Single-fiber myosin heavy chain polymorphism: how many patterns and what proportions?

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Caiozzo, Vincent J., Michael J. Baker, Karen Huang, Harvey Chou, Ya Zhen Wu, and Kenneth M. Baldwin. Single-fiber myosin heavy chain polymorphism: how many patterns and what proportions? Am J Physiol Regul Integr Comp Physiol 285: R570–R580, 2003.—Previous studies have reported the existence of skeletal muscle fibers that coexpress multiple myosin heavy chain isoforms. These surveys have usually been limited to studying the polymorphic profiles of skeletal muscle fibers from a limited number of muscles (i.e., usually <4). Additionally, few studies have considered the functional implications of polymorphism. Hence, the primary objective of this study was to survey a relatively large number of rat skeletal muscle/muscle regions and muscle fibers (n~5,000) to test the hypothesis that polymorphic fibers represent a larger fraction of the total pool of fibers than do so-called monomorphic fibers, which express only one myosin heavy chain isoform. Additionally, we used Hill’s statistical model of the force-velocity relationship to differentiate the functional consequences of single-fiber myosin heavy chain isoform distributions found in these muscles. The results demonstrate that most muscles and regions of rodent skeletal muscles contain large proportions of polymorphic fibers, with the exception of muscles such as the slow soleus muscle and white regions of fast muscles. Several muscles were also found to have polymorphic profiles that are not consistent with the I→IIA→IIX→IIB scheme of muscle plasticity. For instance, it was found that the diaphragm muscle normally contains IIX fibers. Functionally, the high degree of polymorphism may 1) represent a strategy for producing a spectrum of contractile properties that far exceeds that simply defined by the presence of four myosin heavy chain isoforms and 2) result in relatively small differences in function as defined by the force-velocity relationship.

isoform; hybrid fiber; polymorphic fiber; force-velocity relationship; fiber type

During the late 1960s and early 1970s, a number of key papers were published regarding the identification and classification of muscle fiber types (3, 4, 9, 25). In particular, the findings of Brooke and Kaiser (9), Barnard et al. (4), and Peter et al. (25) represent some of the most highly cited articles of that time, and each provides an important historical perspective about the identification and classification of muscle fiber types. During the past 10 years, several methodological advances have had a significant impact on the study of muscle fiber types. The first of these was the development of immunohistochemical techniques for identifying various myosin heavy chain (MHC) isoforms (23). Second, refined techniques for the electrophoretic separation of MHC isoforms (36) provided an improved method for identifying at least four adult MHC isoforms (i.e., slow type I, fast type IIA, fast type IIX, and fast type IIB MHC). Finally, electrophoretic methods have been developed to examine the MHC isoform composition of single fibers. Importantly, the single-fiber electrophoretic approach overcomes some of the inherent limitations associated with immunohistochemistry (e.g., MAb nonspecificity, inability to estimate relative proportions of MHC isoforms within a given fiber, and unequivocal identification of the fast type IIX MHC isoform).

Some of the key observations made using the single-fiber electrophoretic approach are as follows. First, it has been shown (11, 13–15, 19, 26–30, 33, 35, 40) that some muscles contain so-called hybrid or polymorphic fibers (i.e., fibers that coexpress >1 MHC isoform) under normal conditions. Second, in some rodent muscles, perturbations of thyroid and loading state appear to increase the degree of polymorphism (11, 12). Finally, Pette and colleagues (1, 19, 22, 26–30, 33, 35, 39, 40) have used this approach to develop the concept that transitions in MHC isoform expression are, for the most part, obligated to follow a sequential scheme summarized as I→IIA→IIX→IIB.

Recently, we made several novel observations regarding MHC polymorphism in single fibers of rodent skeletal muscle (11–13, 15). Using the combined intervention of hyperthyroidism and mechanical unloading, we found that large numbers of single fibers (~65% of the total population of fibers) in the rodent soleus muscle were capable of displaying a high degree of polymorphism as evidenced by the coexpression of all four adult MHC isoforms (11, 12). The findings from these studies also provided evidence to suggest that...
rodent skeletal muscle fibers are not obligated to follow the sequential scheme proposed by Pette and colleagues (see Ref. 26). Collectively, these findings suggest that the genetic regulation of MHC isoforms is much more complex than previously thought.

The high degree of polymorphism noted above prompted us to ask a question somewhat analogous to that proposed by Brooke and Kaiser (9). However, rather than addressing the question of “Muscle fiber types: how many and what kind?”, the objective of this study was to answer the following question: “Single-fiber MHC polymorphism: how many patterns and what proportions?” In an attempt to address this issue, we examined the MHC isoform compositions of single fibers (~5,000 fibers) taken from a spectrum of rodent muscles/muscle regions (~13 different muscles/muscle regions) commonly studied. Additionally, we used these data in conjunction with Hill’s (20) statistical model of the force-velocity relationship and the single-fiber mechanical data of Bottinelli et al. (8) to address issues related to the mechanical importance of polymorphism within individual fibers.

The findings of this study have important implications with respect to 1) the complexity of MHC isoform gene regulation, 2) the I–IIXA→IIA→IIA/IIX→IIX→IIX/IIB→IIB MHC isoform transition scheme, and 3) the functional significance of MHC isoform polymorphism as illustrated by the force-velocity relationship.

METHODS

Collection of muscle samples. All experiments described in this study were approved by our Institutional Animal Care and Use Committee (IACUC) before experimentation. In experiment 1, muscle samples were harvested from five to seven female Sprague-Dawley rats (mean body wt = 292 g; Bantin-Kingman, Fremont, CA). These animals were estimated by the vendor to be >100 days of age at the time of study. The muscles analyzed in this study were the I–IIXA→IIA→IIA/IIX→IIX→IIX/IIB→IIB MHC isoform transition scheme, and 3) the functional significance of MHC isoform polymorphism as illustrated by the force-velocity relationship.

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Isolation of single fibers. The midportion of each muscle/region (~5 mm) was used for determining the MHC isoform composition of single fibers. The techniques used in the current study are similar to those published previously (11–13, 15, 42). Typically, 40–50 individual muscle fibers/sample were isolated. Overall, 4,081 fibers were analyzed in the first phase of study (experiment 1). Each muscle segment used for single-fiber MHC isoform analyses was cut into small strips and then stored in a glycerol relaxing solution (50% glycerol, 2 mM EGTA, 1 mM MgCl₂, 4 mM ATP, 10 mM imidazole, 100 mM KCl, pH 7.0, −20°C). Single fibers were isolated by microdissection with the use of microsurgical forceps (Super Fine Dumont tweezers; Biomedical Research Instruments, Rockville, MD) and a dissection microscope (Technival 2, ausJena, Germany) with back lighting. Isolated fibers were then transferred into individual polypropylene microcentrifuge tubes (500 µL) that contained 30 µL of denaturing sample buffer (62.5 mM Tris (pH 6.8), 1.0% (wt/vol) SDS, 0.01% (wt/vol) bromophenol blue, 15.0% (vol/vol) glycerol, and 5.0% (vol/vol) b-mercaptoethanol). Each sample was heated (70°C for 2 min) and placed into a sonicator for 60 min. Approximately 15 µL of each sample were then loaded into a well of the gel, and electrophoresis was performed as described below.

Discontinuous PAGE separation of MHC isoforms. MHC isoforms were separated by use of techniques described previously (10–13). The separating gel consisted of 8% acrylamide, 0.16% bis-acrylamide, 30% glycerol, 0.4% SDS, 0.2 M Tris (pH 8.8), and 0.1 M glycine. This solution was degassed for ~15 min, and polymerization was then initiated by adding TEMED (0.05% final concentration) and ammonium persulfate (APS; 0.1% final concentration) to the separating gel solution. The separating gel was poured, layered with ethyl alcohol, and given ~30 min to polymerize. The stacking gel solution contained 4% acrylamide, 0.08% bis-acrylamide, 30% glycerol, 70 mM Tris (pH 6.7), 4 mM EDTA, and 0.4% SDS. This solution was also degassed for 15 min before adding TEMED (0.05% final concentration) and APS (0.1% final concentration). It was then layered onto the separating gel. The running buffer contained 0.1 M Tris, 0.15 M glycine, and 0.1% SDS. An SG-200 vertical slab gel system (CBS Scientific, Del Mar, CA) was used for electrophoresis. Gels were run for ~24 h and at 270 V. This method separated the fast type IIA, fast type IIX, fast type IIB, and slow type I MHC isoforms (progressive order of migration). MHC protein isoform bands obtained from single fibers were stained with the use of a silver stain kit (Bio-Rad, Richmond, CA). A densitometer (Molecular Dynamics, Sunnyvale, CA) was used to scan and quantify the MHC isoform bands.

Polymorphism index. A simple index was developed to express the proportion of polymorphic fibers relative to the total population of fibers. The polymorphism index was simply defined as

\[ \text{Polymorphism index} = \frac{(\text{no. of polymorphic fibers})}{(\text{total no. of fibers})} \times 100 \]

Additional analyses of DIA single-fiber MHC isoform composition. The single-fiber electrophoretic MHC protein analyses yielded several interesting findings with respect to the DIA muscle (see Fig. 1A). First, we observed that there was a high degree of polymorphism and a significant proportion of fibers (~10%) that appeared to coexpress the slow type I and fast type IIX MHC isoforms (see Results). In this context, we performed supplemental experiments (referred to as experiment 2) that included both Western blots and RT-PCR analyses of single fibers to further confirm or reject the presence of the I/IIX fibers in the DIA. Second, the single-fiber MHC isoform profile of the DIA muscles observed in the current study (see Fig. 1A) were quite different from those previously published by Sieck et al. (32), who also used Sprague-Dawley rats. These are several potential reasons for these discrepancies, such as 1) gender differences (i.e., male vs. female rats), 2) possible regional differences, and 3) different breeders/vendors. These latter two possibilities were addressed by examination of the single-fiber MHC isoform distributions in the dorsal and ventral regions of the costal DIA (referred to
as experiment 3) and by contrasting the distributions in animals obtained from Bantin-Kingman and Harlan (defined as experiment 4). Possible regional differences (experiment 3) were examined by separating five hemidiaphragm muscles into two regions defined as 1) dorsal costal DIA and 2) ventral costal DIA. Approximately 220 single fibers were isolated from each region, and the MHC isoform composition was determined as described above. With respect to potential variations due to breeders, it should be noted that Sprague-Dawley rats are an outbred strain and are bred for maximum heterozygocity. Hence, we compared the MHC isoform composition of single fibers from the costal hemidiaphragms of animals obtained from Bantin-Kingman (experiments 1 and 3) with those from Harlan (experiment 4). This involved obtaining five animals from Harlan and isolating fibers from the ventral (n = 198) and dorsal (n = 204) regions of the costal hemidiaphragm muscles. Collectively, experiments 2–4 involved analyses on 1,024 fibers from the DIA muscle.

Western blot analyses of I/IIX fibers in the DIA muscle. As noted above, a second experiment was performed to provide a
higher degree of assurance that the I/IIX fibers observed in the DIA muscle did not also express the fast type IIA MHC isoform. This second experiment employed a more sensitive approach utilizing both Western blot and chemiluminescent techniques. Two hundred single fibers were isolated from the DIA muscle (n = 5), and each fiber was halved. One segment of the fiber was used for electrophoretic determination of MHC composition. Fibers that appeared to be I/IIX fibers (~20 fibers) on the basis of electrophoresis were identified, and the remaining one-half of that fiber was used for Western blot analyses.

The Western blot analyses were performed as follows. A volume of 15 μl of sample buffer was loaded into each lane of a gel, and electrophoresis was performed. After electrophoresis, the MHC isoforms were transferred onto nitrocellulose paper, using a constant voltage of 110 V for 2 h. The blotting buffer contained 25 mM Tris, 192 mM glycine, and 20% methanol. The temperature of the blotting chamber was maintained at 4°C by use of an ice bath. After completion of protein transfer, the nitrocellulose paper was placed into a blocking solution that contained 5% nonfat milk in PBS. The nitrocellulose paper was then incubated in a solution containing the primary monoclonal antibody (1:36,000; MY-32, Molecular Research Center, Cincinnati, OH), vortexed and maintained at 4°C for 1 h. After incubation, the MHC isoforms were transferred onto nitrocellulose paper, sectioned and then immediately freeze dried. Single fibers were then isolated and placed into 100 μl of TRI-Reagent solution (Molecular Research Center, Cincinnati, OH), vortexed briefly (10–15 s), and stored at −80°C.

The TRI-Reagent solution was subsequently thawed at room temperature, and after adding 22 μl of chloroform, each sample was vortexed (10–15 s) and stored for 5 min at room temperature. Samples were then centrifuged at 12,500 g for 20 min (4°C). After centrifugation, total RNA was precipitated from the aqueous phase with isopropanol, and after a washing with ethanol it was dried with the use of a centrifip. The total amount of extracted RNA was reverse transcribed in a 20-μl reaction volume, using Superscript II and oligo(dT) according to the supplied instructions (GIBCO, Life Technology). At the end of the RT reaction, the tubes were heated at 72°C for 15 min to stop the reaction and were stored frozen at −80°C until use in the PCR reactions.

PCR was used to amplify MHC cDNA isoforms by use of specific primers for each isoform. The 5′-oligonucleotide primer was common to each of the MHC mRNA isoforms and contained the following sequence: 5′-GAAGGGCCAGAAGGCCCATC-3′. The 3′-oligonucleotide primers were derived from the 3′-untranslated regions of the four different MHC isoforms where the sequences are known to be highly specific. The sequences for the 3′-oligonucleotide primers are shown in Table 1.

Table 1. Sequences for 3′-oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence* (5′ → 3′)</th>
</tr>
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<tbody>
<tr>
<td>Type I (β-MHC)</td>
<td>5′-GGT CTC AGG GCT TCA GAG CC-3′</td>
</tr>
<tr>
<td>Type IIA</td>
<td>5′-TCT ACA GCA TCA GAG CTG CC-3′</td>
</tr>
<tr>
<td>Type IIX</td>
<td>5′-GTT CAC TTT CCT GCT TTT GA-3′</td>
</tr>
<tr>
<td>Type IIB</td>
<td>5′-GGT AGA TTA TTT CTT CTG TCA GC-3′</td>
</tr>
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Band identity was based on molecular size (by comparison with a DNA 100-bp ladder)."
where \( f_x \) is the fraction of that given MHC isoform in the fiber and \( V_{\text{max}} \) is the maximal shortening velocity associated with that given MHC isoform.

By use of the approach described above, ~20 data points (ranging from 1 to 100% \( P_x \)) were used to fit the Hill equation. The mean difference between the real (modeled) and predicted (value derived from Hill equation) \( V \) at the lowest fitted value (i.e., 1% \( P_x \)) was 3.1%. The mean ± SE coefficient of determination for fitting the Hill equation was 0.9996 ± 0.0002. The lowest coefficient of determination was 0.995.

**Statistical analyses.** Analysis of the regional distribution of specific fiber types in the DIA muscle was performed by use of a \( t \)-test. Overall differences in fiber type distribution of the DIA were determined using a chi-square test. In all statistical tests, significance was defined as \( P \leq 0.05 \).

**RESULTS**

To properly characterize single-fiber MHC polymorphism, three variables must be considered: 1) the different types of polymorphic fibers, 2) the relative proportion of each type of polymorphic fiber, and 3) the ratio of MHC isoform coexpression within a given type of polymorphic fiber. These three variables are integrated into the graphical representations of single-fiber MHC isoform compositions shown in Fig. 1.

**Proportions of polymorphic fibers.** The proportion of polymorphic fibers was highly variable from one muscle to another (~3–78% of the total population of fibers). However, as shown in Fig. 1, the majority of the muscles/regions sampled in this study exhibited a significant degree of single-fiber MHC polymorphism as evidenced by a polymorphism index of >40%. In 18 of these 13 muscles/regions, the proportion of polymorphic fibers was actually greater than the proportion of fibers that expressed only one MHC isoform. Moreover, the IIX/IIB fibers represented the largest pool of any fiber type in the hindlimb musculature.

**Patterns of single-fiber MHC polymorphism.** The patterns of polymorphism were quite variable among the muscles/regions sampled. For instance, in the WMG, WTA, and WVL muscles there was only one type of polymorphic fiber (IIX/IIB), and it represented only a small proportion of the total population of fibers (3–14%). As expected, the remainder of the fibers in these muscles expressed only the fast type IIB MHC isoform. In contrast, muscles like the PLAN and RTA had 9–10 different types of polymorphic fibers. This is remarkable given that there are only 11 possible combinations of MHC isoform coexpression in muscles that express the four adult MHC isoforms commonly found in the hindlimb musculature. The most common polymorphic fiber type among the fast muscles sampled was the IIX/IIB fiber. These fibers were the dominant fiber type in every muscle that had relatively large pools of the fast type IIX and IIB MHC isoforms. Another consistent trend was that muscles containing significant amounts of I and IIA or I and IIX MHC isoforms exhibited substantial numbers of I/IIA and I/IIX fibers, respectively (e.g., DIA, VI, and RVL).

As noted, ~10–12% of the fibers in the DIA muscle coexpressed the slow type I and fast type IIX MHC isoforms (see Fig. 2A). The presence of such polymorphic fibers is not consistent with the sequential scheme of MHC isoform transitions. The DIA muscle was not unique in this respect. Specifically, the PLAN, RF, and RTA muscles also contained fibers that coexpressed specific combinations not consistent with the sequential scheme.

**Western blot and RT-PCR analyses of I/IIX fibers in the DIA.** As noted in METHODS, we were surprised to find such a high percentage of I/IIX fibers in the DIA muscle, especially because previous studies (17, 32) had not reported the presence of such fibers. Hence, we performed further analyses (both Western blots and RT-PCR) in an attempt to either confirm or reject their presence. As described in METHODS, the MHC isoform...
compositions of ~200 fibers from the DIA muscle were determined by electrophoresis, and 23 I/IIX fibers were identified. Subsequently, these fibers were analyzed with the use of a Western blot approach. An example of the Western blot results is shown in Fig. 2B. Note that the MAb used for the Western blot analyses was specific for all three of the fast MHC isoforms, and, as shown in Fig. 2B, only the fast type IIX MHC isoform was detected in a fiber initially identified as I/IIX fibers via electrophoresis. All 23 I/IIX fibers identified by electrophoresis were subsequently shown (by Western blotting) to express only the fast type IIX and not the fast type IIA MHC isoform.

The presence of polymorphic fibers (and specifically I/IIX fibers) in the DIA muscle was also confirmed using RT-PCR. For instance, Fig. 2C demonstrates the coexpression of the slow type I and fast type IIX MHC mRNAs.

Regional distribution of polymorphic fibers in the DIA muscle. In our initial sampling of the costal DIA muscle (experiments 1 and 2), we did not attempt to determine whether there was a regional difference in single-fiber MHC isoform distribution. We were interested in this issue, because previous studies (17, 32) had not reported the same patterns or degree of polymorphism as observed in the current study. Hence, to determine whether this might be due to differences in regional sampling, we conducted another experiment (i.e., experiment 3) whereby single fibers were isolated from the dorsal and ventral regions of the costal DIA. Three key observations evolved from this third experiment.

First, there appeared to be regional differences with respect to the proportion of the slow type I fibers, with the ventral region having a higher proportion (i.e., 30 vs. 10% of the total population; P < 0.001; see Fig. 3). Second, the dorsal region had a higher proportion of fast type IIX fibers (P < 0.001). Finally, both regions contained fibers that coexpressed the slow type I and fast type IIX MHC isoforms (i.e., I/IIX fibers). The dorsal region appeared to have a higher proportion of the I/IIX fibers (P < 0.05).

Breeder-dependent variations in DIA MHC isoform composition. Because Sprague-Dawley rats are an outbred strain and are bred for maximum heterozygocity, it is possible that differences in MHC isoform composition could be dependent on the source of animals (i.e., breeder). To examine this possibility with respect to the DIA muscle, we compared the single-fiber MHC isoform profiles of DIA muscles obtained from Bantin-Kingman (Figs. 1 and 3) and Harlan (Fig. 4). In contrasting the data shown in Figs. 3 and 4, it is clear that there can be significant differences (P < 0.001) between the single-fiber MHC isoform profiles of DIA muscles obtained from different vendors. This may partially or completely explain the differences between our initial findings (i.e., those shown in Figs. 1 and 3) and those of Sieck et al. (32). Interestingly, however, the observations made on the animals obtained from Harlan are consistent in the following ways with those made on Bantin-Kingman animals: 1) the DIA muscle of the female Sprague-Dawley rat contains I/IIX fibers, and 2) the DIA muscle of the female Sprague-Dawley rat contains I/IIX fibers, and 3) there are regional differences in the DIA muscle of the female Sprague-Dawley rat.

Functional consequences of MHC isoform composition. As noted in METHODS, we used Hill’s statistical model of the force-velocity relationship to provide some insights regarding the functional importance of single-fiber MHC polymorphism. The results of these analyses are shown in Fig. 5, A and B. There are several points to be made regarding the results of these analyses. First, as expected, the SOL muscle, with its high percentage of slow fibers, has the slowest predicted V_max. Second, most of the muscles in the rodent hindlimb are fast as evidenced by V_max values that fall within the range of 1.5–1.8 FL/s. Interestingly, even the so-called red regions of the TA, MG, and VL muscles are predicted to have V_max values that are only somewhat slower than those of the fast white regions. Finally, the VI and DIA muscles have V_max values that fall between the extremes as identified by the slow SOL muscle and the fast white regions of muscles like the MG.

DISCUSSION

In revisiting the question initially posed by Brooke and Kaiser (9) but in a contemporary context, single-
fiber MHC polymorphism appears to be a common occurrence among a large number of muscles/muscle regions in the rat. In some muscles, there can be different types of muscle fibers, and polymorphic fibers may collectively represent the majority of fibers. This relatively high degree of polymorphism raises important issues about the complexity of MHC gene expression, especially as it relates to individual myonuclei and the concept of an obligatory scheme of MHC isoform transition. The findings of this study also provide an interesting contrast between the diverse patterns of MHC polymorphism and the functional consequences of such polymorphism. Each of these issues is considered in more detail below (see Are muscle fibers obligated to follow the I\textarrowleft IIA\rightarrow IIX\rightarrow IIB scheme of plasticity? and What is the functional significance of MHC polymorphism?), but first we address important technical considerations underlying the analyses performed in this study.

**Important technical considerations.** Although the issue of MHC polymorphism has been examined in a large number of studies, few studies have tried to quantitatively assess the extent of MHC polymorphism across a broad spectrum of muscles. Rather, most studies examined a small set of muscles or the effects of various interventions on single-fiber MHC isoform distribution. Many of these studies employed immunohistochemical approaches. As noted by our laboratory previously (11), immunohistochemical approaches are limited in several key ways. First, they can only indicate the presence or absence of a given MHC isoform. In polymorphic fibers, such approaches cannot provide any information about the relative distribution of a given MHC isoform. Second, there are no antibodies currently available that specifically recognize the fast type IIX MHC isoform. In many instances, the identification of fast type IIX MHC isoform has been based on the absence of staining. This approach precludes the ability to identify fibers expressing the fast type IIX MHC isoform in combination with other MHC isoforms. Given these key considerations, we believe that single-fiber polymorphism can only be properly studied...
in the rat (as well as other species) with the use of single-fiber electrophoretic approaches similar to those used in the current study.

As noted above, many studies have examined single-fiber MHC isoform polymorphism. Few (if any) of these studies attempted to determine whether such sampling approaches yielded results that were representative of the whole muscle. This same comment also applies to a number of studies that have examined other properties of single fibers, such as $V_{\text{max}}$, stiffness, and calcium sensitivity. Theoretically, such single-fiber sampling procedures should provide reasonable estimates of the property of interest given that two criteria are satisfied: 1) fibers are chosen at random, and 2) a sufficient number of fibers are sampled. To determine whether such criteria were satisfied using approaches similar to those employed in this study, we correlated in four previous studies (11–13, 15) the MHC isoform composition of whole muscle predicted from single-fiber analyses with that actually determined by whole muscle analyses. Collectively, the results from these four studies showed that the coefficients of determination were typically $>0.90$, and the slopes of the single fiber vs. whole muscle relationship approximated 1.0 (i.e., 0.93). Hence, for any given muscle/region, we have a high degree of confidence that the single-fiber profiles shown in Figs. 1, 3, and 4 provide reasonable representations of the various types of fibers, their relative proportions, and the relative distributions of MHC isoforms found within a given pool of fibers.

In this context, several studies have examined the single-fiber MHC isoform distribution of the DIA muscle (17, 32). For instance, Sieck et al. (32) examined the single-fiber MHC isoform distribution of ~700 fibers from the costal region of the DIA and reported that ~85% of all the fibers in the DIA expressed only one MHC isoform. The majority (~12% of all fibers sampled) of the remaining fibers coexpressed the fast type IIX and IIB MHC isoforms. More recently, Bortolotto et al. (5) examined the single-fiber MHC isoform composition of 43 fibers from the DIA muscle of the Wistar-Kyoto strain of rats and observed that ~65% of the fibers expressed only the fast type IIX MHC isoform. Interestingly, Bortolotto et al. also reported the presence of I/IIX polymorphic fibers (~5% of the total pool of 43 fibers studied). In our initial sampling of the DIA muscle, we were struck by two findings: 1) the high degree of polymorphism and 2) the presence of I/IIX fibers.

Given the above, it should be noted that Sprague-Dawley rats were used in both the current study and the study of Sieck et al. (32). Why then is there such a clear discrepancy between the two studies with respect to the single-fiber distributions of MHC isoforms in the DIA muscle? There are two observations that might explain such disparate findings. First, Sieck et al. used adult male Sprague-Dawley rats, whereas we used adult female rats. Second, the breeders/vendors were different. With respect to this latter issue, Sprague-Dawley rats are an outbred strain, and it is entirely possible that there may be variations in the myosin phenotype that are breeder/vendor dependent. To examine this possibility, we performed a fourth experiment on the DIA muscle and obtained animals from Harlan. The distribution that was obtained from this group of animals (i.e., those obtained from Harlan) was quite different ($P < 0.001$; see Figs. 1, 3, and 4) from that of those purchased from Bantin-Kingman. Specifically, the DIA muscles of animals obtained from Harlan had a large proportion of IIX/IIB fibers, something not seen in the animals obtained from Bantin-Kingman. The disparity of single-fiber polymorphism certainly suggests that there can be significant differences in myosin phenotype that are breeder dependent and that comparisons of published studies may be difficult when contrasting studies that use different breeders.

To our knowledge, no previous studies have attempted to determine the functional significance of different patterns of MHC isoform expression at the single-fiber level and across a broad spectrum of muscles. From a logistical perspective, it would be difficult and impractical to actually make mechanical measurements on thousands of fibers. Hence, we modeled the force-velocity relationships of whole muscles/regions of muscles by employing 1) Hill’s statistical model of the force-velocity relationship, 2) the single-fiber data reported by Bottinelli et al. (8), and 3) the actual distributions of MHC isoforms observed at the single-fiber level. Two observations indicate that this represents a reasonable approach. First, the data shown in Fig. 5 predict that the $V_{\text{max}}$ of the PLAN is approximately twofold greater than that of the SOL muscle. This is consistent with actual data published by our laboratory previously (14). Second, in another publication (13), this modeling approach predicted a reduction of 11% in $V_{\text{max}}$, and this approximated the 14% decline actually observed at the whole muscle level. Hence, as a first approximation, this method seems reasonable for determining the functional importance of myosin isoform distributions at the single-fiber level, especially across a broad spectrum of muscles.

There are several important assumptions that should be acknowledged in the application of this model, however. The first is that $V_{\text{max}}$ is assumed to be dependent primarily on the MHC isoform composition and not on other sarcomeric proteins such as the myosin light chains (MLCs). Although this appears to be true for the slow type I, fast type IIA, and fast type IIX fibers, it should be noted that MLCs might influence the activity of the fast type IIB MHC isoform (7). This issue is minimized by simply using the mean value published by Bottinelli et al. (8). The second important consideration is that the $V_{\text{max}}$ fiber was assumed to be determined by the relative percentages of MHC isoforms within a given fiber and the $V_{\text{max}}$ associated with any given MHC isoform. This approach seems reasonable given the findings of Bottinelli et al. (6), who examined the $V_{\text{max}}$ of fibers expressing IIX, IIX/IIB, and IIB MHC isoforms. These investigators observed that the IIX/IIB fibers had $V_{\text{max}}$ values that were intermediate between fast type IIX and IIB fibers and...
dependent on the relative proportions of the fast type IIX and IIB MHC isoforms. Additionally, these findings and the approach taken in the present study are consistent with the cross-bridge model developed by Huxley (21).

With respect to measures of $V_{\text{max}}$, it should be noted that $V_{\text{max}}$ is determined by extrapolation of force-velocity data. As a result, it underestimates the maximal unloaded shortening velocity ($V_o$) determined by so-called slack test techniques. There are several reasons for this (10). The force-velocity relationship represents a composite of all of the individual force-velocity relationships. The importance of this is that at slow shortening velocities, all of the fibers contribute to the shape of the force-velocity relationship, whereas at higher shortening velocities, the slower fibers will be unable to generate any force and thus the shape of the force-velocity relationship in this region will be determined by only the fastest fibers (10). The net result is that at very high shortening velocities (corresponding to loading conditions $<1-2\% P_o$), the force-velocity relationship deviates from a hyperbola such that $V_{\text{max}}$ for the muscle will always be less than that predicted by the presence of the fastest fibers. In muscles where the muscle fibers are homogeneous with respect to contractile properties (e.g., WMG), there will be little difference between measures of $V_{\text{max}}$ and $V_o$. In contrast, the differences between $V_{\text{max}}$ and $V_o$ will be greater in muscles like the rat SOL, where $V_{\text{max}}$ is determined by the large predominance of slow fibers and $V_o$ is probably determined by the presence of the small population of fast fibers (14). Currently, it is not known whether measures of $V_o$ in whole muscle simply reflect that of the fastest fibers. This is unlikely in heterogeneous muscles, given the presence of mechanical linkages between adjacent muscle fibers and the potential for the slower fibers to retard the shortening velocity of the faster fibers.

Are muscle fibers obligated to follow the I→IIA→IIX→IIB scheme of plasticity? Over the course of the past 10–12 years, Pette and colleagues (1, 19, 22, 26–30, 33–35, 39, 40) published a number of original studies (both whole muscle and single fiber) and review articles and proposed that transitions in MHC isoforms generally follow a scheme that can be summarized as follows: I→IIA→IIX→IIB. The development of this scheme evolved from studying muscle fibers under normal steady-state and transitional conditions. However, as shown in Figs. 1–4, we observed pools of fibers in the current study that under normal conditions clearly do not inherently adhere to this scheme. For instance, in the DIA muscle, ~5–20% of the single fibers coexpressed the slow type I and fast type IIX MHC isoforms (see Figs. 1–4). There was also another pool of fibers (I/IIA/IIB) in the DIA muscle that also did not appear to adhere to this model. As shown in Fig. 1, the DIA muscle was not unique in this respect given that the EDL, RMG, MMG, PLAN, RF, RTA, VI, and RVL muscles/regions all contained small pools of fibers that were inconsistent with the scheme presented by Pette and coworkers (1, 19, 22, 26–30, 33–35, 39, 40).

Additionally, some perturbations (e.g., hyperthyroidism + mechanical unloading, spinal transection; neonatal development) have been shown to produce transitions in the patterns of MHC isoform expression that are also inconsistent with the obligatory sequential MHC transition scheme. For example, Caiozzo et al. (11) observed pools of I/II and IIX/IIB polyphasic fibers after 2 wk of a combined intervention of hyperthyroidism plus mechanical unloading. Importantly, time-course analyses demonstrated that the upregulation of the fast type IIA MHC isoform, in response to this intervention, occurred after the upregulation of the fast type IIX and IIB MHC isoforms (11). Findings from Talmage and colleagues (37, 38) reported the existence of I/II fibers after hindlimb suspension and spinal cord transection. With respect to spinal cord transection, Talmage et al. (38) observed large proportions (~15–25%) of I/II fibers 90 and 180 days after spinal cord transection. Di Maso et al. (15) examined transitions in single-fiber MHC isoform expression during neonatal development in eu- and hypothyroid rats. In the hypothyroid animals, the PLAN was observed to contain pools of fibers that coexpressed the combination of embryonic (E), neonatal (N), slow type I, and fast type IIB MHC isoforms. Further, E/N/IIX fibers were also observed in that study.

Collectively, segmental variations in MHC isoform expression (16, 30) and the presence of polyphasic fibers that do not adhere to the sequential scheme (both under steady-state and transitional conditions) provide important pieces of evidence demonstrating that the genetic regulation of MHC isoform composition is sufficiently complex that it may not be simply described by the I→IIA→IIX→IIB model. This means that all fiber transitions do not adhere to this scheme? On the basis of the single-fiber studies to date (including our own), certainly an argument can be made that many fibers appear to follow such a scheme. However, a definitive answer awaits analyses of the genotypic expression of individual myonuclei.

What is the functional significance of MHC polymorphism? The presence of a large proportion of polyphasic fibers in the hindlimb muscles of the rat raises a number of interesting issues related to muscle function and motor unit recruitment. From a functional perspective, the monomorphic pattern of MHC isoform expression might lead to the greatest changes in contractile velocities when 1) recruiting different types of motor units (e.g., slow to fast fatigable) and 2) a fiber undergoes a transition in MHC isoform expression (e.g., slow type I to fast type IIA MHC). In contrast, the polyphasic model potentially might 1) provide smoother transitions in contractile velocities when recruiting additional motor units and 2) minimize the functional significance of MHC isoform transitions. A good example of this latter phenomenon was observed in a study that combined the interventions of hypothyroidism and mechanical overload (13). In that study, it
was observed that the combined intervention upregulated the slow type I MHC isoform content from ~5 to 20% of the total MHC pool. Single-fiber electrophoretic analyses demonstrated that ~65–70% of the fibers expressed the slow type I MHC isoform. Importantly, however, the upregulation of the slow type I MHC isoform was distributed primarily across three different pools of polymorphic fibers, where the fast MHC isoforms represented the majority of the total MHC content. This pattern of distribution (as opposed to a monomorphic model) is hypothesized to minimize the functional consequences of MHC isoform transitions by maintaining the ability of the muscle to produce work and mechanical power (10).

Whether by design or coincidence, polymorphism in rodent skeletal muscle provides a unique design that results not just in four types of cellular motors (i.e., muscle fibers) but has the potential for producing a spectrum of cellular motors that is defined by the types of MHC isoforms and their relative distributions within a given fiber. In this context, one issue that probably will never be resolved is why more than two types of MHC genes evolved. Conceptually, muscles could simply produce a broad spectrum of function by varying the proportions of the slow type I and fast type IIB MHC isoforms within individual fibers. Perhaps comparative approaches, employing cladistical analyses, might be helpful in resolving this issue.

The application of Hill’s statistical model, as used in this study, illustrates intrinsic differences in the force-velocity relationship that are independent of other factors such as architectural properties (e.g., cross-sectional area and fiber length). However, as noted by Lieber and Friden (24), factors such as fiber length may be as important as (if not more so than) MHC isoform composition in determining differences in shortening velocities between muscles.

Perspectives

Mosaicism of skeletal muscle: polymorphism raises important issues about the MHC expression patterns of individual myonuclei. With the advent of histochecmical methods, it was found that most muscles contained fibers that differed in their staining intensities. This variation in staining patterns was described by some as a “mosaic” pattern. For many years, it was assumed that each individual muscle fiber only expressed a single MHC isoform. The single-fiber MHC polymorphism reported herein and by previous investigations across a broad spectrum of species including humans (2, 10–13, 15, 16, 19, 27, 29–31, 33–35, 39, 40, 42) represents another level of mosaicism (one that exists at the protein level) that further complicates the concept of a fiber type. Within this context, a fundamental issue that remains unresolved is how MHC polymorphism is regulated at the myonuclear level. Are there differences in the expression patterns of individual myonuclei that represent an even more complex level of mosaicism? The presence of hundreds to thousands of nuclei per muscle fiber gives rise to several interesting possibilities. For instance, all of the myonuclei may have identical genotypic expression patterns. If this is the case, then polymorphism must be the consequence of multiple MHC gene expression within a given myonucleus. Alternatively, gene expression might differ from one myonucleus to another. Consistent with this latter suggestion, Peucker and Pette (30) reported a segmental difference in MHC mRNA isoforms along the length of IIX/IIB fibers from the rabbit gastrocnemius muscle. Edman et al. (16) also observed segmental variations in MHC isoform expression and ATPase activity in frog skeletal muscle fibers. Such segmental differences support the hypothesis that there are differences in the expression programs of individual myonuclei. In accordance with this suggestion, Hall and Ralston (18) discussed the concept of the “mosaic of domains.” This term implies that individual myonuclei may express protein products that differ from those of other myonuclei within the same muscle fiber. As Hall and Ralston noted, a good example of this is the expression of acetylcholine receptors by the myonuclei associated with the motor end-plate. If it is indeed found that the genotypic expression of myonuclei differs along the length of a fiber, then this will significantly complicate our attempts to understand the roles of neural, hormonal, and mechanical factors in regulating both the genotype and phenotype of skeletal muscle.

DISCLOSURES

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REFERENCES


