triton-skinned single fibers of rat skeletal muscle. Fig. 5 shows the normalized isometric force versus pCa relationships in rat diaphragm muscle fibers containing a) MHC-I and slow TnI and slow TnT, b) MHC-IIa and fast TnI and fast TnT, c) MHC-IIx and fast TnI and fast TnT, and d) MHC-IIb and fast TnI and fast TnT. As summarized in Table 2, the results show that the F_{max} and cooperativity were significantly higher in the MHC-II + fast troponin groups as compared to that of the fibers containing MHC-I and slow troponin. On the other hand, the MHC-I + slow troponin fibers were more sensitive to calcium. No significant differences were detected between the isometric contractility of the diaphragm MHC-IIa and MHC-IIx fibers. The correlation between MHC-II and higher maximum force production is consistent with the findings in previous studies (**6;25**).

Although MHC-IIb was detected in all areas of the rat diaphragm muscle (Fig. 2), we did not obtain any diaphragm fiber that contains solely MHC-IIb. However, a significant number of MHC-IIb only fibers were found in rat EDL and gastrocnemius muscles. Similar to the results from diaphragm MHC-IIa and MHC-IIx fibers that have no significant differences in isometric contractility, Fig. 5B shows that the gastrocnemius muscle fibers containing MHC-IIx and fast troponin or MHC-IIb and fast troponin also had no significant differences in isometric contractility.

To further demonstrate that skinned fibers from different muscles have similar isometric contractile properties as long as they contain the same combination of MHC and TnI/TnT isoforms, Fig. 6A shows that the slow fibers (containing MHC-I and slow troponin) from diaphragm and soleus muscles have essentially identical Ca_{50} and cooperativity. Similarly, Fig. 6B shows that the fibers containing MCH-IIx and fast troponin from diaphragm and gastrocnemius muscles have similar isometric contractile features. In addition, Fig. 6C demonstrates that fibers containing MCH-IIb and fast troponin from gastrocnemius and EDL muscles also have no significant differences in contractility. These features are summarized in Table 2.

Isometric contractility of skinned single fibers from different muscles demonstrated that the F_{max} 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 TnT and TnI isoforms (Table 2). Since 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 ***m***m) in comparison to that at 2.5 ***m***m as used in all other measurements. The results in Table 3 show that the diaphragm fibers had an increased F_{max} at the longer sarcomere length, reaching the level comparable to that of the fibers from other muscles at 2.5 ***m***m sarcomere length. In contrast, an increase of sarcomere length from 2.5 μm to 3.0 μm did not produce significant change in F_{max} and Ca_{50} in soleus (174.7 ± 25 versus 160.8 ± 17 kN/m^2 and 1.89 ± 0.01 versus 1.96 ± 0.02 ***m***M, respectively, n=9) and gastrocnemius (306.00 ± 29 versus 290.7 ± 19 kN/m^2 and 2.23 ± 0.01 versus 2.21± 0.03 ***m***M, respectively, n=9) fibers.

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

DISCUSSION

A polyploidy skeletal muscle fiber can express a single isoform of MHC and/or single class of TnT and TnI. We have previously reported that in the Amish nemaline myopathy, the loss of slow TnT results in lethal neuromuscular abnormalities (**25**). The fact that the absence of only one isoform of TnT causes severe myopathy provides evidence for a critical role of the fiber type-specific troponin isoforms in skeletal muscle development and function. Most skeletal muscles contain both fast and slow fibers at various ratios. To investigate the contribution of troponin isoforms to muscle contractility, one also 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 results revealed that polyploid adult vertebrate skeletal muscle fibers (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, which may coordinate the multiple nuclei in a muscle cell (fiber). This finding justifies the use of a muscle fiber as the functional unit to investigate the differentiation and functional adaptation of muscle during development and under physiological and pathological stress conditions. These results laid a foundation for investigating the functional significance of fast and slow muscle fibers as well as the fast and slow TnT and TnI isoforms in muscle differentiation, adaptation, fatigue and 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 demonstrate a tightly coupled expression of the two troponin subunit isoform genes. In contrast to TnC isoforms that are members of the calmodulin gene family (**10**), TnT and TnI are encoded by striated muscle-specific genes with a co-evolutionary relationship (**24**). Together with the slow and fast fiber-specific expression of TnC isoforms (**38**), the coupled expression of fast or slow TnT and TnI in all fibers analyzed indicates that the regulation of troponin subunit gene is uniformly regulated in a polyploidy muscle fiber. It is important to note that the slow TnI and slow TnT genes are not physically paired in the vertebrate genome (**24;49**). Therefore, their strictly coupled expressions in each and all single fibers examined indicate a determining role of cellular *trans*-regulatory factors other than genomic linkage. The observation supports 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 isoform genes, 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 been classically 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 the fiber typing complicated. It is commonly considered that MHC-I is specific to the slow fibers and MHC-IIx and IIb are specific to fast fibers. The typical slow muscle soleus is known to express both MHC-I and MHC-IIa (Fig. 4) while the classical fast muscle EDL expresses both MHC-IIx and MHC-IIb. Although MHC-IIa is expressed in soleus, our isometric force results showed that MHC-IIa fibers behave like MHC-IIb and MHC-IIx fibers with higher isometric force as compared to that of MHC-I fibers (Table 2). In contrast, we show in the present study that the expression of TnT and TnI isoforms is highly specific to the muscle fiber type and shows a relationship to the contractile features of the fiber. Unlike MHC isoforms, most muscle fibers express only fast or only slow TnT and TnI. For example, while both slow and fast TnT are detected in the homogenate 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 makers 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.

Slow and fast TnT isoforms determine the Ca^{2+}sensitivity and cooperativity of myofilament. 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 muscle soleus contain mixed fast and slow fibers (**4;24;41**). The slow fibers are known to be important in the sustained contraction of muscle (**21;46**). The results in Fig. 5A show that the rat muscle single fibers containing slow troponin had higher Ca^{2+}-sensitivity than that containing fast troponin. In addition, the fibers expressing fast troponin showed a higher cooperativity during the Ca^{2+} activation of contraction than that of fibers expressing 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 F_{max} is lower than MHC-II fibers and Ca^{2+}-sensitivity is lower than that of slow troponin fibers. Therefore, the data suggest that the TnT and TnI isoform rather than MHC isoforms may be a determining factor for the Ca^{2+}-regulation of slow and fast muscle fibers.

To support this observation, we have previously demonstrated that cardiac muscle from transgenic mice over-expressing fast skeletal muscle TnT had an increased cooperativity during Ca^{2+} activation of contraction (**23**). We have also shown in chicken skeletal muscles expressing identical isoforms of TnI and TnC that acidic or basic isoforms of TnT had different Ca^{2+}

sensitivity (**36**). Together with the observation that TnT–tropomyosin interaction is cooperative (**37**), these results indicate TnT isoforms may be a key element of the role of slow and fast troponins in the modulation of Ca^{2+} sensitivity and cooperativity of myofilaments. It is worth noting that the central and C-terminal domains of TnT are conserved in all isoforms and the determinant for acidic and basic TnT isoforms, and therefore the observed functional differences, is the amount of their N-terminal negative charge (**23;36;50**).

Moss and colleagues (**35**) showed that the Hill coefficient was markedly different in the region between 0 and 50% of maximum tension versus that between 50 and 100%, thus giving rise to two different Hill coefficients. This observation supports that the property of troponin complex can affect muscle cooperativity. Our force versus pCa data points were properly fitted by 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 and one isoform of myosin, instead of comparing fibers with undefined TnT and TnI isoforms. While TnC deficiency might alter the cooperativity features, we found in a previous study that when TnC was partially lost, the force versus pCa parameters would be significantly different from those reported in the present paper (**9**). Integrity of the troponin complex in the fibers analyzed in the present study is further supported by the high absolute force levels reported as a decrease in TnC content should decrease maximal Ca-activated force.

Similar isometric contractility of muscle fibers expressing MHC-IIa, IIx and IIb. The single fiber contractility analysis suggests that the myosin isoform contents determine the maximum force of isometric contraction. The data in Table 2 show that maximal forces produced by fibers expressing MHC-II were higher than that containing MHC-I. These results are consistent with the fact that slow myosin has a lower ATPase activity than that of the fast myosin isoenzyme (**2**), which may contribute to the different F_{max} developed. Previous studies have shown that skeletal muscle containing MHC-IIa, IIx, and IIb have different contractile velocities (**6**). On the other hand, our results demonstrated similar maximum steady force produced by the rat muscle fibers containing MHC-IIa, IIx, and IIb (Table 2). It is interesting to note that the moderately different F_{max} of diaphragm and EDL type II fibers is dependent on the resting length of the muscle, suggesting different optimal lengths for the generation of maximum steady-state force, which 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 is the only rhythmic skeletal muscle among the muscles studied here, and posses a remarkably different geometry when compared to other skeletal muscles (**42;43**). In diaphragm muscles, it has been demonstrated that transmission of force occurs not only longitudinally but also transversally/diagonally because of a special architecture of this muscle. Furthermore, there is evidence suggesting that elastic components seem to play an important role in contributing towards force development, maintenance, and relaxation in diaphragm muscles (**18**). It is also important to point out that our study was focused to investigate isometric contractile properties and does not provide information regarding contractile velocity, or isotonic and lengthening properties of contractile function. In addition, since the present study focuses on the function of TnT and TnI isoforms by analyzing 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 with each other 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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References

1. Allen DL, Yasui W, Tanaka T, Ohira Y, Nagaoka S, Sekiguchi C, Hinds WE, Roy RR, Edgerton VR. Myonuclear number and myosin heavy chain expression in rat soleus single muscle fibers after spaceflight. J Appl Physiol 1996;81:145–151. [PubMed: 8828656]
2. Barany M. ATPase activity of myosin correlated with speed of muscle shortening. J Gen Physiol 1967;50:197–216. [PubMed: 4227924]
3. Barton PJ, Townsend PJ, Brand NJ, Yacoub MH. Localization of the fast skeletal muscle troponin I gene (TNNI2) to 11p15.5: Genes for troponin I and T are organized in pairs. Ann Human Genet 1997;61:519–523. [PubMed: 9543552]
4. Betto DD, Zerbato E, Betto R. Type 1, 2A, and 2B myosin heavy chain electrophoretic analysis of rat muscle fibers. Biochem Biophys Res Commun 1986;138:981–987. [PubMed: 2943282]
5. Bottinelli R, Betto R, Schiaffino S, Reggiani C. Maximum shortening velocity and coexistence of myosin heavy chain isoforms in single skinned fast fibres of rat skeletal muscle. J Muscle Res Cell Motil 1994;15:413–419. [PubMed: 7806635]
6. Bottinelli R, Schiaffino S, Reggiani C. Force-velocity relations and myosin heavy chain isoform compositions of skinned fibres from rat skeletal muscle. J Physiol (Lond) 1991;437:655–672. [PubMed: 1890654]
7. Bredman JJ, Weijs WA, Korfage HAM, Brugman P, Moorman AFM. Myosin heavy chain expression in rabbit masseter muscle during postnatal development. J Anat 1992;180:263–274. [PubMed: 1387129]
8. Brotto MA, Nosek TM. Hydrogen peroxide disrupts Ca2+ release from the sarcoplasmic reticulum of rat skeletal muscle fibers. J Appl Physiol 1996;81:731–737. [PubMed: 8872640]
9. Brotto MA, van Leyen SA, Brotto LS, Jin JP, Nosek CM, Nosek TM. Hypoxia/fatigue-induced degradation of troponin I and troponin C: new insights into physiologic muscle fatigue. Pflugers Arch 2001;442:738–44. [PubMed: 11512030]
10. Collins JH. Myosin light chains and troponin C: structural and evolutionary relationships revealed by amino acid sequence comparisons. J Muscle Res Cell Motil 1991;12:3–25. [PubMed: 2050809]
11. Cooke R. The mechanism of muscle contraction. Crit Rev Biochem 1986;21:53–118. [PubMed: 3524992]
12. Eddinger TJ, Moss RL, Cassens RG. Fiber number and type composition in EDL, SOL, and diaphragm muscles with aging in Fisher 344 rats. J Histochem Cytochem 1985;33:1033–1041. [PubMed: 2931475]
13. Godt RE, Lindley BD. Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. J Gen Physiol 1982;80:279–297. [PubMed: 6981684]
14. Gordon AM, Homsher E, Regnier M. Regulation of contraction in striated muscle. Physiol Rev 2000;80:853–924. [PubMed: 10747208]
15. Gordon AM, Huxley AF, Julian FJ. The variation in isometric tension with sarcomere length in vertebrate muscle fibres. J Physiol (Lond) 1966;184:170–192. [PubMed: 5921536]
16. Gosselin LE, Betlach M, Vailas AC, Greaser ML, Thomas DP. Myosin heavy chain composition in the rat diaphragm: Effect of age and exercise training. J Appl Physiol 1992;73:1282–1286. [PubMed: 1447070]
17. Greaser ML, Moss RL, Reiser PJ. Variations in contractile properties of rabbit single muscle fibres in relation to troponin T isoforms and myosin light chains. J Physiol 1988;406:85–98. [PubMed: 3254423]

18. Griffiths RI, Shadwick RE, Berger PJ. Functional importance of a highly elastic ligament on the mammalian diaphragm. Proc R Soc Lond B Biol Sci 1992;249:199–204.
20. Gunning P, Hardeman E. Multiple mechanisms regulate muscle fiber diversity. FASEB J 1991;5:3064–3070. [PubMed: 1835946]
21. Gupta RC, Misulis KE, Dettbarn W-D. Activity dependent characteristics of fast and slow muscle: Biochemical and histochemical considerations. Neurochem Res 1989;14:647–655. [PubMed: 2779725]
22. Hastings KE. Molecular evolution of the vertebrate troponin I gene family. Cell Struct Funct 1997;22:205–211. [PubMed: 9113408]
23. Huang QQ, Brozovich FV, Jin JP. Fast skeletal muscle troponin T increases the cooperativity of transgenic mouse cardiac muscle contraction. J Physiol 1999;520(1):231–242. [PubMed: 10517814]
24. Huang QQ, Jin JP. Preserved close linkage between the genes encoding troponin I and troponin T, reflecting an evolution of adapter proteins coupling the Ca(2+) signaling of contractility. J Mol Evol 1999;49:780–788. [PubMed: 10594179]
25. Jin JP, Brotto MA, Hossain MM, Huang QQ, Brotto LS, Nosek TM, Morton DH, Crawford TO. Truncation by glu180 nonsense mutation results in complete loss of slow skeletal muscle troponin T in a lethal nemaline myopathy. J Biol Chem 2003;278:26159–26165. [PubMed: 12732643]
26. Jin JP, Chen A, Huang QQ. Three alternatively spliced mouse slow skeletal muscle troponin T isoforms: conserved primary structure and regulated expression during postnatal development. Gene 1998;214:121–129. [PubMed: 9651500]
27. Jin JP, Malik ML, Lin JJ. Monoclonal antibodies against cardiac myosin heavy chain. Hybridoma 1990;9:597–608. [PubMed: 1706314]
28. Jin J-P, Yang F-W, Yu Z-B, Ruse CI, Bond M, Chen A. The highly conserved COOH-terminus of troponin I forms a Ca^{2+}-modulated allosteric domain in the troponin complex. Biochemistry 2001;40:2623–2631. [PubMed: 11327886]
29. Johnson BD, Wilson LE, Zhan WZ, Watchko JF, Daood MJ, Sieck GC. Contractile properties of the developing diaphragm correlate with myosin heavy chain phenotype. J Appl Physiol 1994;77:481–487. [PubMed: 7961272]
30. Johnston JJ, Kelley RI, Crawford TO, Morton DH, Agarwala R, Koch T, Schaffer AA, Francomano CA, Biesecker LG. A novel nemaline myopathy in the Amish caused by a mutation in troponin T1. Am J Hum Genet 2000;67:814–821. [PubMed: 10952871]
31. Kischel P, Bastide B, Muller M, Dubail F, Offredi F, Jin JP, Mounier Y, Martial J. Skeletal troponin T isoforms in rat muscles. Am J Physiol Cell Physiol 2005;289:437–443.
32. Leavis PC, Gergely J. Thin filament proteins and thin filament-linked regulation of vertebrate muscle contraction. CRC Crit Rev Biochem 1984;16:235–305. [PubMed: 6383715]
33. Lin, JJC.; Feramisco, JR.; Blose, SH.; Matsumura, F. Monoclonal Antibodies and Functional Cell Lines. Plenum Publishing Co.; New York: 1984.
34. Maughan DW, Molloy JE, Brotto MA, Godt RE. Approximating the isometric force-calcium relation of intact frog muscle using skinned fibers. Biophys J 1995;69:1484–1490. [PubMed: 8534819]
35. Moss RL, Giulian GG, Greaser ML. The effects of partial extraction of TnC upon the tension-pCa relationship in rabbit skinned skeletal muscle fibers. J Gen Physiol 1985;86:585–600. [PubMed: 4056736]
36. Ogut O, Granzier H, Jin JP. Acidic and basic troponin T isoforms in mature fast-twitch skeletal muscle and effect on contractility. Am J Physiol 1999;276:C1162–C1170. [PubMed: 10329966]
37. Ogut O, Jin JP. Cooperative interaction between developmentally regulated troponin T and tropomyosin isoforms in the absence of F-actin. J Biol Chem 2000;275:26089–26095. [PubMed: 10844003]
38. Parmacek MS, Leiden JM. Structure and expression of the murine slow/cardiac troponin C gene. J Biol Chem 1989;264:13217–13225. [PubMed: 2753913]
39. Perry SV. Troponin T: genetics, properties and function. J Muscle Res Cell Motil 1998;19:575–602. [PubMed: 9742444]
40. Pette D, Staron RS. The molecular diversity of mammalian muscle fibers. NIPS 1993;8:153–157.

41. Pette D, Staron RS. Myosin isoforms, muscle fiber types, and transitions. Microsc Res Tech 2000;50:500–509. [PubMed: 10998639]

42. Poole DC. Optimum sarcomere length in mammalian diaphragm. J Appl Physiol 1997;82:1712–1714. [PubMed: 9204377]

43. Poole DC, Mathieu-Costello O. Capillary and fiber geometry in rat diaphragm perfusion fixed in situ at different sarcomere lengths. J Appl Physiol 1992;73:151–159. [PubMed: 1506362]

44. Reiser PJ, Greaser ML, Moss RL. Developmental changes in troponin T isoform expression and tension production in chicken single skeletal muscle fibres. J Physiol 1992;449:573–588. [PubMed: 1522524]

45. Ruff RL, Whittlesey D. Ca-, Sr-tension relationships and contraction velocities of human muscle fibers. Muscle Nerve 1991;14:1219–1226. [PubMed: 1766453]

46. Salmons S, Vrbová G. The influence of activity on some contractile characteristics of mammalian fast and slow muscles. J Physiol (Lond) 1969;201:535–549. [PubMed: 5767881]

47. Sosnicki AA, Lutz GJ, Rome LC, Goble DO. Histochemical and molecular determination of fiber types in chemically skinned single equine skeletal muscle fibers. J Histochem Cytochem 1989;37:1731–1738. [PubMed: 2530270]

48. Talmadge RJ, Roy RR. Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. J Appl Physiol 1993;75:2337–2340. [PubMed: 8307894]

49. Tiso N, Rampoldi L, Pallavicini A, Zimbello R, Pandolfo D, Valle G, Lanfranchi G, Danieli GA. Fine mapping of five human skeletal muscle genes: alpha-tropomyosin, beta-tropomyosin, troponin-I slow-twitch, troponin-I fast-twitch, and troponin-C fast. Biochem Biophys Res Commun 1997;230:347–350. [PubMed: 9016781]

50. Wang J, Jin JP. Primary structure and developmental acidic to basic transition of 13 alternatively spliced mouse fast skeletal muscle troponin T isoforms. Gene 1997;193:105–114. [PubMed: 9249073]

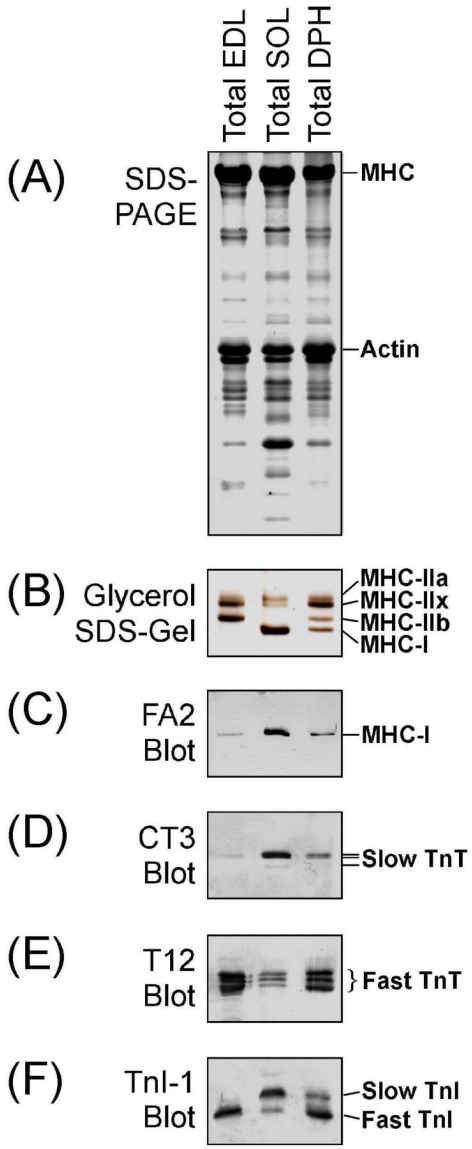
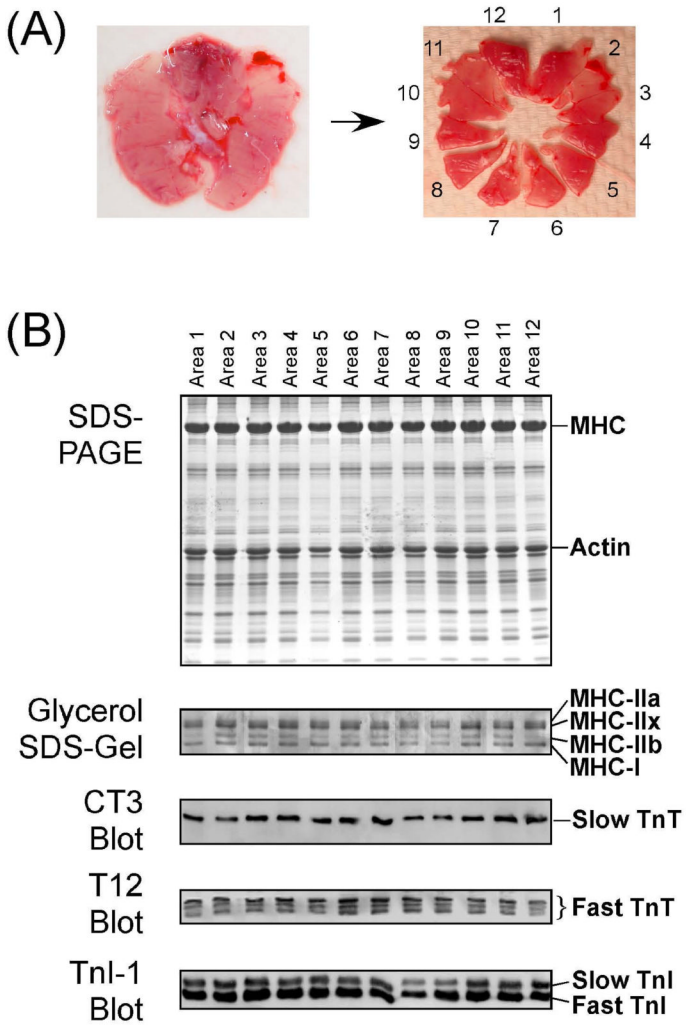


Fig. 1.

Identification of TnT, TnI and MHC isoforms in muscle homogenate. (A) Total protein extracts of adult rat EDL, soleus (SOL) and diaphragm (DPH) muscles were resolved by 14% SDS-PAGE with acrylamide:bisacrylamide = 180:1 and stained with Coomasie Brilliant Blue R250. (B) Dilutions of the same samples were analyzed by 8% SDS-PAGE with acrylamide:bisacrylamide = 50:1 and 30% glycerol followed by silver stain to reveal the four MHC isoforms. The samples were also analyzed by Western blots using anti-MHC-I mAbs FA2 (C), anti-slow skeletal muscle TnT mAb CT3 (D), anti-fast skeletal muscle TnT mAb T12 (E), and anti-TnI mAb TnI-1 (F). The results demonstrate an effective identification of the MHC, TnT and TnI isoforms by our experimental procedures. Similar to that seen in mouse

soleus (**26**), three alternatively spliced slow TnT bands were found in the rat soleus by the CT3 Western blot. The two high molecular weight and one low molecular weight rat slow TnT shown in this figure correspond to the recently sequenced rat slow TnT isoform 1, 2, and 4 (**31**).

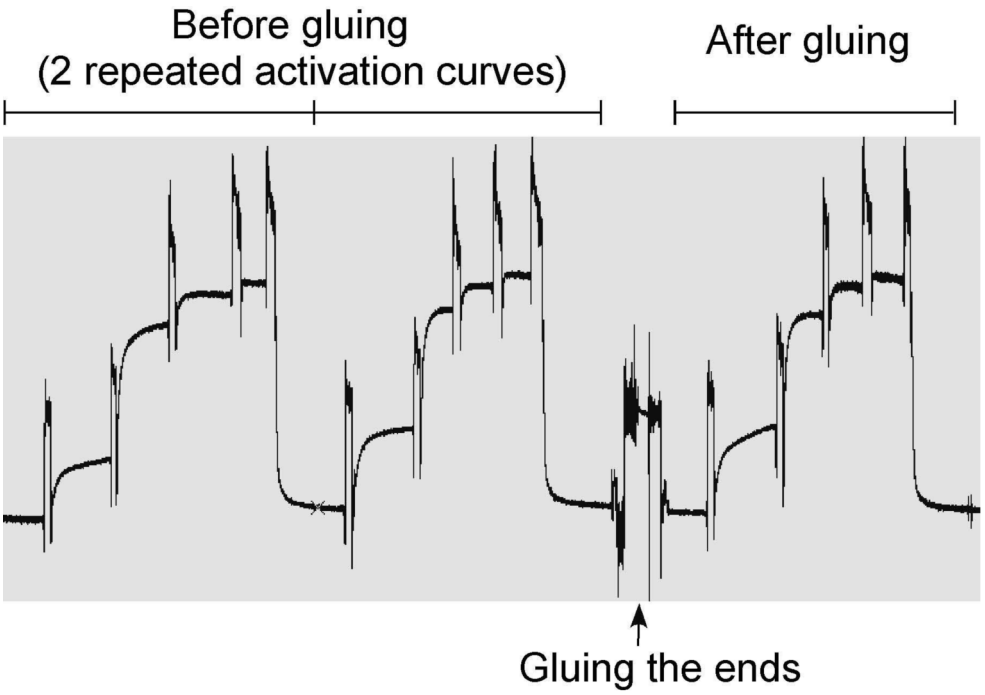


Brotto et al., Fig. 2

Fig. 2.

Expression of TnT, TnI and MHC isoforms in different parts of diaphragm. (A) The rat diaphragm (head view with the dorsal side on top) was dissected into twelve 30° sectors for the analysis of muscle protein contents. (B) Total protein extracts from the 12 diaphragm muscle samples were analyzed by 14% SDS-PAGE with acrylamide:bisacrylamide = 180:1 and stained with Coomasie Brilliant Blue R250 to examine the total protein contents, by 8% SDS-PAGE with acrylamide:bisacrylamide = 50:1 and 30% glycerol followed by silver stain to reveal the MHC isoforms contents, and by Western blots using anti-slow skeletal muscle TnT mAb CT3, anti-fast skeletal muscle TnT mAb T12, and anti-TnI mAb TnI-1 to examine the TnT and TnI isoforms. The results demonstrate that the 12 areas of diaphragm muscle had

no significant difference in protein contents. Most areas of diaphragm muscle had similar patterns of MHC isoform expression except for the dorsal region (Sectors 1 and 12). Different sectors of the rat diaphragm muscle express similar ratios of slow and fast skeletal muscle TnT, and of slow and fast skeletal muscle TnI. These ratios were also similar to that detected in the total diaphragm muscle homogenate (Fig. 1).

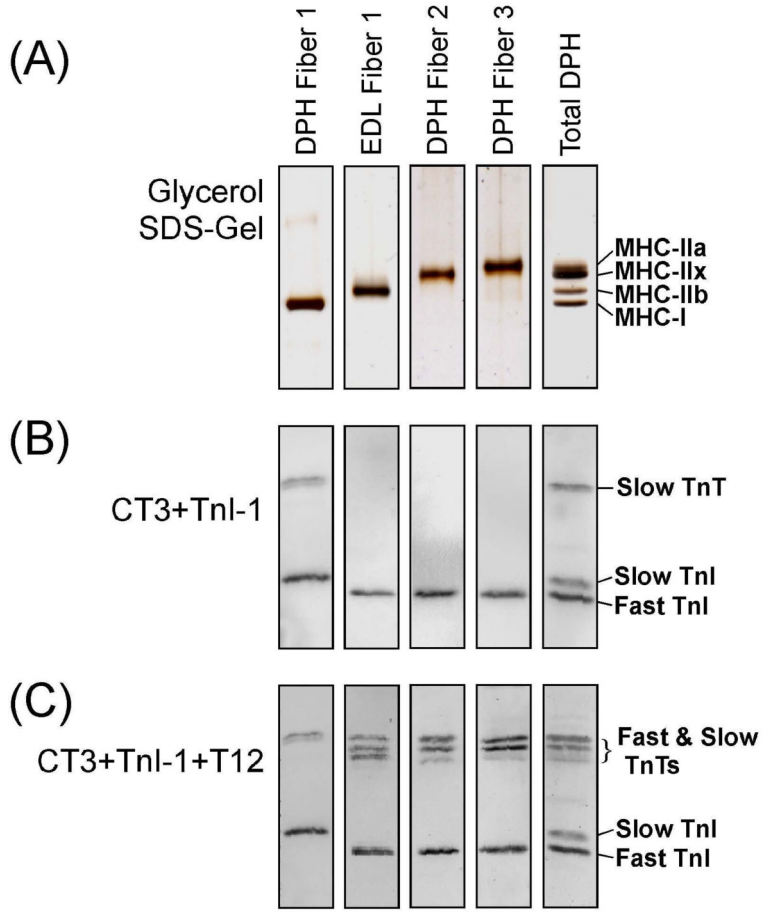


Brotto et al., Flg. 3

Fig. 3.

Representative recording for the Ca^{2+}-activated force development in wrapped EDL single fiber before and after gluing. The traces show the Ca^{2+}-activated force development of a representative rat EDL single fiber. A series of measurements was first performed after securely mounting the fiber by the wrapping method. The spikes are artifact due to moving the fiber between troughs containing buffers with difference pCa. After recording the force development at four serial increases of $[Ca^{2+}]$, the fiber was washed in pCa8.5 buffer and the process was repeated. The results demonstrate that there was no significant change between the two rounds. In addition to show the reliable fiber mounting by the wrapping method, the results demonstrate that there was no run-down in fiber contractility under the experimental

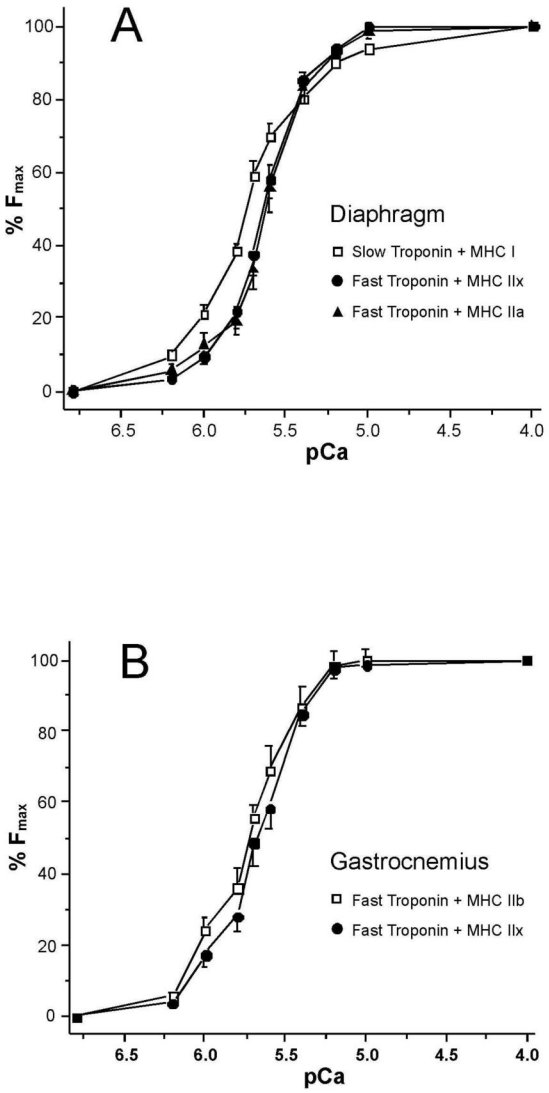
conditions. The wrapped ends of the fiber were then glued using a tissue adhesive composed of methoxypropyl cyanoacrylate monomer and polymeric modifiers (TISSUMEND II, Veterinary Products Laboratory, Phoenix, AZ) and another round of Ca^{2+} activation was measured. The results showed no difference for the Ca^{2+}-activated forces recorded before and after gluing.



Brotto et al., Fig. 4

Fig. 4.

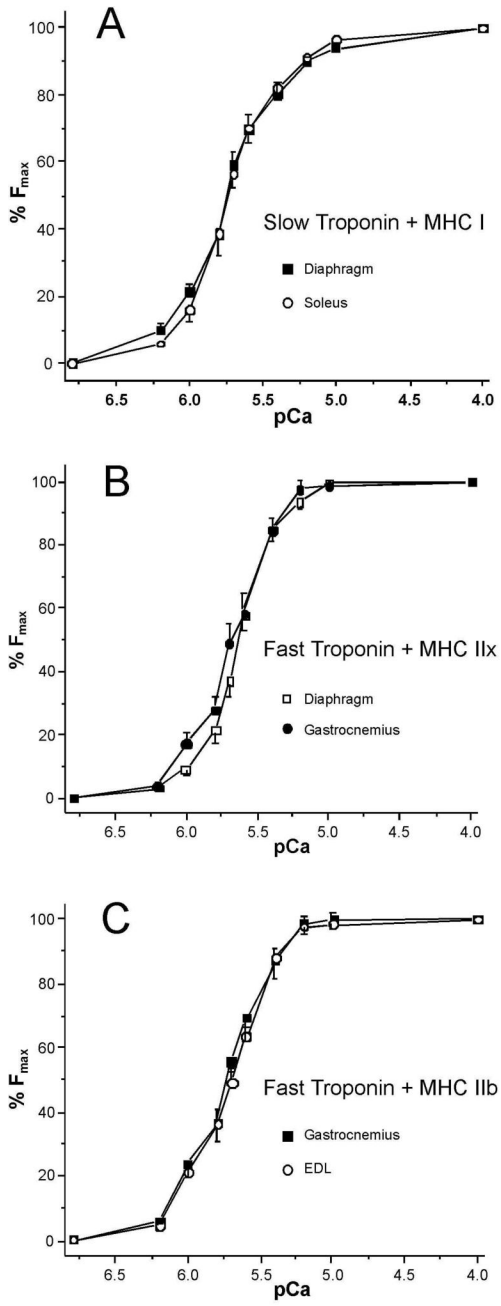
Rat single skeletal muscle fibers expressing representative combinations of MHC and TnT and TnI isoforms. The silver stained SDS-glycerol-gel (A), Western blots using a mixture of anti-slow TnT mAb CT3 and anti-TnI mAb TnI-1 (B), and Re-probing of the same nitrocellulose in (B) with anti-fast TnT mAb T12 (C) on extracts from single rat diaphragm (DPH) and EDL muscle fibers demonstrate four representative simple combinations of MHC, TnT and TnI isoforms. Total diaphragm muscle MHC were used as controls. Multiple fast TnT bands are present in every fast TnT single fibers, representing alternatively spliced variants with differences in the N-terminal variable region (**50**).



Brotto et al., Fig. 5

Fig. 5.

Isometric force versus pCa relationship of representative diaphragm and gastrocnemius muscle fibers. (A) The diaphragm fibers containing MHC-I and slow troponin, MHC-IIa and fast troponin, or MHC-IIx and fast troponins were compared. The normalized force-pCa curves show that cooperativity of activation was higher in the MHC-II + fast troponin groups as compared to that of fibers expressing MHC-I and slow troponin. However, the MHC-I + slow troponin fibers were more sensitive to calcium (Table 2). No significant differences were detected between the MHC-IIa and MHC-IIx groups. (B) Gastrocnemius muscle fibers containing MHC-IIa + fast troponin or MHC-IIx + fast troponin also showed no significant differences in the contractile properties.



Brotto et al., Fig. 6

Fig. 6.

Isometric force versus pCa relationship of slow and fast fibers from different muscles. (A) Slow fibers (containing MCH-I and slow troponin) from diaphragm or soleus muscles showed very similar Ca_{50} and cooperativity. (B) Fibers containing MCH-IIx and fast troponin from diaphragm or gastrocnemius muscles have very similar isometric contractile features. (C) Fibers containing MCH-IIb and fast troponin from gastrocnemius or EDL muscles also have very similar isometric contractile properties.

Table 1:

F_{max} and Ca_{50} for wrapped EDL and soleus muscle fibers before and after gluing.

EDL	F_{max} (in kN/m^2)	Ca_{50} (in μM)
Before glue (10)	305.00 ± 29.58	2.31 ± 0.05
After glue (10)	270.00 ± 35.00	2.48 ± 0.1
Soleus	F_{max} (in kN/m^2)	Ca_{50} (in μM)
Before glue (6)	166.00 ± 22.78	1.81 ± 0.08
After glue (6)	155.00 ± 35.00	1.86 ± 0.1

Data are presented as averages ± SEM. The numbers into parenthesis are the number of fibers used. No statistical significance was present in EDL (p = 0.189) and in solues (p = 0.236) fibers.

Table 2:

Isometric contractile parameters of Ca^{2+}-activated force of single fibers at sarcomere length of 2.5 μm for four distinct fiber types from four different rat muscles.

Muscle	Fiber types	F_{max} (kN/m^2)	Ca_{50} (μM)	Hill Coefficient
Diaphragm (40)	Slow Troponin + MCH-I (8)	155.23 ± 34.49	1.83 ± 0.02	2.0 ± 0.2
	Fast Troponin + MCH-IIa (15)	214.03 ± 27.83	2.51 ± 0.002	4.75 ± 0.31
	Fast Troponin + MHC-IIx (17)	214.82 ± 33.71	2.61 ± 0.002>	5.04 ± 0.60
Gastrocnemius (16)	Fast Troponin + MHC-IIb (7)	321.04 ± 38.04	2.32 ± 0.005	4.70 ± 0.59
	Fast Troponin + MHC-IIx (9)	291.65 ± 44.30	2.20 ± 0.02	4.72 ± 0.43
Soleus (10)	Slow Troponin + MHC-I (10)	169.74 ± 34.50	1.92 ± 0.01	1.95 ± 0.25
EDL (14)	Fast Troponin + MHC-IIb (14)	297.92 ± 39.98	2.30 ± 0.003	4.41 ± 0.24

Data are presented as mean ± SEM. The numbers in parenthesis are the number of fibers analyzed. Statistical analyses found 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 produced higher levels of force than slow diaphragm and soleus ($p < 0.01$). Hill Coefficients were greater in fast troponin fibers ($p < 0.02$), while slow troponin fibers were more sensitive to Ca^{2+} ($p < 0.05$). No significant differences were found between the contractile parameters of slow troponin + MHC-I fibers from diaphragm and soleus muscles.

Table 3:

F_{max} and Ca_{50} for fast fibers from diaphragm muscles at sarcomeric lengths of 2.5 and 3.0 μm.

Fiber types	SL = 2.5 μm		SL = 3.0 μm	
	F_{max} (kN/m^2)	Ca_{50} (μM)	F_{max} (kN/m^2)	Ca_{50} (μM)
Fast Tn + MCH-IIa (3)	204.83 ± 17.67	2.48 ± 0.06	293.22 ± 21.56	2.55 ± 0.07
Fast Tn + MHC-IIx (3)	209.12 ± 23.71	2.59 ± 0.05	289.30 ± 13.72*	2.63 ± 0.08

Data are presented as mean ± SEM. The numbers in parenthesis are the number of fibers used. Average F_{max} was higher when sarcomere length (SL) was set at 3.0 μm as compared to that at 2.5 μm ($p < 0.001$). The 2.5 μm F_{max} values obtained in this set of experiments was comparable to that obtained for the fast fibers of the other muscles studied (Table 2).