Electrophoretic and functional identification of two troponin C isoforms in toad skeletal muscle fibers

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O’Connell, Brett, Ronnie Blavez, and Gabriela M. M. Stephenson. Electrophoretic and functional identification of two troponin C isoforms in toad skeletal muscle fibers. Am J Physiol Cell Physiol 290: C515–C523, 2006. First published September 21, 2005; doi:10.1152/ajpcell.00307.2005.—The differential sensitivity of frog twitch and slow-tonic fibers to Ca2⁺ and Sr2⁺ suggests that these two fiber types express different troponin C (TnC) isoforms. To date, only one TnC isoform from anurans (resembling the mammalian fast-twitch isoform) has been isolated and characterized. In this study, we examined the possibility that anuran striated muscle contains more than one TnC isoform. Toward this end, we determined the TnC isoform composition of 198 single fibers from the rectus abdominis of the cane toad (a mixed slow-tonic and twitch muscle) and of toad cardiac muscle using a method that enables the identification of TnC isoforms on the basis of the effect of Ca2⁺ on their electrophoretic mobility. The fibers were typed according to their myosin heavy chain (MHC) isoform composition. The data indicate that striated muscle of the cane toad contains two TnC isoforms, one of which (TnC-t) is present in all fibers displaying only twitch MHC isoforms and the other of which (TnC-Tc) is present in fibers displaying the tonic MHC isoform and in cardiac muscle. For a subpopulation of 15 fibers, the TnC isoform composition was also compared with Ca2⁺ and Sr2⁺ activation characteristics. Fibers containing the TnC-Tc isoform were ~3-fold more sensitive to Ca2⁺, ~40-fold more sensitive to Sr2⁺, and responded to a ~4.6-fold broader range of [Ca2⁺⁺] than did fibers containing the TnC-t isoform. The Ca2⁺ activation properties of toad fibers containing the TnC-Tc/c isoform appear to be consistent with the previously reported physiological characteristics of amphibian slow-tonic muscle fibers.

myofibrillar proteins; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; alanine SDS-PAGE; hybrid fibers; Ca2⁺-binding proteins; single fiber; muscle protein polymorphism; fiber type

IN THE FIRST PART OF THEIR comprehensive review of vertebrate slow-tonic muscle, Morgan and Prosek (13) hypothesized that amphibian skeletal muscles (used as a template for discussion because of their mixed twitch and slow-tonic fiber composition) contain, in different proportions, twitch fibers (producing “all-or-none” contractile responses induced by propagated action potentials), slow-twitch fibers (producing slow, graded responses induced by graded membrane depolarizations), and intermediate fibers (e.g., fibers that maintain contracture but are also able to twitch). Unlike twitch fibers, which have been found in all vertebrate skeletal muscles studied to date, regardless of the species and/or the size of the animal or the location and/or function of the muscle, slow-twitch fibers have been detected in only a limited number of specialized muscles from amphibians, reptiles, birds, fish, and mammals. Muscles containing slow-tonic fibers are able to maintain slow, prolonged contractures with minimum consumption of metabolic energy, which renders them useful for holding posture against gravitational force (e.g., rectus abdominis muscle in anurans, the anterior latissimus dorsi muscle that helps the chicken to hold its wings close to its body), slow cruising (e.g., fish red muscles), and execution of fine complex movements (e.g., extraocular muscles that point the eye toward a desired target so that its image is formed on a specialized region of the retina) (for review, see Ref. 13).

According to the current dogma, in both slow-tonic and twitch muscle fibers, the complex interaction between myosin and actin leading to force generation is regulated by Ca2⁺ via the troponin-tropomyosin system associated with the thin filament. Compelling evidence demonstrates that slow-twitch fibers differ from twitch fibers with respect to structural and physiological characteristics of all intracellular compartments known to play key roles in the Ca2⁺ regulation process. They contain a more sparse transverse (T)-tubular system, a poorly developed sarcoplasmic reticulum (SR), fewer contacts between the SR and the T-tubular system and between the SR and myofilaments, and less ordered sarcomeres and filament alignments. Consequently, in slow-twitch fibers, Ca2⁺ travels a longer distance between the SR-Ca2⁺ release sites and the Ca2⁺-binding sites on the myofilaments and is taken up by the SR at a slower rate (20).

Notwithstanding the persuasiveness of the evidence supporting the aforementioned differences, most investigations into the mechanism of Ca2⁺ regulation in vertebrate skeletal muscle contraction performed to date have concentrated on twitch muscle preparations. As a result, the currently available data regarding the regulation of slow-tonic muscle fibers by Ca2⁺ are scarce and, at times, inconsistent. For example, it is not clear whether slow-twitch and twitch amphibian muscle fibers contain the same troponin C (TnC) isoform. This lack of clarity is surprising, given that amphibians (particularly anurans), the group of animals in which slow-tonic muscle was first identified, have the largest number of such muscles and have generated most of the physiological data currently available on slow-twitch muscle and isolated muscle fibers. The two TnC purification procedures performed to date on the hindlimb skeletal muscle of the bullfrog Rana catesbeiana (25) and the frog Rana esculenta (27) have produced only one TnC isoform. However, fiber-type differences with respect to both Ca2⁺ sensitivity (e.g., 11) and Sr2⁺ sensitivity (6) of the contractile apparatus have been shown to exist between slow-tonic and twitch fibers from the cruralis muscle of the adult frog Rana...
pipiens (11) and from the iliofibularis muscle of the adult African clawed toad Xenopus laevis (6). This evidence raises the possibility, not addressed to date, that the TnC isoforms expressed in slow-tonic and twitch muscle fibers of anurans are different.

Recently we developed a rapid method for identifying fast and slow TnC isoforms in rat skeletal muscle fibers on the basis of the principle that the electrophoretic mobility of Ca²⁺-binding proteins such as TnC in SDS gels is altered by Ca²⁺ (16). As previously reported, by determining TnC isoform composition, myosin heavy chain (MHC) isoform composition, and Ca²⁺- and Sr²⁺-activation characteristics in the same fiber segment, we produced compelling evidence that fiber-type differences with respect to the sensitivity to Sr²⁺ of the contractile apparatus are closely related to the TnC isoform present in rat diaphragm fibers (17). In the present study, we applied the same experimental strategy as that used in the rat study (i.e., determination of MHC isoform, TnC isoform, and Ca²⁺ and Sr²⁺ activation characteristics of the same fiber segment) to single fibers of the rectus abdominis (RA) muscle of the adult cane toad Bufo marinus. As previously demonstrated in our laboratory (15), this muscle contains both fibers that express exclusively twitch MHC isoforms and a large proportion of fibers that express the tonic MHC isoform in combination with different proportions of twitch MHC isoforms.

Our results indicate that cane toad skeletal muscle contains two electrophoretically distinguishable TnC isoforms, one of which (TnC-T/tc) is expressed in fibers containing the tonic MHC isoform and in cardiac muscle and the other of which (TnC-t) is expressed in all fibers containing only twitch MHC isoforms and in some of the fibers coexpressing twitch and tonic MHC isoforms. Fibers containing different TnC isoforms display clear differences with respect to the sensitivity of the contractile apparatus to both Ca²⁺ and Sr²⁺.

MATERIALS AND METHODS

Animals and muscle. Adult cane toads (Bufo marinus, 250–380 g body wt) were killed by double pithing in accordance with procedures approved by the Animal Experimentation and Ethics Committee at Victoria University. The rectus abdominis muscle (see Fig. 1), previously shown to contain a wide variety of MHC isoform-based fiber types (15), was dissected and then pinned in a petri dish on a silicone elastomer (Sylgard 184) base under paraffin oil. Unless indicated otherwise, all procedures described herein were performed at room temperature (RT; 21 ± 1°C).

Preparation of single fibers. Single fibers (n = 198) were isolated with fine jewelers’ forceps under oil from a region close to the linea alba of the RA muscle (Fig. 1) using a dissecting microscope (Olympus) as previously described (17). The cross-sectional area (CSA) and volume of each single-fiber segment (assumed to be cylindrical) were determined using the width (mean of 5 values) and length of the fiber segment measured with the aid of a video monitoring system (Panasonic). An attempt was made to distinguish twitch from tonic fiber types on the basis of their microscopio appearance under oil. Toward this end, the two arms of a cold light source (Meiji Techno) were positioned at 90° angles with respect to the fiber length and the viewer’s perspective. For a subpopulation of 38 fibers, the degree of relative transparency or opacity was also recorded and was later compared with the MHC isoform composition (see RESULTS). Each fiber segment was mounted at slack length between a force sensitive transducer (model 801; Sensonor, Horten, Norway) and a pair of fine Barcroft forceps on the apparatus used for mechanical measurements.

Before the force activation experiments were conducted, the mounted fiber was chemically skinned by incubation for 10 min in a relaxing solution [10⁻⁹ M Ca²⁺ concentration ([Ca²⁺]) and see composition below] containing 2% (vol/vol) Triton X-100, followed by two 2.5-min washes in relaxing solution.

Solutions for force activation experiments. Details regarding the preparation of the solutions used in these experiments can be found in earlier studies (1, 29). These solutions included a set of 13 strongly buffered (50 mM total EGTA) Sr²⁺ solutions (pSr range of 3.2 to >9, where pSr = −log₁₀ Sr²⁺ concentration ([Sr²⁺])) and a set of 11 strongly buffered (50 mM total EGTA) Ca²⁺ solutions (pCa range of 4.8 to >9, where pCa = −log₁₀[Ca²⁺]). In addition to the 50 mM Sr²⁺-EGTA or the Ca²⁺-EGTA buffer, all solutions contained (in mM) 117 K⁺, 37 Na⁺, 1 free Mg²⁺, 60 HEPES, pH 7.10 ± 0.01, 8 total ATP, and 10 creatine phosphate. The apparent affinity constants for Sr²⁺ and Ca²⁺ binding to EGTA used in this study (1.53 × 10⁶ M⁻¹ and 4.78 × 10⁶ M⁻¹, respectively) were the same ones measured earlier under the same conditions (23, 28). The pH, osmolality, and ionic strength of all solutions at RT were 7.10 ± 0.01, 255 ± 5 mosmol/kgH₂O, and 225 ± 2 mM, respectively.

Protocol for isometric force activation experiments. For each individual fiber segment, the isometric force activation characteristics were determined from the steady-state isometric force responses developed by the fiber in the strongly Sr²⁺- and Ca²⁺-buffered solutions as described by O’Connell et al. (17). The experiments were performed with the fiber segments at slack length, with the average sarcomere length measured in 6 fibers being 2.12 ± 0.05 μm (mean ± SE). After being chemically skinned and washed (see description above), each preparation was first equilibrated for 2 min in a relaxing solution (pCa, pSr >9) and then sequentially activated in the Ca²⁺- and Sr²⁺-buffered solutions after being exposed briefly to the appropriate maximal Ca²⁺ or Sr²⁺-activating solution. At the end of the activation protocol, the fiber segment was placed in SDS-PAGE solubilizing buffer for electrophoretic analysis of MHC and TnC isoform composition.

Determination of Ca²⁺ and Sr²⁺ activation characteristics of single-fiber segments. For each fiber segment, the relationship between isometric force and [Ca²⁺] or [Sr²⁺] was determined by plotting the steady-state force responses (pF) developed by the fiber at different [Ca²⁺] or [Sr²⁺], expressed as a percentage of CaFmax or SrFmax (%maximum force), respectively, against the pCa or pSr values of the solutions. To correct for the slight deterioration in the force production associated with the repeated activation of skinned fiber preparations, a simple interpolation protocol was used to esti-
mate the values of CaF\textsubscript{\text{max}} (or SrF\textsubscript{\text{max}}) corresponding to the force responses obtained in a particular pCa (or pSr) solution as described previously (19). The P\textsubscript{\text{t}}-pCa and P\textsubscript{\text{t}}-pSr data points were then best fitted using theoretical Hill curves and the nonlinear regression analysis protocols provided by Prism software (version 4.02; GraphPad, San Diego, CA).

The following equation was used to calculate the theoretical Hill curves:

\[
P_t = \frac{nH}{1 + 10^{(x_{\text{pCa}} - x_{\text{pCa}_{50}})}}
\]

where \(nH\) is the associated Hill coefficient, \(x\) is pCa or pSr, and \(x_{50}\) is the pCa or pSr value at which 50% of CaF\textsubscript{\text{max}} (pCa\textsubscript{50}) or SrF\textsubscript{\text{max}} (pSr\textsubscript{50}) was reached. The following Ca\textsuperscript{2+} and Sr\textsuperscript{2+} activation parameters were determined for each skinned fiber segment: maximum Ca\textsuperscript{2+} and Sr\textsuperscript{2+}-activated force per CSA (CaF\textsubscript{\text{max}}/CSA, SrF\textsubscript{\text{max}}/CSA; kN/m\textsuperscript{2}, parameters closely related to the number of Ca\textsuperscript{2+} or Sr\textsuperscript{2+}-induced strong cross bridges per half sarcomere), pCa\textsubscript{10} (indicator of sensitivity to Ca\textsuperscript{2+}), pSr\textsubscript{10} (indicator of sensitivity to Sr\textsuperscript{2+}), \(n_C\) and \(n_S\) (indicators of the slope of the activation curves and the minimum number of cooperative regulatory sites per functional unit), CaF\textsubscript{\text{max}}/CSA and SrF\textsubscript{\text{max}}/CSA were determined from the amplitude of the first force response developed by the fiber segment in the maximally Ca\textsuperscript{2+}- or Sr\textsuperscript{2+}-activated solutions and from its estimated CSA measured in paraffin oil before exposure to aqueous solutions. The major advantages of measuring the CSA under oil before exposing the fiber to aqueous solutions are that under oil, the CSA of the skinned fiber resembles the CSA of the contractile apparatus in the intact fiber and the fiber assumes a quasicylindrical shape with a relatively consistent width along its length. By comparison, when exposed to an aqueous solution, the fiber swells and its cross section is more irregular, displaying marked nonuniformities along the fiber length.

**SDS-PAGE analysis of MHC isoforms.** In this study, the SDS-PAGE solubilizing buffer (SB) contained 80 mM Tris·HCl (pH 6.8), 2.3% (wt/vol) SDS, 710 mM β-mercaptoethanol, 10 mM DTT, 12.5% (vol/vol) glycerol, 13.6% (wt/vol) sucrose, 0.01% (wt/vol) bromophenol blue, 0.1 mM PMSF, 0.002 mM leupeptin, and 0.001 mM pepstatin. The samples (0.4 μl of fiber/μl of SB or 0.07 μg of muscle homogenate protein/μl of SB) were left overnight at RT, boiled for 3 min the next day, and then stored at −85°C. MHC isoform analyses were performed in 4-μl samples [containing ~1.6 nl of fiber (for single-fiber analysis) and ~0.28 μg of protein (for muscle homogenate analysis)] using 0.75-mm-thick slab gels, the Hofer Mighty Small gel apparatus (GE Healthcare UK), and the alanine-SDS-PAGE protocol described previously (3, 14). The separating gel (T = 7.6%, C = 1.2%, where T = [acrylamide] + [bis-acrylamide] expressed as %wt/vol and C = [bis-acrylamide]/[acrylamide] + [bis-acrylamide] expressed as %) contained 435 mM Tris·HCl, pH 8.8, 7.5 mM alanine, 40% (vol/wt) glycerol, 0.3% (wt/vol) SDS, 0.05% (wt/vol) ammonium persulfate, and 0.05% (vol/vol) N,N,N’,N’-tetramethylthlenediamine (TEMED), and the stacking gel (T = 4%, C = 2.6%) contained 125 mM Tris·HCl, pH 6.8, 4 mM EDTA, 40% (vol/wt) glycerol, 0.3% (wt/vol) SDS, 0.1% (wt/vol) ammonium persulfate, and 0.05% (vol/vol) TEMED. Electrophoresis was performed at constant voltage (150 V) for 28 h at 4-5°C with a running buffer containing 0.1% (wt/vol) SDS, 25 mM Tris, and 75 mM alanine. The gels were stained with silver according to the Bio-Rad method, scanned using a Molecular Dynamics personal densitometer, and analyzed using ImageQuant software (version 5.2; GE Healthcare UK).

The MHC isoform profile of single fibers (see, e.g., Fig. 2B, lane 2) was established using a laboratory MHC marker containing all four MHC isoforms previously detected in skeletal muscles of adult toads: *Bufo marinus* heavy chain (BmHc1) (BmHc1), 2 (BmHc2), 3 (BmHc3), and tonic (BmHct) (Fig. 2B, lane 1; see also Refs. 14 and 15). Herein as in the Nguyen and Stephenson studies (14, 15), fiber types are named according to the MHC isoforms present: fibers expressing a single-twitch MHC isoform type (pure fibers) are referred to as t1, t2, or t3 fibers, and fibers expressing two or more MHC isoforms (hybrid fibers) are designated, e.g., t2 + t3, t1 + t2 + t3.

**SDS-PAGE analysis of TnC isoforms.** Guided by the data obtained as part of a set of preliminary experiments (summarized in RESULTS), we determined the TnC isoform composition in single toad fiber segments using the distinctive upward curve of TnC bands associated with the [Ca\textsuperscript{2+}] gradient produced by the diffusion of EGTA from an adjacent lane into the sample lane (see Fig. 2C, lanes 1–4) and by two TnC isoform markers; the twitch TnC isoform (TnC-t) purified in our laboratory from toad hindlimb skeletal muscle using the method of Potter (18) (Fig. 2C, lane 2) and a toad heart homogenate in which the...
Table 1. MHC isoform composition, diameter, and TnC isoform composition of single fibers from cane toad RA muscles

<table>
<thead>
<tr>
<th>MHC Isomform-Based Fiber Type</th>
<th>Diameter, μm</th>
<th>TnC Isoform Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure twitch fibers, n = 37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1, n = 1; 0.5%</td>
<td>40.30</td>
<td>TnC-t</td>
</tr>
<tr>
<td>t2, n = 12; 6.1%</td>
<td>86.82±2.87 (14.68)</td>
<td>TnC-t</td>
</tr>
<tr>
<td>t3, n = 24; 12.1%</td>
<td>50.58±2.11 (20.45)</td>
<td>TnC-t</td>
</tr>
<tr>
<td>Hybrid twitch fibers, n = 79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1 + t2, n = 19; 9.6%</td>
<td>72.64±3.49 (20.93)</td>
<td>TnC-t</td>
</tr>
<tr>
<td>t2 + t3, n = 58; 29.3%</td>
<td>60.99±1.56 (19.52)</td>
<td>TnC-t</td>
</tr>
<tr>
<td>t1 + t2 + t3, n = 2; 1%</td>
<td>49.45±13.55</td>
<td>TnC-t</td>
</tr>
<tr>
<td>Hybrid twitch-tonic fibers, n = 82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1 + t, n = 52; 26.3%</td>
<td>50.46±1.44 (20.56)</td>
<td>TnC-T</td>
</tr>
<tr>
<td>t3 + t, n = 4; 2%</td>
<td>54.07</td>
<td>TnC-T/c</td>
</tr>
<tr>
<td>Fiber 1</td>
<td>54.95</td>
<td>TnC-T/c</td>
</tr>
<tr>
<td>Fiber 2</td>
<td>51.87</td>
<td>TnC-T/c</td>
</tr>
<tr>
<td>Fiber 3</td>
<td>52.01±2.16 (17.64)</td>
<td>TnC-T/c</td>
</tr>
<tr>
<td>Fibers 1–17</td>
<td>52.38±2.16 (23.62)</td>
<td>TnC-T/c</td>
</tr>
<tr>
<td>Fibers 18–26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numerical values are means ± SE (coefficients of variation are shown in parentheses). MHC, myosin heavy chain; TnC, troponin C; RA, rectus abdominis; TnC-T/c, twitch-tonic cardiac isoform.

tonic/cardiac (TnC-T/c) band is distinct (see Fig. 2C, lane 1). Single-fiber samples (10 μl containing ~4 nl of fiber) were run on 0.75-mm-thick slab glycine-SDS gels using the Hoefer Mighty Small gel apparatus. The separating gel (T = 16%, C = 2.6%) contained 750 mM Tris·HCl, pH 9.3, 10% (vol/vol) glycerol, 0.1% (wt/vol) SDS, 0.04% (wt/vol) ammonium persulfate, and 0.116% (vol/vol) TEMED. The stacking gel (T = 4%, C = 4.76%) contained 125 mM Tris·HCl, pH 6.8, 10% (vol/vol) glycerol, 0.1% (wt/vol) SDS, 0.1% (vol/vol) ammonium persulfate, and 0.1% (vol/vol) TEMED. Electrophoresis was performed at constant current (10 mA/gel) for 4.5 h at RT. The samples were loaded in such a way that each single-fiber sample was adjacent to one lane to which 5 μl of SB containing 10 mM EGTA was added (i.e., 50 nmol EGTA/well), causing an upward curve of TnC bands in the single-fiber sample (see Fig. 2C, lanes 3 and 4). The gels were stained with silver according to the Hoefer method, scanned using a Molecular Dynamics personal densitometer, and analyzed with ImageQuant software, version 5.2.

Statistical analysis. All of the numerical data are presented as means ± SE. For all numerical parameters presented in Tables 1 and 2, the coefficients of variation (CV; standard deviation divided by the mean and expressed as percentages) are also included and median values are included for the parameters shown in Table 2. All parameters were analyzed for normality (i.e., gaussianity) using SPSS software (version 12.0.1; SPSS, Chicago, IL), and tested for statistical significance using Prism software version 4.02. Normality was accepted when kurtosis fell in the range +2 to −2. Statistical significance was tested using one-way ANOVA with Tukey’s posttest (for data in Table 1), the two-tailed unpaired t-test (for parameters with gaussian populations in Table 2), and the Mann-Whitney U-test (for parameters with nongaussian populations in Table 2).

RESULTS

In this study, we electrophoretically determined the MHC and TnC isoform composition of a large population of single-fiber segments (198 fibers) from the RA muscle of the adult cane toad. A subset of these fibers (15 fibers) were also examined for Sr2+ and Ca2+ activation characteristics before electrophoretic analysis was performed. Experimental details are described in MATERIALS AND METHODS, and a representative set of contractile (Fig. 2A) and electrophoretic data (Fig. 2, B, lane 2, and C, lane 3) obtained from one of the fibers (type t1 + T) is presented in Fig. 2.

Fiber-type identification. Table 1 lists the MHC-based fiber types detected in this study. Representative electrophoretograms of the MHC isoform profile for each of these fiber types are shown in Fig. 3 (top). As shown in Table 1, 116 (~58%) of 198 fibers analyzed contained only twitch MHC isoforms. Thirty-seven (~32%) of these fibers were pure twitch fibers (containing only one twitch MHC isoform) (Fig. 3, lanes A–C), and 79 (~68%) of the fibers were twitch hybrid fibers (containing 2 or 3 twitch MHC isoforms) (Fig. 3, lanes D–F). Of the latter type, 77 fibers (~97%) contained two twitch MHC isoforms (BmHC2 and BmHC3 being the most common combination), and two (~3%) contained all three twitch MHC isoforms. The remaining 82 fibers (~42% of all fibers) were twitch-tonic hybrids that contained either BmHCT and one twitch MHC isoform (56 fibers, or ~68% of twitch-tonic hybrids) (Fig. 3, lanes G, H, and K) or BmHCT and two twitch MHC isoforms (26 fibers, or ~32% of twitch-tonic fibers) (Fig. 3, lanes I and J). Consistent with previous results at our laboratory (15), no pure tonic fibers (T fibers) were detected among the 198 fibers examined in this study.

Table 1 also includes the mean fiber diameter values for each of the fiber types examined. The twitch fibers (comprising both pure and hybrid fibers) were significantly larger in diameter (61.08 ± 1.30 μm, n = 116) than twitch-tonic hybrids (50.85 ± 1.13 μm; n = 82) (P < 0.001). A smaller but significant difference also was observed between twitch hybrid (64.37 ± 0.16 μm n = 79) and pure twitch fibers (56.52 ± 0.22 μm; n = 37) (P < 0.05). Note that the diameters of the
fibers containing the tonic MHC isoform fell well within the range of diameters of the slow-tonic amphibian fibers reported to date (15–130 μm) (13).

During the single-fiber isolation step, which was performed under oil (see MATERIALS AND METHODS), two types of fibers were distinguished on the basis of their relative transparency under illumination conditions mimicking those under dark-field illumination. On the basis of previous reports that amphibian tonic fibers are more transparent than twitch fibers, when observed in aqueous solutions in dark-field illumination (7), the tonic or twitch identity of 38 fiber segments was predicted from their transparency. The predicted identity was confirmed by MHC isoform analysis in only 32 (84%) of the fibers.

Electrophoretic evidence of two TnC isoforms. Preliminary experiments showed that 1) the recently described (16) EGTA gradient protocol for electrophoretic identification of TnC isoforms in rat muscle preparations could also be used to identify TnC isoforms in cane toad muscle preparations (Fig. 2C), 2) the TnC isoform band present in toad cardiac muscle (Fig. 2C, lane 1) displays a higher electrophoretic mobility than the TnC isoform band detected in the myofibrillar protein fraction purified from toad hindlimb skeletal muscles (Fig. 2C, lane 2) using the method of Potter (18), and 3) the electrophoretic mobilities of the TnC isoforms in the cardiac and hindlimb toad muscle preparations were the same as those of the TnC bands in fibers containing predominantly BmHCT or only BmHC twitch isoforms, respectively. On the basis of these findings, a toad cardiac muscle homogenate and the TnC fraction purified from toad hindlimb muscle were subsequently used in conjunction with the EGTA-induced upward curvature as markers for identifying the TnC isoforms present in single toad muscle fiber segments containing tonic and/or twitch MHC isoform types.

The TnC isoform compositions of the fibers analyzed in this study are listed in Table 1, and representative electrophoretograms of the low molecular weight myofibrillar proteins for each fiber type listed in the table are shown in Fig. 3, bottom. As shown in Fig. 3 (lanes A–F), all of the twitch fibers contained only one curved protein band, identifiable as twitch TnC on the basis of its migration being identical to that of the TnC-t isoform marker (Fig. 2C, lane 2). About 85% of the twitch-tonic hybrids examined also showed only one protein band (Fig. 3, lanes G, J, and K), which was identifiable as TnC-T/c on the basis of its migration being identical to that of the TnC-T/c isoform marker (Fig. 2C, lane 1). A small proportion (5% of the total number of fibers) of the twitch-tonic hybrids displayed two curved bands, with one identifiable as TnC-t and the other identifiable as TnC-T/c (Fig. 3, lanes H and I).

The relative proportions of the tonic (left y-axis) and twitch (right y-axis) MHC isoform types and the type of electrophoretically detectable TnC isoform (x-axis) in each of the 82 twitch-tonic hybrid RA muscle fibers listed in Table 1 are presented as a scatterplot in Fig. 4. Notably, the tonic TnC isoform was detected in all twitch-tonic fibers examined in this study, even when the relative proportion of the tonic MHC isoform present in the fiber was as low as 10%. By comparison,
the twitch TnC isoform was absent from fibers containing less than ~39% twitch MHC isoform types and was detected in only 12 (~25%) of the 49 fibers containing >39% twitch MHC isoform types.

**Functional evidence of two TnC isoforms.** The population of 15 fibers that were analyzed for Ca\(^{2+}\) and Sr\(^{2+}\) activation characteristics comprised five fibers displaying only TnC-t (TnC-t fibers) and 10 fibers displaying only TnC-T/c (TnC-T/c fibers). All of the TnC-t fibers contained only BmHHC2 and BmHHC3 (type t2 + t3). Of the 10 TnC-T/c fibers, 9 contained BmHHC1 and BmHHCt (type t1 + t) and 1 contained BmHHC1, BmHHC3, and BmHCT (type t1 + t3 + T fiber). As predicted from their low proportion in adult RA muscle (~5%) (see Table 1), no fibers containing both TnC-t and TnC-T/c isoforms were found among the 15 fibers dissected specifically for this part of the study.

Figure 5 shows representative P\(_r\)-pCa-pSr curves and electrophoretograms for TnC-t fibers (top) and TnC-T/c fibers (bottom). A summary of the contractile activation parameters obtained for the 15 fibers, grouped according to their TnC isoform composition, is presented in Table 2. Note that although the contractile activation characteristics of the t1 + t3 + T fiber were similar to those of the other nine TnC-T/c fibers (with the exception of FCA\(_{\text{max}}\)/CSA), this fiber was not included in the comparison of the two TnC isoform-based fiber groups, because it displayed a MHC isoform composition different from that of the other fibers in the group.

As calculated using the individual pCa\(_{50}\) values shown in Table 2, toad RA TnC-T/c fibers displayed threefold greater sensitivity than TnC-t fibers to Ca\(^{2+}\). The difference in sensitivity to Ca\(^{2+}\) between the two fiber groups was found to be even higher (~7-fold) when the [Ca\(^{2+}\)] that induced 10% FCA\(_{\text{max}}\) were compared (0.09 ± 0.01 μM for the TnC-T/c fibers vs. 0.59 ± 0.04 μM for the fibers containing TnC-t). The mean value of the Ca\(^{2+}\) activation range (indicated by the ratio of [Ca\(^{2+}\)] to that producing 90% FCA\(_{\text{max}}\) to that producing 10% FCA\(_{\text{max}}\) for TnC-T/c fibers (8.63 ± 1.85) was ~4.6-fold that of TnC-t fibers (1.89 ± 0.09). TnC-T/c fibers displayed markedly greater (~40-fold) sensitivity than TnC-t fibers to Sr\(^{2+}\); this result is in good agreement with the findings previously reported by Horiuti (6), who stated that the contractile system in slow-twitch fibers is “several tens of times more sensitive” to Sr\(^{2+}\) than that of twitch fibers. The value of the parameter indicating the relative sensitivity to Ca\(^{2+}\) and Sr\(^{2+}\) (pCa\(_{50}\) - pSr\(_{50}\), Δ50) of the contractile apparatus for each fiber group was Δ50 < 0.5 for TnC-T/c fibers and Δ50 > 1 for TnC-t fibers. Table 2 shows that both n\(_{ca}\) and n\(_{sr}\) values for TnC-T/c fibers were significantly lower than the values for TnC-t fibers (P < 0.05), suggesting that the degree of cooperative activation along the thin filament was lower for fibers containing TnC-T/c than for those containing TnC-t fibers.

Toad RA fibers containing TnC-T/c did not differ significantly from those containing TnC-t with respect to either FCA\(_{\text{max}}\)/CSA or FSR\(_{\text{max}}\)/CSA (Table 2). A similar result was previously reported for tonic and twitch fibers from the garter snake (21). We suggest that the higher FCA\(_{\text{max}}\)/CSA value recorded for the t1 + t3 + T fiber type (bottom row in Table 2) compared with the FCA\(_{\text{max}}\)/CSA values obtained for the other fibers (type t1 + T) containing TnC-T/c may not be related to the difference in the MHC isoform composition, because the FCA\(_{\text{max}}\)/CSA value obtained for another t1 + t3 + T fiber examined as part of another study was similar to that of the t1 + T group (243.25 kN/m\(^2\)).

**DISCUSSION**

In the present study, we have provided evidence that the rectus abdominis muscle of the anuran *Bufo marinus* (cane toad) contains two TnC isoforms, one of which (TnC-T/c) is present in fibers that display the tonic MHC isoform and in cardiac muscle and the other of which (TnC-t) is present in all fibers that display only twitch MHC isoform types without regard to their number or identity. The electrophoretic bands associated with the TnC-T/c and TnC-t isoforms were identified in single muscle fibers on the basis of a distinctive pattern of Ca\(^{2+}\), induced alteration in their migration (similar to that previously described for rat fast-twitch and slow-twitch/cardiac TnC isoform bands) (16) and on the basis of the difference in their relative mobility, with TnC-T/c being faster than TnC-t. The contractile apparatus in fibers containing the TnC-T/c isoform was found to have a significantly higher sensitivity to both Ca\(^{2+}\) and Sr\(^{2+}\) and to generate force responses over a wider range of [Ca\(^{2+}\)] than that in fibers containing the TnC-t isoform.

To date, only a small number of studies have compared the sensitivity of slow-twitch and twitch muscle fibers to Ca\(^{2+}\) (6, 11, 21, 24). These studies, in which slow-twitch and twitch fibers were distinguished by criteria other than MHC isoform composition, namely, by microscopic appearance under dark-field illumination (6, 11, 24), Nomarski optics (21), size and mechanical responsiveness to ChCl (24), or SDS-PAGE analysis of low molecular weight muscle proteins (~40,000) (11), have produced inconsistent results. Thus, according to Ste-
phenson and Williams (24) and Horiuti (6), slow-tonic and twitch fibers display similar sensitivity to Ca$^{2+}$ (e.g., pCa$_{50}$ 5.83 for slow-twitch fibers and pCa$_{50}$ 5.73 for twitch fibers) (24), whereas according to Martyn et al. (11), they differ, with slow-twitch fibers being 5.8-fold more sensitive than twitch fibers to Ca$^{2+}$.

The group of fibers for which contractile activation characteristics were determined in this study comprised three MHC-based fiber types (t2 + t3, t1 + T, and t1 + t3 + T), but the fibers fell into two categories according to TnC isoform composition: TnC-T/c fibers and TnC-t fibers. TnC-T/c fibers were found to be more sensitive than TnC-t fibers to Ca$^{2+}$ (as indicated by higher pCa$_{50}$ values), 2) to generate measurable force (10% F$_{max}$ with our force measuring system) at Ca$^{2+}$ concentrations as low as 0.09 μM, a value ~7-fold lower than [Ca$^{2+}$] that induced the same level of force in TnC-t fibers (0.59 μM), 3) to develop contractile responses over an ~4.6-fold broader range of [Ca$^{2+}$] than the fibers from the TnC-t group, and 4) to display force-pCa curves twice as shallow as those produced by TnC-t fibers. It is worth noting that Martyn et al. (11) reported a similar ratio (~2) between the mean n$_{Ca}$ values for twitch and slow-twitch fibers of the frog skeletal muscle.

On the basis of their similar electrophoretic mobility, we assumed that the same TnC isoform is expressed in both cardiac muscle and in fibers containing the Bufo marinus tonic MHC isoform (BmHCT); hence our designation of this protein species as TnC-T/c. This assumption is further supported by the previous finding of a single cDNA encoding the slow-cardiac TnC isoform in Xenopus laevis muscle (30).

In the present study, we did not investigate the contractile activation characteristics of the toad cardiac muscle. However, we used the mean values of K$_{1/2}$ (pCa$_{50}$) and n$_{H}$ for frog cardiac muscle in Table 1 of the report published by Harrison and Bers (5) to gain more insight into the physiological role of TnC-T/c. The mean pCa$_{50}$ value for toad RA fibers containing TnC-T/c (6.59) in the present study is markedly higher than the K$_{1/2}$ value reported for frog heart (5.62), a difference that could be due simply to differences in experimental conditions or to structural differences between the TnC isoform expressed in frog cardiac muscle and toad TnC-T/c. However, if we assume that such differences do not exist, then we can conclude that the higher sensitivity of the contractile apparatus to Ca$^{2+}$ in TnC-T/c fibers than in cardiac muscle is closely related not to the TnC isoform but to the pattern of interaction of TnC fibers with isoforms of other myofibrillar proteins that are not shared by the slow-twitch fibers and cardiac muscle.

In this context, it is also worth noting that the mean value of n$_{Ca}$ determined for TnC-T/c fibers of toad RA muscle in the present study (2.29) is almost identical to that reported for frog heart muscle (2.3) by Harrison and Bers (5). If one assumes that there is no physiologically meaningful difference between the frog cardiac TnC and the TnC-T/c isoform described herein, this similarity could be viewed as compelling evidence that the minimum number of cooperative regulatory sites per functional unit in the two types of muscle cells is determined by the TnC isoform. This conclusion becomes less convincing, however, if one considers the evidence produced by Schachat et al. (22), who studied rabbit psoas muscles fibers, that it is the troponin T-tropomyosin (TnT-Tm) complement rather than the molecular form of TnC that determines the n$_{H}$ value in vertebrate skeletal muscle. On this basis, Schachat et al. (22) suggested that the magnitude of n$_{H}$ for a fiber could be predicted on the basis of its TnT-Tm isoform combination (e.g., fast-twitch fibers containing TnT2f-a2-Tm would display the highest n$_{H}$ value) and vice versa. Analysis of properly identified TnT-Tm isoforms in TnC-T/c fibers and TnC-t fibers is required to establish whether this parameter is as closely related to n$_{H}$ values in anuran muscle as has been reported for mammalian muscle.

Currently, we have no information regarding TnC-T/c fibers with respect to various electrophysiological and morphological properties that are commonly associated with slow-twitch fibers (for review, see Ref. 13). However, on the basis of their location in the pelvic region of the RA muscle, low Ca$^{2+}$-stimulated ATPase activity (15) and tonic fiber-like Ca$^{2+}$ activation properties, we assume herein that these fibers are identical to the anuran slow-tonic muscle fibers, for which there is an abundance of physiological and electron microscopic data. This assumption enables us to speculate about the relationship between the properties of the regulatory contractile machinery in the TnC-T/c fibers described in the present study and the properties of key excitation-contraction coupling systems reported by other laboratories with regard to anuran slow-twitch muscle fibers.

As indicated by our results, TnC-T/c fibers develop measurable mechanical responses (10% F$_{max}$) at [Ca$^{2+}$] (~90 nM) lower than the generally quoted value for the resting [Ca$^{2+}$] in twitch fibers (~100 nM) (20). This characteristic is consistent with previous reports of slow-twitch fibers having a relatively low threshold for contraction (15 mM K$^+$ tonic fibers vs. 25 mM K$^+$ twitch fibers) (8) and an underdeveloped SR compartment (13), which may mean less releasable Ca$^{2+}$. Furthermore, TnC-T/c fibers produce force responses over a relatively broad range of [Ca$^{2+}$] levels, which is consistent with the ability of slow-twitch fibers to develop graded contractions in response to graded membrane depolarizations. No obvious relationship between the molecular properties of the TnC-T/c isoform and the ability of slow-twitch fibers to maintain contracture (tonus) for prolonged periods can be inferred. However, one could suggest such a relationship on the basis of evidence that TnC isoform-related differences in Ca$^{2+}$ sensitivity are associated with differences in the rates of Ca$^{2+}$ dissociation from the regulatory sites (4) and on the basis of the three-state model of thin filament activation (Ref. 12; for review, see also Ref. 4). According to this model, longer retention of Ca$^{2+}$ on the regulatory sites of TnC-T/c would equate to longer time spent by the regulatory system in the “closed” state, which in turn would allow the development of force-producing cross-bridges over a longer period.

What is the relationship between amphibian slow cardiac TnC isoform reported in this study and the mammalian slow-twitch cardiac TnC isoform (TnC-s/c)? Does TnC-T/c have only one functional regulatory site? It is tempting to suggest that TnC-T/c and TnC-s/c have structural similarities on the basis of their similar relative affinities for Ca$^{2+}$ and Sr$^{2+}$ (A$_{50}$ <0.5) and the common contractile activation characteristics displayed by the fibers in which they are expressed (high sensitivity to Sr$^{2+}$ and shallower force-pCa-pSr curves). However, the definitive answers to the above questions, which are highly relevant to the understanding of the process of Ca$^{2+}$ regulation of vertebrate striated muscle contraction in an evo-
lutionary sense, ultimately must await sequencing data for TnC-T/c.

This study has revealed an interesting difference between amphibian and mammalian twitch muscle with respect to TnC polymorphism. Unlike mammalian twitch muscle fibers, which display one of two TnC isoforms or a combination thereof (16, 17), all toad twitch fibers examined in the present study displayed only one TnC isoform, regardless of the identity or the number of twitch MHC isoforms expressed in the fiber. We note that differences between amphibian and mammalian twitch muscles with respect to other parameters relevant to the process of Ca\(^{2+}\) regulation of muscle contraction also have been reported by others (see, e.g., Ref. 31). Such differences may limit the applicability to amphibian muscle of some of the current theories regarding Ca\(^{2+}\) regulation of skeletal muscle contraction, because these theories have been developed almost exclusively on the basis of data obtained with mammalian muscle preparations (see Refs 4 and 20). Furthermore, we suggest that conclusions drawn from the data obtained with amphibian slow-tonic muscle preparations should not be extrapolated to slow-twitch fibers in highly specialized mammalian muscles without further testing.

In the present study and in our previous investigation of single fibers from the RA muscle of cane toad (15), we used electrophoretic analysis of MHC isoform composition as the method of fiber typing. On the basis of the data produced using this method, the population of 152 fibers examined by Nguyen and Stephenson (15) yielded 10 fiber types comprising 3 pure twitch fiber types, 3 hybrid twitch fiber types, and 4 hybrid twitch-twitch fiber types, but no pure tonic fibers. The identity of the pure twitch fibers and the twitch-twitch hybrids containing predominantly the tonic isoform was further confirmed by the relative magnitude of the Ca\(^{2+}\)-stimulated Mg\(^{2+}\)-ATPase activities determined in fibers from each group (t1 > t2 > t3 > Tpredominant). With the exception of one fiber type (type t2 + t3 + T), all of the fiber types detected in the ATPase study also were detected in similar proportions among the 198 fibers examined in the present study. Consistent with our previous report, no pure tonic fibers were detected among the relatively large population of single fibers dissected from the cane toads used in the present study. Our failure to find pure tonic fibers among the 412 total fibers of toad RA muscle examined in our two studies challenges the validity of the concept of “true slow fibers” put forward in the concluding section of the review of vertebrate slow muscle by Morgan and Proske (13), because one would expect a true slow fiber to contain only the tonic MHC isoform.

For any given muscle, the number of fiber types reported in a certain investigation depends on the criterion or method used to distinguish the fibers. Most studies of slow-tonic muscle consider only two types of amphibian fibers: slow-tonic and twitch. These two fiber types have been reported to differ with respect to several criteria, such as appearance when visualized using electron microscopy (26), the degree of transparency under light microscopy (2, 6, 7, 11, 24), size (24), location (6), and sensitivity to Sr\(^{2+}\) (6). A larger number of fiber types (up to 6 fibers in Ref. 9) were identified in amphibian muscles when the fibers were distinguished using a combination of histochemical and physiological methods (for review, see Ref. 10), SDS-PAGE analysis of low molecular weight muscle protein isoforms (11), or SDS-PAGE analysis of MHC isoform composition using an electrophoretic protocol developed for mammalian muscle (9). Performing single-fiber analysis of MHC isoform composition using an electrophoretic method developed specifically for toad muscle (14) enabled us to distinguish not only pure twitch but also hybrid twitch and hybrid twitch-tonic fiber types in toad RA muscle. It is clear that this MHC-based method of fiber typing used here and in our previous study (15) has a higher resolving power than other fiber-typing methods, but does this mean that it is superior to all others? Also, how well do MHC-based fiber types relate to the fiber types identified using other criteria? These two not-unrelated-questions are addressed briefly in the remainder of this section.

The value of a fiber-typing criterion or method is determined by the context in which it is used. For example, two of the criteria listed above (namely, size and transparency in dark-field microscopy) have been used previously, either separately or in combination, by several groups of researchers to distinguish twitch and slow-twitch fibers during dissection. In the present study, we compared the diameters and the microscopic appearance of fibers containing only twitch MHC isoforms with those of fibers containing the slow-tone MHC isoform (namely, the twitch-tonic hybrids). Although statistically significant, the fiber type-related size differences found in the present investigation were clearly not large enough to enable clear-cut visual differentiation of the two fiber groups. Furthermore, the fiber-type identity predicted on the basis of microscopic appearance (transparent appearance for tonic fibers and opaque appearance for twitch fibers) was confirmed by performing MHC isoform analyses in only 84% of the fibers studied. Taken together, these results suggest that although these characteristics are easy to determine, they are not fully reliable in studies of the regulatory contractile systems in amphibian muscle fibers. A note of caution therefore is inserted here: despite its reliability, MHC isoform composition may not be the best criterion for distinguishing twitch from slow-tonic fibers if the time available is limited, because electrophoretic analysis of MHC isoform composition in single-fiber segments is a lengthy and labor-intensive procedure.

To assess the extent of the match between fiber types established on the basis of MHC isoform composition and those established according to criteria other than the two discussed above, one would have to compare the MHC composition with any of the other fiber type-defining characteristics in the same fiber segment. For example, using this approach, we (17) previously showed on the basis of the close relationship between MHC isoform type composition, TnC isoform composition, and sensitivity to Sr\(^{2+}\) of the contractile apparatus in rat skeletal muscle fibers that knowledge of any of these three parameters for a given rat muscle fiber would allow the prediction of the other two. In the present study, we also found a close correlation between the TnC isoform composition of a fiber and its sensitivity to Sr\(^{2+}\) (as well as Ca\(^{2+}\)), but the relationship between MHC and TnC isoform composition in toad RA muscle fibers proved to be more complex. Thus all 82 twitch-tonic hybrids from RA muscle examined herein displayed the TnC-T/c isoform, even though the proportion of BmHCT in the fiber was as low as 10%, no fibers containing <39% twitch MHC isoform type displayed the TnC-t isoform, and only 25% of the fibers containing >39% twitch MHC isoform type displayed both TnC-T/c and TnC-t isoforms. This
result leads to two conclusions of practical and cognitive significance: 1) for amphibian twitch-tonic hybrid fibers, pSRs50 is the most reliable indicator of their TnC isoform composition; and 2) amphibian and mammalian skeletal muscles may differ with respect to the pattern of structural articulation between MHC and TnC isoforms.

In summary, in this study, we have shown compelling evidence that cane toad skeletal muscle (and, by extrapolation, anuranskeletal muscle), like the twitch skeletal muscle of higher vertebrates, contains two TnC isoforms (one of which may be shared with the cardiac muscle) that can be distinguished electrophoretically by SDS-PAGE and functionally by the marked difference in sensitivity to Sr2+ of the contractile system of the fibers in which they are expressed. The Ca2+ activation properties of toad fibers containing the tonic cardiac TnC isoform appear to be consistent with several physiological characteristics of amphibian slow-tonic muscle fibers.

GRANTS

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