A single-fibre study of the relationship between MHC and TnC isoform composition in rat skeletal muscle

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In the present study, we investigated the possibility that MHC (myosin heavy chain) and TnC (troponin C) isoforms exist in specific combinations in rat-skeletal-muscle fibres. Single fibres (numbering 245) from soleus (predominantly slow-twitch) and sternomastoid (predominantly fast-twitch) muscles of adult rats were analysed for MHC and TnC isoform composition, using alanine-SDS/PAGE for separating MHC isoforms, and a novel method (based on the previously reported influence of Ca^{2+} on the mobility of Ca^{2+}-binding proteins in SDS gels) for unequivocal identification of TnC isoforms in single-fibre segments. In this study, all fibres that contained only one MHC isoform (slow or fast) contained only the matching TnC isoform and all fibres that contained multiple fast MHC isoforms contained only the fast TnC isoform. Fibres expressing both slow and fast MHC isoforms displayed either both TnC isoforms or only one TnC isoform of a type depending on the relative proportion of fast/slow MHC present. Our results suggest a close relationship between MHC and TnC isoform composition in non-transforming skeletal muscles of adult rat.

Key words: fibre type, gel electrophoresis, myofibrillar proteins, SDS/PAGE, single fibre, troponin.

INTRODUCTION

It is now widely accepted that the binding of Ca^{2+} to TnC (troponin C) initiates a complex process, known as 'thin-filament activation', which involves the movement of tropomyosin along the thin filament to expose the myosin binding sites on actin [1]. Ongoing investigations into the mechanism underlying this process have produced evidence that the thin filament may exist in three 'activation' states which differ with respect to the extent/nature of myosin head binding to actin: blocked (no binding), closed (weak binding) and open (strong binding in a force producing manner) [2]. According to recent findings from X-ray diffraction and fluorescence resonance-energy-transfer studies [2,3], the equilibrium between the blocked and closed states is regulated by Ca^{2+} binding to TnC, whereas the transition from the closed to the open states is affected by the myosin heads bound to actin at certain stages of the actomyosin-ATP hydrolysis cycle. Furthermore, it appears that there are mutual influences between the binding of Ca^{2+} to TnC and the transition of myosin heads between conformational states associated with specific steps in the ATP hydrolysis reaction (see [1] for a comprehensive review). Taken together, these findings suggest that the process of contractile activation in skeletal muscle may be modulated by a subtle functional interplay between myosin and TnC.

In mammals, both MHC (myosin heavy chain, the major component of myosin heads and the site of ATP hydrolysis and actin binding) and TnC are polymorphic proteins. Structural differences between MHC isoforms have been found to influence a number of contractile characteristics of a muscle cell, such as maximum shortening velocity, peak mechanical power and optimal velocity of shortening [4], ATP consumption rate during isometric contraction and tension cost [5]. Based on the maximum shortening velocity and ATP hydrolysis rate conferred to a fibre, the four MHC isoforms detected in the limb muscles of small adult mammals have been ranked fastest to slowest, MHC IIb > IId/x > IIa > I [6]. In contrast with the MHC protein, mammalian TnC is known to exist only as two isoforms, one fast (TnC-f) and one slow/cardiac (TnC-s/cTnC), which differ on the basis of the number of active Ca^{2+}-binding sites [7] and the degree to which Ca^{2+}-binding increases the affinity of TnC for troponin I (TnI) [8]. These differences have been shown to affect properties of the contractile apparatus such as sensitivity to Ca^{2+} [9], pH sensitivity of contraction [10], sarcomere length sensing [11] and rigor cross-bridge-activated optimal tension [12].

Although the current knowledge gives no indication that MHC and TnC share a structural relationship, the polymorphic nature of these proteins coupled with compelling evidence that the function of each one is influenced by the actions of the other suggests that mammalian skeletal-muscle fibres may contain specific combinations of TnC and MHC isoforms. To date, there has been no large-scale systematic investigation into the MHC and TnC isoform composition in single fibres of non-transforming mammalian skeletal muscle (i.e. a muscle that has not undergone changes in muscle-protein isoform composition induced by experimental manipulations or pathological conditions), which might shed light on this possibility. In the present study, we proposed to fill this cognitive gap by performing electrophoretic MHC and TnC isoform analyses on a large number of single fibres comprising a broad range of MHC isoform-based fibre types.

The lack of substantial information regarding the relationship between MHC and TnC isoform composition in single muscle fibres may be due, in part, to the difficulties involved with the identification of TnC isoforms on electrophoretograms of low-molecular-mass (< 42 kDa) muscle proteins. Indeed, as illustrated by other studies [13,14] and discussed in detail in this paper, there is a relatively high risk of misidentification of other protein species for TnC isoforms in single muscle fibres, when protein bands are identified solely on the basis of relative electrophoretic migration.

In the present work, we examined the relationship between the MHC and TnC isoform composition in single fibres from non-transforming rat skeletal muscle using alanine-SDS/PAGE [15] for reproducibly separating MHC isoforms, and a simple,

Abbreviations used: DTT, dithiothreitol; MHC, myosin heavy chain; MLC, myosin light chain; RT, room temperature; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; TnC, troponin C.

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rapid and inexpensive strategy for unequivocal identification of TnC isoforms. This strategy involves the use of a novel method for identifying TnC isoforms in single-muscle-fibre segments, developed by us on the basis of the previously reported influence of Ca^{2+} on the migration of Ca^{2+}-binding proteins in SDS gels [16]. The fibres were dissected from the sternomastoid (predominantly fast-twitch) and soleus (predominantly slow-twitch) muscles of the adult rat.

EXPERIMENTAL

Animals and muscles

Rats (male Sprague–Dawley, aged 13–14 weeks) were killed by halothane overdose, in accordance with the guidelines of the Animal Ethics Committee of Victoria University. Soleus and sternomastoid muscles were excised and immediately placed under paraffin oil.

Single-fibre preparation

Single fibres were isolated randomly under paraffin oil using a dissecting microscope as described previously [17]. The volume of the fibre was calculated by measuring its length and width (mean of 3–4 values along the length) using a video-monitor set-up (Olympus). Each fibre was then placed in SDS/PAGE solubilization buffer (1 μl/0.4 nl of fibre volume) containing 80 mM Tris/HCl (pH 6.8), 2.3 % (w/v) SDS, 710 mM 2-mercaptoethanol, 10 mM dithiothreitol (DTT), 12.5 % (v/v) glycerol, 13.6 % (w/v) sucrose, 0.01 % (w/v) Bromophenol Blue, 0.1 mM PMSF, 0.002 mM leupeptin and 0.001 mM pepstatin. The samples were left overnight at RT (room temperature, 21 °C) and boiled for 3 min the following morning. Samples were stored at − 80 °C until required for gel electrophoresis.

MHC isoform analysis

MHC isoforms were separated using alanine-SDS/PAGE as described previously [15]. Briefly, electrophoresis was carried out on 0.75-mm-thick slab gels using the Hoefer Mighty Small gel apparatus. The separating gel [T = 7.6 %, C = 1.2 %, 425 mM Tris/HCl (pH 8.8), 75 mM alanine, 40 % (v/v) glycerol, 0.3 % (w/v) SDS, 0.05 % (w/v) ammonium persulphate and 0.1 % (v/v) TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine)] was allowed to set at RT for 2–4 h, after which the stacking gel [T = 4 %, C = 2.6 %, 125 mM Tris/HCl (pH 6.8), 4 mM EDTA, 40 % (v/v) glycerol, 0.3 % (w/v) SDS, 0.1 % (w/v) ammonium persulphate and 0.05 % (v/v) TEMED] was cast and allowed to set for 40 min. To each electrophoretic well, 4 μl of sample, containing approx. 1.6 nl fibre, was applied. The gels were run at constant voltage (150 V) for 28 h at 4 °C with a running buffer containing 0.1 % (w/v) SDS, 25 mM Tris and 175 mM alanine. The gels were stained with silver according to the Bio-Rad method, scanned using a Molecular Dynamics Personal Densitometer and analysed with ImageQuaNT software.

TnC isoform analysis

Samples (10 μl containing approx. 4 nl fibre) 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 %, 75 mM Tris, 10 % (v/v) glycerol, 0.1 % (w/v) SDS, 0.04 % (w/v) ammonium persulphate, 0.116 % (v/v) TEMED] was allowed to set overnight at RT. The stacking gel [T = 4 %, C = 4.76 %, 125 mM Tris, 10 % (v/v) glycerol, 0.1 % (w/v) SDS, 0.1 % (w/v) ammonium persulphate, 0.1 % (v/v) TEMED] was cast the following day and allowed to set for 25 min. Electrophoresis was carried out at constant current (10 mA/gel) for 4.25 h at RT with a running buffer composed of 0.1 % (w/v) SDS, 50 mM Tris and 380 mM glycine. The gels were silver-stained by the Hoefer method and scanned as described in the previous section.

The samples were loaded in such a way that each single-fibre sample was adjacent to one lane of solubilization buffer (5 μl) containing 10 mM EGTA (i.e. 50 nmol EGTA/well). In this way, TnC bands could be identified by a characteristic upward bend caused by the diffusion of EGTA from the adjacent lane (see the Results section). Purified rat TnC isoforms used as reference in this study were prepared in our laboratory as described in [18].

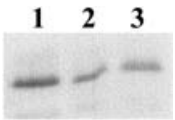

Figure 1 Effect of EGTA on the migration of the rat TnC-f isoform in SDS/polyacrylamide gels

Lanes 1 and 2, purified rat TnC-f (approx. 0.04 μg). Lane 3, purified rat TnC-f (approx. 0.034 μg) + 50 nmol of EGTA.

RESULTS

Identification of TnC isoforms in single fibres using SDS/PAGE and EGTA

Based on reports that the electrophoretic mobilities of TnC in urea gels [19] and calmodulin in SDS gels [16] increase or decrease in the presence or absence of calcium respectively, it has become a common practice to add $CaCl_2$ or EGTA to electrophoretic samples to identify Ca^{2+}-binding proteins (see e.g. [13,14,20–25]). In this study, we have confirmed that the addition of EGTA to samples of both fast and slow rat TnC causes a decrease in the electrophoretic mobility of the protein band in SDS gels. Figure 1 shows an example where the original migration of rat TnC-f (lane 1) was altered by the addition of 50 nmol EGTA (lane 3). More importantly, the results shown in Figure 1 reveal that the gradient of Ca^{2+} concentration produced by the diffusion of EGTA from lane 3 to the adjacent lane during electrophoresis caused a distortion in the TnC band in lane 2, such that half of the band curved upward. This consistently reproducible feature was further exploited by us to enable identification of TnC isoforms in single rat muscle fibres, as illustrated in Figure 2.

Figure 2(a) shows the low-molecular-mass protein profile of a single fibre (lane 2), which is flanked by the rat TnC marker (a mixture of fast and slow rat TnC isoforms) in lane 1, and the rat TnC marker with 50 nmol EGTA in lane 3. The electrophoretogram of the fibre clearly shows two protein bands that curve up from the positions of the TnC bands in lane 1 to the positions of the EGTA-shifted TnC bands in lane 3, indicating that the fibre examined on this gel contains both TnC isoforms. Importantly, EGTA affected the migration of TnC bands exclusively.

The value of the EGTA strategy for identification of TnC in rat single muscle fibres is further illustrated by the results shown in Figures 2(b) and 2(c). In Figure 2(b), it can be seen that the fibre shown in lane 2 contains the fast TnC (TnC-f) isoform, which curves from a position identical with that of the TnC-f in the TnC marker without EGTA (lane 1) to a position of the TnC-f band in the EGTA-treated TnC marker (lane 3). It is important to note that the fibre shown in lane 2 also contains a band, indicated by the

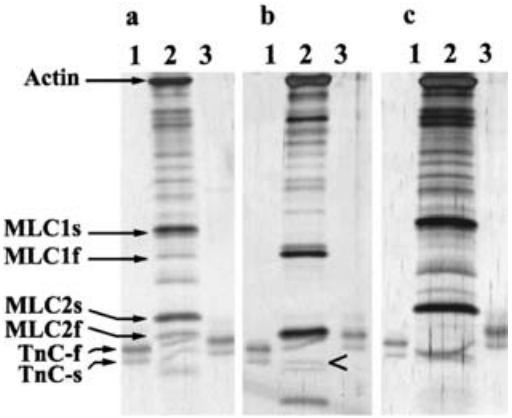

Figure 2 Identification of TnC isoforms in single fibres using EGTA diffusion from an adjacent lane to cause TnC bands to curve upward

All single fibre samples contained approx. 3–4 nl of fibre. Purified rat TnC isoform markers contained approx. 0.04 μg of TnC-f and approx. 0.02 μg of TnC-s. In (**a**–**c**): lane 1, purified rat TnC marker; lane 2, single fibre; lane 3, purified rat TnC marker + 50 nmol of EGTA. The arrowhead in (**b**) points to a protein of unknown identity which on this gel co-migrated with TnC-s, and whose electrophoretic mobility was unaffected by EGTA.

arrowhead, that exhibits identical electrophoretic migration to that of TnC-s in lane 1. Based on this band, it would be assumed that this fibre also contained TnC-s. However, as seen in Figure 2(b), migration of the band indicated by the arrowhead was unaffected by the EGTA diffusion from lane 3, and therefore could not be TnC-s. Figure 2(c) shows an electrophoretogram of a single fibre (lane 2) which, judging by the migration of TnC-s in lane 1 and the curve of the band induced by EGTA diffusion from lane 3, expresses only TnC-s. Notably, the upward curve of the TnC-s reveals the presence of two other bands that co-migrated with TnC, but were not affected by EGTA. Based on this result, it would be inappropriate to use a gel slice containing this TnC-s band for amino acid sequencing or quantification purposes.

Detection of TnC isoforms in single muscle fibres

The conclusions drawn from electrophoretic analyses of TnC isoform composition in single muscle fibres when the protein bands are visualized by silver staining depend on (i) sensitivity of TnC to the stain, (ii) amount of TnC present in the single fibre segments and (iii) maximum level of staining intensity which is compatible with optimal resolution of all protein bands of interest on the gel. As seen in Figure 3 (upper panel), the smallest amount of purified TnC detectable by our silver-staining procedure was 0.5 ng for both TnC-f and TnC-s.

Using a visual comparison of the intensities of the TnC bands in several 4 nl segments of pure fibres with those of known amounts of purified TnC isoforms, we estimated that a single-fibre segment of approx. 4 nl (see e.g. Figure 3, lower panel) contains 8–10 ng TnC. It is worth noting that this value is similar to that calculated by us (8 ng/4 nl fibre) from the TnC content of rabbit skeletal muscle (93 pmol/mg wet weight) reported earlier by Yates and Greaser [26] using amino acid analysis of myofibrillar proteins separated by SDS/PAGE. Based on these results, and as shown by the results presented in Figure 3 (lower panel, right lane), one would predict that in a mixed TnC-f/TnC-s sample containing 10 ng of total protein (such as a 4 nl fibre segment containing both TnC-f and TnC-s), one should be able to detect both TnC isoform bands as long as neither of them was $<5\,\%$ (0.5/10 ng) of the total. A direct implication of this finding is that if the electrophoretogram of a 4 nl fibre segment displays only one TnC isoform band, then the relative proportion of that isoform in the fibre should be at least 95 % of the total.

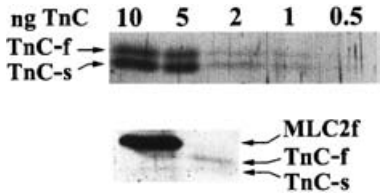

Figure 3 Detection limit of silver-stained TnC isoform bands (rabbit TnC-f and human cardiac TnC-s) resolved by SDS/PAGE (upper panel) and electrophoretic estimation of the TnC content of a 4 nl fibre segment using a mixture of known amounts of purified TnC isoforms as reference (lower panel)

Upper panel: The amount of protein applied for each TnC isoform (ranging from 10 to 0.5 ng) is shown above the lane. Lower panel: MLC2/TnC region of a 4 nl fast-twitch single-fibre segment (left lane); mixed sample containing 10 ng of rabbit TnC-f and 0.5 ng of human cardiac TnC-s (right lane).

Table 1 TnC isoform composition of the pure and hybrid MHC-based fibre types obtained from soleus and sternomastoid muscles of adult rat

The values listed represent the number (proportion) of fibres of a given fibre type containing the indicated TnC isoform(s).

	TnC isoforms present		
MHC isoforms present	TnC-f	TnC-f + TnC-s	TnC-s
Pure fibres			
IIa ($n = 4$)	4 (100%)	0	0
IIb ($n = 68$)	68 (100%)	0	0
IId ($n = 10$)	10 (100%)	0	0
I ($n = 22$)	0	0	22 (100%)
Fast–fast hybrid fibres			
IIa + IIb ($n = 4$)	4 (100%)	0	0
IIa + IId ($n = 15$)	15 (100%)	0	0
IIb + IId ($n = 77$)	77 (100%)	0	0
IIa + IIb + IId ($n = 13$)	13 (100%)	0	0
Fast–slow hybrid fibres			
IIa + I ($n = 11$)	3 (27%)	5 (46%)	3 (27%)
IId + I ($n = 3$)	0	3 (100%)	0
IIa + IId + I ($n = 9$)	2 (22%)	7 (78%)	0
IIb + IId + I ($n = 4$)	2 (50%)	2 (50%)	0
IIa + IIb + IId + I ($n = 5$)	3 (60%)	2 (40%)	0

Classification of MHC isoform-based fibre types

In this study, we examined 245 single fibres of which 218 were from the sternomastoid muscle and 27 from the soleus. The pool of sternomastoid fibres comprised 13 MHC isoform-based fibre types (listed in Table 1), whereas the population of soleus fibres was composed of only three of the fibre types listed in Table 1 (type I, 21 fibres; type IIA + I, 5 fibres; type IIA + IID + I, 1 fibre). In Figure 4, representative electrophoretograms of fibres classified as 'pure' types (IIB, IID/X, IIA and I), which contained only one MHC isoform (Figure 4, upper panel), fibres classified as 'fast–fast hybrids', which contained various combinations of fast MHC isoforms (Figure 4, middle panel), and 'fast–slow hybrids' containing both the slow MHC I isoform and one or several fast MHC isoforms (Figure 4, lower panel), are shown.

TnC isoforms detected in pure and hybrid fibres

As listed in Table 1, and shown by the examples in Figure 5, all pure slow type I fibres ($n = 22$, Figure 5a) displayed only TnC-s,

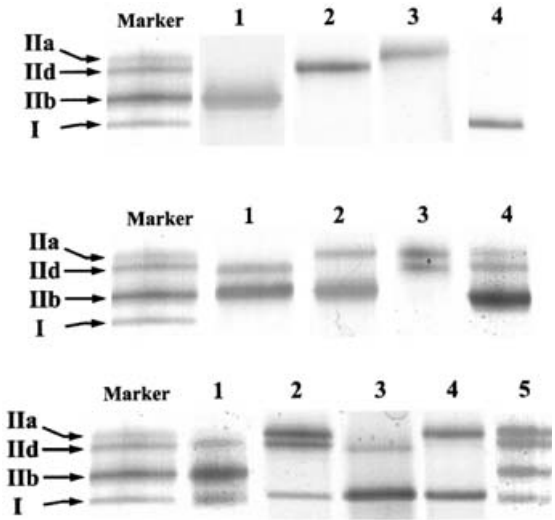

Figure 4 Representative electrophoretograms of the 13 MHC isoform-based fibre types found in this study

All lanes contained approx. 1.6 nl of fibre. Top panel shows 'pure' fibres: lane 1, IIB; lane 2, IID; lane 3, IIA; lane 4, I. Middle panel presents 'fast–fast hybrid' fibres: lane 1, IIB + IID; lane 2, IIA + IIB; lane 3, IIA + IID; lane 4, IIA + IIB + IID. Lower panel displays 'fast–slow hybrid' fibres: lane 1, I + IIB + IID; lane 2, IIA + IID + I; lane 3, IID + I; lane 4, IIA + I; lane 5, IIA + IIB + IID + I. The MHC marker was a sample prepared by mixing rat diaphragm, extensor digitorum longus and soleus muscle homogenates.

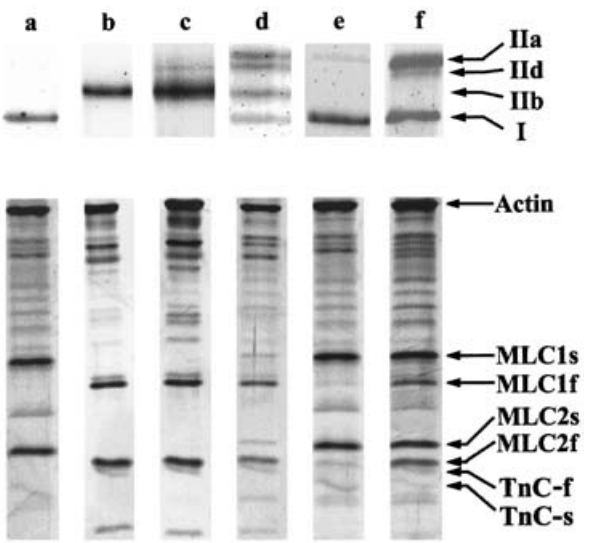

Figure 5 Examples of the MHC and TnC isoform combinations detected in the single fibres examined in this study

All MHC lanes (top panels) contained approx. 1.6 nl of fibre, all low-molecular-mass lanes (bottom panels) contained approx. 3–4 nl of fibre. Lane a, pure type I fibre with TnC-s; lane b, pure type IIB fibre with TnC-f; lane c, 'fast–fast hybrid' fibre (type IIB + IID) with TnC-f; lane d, 'fast–slow hybrid' fibre (type IIA + IIB + IID + I) with TnC-f; lane e, 'fast–slow hybrid' fibre (type IIA + I) with TnC-s; lane f, 'fast–slow hybrid' fibre (type IIA + IID + I) with TnC-f and TnC-s. For abbreviations see legend to Figure 2.

and all pure fast ($n = 82$, Figure 5b) and fast–fast hybrid fibres ($n = 109$, Figure 5c) displayed only TnC-f. Clearly, in each of these fibres, the type of TnC isoform present 'matched' that of the MHC isoform(s). In contrast, fibres classified as fast–slow hybrids ($n = 32$, Figures 5d–5f) were found to display TnC-f only or TnC-s only or both TnC isoforms depending, largely, on the proportion of fast and slow MHC isoforms present (as illustrated by Figures 5 and 6). For example, in the fibres shown in Figures 5(d)–5(f), displaying TnC-f (Figure 5d), TnC-s (Figure 5e) and both TnC-f and TnC-s (Figure 5f), the proportions of MHC isoforms were 89 % fast/11 % slow, 7 % fast/93 % slow and 58 % fast/42 % slow respectively.

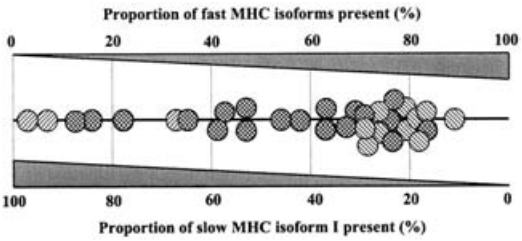

Figure 6 Relationship between the proportion of fast/slow MHC isoforms present and the type of TnC isoforms detected in 'fast–slow hybrid' fibres

Each single fibre (represented by a circle) is placed on the scale according to the proportion of fast/slow MHC present. The line pattern of the circles is indicative of the TnC isoform composition: crossed lines, TnC-f and TnC-s; upward-right slanted lines, TnC-s; upward-left slanted lines, TnC-f.

Further insight into the relationship between the relative proportion of fast and slow MHC isoforms and the TnC isoform composition in adult rat-skeletal-muscle fibres is provided by the graphical representation of the fast–slow hybrid fibre analysis shown in Figure 6. In this diagram, each fibre is represented by a circle, the proportion of fast and slow MHC isoforms contained in the fibre is indicated by the relative position of the circle on the horizontal scale and the type of TnC isoform(s) detected in the fibre is represented by the line pattern of the circle.

As seen in Figure 6, the 12 fast–slow hybrid fibres in which the proportions of the fast and slow MHC isoform types were approx. > 30 % displayed both TnC-f and TnC-s. Since the types of TnC isoforms present in these fibres matched those of the MHC isoforms, they are hereafter referred to as 'matched fibres'. The population of fast–slow hybrids in which one of the MHC isoform types was approx. < 30 % included two groups of fibres: one comprising fibres that displayed two MHC and two TnC isoform types (i.e. matched fibres), and a second containing fibres that displayed a single TnC isoform type, which was matched with one of the MHC isoform types present and mismatched with the other. In these latter fibres, hereafter referred to as 'mismatched fibres', which originated from either the soleus or the sterno-mastoid muscle, the TnC isoform was always matched with (of the same type as) the dominant (approx. > 70 %) MHC isoform type, and was mismatched with (the opposite type of) the less abundant MHC isoform type. The population of fast–slow hybrids containing approx. < 30 % fast MHC isoforms included three type IIA + I fibres (all mismatched) and three type IID + I fibres (all matched). The subgroup of fibres containing approx. < 30 % slow MHC isoform was composed of three type IIA + I fibres (all mismatched), five type IIA + IID + I fibres (three matched and two mismatched), three type IIB + IID + I fibres (one matched and two mismatched) and four type IIA + IIB + IID + I fibres (one matched and three mismatched).

DISCUSSION

The major finding of this study is that, in skeletal muscles of adult rat, fibres containing exclusively fast MHC isoforms (one or several) displayed only TnC-f, fibres containing exclusively the slow MHC isoform displayed only TnC-s and fibres containing

both fast and slow MHC isoforms displayed either both TnC isoforms or one TnC isoform matching the most abundant MHC isoform type. These results are consistent with the idea that MHC and TnC isoforms co-exist in specific combinations in fibres from non-transforming mammalian skeletal muscle. The strength of this conclusion comes from the large number (245) of single fibres examined, the breadth of the fibre types included in the study (13 out of 15 theoretical possibilities) and from the reproducibility and effectiveness of the microelectrophoretic methods used for analysing MHC and TnC isoform composition in single-fibre segments.

Our results contradict those of Danieli-Betto et al. [27] and Geiger et al. [28], who reported cases of mismatched MHC and TnC isoforms in both pure fast-twitch (TnC-f and TnC-s co-existing with MHC IIa) [27] and pure slow-twitch (TnC-s and TnC-f or TnC-f alone co-existing with MHC I) [27,28] single fibres of the adult rat. The discrepancy between our results and those of Danieli-Betto et al. [27] and Geiger et al. [28] may be explained if one considers that, in the latter studies, TnC bands on SDS gels were identified only on the basis of electrophoretic migration, without reference to appropriate markers (such as purified rat TnC isoforms). The value of this strategy is limited considering that TnC isoform bands have the potential to co-migrate with neighbouring protein species of known or unknown identity. Evidence supporting this point comes from the studies of Gulati et al. [14], who showed that TnC-f co-migrated with MLC2 (myosin light chain 2) in hamster trabeculae, and Babu et al. [13] and Metzger [10], who reported the co-migration of both TnC-f and TnC-s isoform bands with MLC2 in rabbit trabeculae and soleus muscle fibres respectively. Many of the rat fibres examined in this study (117 of 245) contained either one or two bands of unknown identity which exhibited electrophoretic mobilities similar to the TnC isoforms. The exact position of these bands varied from gel to gel; however, when present, they would often co-migrate with TnC-s (as shown by the example in this study) or, less frequently, with TnC-f. Taken together, our results and those of others [10,13,14] indicate that protein species co-migrating with TnC can be easily misidentified as TnC, unless appropriate measures are taken (such as those taken in this study). Therefore, we suggest that the discrepancy between our results and those of previous studies [27,28] with regard to MHC and TnC isoform composition in single rat muscle fibres could be the result of misidentification of TnC isoform bands in the latter.

As the results of this study show, the misidentification of TnC bands in single fibres due to co-migration of TnC with other protein species can be avoided by allowing the diffusion of EGTA from a lane adjacent to the single fibre sample to produce a characteristic upward curve of TnC bands only. The notable advantage of this method is that, unlike previously reported methods using Ca^{2+}/EGTA to identify TnC bands, it requires sample for one electrophoretic well only. Thus a combined TnC and MHC electrophoretic analysis can be successfully performed on fibres of volume as small as 5.6 nl, a feature of substantial benefit in studies involving the identification of MHC and TnC isoforms in very small fibres from atrophied muscles or from small animals. The EGTA diffusion strategy also has the potential to be of great benefit in studies involving TnC extraction/reconstitution experiments, in which SDS/PAGE is commonly used to assess the success of the extraction/replacement protocols. Finally, since the method allows a TnC-f band to be visualized without being shifted fully into the position of MLC2f, it allows for accurate quantification of MLC isoforms whenever required.

Based on the number of MHC and TnC isoforms known to exist in skeletal muscles of adult rat (4 and 2 respectively) and assuming that these isoforms could combine in a non-restricted manner, one would expect that analyses of MHC and TnC isoform composition of a large pool of single fibres from rat skeletal muscles would produce 15 combinations of MHC isoforms and 45 combinations of MHC and TnC isoforms (shown in Table 2). In the present study, we examined 245 single fibres, randomly isolated from soleus and sternomastoid muscles of nine adult rats and found 13 combinations (approx. 87 % of theoretical possibilities) of MHC isoforms (one of which, IIa + IIb, was not detected before in non-transforming mammalian muscle), but only 18 MHC and TnC isoform combinations (approx. 40 % of theoretical possibilities; see Table 2 for details). This finding is consistent with the idea that the combinations of MHC and TnC isoforms follow a specific pattern in fibres from non-transforming mammalian skeletal muscle.

Table 2 Theoretically possible MHC–TnC isoform combinations

The MHC–TnC isoform combinations detected in this study in a pool of 245 single fibres from non-transforming rat skeletal muscle represent only a small proportion of the theoretically possible combinations between four MHC and two Tn C isoforms. Symbols used for combinations: +, theoretically possible and detected; 0, theoretically possible, but not detected.

	TnC isoform		
MHC isoform	TnC-f	TnC-f + TnC-s	TnC-s
Pure slow			
I	0	0	+
Pure fast			
IIa	+	0	0
IId	+	0	0
IIb	+	0	0
Fast–fast hybrid			
IIa + IIb	+	0	0
IIa + IId	+	0	0
IIb + IId	+	0	0
IIa + IIb + IId	+	0	0
Slow–fast hybrid			
IIa + I	+	+	+
IIb + I	0	0	0
IId + I	0	+	0
IIa + IIb + I	0	0	0
IIa + IId + I	+	+	0
IIb + IId + I	+	+	0
IIa + IId + IIb + I	+	+	0

In this study, 20 of the 32 fast–slow hybrid fibres displayed a marked difference between the proportions of MHC isoform types present, with one type being approx. $\leqslant$ 30 % of the total. Interestingly, some of these fibres appeared to lack the TnC isoform corresponding to the least abundant MHC isoform type. The simplest explanation for this finding is that in these fibres, the apparently missing TnC isoform was actually present, but in amounts that were below the detection limit of the staining method used, which under our conditions was 0.5 ng of TnC/electrophoretic well (equivalent to 5 % of the total TnC content of a 4 nl fibre segment, estimated to be approx. 10 ng). This possibility could be investigated, e.g. by loading more sample to increase the amount of TnC in the well above 0.5 ng; however, this is not usually feasible due to the relatively small size of rat-skeletal-muscle fibre segments. An alternative strategy would be to compare TnC-isoform-related functional characteristics (such as sensitivity of the contractile apparatus to Sr^{2+} activation) of fast–slow hybrid fibres displaying only one TnC isoform with those of fibres displaying both. We are currently in the process of undertaking this technically complex study.

In summary, in this study, we described a method that enables definitive identification of TnC isoforms in small segments of

pure and hybrid single muscle fibres. Using this method in conjunction with a method for reproducible separation of MHC isoforms in single fibres, we have found that fibres from the sternomastoid and soleus muscles of the adult rat display only a relatively small number of the possible theoretical combinations of MHC and TnC isoforms, which follow an apparent pattern. Based on these results we conclude that in single fibres from non-transforming mammalian skeletal muscle, MHC and TnC isoforms exist in specific combinations that may be related to the functional interplay of these two proteins.

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