Force-calcium relationship depends on myosin heavy chain and troponin isoforms in rat diaphragm muscle fibers

PAIGE C. GEIGER, MARK J. CODY, AND GARY C. SIECK
Departments of Anesthesiology and Physiology and Biophysics, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Geiger, Paige C., Mark J. Cody, and Gary C. Sieck. Force-calcium relationship depends on myosin heavy chain and troponin isoforms in rat diaphragm muscle fibers. J. Appl. Physiol. 87(5): 1894-1900. 1999.—The present study examined Ca$^{2+}$ sensitivity of diaphragm muscle (Dia$_m$) fibers expressing different myosin heavy chain (MHC) isoforms. We hypothesized that Dia$_m$ fibers expressing the MHC$_{slow}$ isoform have greater Ca$^{2+}$ sensitivity than fibers expressing fast MHC isoforms and that this fiber-type difference in Ca$^{2+}$ sensitivity reflects the isoform composition of the troponin (Tn) complex (TnC, TnT, and TnI). Studies were performed in single Triton-X-permeabilized Dia$_m$ fibers. The Ca$^{2+}$ concentration at which 50% maximal force was generated (pCa$_{50}$) was determined for each fiber. SDS-PAGE and Western analyses were used to determine the MHC and Tn isoform composition of single fibers. The pCa$_{50}$ for Dia$_m$ fibers expressing MHC$_{slow}$ was significantly greater than that of fibers expressing fast MHC isoforms, and this greater Ca$^{2+}$ sensitivity was associated with expression of slow isoforms of the Tn complex. However, some Dia$_m$ fibers expressing MHC$_{slow}$ contained the fast TnC isoform. These results suggest that the combination of TnT, TnI, and TnC isoforms may determine Ca$^{2+}$ sensitivity in Dia$_m$ fibers.

Myosin heavy chain; troponin; diaphragm muscle; single fibers; calcium sensitivity; cooperativity

DIAPHRAGM MUSCLE (Dia$_m$) fibers express different myosin heavy chain (MHC) isoforms, which form the basis for fiber-type classification (11, 33, 36). Differences in muscle fiber contractile properties are associated with MHC isoform expression. For example, fibers expressing the MHC$_{slow}$ isoform have a slower maximum shortening velocity ($V_{max}$) than fibers expressing fast MHC isoforms (MHC$_{2A}$, MHC$_{2X}$, and MHC$_{2B/2X}$) (1, 6, 12, 30, 34, 35). Although controversial (8), it has been reported that fibers expressing fast MHC isoforms have a greater specific force (force per cross-sectional area) than fibers expressing the MHC$_{slow}$ isoform (1, 6, 25, 34, 35).

The force generated by muscle fibers depends on myoplasmic calcium concentration ([Ca$^{2+}$]; represented as pCa or $-\log$ [Ca$^{2+}$]), and Ca$^{2+}$ sensitivity is often indexed as the pCa at which 50% of maximum force is generated (pCa$_{50}$). Generally, it has been reported that slow fibers have a higher pCa$_{50}$ than fast fibers (15, 17). However, with respect to Dia$_m$, an apparent controversy exists regarding fiber-type differences in Ca$^{2+}$ sensitivity. For example, in the rabbit Dia$_m$, Kerrick (15) found that fibers expressing the MHC$_{slow}$ isoform (as determined electrophoretically) had greater Ca$^{2+}$ sensitivity than fibers expressing fast MHC isoforms. In contrast, Eddinger and Moss (6) reported that in the rat Dia$_m$, the force-pCa curve for slow fibers (as determined histochemically and electrophoretically) was shifted rightward compared with that of fast fibers. In another study in the rat Dia$_m$, Danieli-Betto et al. (5) reported no significant differences in the pCa$_{50}$ of fibers expressing MHC$_{slow}$ and MHC$_{2A}$ isoforms (as determined electrophoretically). It is unclear why fiber-type differences in Ca$^{2+}$ sensitivity should be unique for the rat Dia$_m$. Therefore, the purpose of the present study was to examine the force-pCa relationship of rat Dia$_m$ fibers expressing different MHC isoforms. We hypothesize that, as in other skeletal muscles, the pCa$_{50}$ of Dia$_m$ fibers expressing the MHC$_{slow}$ isoform is higher than that of fibers expressing fast MHC isoforms, with no differences in Ca$^{2+}$ sensitivity among fibers expressing MHC$_{2A}$/MHC$_{2X}$/MHC$_{2B/2X}$ isoforms.

It has been suggested that the presence of different isoforms of troponin C (TnC) may contribute to fiber-type differences in Ca$^{2+}$ sensitivity (13, 23, 40). It has also been suggested that expression of TnT and TnI isoforms (12, 29, 32, 39) is important in determining fiber-type differences in Ca$^{2+}$ sensitivity. Therefore, we further hypothesize that the greater Ca$^{2+}$ sensitivity of Dia$_m$ fibers expressing the MHC$_{slow}$ isoform is associated with a unique combination of troponin (Tn) isoforms compared with fibers expressing fast MHC isoforms.

METHODS

Animal preparation and single-fiber dissection. Adult male Sprague-Dawley rats (body wt ~300 g) were injected intramuscularly with ketamine (60 mg/kg) and xylazine (2.5 mg/kg), and the right side of the Dia$_m$ was excised. Muscle fiber bundles were stretched ~20% to optimal length, pinned on cork, and placed in a relaxing solution, at 5°C for 24 h, consisting of (in mM) 59.0 KAc; 6.7 MgAc$_2$; 5.6 Na-ATP; 10 EGTA, 2.0 1,4-dithiothreitol, 15.0 CrP, and 50 mM 1-methyl imidazole, as well 1 mg/ml creatine phosphokinase, for a total ionic strength of 200 mM at a pH of 7.0. The fiber bundles were then stored in relaxing solution containing 50% glycerol (vol/vol) for 2–3 wk.

Before single-fiber dissection, a fiber bundle was placed in relaxing solution containing 1% Triton X-100 to permeabilize the plasma membrane. The fiber bundle remained in this skimming solution for 20 min, during which time single fibers were dissected under a dissecting microscope. The single fibers were then transferred to 50% glycerol relaxing solution before force measurement.

Force measurements. Activating and relaxing solutions used for force measurements were determined by the computer program described by Fabiato and Fabiato (7) with...
stability constants listed by Godt and Lindley (10). The solutions contained the following (in mM): 10.0 EGTA, 1.0 free Mg²⁺, 5.0 MgATP, 15.0 creatine phosphate, 50.0 imidazole, and 2.0 1,4-dithiothreitol, as well as creatine phosphokinase at 1 mg/ml for a total ionic strength of 150 mM. The relaxing solution had a pCa of 9.0. In the activating solutions, pCa ranged from 7.0 to 4.0.

To maintain noncompliant attachments of the fibers to a force transducer and servo-controlled motor (see below), the ends of the fibers were fixed by exposing them to a 5% glutaraldehyde solution. Aluminum foil T clips were also attached to the fiber ends to reduce compliance. The fibers were then placed in a temperature-controlled flow-through acrylic chamber (volume, 120 µl) located on the stage of an inverted microscope (Olympus IMT-2). Two small stainless steel hooks were used to mount the fiber horizontally in the chamber. One end of the fiber was attached to a force transducer (Aksjeselskapet, AE-801) with a resonant frequency of 5 kHz, whereas the other end was attached to a servomotor (General Scanning, G120DT) with a step time of 800 µs. First-order laser diffraction (He-Ne laser; UDT Sensors, LSC 30D) was used to set and maintain sarcomere length at 2.5 µm. During experiments, stability of sarcomere lengths was maintained according to the techniques of Brenner (3) as modified by Sweeney et al. (38). LabView-based software and a data-acquisition board were used to record signals. The length and width of the fiber were measured by using a reticle in the eyepiece. The fiber was sequentially exposed to solutions containing progressively higher free Ca²⁺ concentrations. A baseline force was recorded with the fiber in relaxing solution (pCa 9.0) followed by recordings of force generated in solutions ranging in pCa from 7.0 to 4.0.

Single-fiber gel electrophoresis. MHC isoform composition of single skeletal muscle fibers was identified by SDS-PAGE according to methods previously described (36). Single fibers were placed in 25 µl of SDS sample buffer containing 62.5 mM Tris·HCl, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.001% (wt/vol) bromophenol blue at pH 6.8. The samples were denatured by boiling for 2 min. A modified procedure by Sugiiura and Murakami (37) was used to prepare the gradient gels. The stacking gel contained a 3.5% acrylamide concentration (pH 8.8) with 25% glycerol (8 × 10 cm, 0.75 mm thick; Hoefer SE250). Control samples of Dia₇ fibers in a 1:200 dilution (~9.0 ng/µl) were run on the gels for comparison of migration patterns of the MHC isoforms. The concentration of protein in Dia₇ bundles was determined by the Lowry et al. method (18). Sample volumes of 10 µl were loaded per lane. The gels were stained with silver according to the procedure described by Oakley et al. (26).

Western analysis was performed to confirm the identification of MHC isoforms by migration patterns. Rat Dia₇ bundles were run on SDS-PAGE according to the procedure described above. The protein was then transferred to nitrocellulose overnight at 1 A. After transfer, the nitrocellulose sheet was divided into five sections for protein detection via immunologic reaction with antibodies specific for the different MHC isoforms. One nitrocellulose segment was stained with colloidal gold to visualize protein bands and ensure adequate protein transfer. The four additional segments were stained with one of the following mouse monoclonal or polyclonal antibodies: NCL (Novocastra, IgG), which reacts with MHCslow; SC.71 (ATTG, IgG), which reacts with MHCslow; BF-F3 (Schiaffino, IgM), which reacts with MHCslow; and BF-35 (Schiaffino, IgG), which reacts with all but the MHC₂₇ isoform. The specificity of these isoforms was previously determined (16, 33). Each segment was stained with a biotinylated secondary antibody specific to IgG (NCL, SC.71, BF-35) or IgM (BF-F3) and visualized with alkaline-phosphatase (Vectastain ABC-kit, Vector Laboratories).

The Tn composition of rat Dia₇ fibers was determined by SDS-PAGE by using the method as described by Giulian et al. (9). The separating gel contained a 15% acrylamide concentration (pH 8.8), and the stacking gel contained a 9% acrylamide concentration (pH 6.8). Single fibers were dissected from Dia₇ bundles, placed in 25 µl of SDS sample buffer, and stored at −70°C before electrophoresis. Samples were loaded on the gels in 10-µl volumes. Tn gels were silver stained according to the procedure described by Giulian et al. with slight modifications. The gels were fixed in 10% glutaraldehyde for up to 12 h to improve the retention of Tn isoforms on the gels (22). The gels were then continuously washed in distilled water until the color and odor of glutaraldehyde were no longer detectable in the gel (6–12 h). Rat soleus and extensor digitorum longus (EDL) muscles were excised, and each muscle sample was placed in 10 vol of SDS sample buffer and scissor minced. These samples were then diluted 1:200 and run on gels for comparison of the migration pattern of fast (EDL) and slow (soleus) Tn isoforms. In addition, a combination of ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin, and aptatin (C3187, Sigma Chemical) was used on the gels as a molecular weight marker. In this manner, TnC and TnI isoforms were determined on the basis of electrophoretic migration patterns. The identification of TnT isoforms by migration patterns was confirmed with Western analysis. A skeletal TnT monoclonal antibody (T6277, Sigma Chemical) and a TnT antibody for sarcomeric Tm (9283, Sigma Chemical) were used in the Western analysis. Control samples of rat Dia₇, EDL, and soleus muscles were analyzed as well as rat Dia₇ single fibers expressing different MHC isoforms. A biotinylated IgG secondary antibody was used, followed by peroxidase-conjugated streptavidin and visualization with 3,3′-diaminobenzidine.

RESULTS

MHC isoform expression. In single dissected Dia₇ fibers, MHC isoform expression was readily identified by SDS-PAGE and Western analysis (Fig. 1). The MHC isoform composition of 165 Dia₇ fibers was evaluated in the present study. Most of these Dia₇ fibers (~82%) expressed a single MHC isoform. In ~18% of all Dia₇ fibers, coexpression of MHC isoforms was detected, with coexpression of the MHC₂₇ and MHC₂X isoforms predominating. In the present study, none of the dissected fibers displayed singular expression of MHC₂B. This is consistent with our previous observation of a relatively low incidence (~3–8%) of fibers expressing only MHC₂B in the rat Dia₇ (36). Coexpression of the MHC₂A and MHC₂X isoforms was detected in one Dia₇ fiber.

Force-Ca²⁺ relationship. The dependency of force generation on myoplasmic [Ca²⁺] was systematically evaluated in 45 Dia₇ fibers. The force-pCa relationships of Dia₇ fibers expressing different MHC isoforms are shown in Fig. 2A. The force-pCa curve of fibers
expressing the MHC slow isoform was shifted leftward compared with curves for fibers expressing the fast MHC isoforms (MHC2A, MHC2X, and MHC2B/2X). These fiber-type differences in Ca\(^{2+}\) sensitivity were reflected by differences in the pCa\(_{50}\) (Table 1). Fibers expressing the MHC slow isoform displayed significantly higher pCa\(_{50}\) compared with fast fibers (Table 1, Fig. 2 B). In fibers expressing MHC 2A, MHC 2X, and MHC 2B/2X isoforms, no significant differences in the pCa\(_{50}\) values were found.

The threshold for force initiation, designated as the point when extrapolated force values reached 0.10\% of maximum force, occurred at different myoplasmic Ca\(^{2+}\) across Dia\(_{m}\) fibers expressing different MHC isoforms (Fig. 2, Table 1). The force threshold occurred at the highest pCa value in fibers expressing the MHC\(_{slow}\) isoform, and the lowest pCa values in fibers expressing the MHC\(_{2X}\) and MHC\(_{2B/2X}\) isoforms. The force threshold of fibers expressing the MHC\(_{2A}\) isoform was intermediate.

The steepness of the force-pCa curve (i.e., the Hill coefficient, \(n\)) (14) is commonly used to reflect the cooperativity of Ca\(^{2+}\) binding to TnC, the regulatory protein for myosin binding to actin (13, 23, 27). The steepness of the force-pCa curve at pCa values above the pCa\(_{50}\) (\(n_1\)) and pCa values below the pCa\(_{50}\) (\(n_2\)) varied across Dia\(_{m}\) fibers expressing different MHC isoforms (Fig. 3, Table 1). Values for \(n_1\) and \(n_2\) were lowest in fibers expressing the MHC\(_{slow}\) isoform, followed by fibers expressing MHC\(_{2A}\), and MHC\(_{2X}\) either alone or in combination with MHC\(_{2B}\). These differences were reflected in the Hill plots shown in Fig. 3. No significant differences were found in the Hill coefficients \(n_1\) and \(n_2\) in fibers expressing the MHC\(_{2A}\), MHC\(_{2X}\) and MHC\(_{2B/2X}\) isoforms (Table 1).

Tn isoform expression. The electrophoretic migration patterns of Tn isoforms expressed in different Dia\(_{m}\) fiber types are shown in Fig. 4. In a total of 111 Dia\(_{m}\) fibers expressing the MHC\(_{2A}\), MHC\(_{2X}\), and MHC\(_{2B/2X}\) isoforms, only the TnC-f isoform was expressed (Table 2). In 24 Dia\(_{m}\) fibers expressing the MHC\(_{slow}\) isoform, both the TnC-s and TnC-f isoforms were expressed, but in all cases, these isoforms were expressed singularly (Table 2). This sample is not meant to reflect the overall

<table>
<thead>
<tr>
<th>MHC Isoform</th>
<th>n</th>
<th>Threshold pCa</th>
<th>pCa(_{50})</th>
<th>(n_1) (pCa(_{pCa&gt;50}))</th>
<th>(n_2) (pCa(_{pCa&lt;50}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>12</td>
<td>7.40 ± 0.06</td>
<td>6.04 ± 0.02</td>
<td>2.25 ± 0.11</td>
<td>1.87 ± 0.13</td>
</tr>
<tr>
<td>2A</td>
<td>9</td>
<td>6.87 ± 0.08*</td>
<td>5.89 ± 0.03*</td>
<td>3.05 ± 0.20*</td>
<td>2.43 ± 0.35*</td>
</tr>
<tr>
<td>2X</td>
<td>16</td>
<td>6.59 ± 0.04†</td>
<td>5.92 ± 0.02*</td>
<td>4.18 ± 0.18*</td>
<td>3.13 ± 0.30*</td>
</tr>
<tr>
<td>2B/2X</td>
<td>8</td>
<td>6.56 ± 0.07†</td>
<td>5.91 ± 0.04*</td>
<td>4.14 ± 0.15*</td>
<td>3.45 ± 0.49*</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of fibers; Dia\(_{m}\), diaphragm muscle; MHC, myosin heavy chain; pCa\(_{50}\), Ca\(^{2+}\) concentration at which 50\% maximal force was generated. *Significantly different (P < 0.05) from fibers expressing MHC\(_{slow}\). †Significantly different (P < 0.05) from fibers expressing MHC\(_{2A}\).
percentage of slow Dia\textsubscript{m} fibers expressing the fast TnC isoform.

Differences in TnT and TnI isoforms were also observed between fibers expressing MHC\textsubscript{slow} and the fast MHC isoforms (Fig. 4, Table 2). Rat Dia\textsubscript{m} fibers expressing the MHC\textsubscript{slow} isoform expressed slow TnT isoforms (Table 2), previously identified as TnT\textsubscript{1s} and TnT\textsubscript{2s} (32) and confirmed with Western analysis in the present study. The TnT\textsubscript{2s} isoform displayed greater electrophoretic migration than TnT\textsubscript{1s} in SDS gels (Fig. 4). Rat Dia\textsubscript{m} fibers expressing fast MHC isoforms expressed a fast TnT isoform (Table 2) on the basis of migration pattern and Western analysis. The TnT isoform present in fast fibers from the rabbit Dia\textsubscript{m} has been previously identified as TnT\textsubscript{1f} (32).

Fibers expressing the MHC\textsubscript{slow} isoform expressed TnI-s, whereas fast fibers expressed TnI-f. In addition, two Tm isoforms, \( \alpha \)Tm and \( \beta \)Tm, were expressed in fast and slow fibers. However, the \( \alpha \)Tm expressed in slow fibers appeared to be different from the \( \alpha \)Tm expressed in fast fibers on the basis of electrophoretic migration patterns and Western analysis.

**DISCUSSION**

The results of the present study clearly indicate that rat Dia\textsubscript{m} fibers expressing the MHC\textsubscript{slow} isoform have greater Ca\textsuperscript{2+} sensitivity of force generation than fibers expressing fast MHC isoforms. These results are in general agreement with several previous studies in limb skeletal muscles (5, 12, 17) as well as in the rabbit Dia\textsubscript{m} (15). Furthermore, the results of the present study provide new information demonstrating that Ca\textsuperscript{2+} sensitivity does not vary across Dia\textsubscript{m} fibers expressing MHC\textsubscript{2A}, MHC\textsubscript{2X}, and MHC\textsubscript{2B/2X} isoforms. The greater Ca\textsuperscript{2+} sensitivity of Dia\textsubscript{m} fibers expressing the MHC\textsubscript{slow} isoform was associated with the expression of slow isoforms of the Tn complex. However, a small proportion of slow Dia\textsubscript{m} fibers expressed the fast isoform of TnC, indicating that Tn isoform composition is not the only determinant of Ca\textsuperscript{2+} sensitivity in Dia\textsubscript{m} fibers.

Previous studies examining Ca\textsuperscript{2+} sensitivity of rat Dia\textsubscript{m} fibers have been equivocal. For example, Eddinger and Moss (6) showed that the force-pCa curve of slow fibers was shifted to the right compared with that of fast fibers (cf. Fig. 5A in Eddinger and Moss (6)). However, these investigators stated that fast Dia\textsubscript{m} fibers were less sensitive to Ca\textsuperscript{2+} than slow fibers. Because pCa\textsubscript{50} values were not reported, the basis for this stated fiber-type difference in Ca\textsuperscript{2+} sensitivity was unclear. In another study, Danieli-Betto et al. (5) reported no significant difference in Ca\textsuperscript{2+} sensitivity between fast and slow fibers in the rat Dia\textsubscript{m}. However, in this study only two slow Dia\textsubscript{m} fibers were compared with only five fast fibers, which were all classified as type 2A.

The results of the present study demonstrated that Ca\textsuperscript{2+} sensitivity was comparable across rat Dia\textsubscript{m} fibers expressing MHC\textsubscript{2A}, MHC\textsubscript{2X}, and MHC\textsubscript{2B/2X} isoforms. These results contrast with those of Eddinger and Moss (6), who reported that histochemically classified type IIa fibers were less sensitive to Ca\textsuperscript{2+} than type IIb fibers. It is likely that most of the fibers histochemically classified as type IIb in the rat Dia\textsubscript{m} actually express the MHC\textsubscript{2x} isoform (11, 33, 36). For example, we...
previously reported that only ~3–8% of all fibers in the rat Diam singularly express the MHC2B isoform, whereas ~12% coexpress MHC2X and MHC2B isoforms (36). However, it is unlikely that the ambiguity of histochemical subclassification of fast fiber types accounted for the apparent discrepancy between the results of the present study and those of Eddinger and Moss (6). It is of interest that although Diam fibers expressing different fast MHC isoforms do not vary in the Ca$^{2+}$ sensitivity of force generation they do vary with respect to maximum shortening velocity and maximum specific force (34, 35), consistent with observations in several other skeletal muscles (1, 6, 25).

It has been suggested that the presence of different isoforms of TnC may contribute to fiber-type differences in Ca$^{2+}$ sensitivity (13, 23). The slow or cardiac TnC isoform (TnC-s) has only one low-affinity Ca$^{2+}$-binding site, yet this binding site has a higher affinity for Ca$^{2+}$ than the two low-affinity binding sites present on the TnC-f isoform found in fast skeletal muscle fibers (27, 40). The observation in the present study that the onset of force in Diam fibers expressing the MHCslow isoform occurred at lower myoplasmic Ca$^{2+}$ concentrations (higher pCa values) is consistent with a higher Ca$^{2+}$-binding affinity of the TnC-s isoform. This suggests a possible role for fiber-type specific isoforms of TnC in conferring Ca$^{2+}$ sensitivity. However, experiments that substitute native TnC in skeletal muscle fibers with the opposite TnC isoform have produced controversial results. For example, in an initial study by Moss et al. (23), it was shown that substitution of the TnC-s isoform into fast fibers resulted in a reduction of Ca$^{2+}$ sensitivity. However, in a subsequent study, these investigators concluded that this result was the consequence of incomplete reconstitution of TnC rather than a shift in the force-pCa relationship (21, 24) because partial extraction of TnC has been shown to cause a reduction in Ca$^{2+}$ sensitivity (22). The role of TnC isoforms in conferring Ca$^{2+}$ sensitivity is also challenged by the observation of the present study that a small population of Diam fibers expressing the MHCslow isoform contained the TnC-f isoform. This observation is consistent with the previous report of Danielli-Betto et al. (5), who also found that slow fibers of the rat Diam expressed both TnC-s and TnC-f. Together, these results indicate that the expression of different TnC isoforms is not the sole determinant of fiber-type differences in Ca$^{2+}$ sensitivity.

The results of the present study suggest that the expression of other Tn isoforms (TnT, Tnl) or tropomyosin (Tm) isoforms may be important in determining fiber-type differences in Ca$^{2+}$ sensitivity. In the present study, distinct isoforms of TnT were found in fibers expressing the MHCslow isoform. The presence of two slow TnT isoforms in these fibers is in agreement with Schachat et al. (31), who found that slow fibers from the rabbit Diam express TnT1s and TnT2s isoforms. These same investigators found the fast TnT isoform, TnT1f, in fast fibers from the rabbit Diam, although they did not indicate the MHC isoform of these fibers. In the study by Schachat et al. (32), differences in Ca$^{2+}$ sensitivity were attributable to the combination of TnT and Tm isoforms. These investigators reported that among rabbit fast fibers from a variety of muscles, those that expressed a combination of the TnT2f and $\alpha_1$ Tm isoforms had greater Ca$^{2+}$ sensitivity than fast fibers expressing a combination of the TnT1f and $\alpha_1$ Tm/Tm isoforms. This is in agreement with the results of the present study in which fibers expressing different fast MHC isoforms but the same TnT and Tm isoforms did not have a significant difference in Ca$^{2+}$ sensitivity.

Similar differences in TnT isoforms have been found in other skeletal muscles when fast and slow fibers were compared. For example, Greaser et al. (12) reported that rabbit plantaris muscle fibers expressing the MHCslow isoform contained a slow TnT isoform, whereas plantaris fibers expressing fast MHC isoforms contained a fast TnT isoform. These investigators concluded that this difference in TnT isoform composition accounted for the greater Ca$^{2+}$ sensitivity of slow vs. fast plantaris muscle fibers. Furthermore, they found that among fast plantaris muscle fibers several TnT isoforms were expressed and that Ca$^{2+}$ sensitivity was dependent on the molecular weight of these fast TnT isoforms. Reiser et al. (29) also found that variations in TnT isoforms in adult chicken skeletal muscles corresponded with variations in Ca$^{2+}$ sensitivity among fast muscle fibers. However, a comparison between fast and slow fibers in this study was conflicting. Slow fibers from the anterior latissimus dorsi muscle, expressing only the TnT slow isoform, had higher pCa50 values than fast fibers from the posterior latissimus dorsi muscle but lower pCa50 values than fast fibers from the pectoralis major muscle (29). In this study, the fast posterior latissimus dorsi and the pectoralis major muscles expressed variable amounts of fast TnT isoforms.

In addition to TnT and TnC, a role for Tnl in conferring Ca$^{2+}$ sensitivity has also been considered. For example, Van Eyk et al. (39) examined a variety of Tnl mutants and fragments with different affinities for actin-Tm and the TnT-TnC complex. Results of this study indicate a possible role for Tnl residues 96–148 in determining Ca$^{2+}$ sensitivity of the thin filament. However, Tnl recombinants used in this study were from fast rabbit and chicken muscle, and a comparison with slow Tnl was not made. Results from the present study indicate differences in Tnl isoforms between fast and slow fibers in the rat Diam, but future studies are needed to determine whether Tnl isoforms are directly involved in conferring fiber-type differences in Ca$^{2+}$ sensitivity.

In the biphasic force-pCa relationship, molecular cooperativity occurs predominantly in the activation of force up to half-maximal with little cooperativity present at higher Ca$^{2+}$ concentrations (20). Therefore, the discussion of cooperative processes will be limited to conditions of low Ca$^{2+}$ concentration ($n_1$). In the present study, rat Diam fibers expressing the MHC2A, MHC2X, and MHC2B/2X isoforms displayed significantly higher $n_1$ values than did fibers expressing the MHCslow isoform. Previous studies in the rat Diam (6) as well as in...
limb skeletal muscles (12, 23) have also reported that fast fibers have higher n1 values than slow fibers. Furthermore, Eddinger and Moss (6) reported that, in the rat Dia, fibers histochemically classified as IIb had higher n1 values than fibers classified as IIa. In the present study fibers expressing the MHC2x and MHC2B/2X isoforms did not have significantly greater n1 values than fibers expressing the MHC2a isoform (Table 1). In limb muscles, differences in n1 between fibers expressing the MHC2a isoform and other fast MHC isoforms were not explored. Therefore, it appears that fibers expressing fast MHC isoforms have greater cooperativity of force development than fibers expressing the MHCslow isoform.

A possible role for TnC isoforms in conferring fiber-type specific differences in the steepness of the force-pCa relationship has also been considered. However, stochiometric substitution of TnC isoforms in skeletal muscle did not affect the slopes of the force-pCa relationship in fast and slow fibers (24). Expression of different TnT isoforms has been associated with differences in cooperativity of muscle fibers. In the study by Schachat et al. (32), the greatest Hill coefficient value in a variety of fast rabbit muscles was found in fibers expressing the greatest fraction of both TnT2x and α2Tm. Another theory proposed to explain thin-filament cooperativity involves differences in the response of the thin filament to strong-binding myosin cross bridges. In these experiments, use of a myosin derivative with strong binding properties, N-ethylmaleimide S1, increased Ca2+-sensitivity and reduced the slope of the tension-pCa curve to a greater extent in slow muscles than in fast muscles, indicating that slow muscles respond more readily to strongly bound cross bridges (41). Thus, on the basis of these results, it would appear the need for cooperativity with thin-filament activation is less prominent in slow fibers.

Modulation of Ca2+-sensitivity and the slope of the force-pCa relationship by cross-bridge cycling kinetics should also be considered in this discussion. On the basis of Brenner’s model (2) for regulation of muscle contraction, the transition of cross bridges from a non-force-generating state to a force-generating state is described by the apparent rate constant fapp, the reverse transition from a force-generating state to a non-force-generating state is described by the apparent rate constant gapp. Estimates of these rate constants, and thus cross-bridge cycling kinetics, can be obtained from measuring the rate of tension redevelopment, ktr, after a quick release and restretch where ktr = fapp + gapp. Brenner reported that fapp is Ca2+-sensitive and that an increase in the ratio of fapp to gapp (fapp/gapp) would be expected to shift the force-pCa curve to the left, with a concomitant increase in the slope at low Ca2+ levels (2). Thus Brenner concluded that differences in Ca2+-sensitivity are not necessarily attributed solely to the binding affinity and cooperative properties of TnC but that cross-bridge cycling kinetics could also play a role.

However, additional studies indicate the molecular mechanisms regulating Ca2+-sensitivity may depend on the level of activation. For example, at maximal Ca2+ activation the rate of tension redevelopment is determined by cross-bridge cycling kinetics and depends on MHC isoform expression in fast and slow mammalian skeletal muscle (19). In contrast, more recent studies reveal that ktr varies with the kinetics of thin-filament activation at submaximal Ca2+ activation. For example, in a study by Regnier and colleagues (28), an increase in the Ca2+-affinity of TnC with calmidazolium results in an increase in ktr at submaximal Ca2+ levels with no change in ktr at maximal Ca2+ activation. In addition, when native TnC is replaced with cardiac TnC or an activated form of TnC (aTnC), reducing the Ca2+-binding affinity of TnC, the Ca2+-sensitivity of ktr is reduced or eliminated, respectively (4). On the basis of these experiments, the Ca2+-binding kinetics of the TnC isoform present determine the degree to which changes in submaximal Ca2+ can affect cross-bridge cycling kinetics. These studies make cross-bridge cycling rates an unlikely mechanism for the Ca2+-sensitivity of force development at submaximal [Ca2+]i.

In conclusion, results from this study indicate that fibers expressing the MHCslow isoform have greater Ca2+-sensitivity than fibers expressing fast MHC isoforms. Furthermore, the Ca2+-sensitivity and cooperativity of force generation were not different across Dia, fibers expressing MHC2a, MHC2x, and MHC2B/2X isoforms. This fiber-type difference in Ca2+-sensitivity may be attributable to the isoforms of the Tn complex present. Slow fibers typically express slow Tn isoforms, although results from this study reveal that both fast and slow Tn isoforms can be singularly expressed in these fibers. Because the population of slow fibers expressing the fast TnC isoform in this study was small, and in light of new experiments indicating that the Ca2+-binding kinetics of TnC isoforms affect the rate of tension redevelopment, TnC isoforms cannot be ruled out as a determinant of Ca2+-sensitivity. However, the results of TnC substitution studies have been inconclusive, and a predominant role for TnC isoforms in determining Ca2+-sensitivity has not been clearly demonstrated. Although it is likely that thin-filament activation affects the Ca2+-sensitivity of force development, future studies are needed to further define the integrative roles of TnC, TnT, and TnI isoforms in this regulation.

The authors thank Rebecca Macken for technical assistance in these studies and Dr. Philippe Housmans for useful comments. This research was supported by National Heart, Lung, and Blood Institute Grants HL-34817 and HL-37680.

Address for reprint requests and other correspondence: G. C. Sied, Anesthesia Research, Mayo Clinic and Foundation, 200 First St. SW, Rochester, MN 55905.

Received 3 November 1998; accepted in final form 19 July 1999.

REFERENCES


2. Brenner, B. Effect of Ca2+ on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: Implications for regulation of
1900 FORCE-CALCIUM RELATIONSHIPS IN RAT DIAPHRAGM FIBERS


