Histochemical, biochemical, and contractile properties of red, white, and intermediate fibers

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Recent results from histochemical (7, 8, 11, 12, 15, 18) and electron microscopic (10) studies suggest the existence of three fiber types in mammalian skeletal muscle. Although different classifications have been used, many investigators now classify fibers as "red," "white," and "intermediate." Muscles which appear red are thought to be slow contracting, whereas white muscles are fast contracting. Since muscles composed of histochemically red and intermediate fibers are both red in appearance, much confusion has arisen concerning the interrelationships among contraction times, fiber type, and general appearance of skeletal muscle. Further confusion arises from the fact that most red muscles are composed of a mixture of histochemically red and intermediate fibers in addition to some white fibers. The importance of this point has been overlooked because it is generally assumed that intermediate fibers are simply variants of red fibers. The soleus, a slow-twitch muscle, is generally assumed that intermediate fibers are simply variants of red fibers. The soleus, a slow-twitch muscle, is composed predominately of intermediate fibers in the rat and solely of intermediate fibers in the guinea pig (8). Therefore, it has been suggested that the intermediate fiber has a slow contraction time, whereas both the red and white fibers have fast contraction times (8, 11). Bárány and co-workers (1, 2) reported that the specific activity of myosin ATPase is correlated with the contraction time of muscle, and Guth and Samaha (11) demonstrated that actomyosin ATPase measured biochemically is correlated with the histochemical myofibrillar ATPase.

The present investigation was therefore undertaken to study the relationships between histochemical properties, actomyosin, and myosin ATPase activity, and the isometric contractile properties of mammalian skeletal muscle.

METHODS

Adult (>700 g) male Hartley guinea pigs were used. The muscles selected for study were the soleus, flexor hallucis longus (FHL), flexor digitorum longus (FDL), medial gastrocnemius (MG), and the portion of the vastus lateralis (RV), which is grossly red (RV).

Histochemistry. The muscles used for histochemical analysis were rapidly removed from the animals and trimmed free of fat and connective tissue. The belly of each muscle was ablated and placed on a mounting chuck for quick freezing with liquid nitrogen. Serial sections (6 μ) were then cut from each muscle and placed on cover glasses for staining. NADH-diaphorase (NADH-D) activity was determined by the method of Novikoff et al. (14) and myosin ATPase activity by the method of Padykula and Herman (16) and a modification of Guth and Samaha (11) method. Photographs were taken from each slide and enlarged to permit classification of individual fibers. Cross sections of the muscles were analyzed from more than 50 soleus muscles, 4 FHL, 4 FDL, 10 vastus lateralis (red portion), and 13 MG. Individual fibers were classified as red, white, or intermediate. Intermediate fibers are characterized by their light staining with myosin ATPase and their distinct pattern of small, uniformly dispersed diimormazan granules in the NADH-diaphorase reaction. Both red and white fibers are dark staining with myosin ATPase. The division into red and white is subjectively made from the NADH-D reaction. Red fibers are characterized by an abundance of coarse diimormazan granules which are most concentrated in the subsarcolemmal region. White fibers are characterized by few diimormazan granules which are also most prominent in the subsarcolemmal region. These histochemical characteristics are shown in Fig. 1, A and B. Further details concerning the histochemical techniques and methods of fiber classification have been published elsewhere (8).

Biochemistry. Actomyosin and myosin ATPase activities were determined at pH 6.8 and 9.4. At least three separate
determinations were done for each muscle. Actomyosin was prepared from approximately 1 g of muscle. The muscle was homogenized in a Polytron homogenizer (Brinkmann Instruments, Santa Monica, Calif.). Natural actomyosin was extracted in 0.6 M KCl and sedimented in 0.05 M KCl (pH 6.8) as described previously (9). Adenosine triphosphatase activity determined in the presence of Tris-maleate buffer (0.02 M), MgSO₄ (1.0 mM), KCl (0.03 M), CaCl₂ (0.02 mM), ATP (1.0 mM), and actomyosin (0.1 mg/ml) was expressed as actomyosin ATPase activity. Adenosine triphosphatase activity measured in the presence of Tris-maleate buffer (0.02 M), KCl (0.6 M), CaCl₂ (0.01 M), ATP (1.0 mM) and actomyosin (0.1 mg/ml) was expressed as myosin ATPase. Determinations at 25°C were done at pH 6.8 and 9.4. The reaction was started by the addition of ATP and terminated after 20 min by the addition of trichloroacetic acid. Protein concentration was measured by the Lowry method (13) and inorganic phosphate was measured by the method of Rockstein and Herron (17). Both ATPase activities were expressed per milligram of natural actomyosin protein.

**Contractile properties.** The guinea pigs were anesthetized with pentobarbital (40 mg/kg ip) and the skin was removed from the right hindleg. The superficial muscles were removed from the lower leg, and the muscles to be tested were each isolated, with caution being taken not to disrupt the blood and nerve supply. The distal tendon of the muscle was then fastened to an isometric transducer as described previously (3). Measurements were obtained from the red vastus by separating the red and white portions and removing the red portion from the femur up to its origin on the greater tuberosity. This muscle inserts directly into the patella with a very short tendon; thus, the patella was removed from the tibia and used to fasten the muscle to the isometric transducer. The preparations were continuously moistened with mammalian Ringer solution (37°C) and kept warm (36-38°C) by a lamp. When soleus muscles were tested the underlying muscles were denervated to eliminate their influence on the contraction time of the soleus. The muscles were stimulated via their nerves with a Grass stimulator (Grass Instruments, Quincy, Mass.) by monophasic pulses of 0.2 msec duration. After the surface temperature had reached 37°C the muscle was stretched to a position which produced the maximum isometric twitch tension. A check was then made to assure that the voltage was supramaximal. Several individual twitches were recorded from each muscle. All measurements were recorded on a Texas Instruments Oscillo/riter recorder (Texas Instruments Inc., Houston, Texas). Measurements were made on at least five of each of the different muscles.

**RESULTS**

**Histochemistry.** The existence of three different histochemical types of fibers as demonstrated with NADH-D in guinea pig skeletal muscle is shown in Fig. 1A. Sections taken from the soleus (Fig. 2, A and B) show moderate staining with NADH-diaphorase and light staining with myosin ATPase, demonstrating that this muscle is composed of only intermediate fibers. Analysis of cross sections of four FHL muscles shows that this muscle is composed of 73% white, 10% red, and 17% intermediate fibers (Table 1). Figure 3, A and B, shows the high percentage of white fibers and the low percentage of both red and intermediate fibers in the FHL. Histochemical studies of the FDL (Table 1; Fig. 4, A and B) show that it also consists predominately of white fibers (54%) with only 35% red and 11% intermediate fibers. Conversely the red portion of the vastus (Fig. 5, A and B) is composed of 78% red and only 18% and 4% white and intermediate fibers, respectively. The cross section from the MG contained 51% red fibers, 30% white fibers, and 12% intermediate fibers. The peripheral area of this muscle consists of a higher proportion of white fibers than does the central area (Fig. 6, A and B). Most of the intermediate fibers are also located in the central red region of the medial gastrocnemius.

**Contractile properties.** The twitch characteristics of the various muscles are given in Table 1. The time-to-peak tension (82.3 ± 1.3 msec) and half-relaxation time (113.0 ± 1.9 msec) recorded for the soleus muscles demonstrate that this is indeed a slow-twitch muscle when compared to the times-to-peak tension and half-relaxation times for the FHL, FDL, RV, and MG, which were all in the neighborhood of 20 msec.

**Actomyosin and myosin ATPase activity.** The specific activities of actomyosin and myosin ATPase at pH 6.8 and 9.4 are given in Table 1. The myosin ATPase activity for the soleus (pH 6.8) was 0.12 ± 0.02 μmoles Pi/min per mg. The mean values for the other four muscles ranged from 0.12 ± 0.05 μmoles Pi/min per mg protein for the MG to 0.23 ± 0.02 μmoles Pi/min per mg protein for the red vastus. The actomyosin ATPase activity showed a similar relationship, i.e., the ATPase of actomyosin from the soleus was 0.05 ± 0.01 μmoles Pi/min per mg, and the values for the other four muscles ranged from 0.12 ± 0.03 μmoles Pi/min per mg for the FDL to 0.19 ± 0.02 μmoles Pi/min per mg for the FHL.

At pH 9.4 the myosin ATPase activity for the soleus increased to 0.14 ± 0.01 μmoles Pi/min per mg. However, the activity in the other four muscles all showed increases with values ranging from 0.29 ± 0.02 μmoles Pi/min per mg for the FDL to 0.34 ± 0.02 μmoles Pi/min per mg for the RV.

**DISCUSSION**

Several investigators (7, 8, 11, 12, 13, 18) have used histochemical techniques to identify three different fiber types. However, a careful survey of the techniques used by different authors reveals inconsistencies in the identification of different fiber types (8). In 1962 Stein and Padykula (18) described three fiber types in which type A was the classical white fiber and types B and C were thought to be two different types of red fibers. The classification was made primarily on the intensity of succinate dehydrogenase staining. Their paper also contained data showing that unfixed ATPase activity was heavy in both the A and C fibers but light in the B fibers. However, this point was not emphasized as being useful in the classification of fibers. Other investigators (6) have more recently divided fibers into red, white, and intermediate according to the intensity of staining produced by various mitochondrial enzymes.
See facing page for legend.
TABLE 1. **Guinea pig skeletal muscle characteristics**

<table>
<thead>
<tr>
<th>Fiber types, %</th>
<th>Soleus</th>
<th>FHL</th>
<th>FDL</th>
<th>MG</th>
<th>RV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>0</td>
<td>9.7</td>
<td>35.5</td>
<td>50.2</td>
<td>78.2</td>
</tr>
<tr>
<td>White</td>
<td>0</td>
<td>72.8</td>
<td>33.8</td>
<td>38.6</td>
<td>17.7</td>
</tr>
<tr>
<td>Intermediate</td>
<td>100</td>
<td>17.3</td>
<td>10.7</td>
<td>11.7</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Values are means ± SE.

In 1968 Edgerton (7) suggested that the division of fibers into red, white, and intermediate according to the intensity of mitochondrial staining may sometimes be difficult and may sometimes have to be arbitrary when only stains for mitochondrial enzymes are used. He suggested that there may be a continuous spectrum of fibers containing various amounts of mitochondria. However, he also observed that in serial sections from rat plantaris and gastrocnemius muscles all of the fibers classified as red or white by mitochondrial enzyme activity stained dark with myosin ATPase, whereas some of the intermediate (moderate mitochondrial enzyme activity) fibers were dark but most of them were light with the ATPase reaction. In the rat soleus, a slow contracting muscle, most of the fibers (approximately 80%) were classified as intermediate; the remaining 20% were classified as red according to mitochondrial enzyme staining. However, all of the intermediate fibers in the soleus stained light with myosin ATPase, whereas the red fibers were dark staining.

Further investigations by Edgerton and Simpson (8) have clarified the histochemical identification of a specific fiber which they have called the intermediate fiber. This is the light-staining myosin ATPase fiber which was described previously by Edgerton (7) and subsequently by Guth and Samaha (11). Not only does this fiber type stain lightly with myosin ATPase but the pattern of mitochondrial staining is also distinct. Under high power magnification the intermediate fiber is easily distinguished as can be seen in Fig. 1A.

Because the soleus has a slow contraction time and is composed predominantly of intermediate fibers, some investigators have suggested that it is the intermediate fiber which is slow contracting (8, 11). More recently Guth and Samaha (11) also reported that the actomyosin ATPase activity (both histochemical and biochemical) was low in the intermediate fiber and high in both the red and white fibers. Their data are consistent with the proposal that the intermediate fiber has a slow contraction time, and they presumed that both white and red fibers are fast contracting.

The data obtained in the present study clearly demonstrate that in skeletal muscle of guinea pigs the intermediate fiber is the slow twitch fiber, whereas red and white fibers are both fast twitch fibers. Although the time-to-peak tension is related to the active state duration as well as the velocity of shortening, Close (5) has reported that the differences in twitch times may be attributed to differences in the intrinsically slow fibers. Hence, the data presented in Table 1 may reflect different intrinsic speeds of shortening of red, white, and intermediate fibers.

Muscles composed predominantly of white or of red fibers have fast contraction times, whereas the soleus which is composed of intermediate fibers has a slow contraction time. Since the soleus and red vastus are both red in appearance, the generalization that red muscles have slow contraction times is not valid. Examples of fast contracting red muscles have also been reported in other species, e.g., the thyroarytenoid of the rabbit (8).

Our data on the specific activity of myosin ATPase confirm the reports of Bárány et al. (1, 2) that Ca^{2+}-activated myosin ATPase is reciprocally related to contraction time. At pH 6.8 the specific activity of myosin ATPase for the soleus was only 0.12 ± 0.02 μmoles Pi/min per mg, whereas values ranging from 0.21 ± 0.03 to 0.23 ± 0.02 μmoles Pi/min per mg were recorded for the fast twitch muscles. The highest myosin ATPase activity at pH 6.8 was recorded for the RV which also had the fastest time-to-peak tension. Hence, the high and uniform activity of myosin ATPase of fast muscles (at both pH 6.8 and 9.4) accurately reflects the contraction time and does not distinguish muscles which contain varying proportions of red and white fibers. On the other hand, the ATPase activity of natural actomyosin at pH 6.8 or 9.4 does not correlate consistently with the contraction times of the muscles studied (Table 1) except that the activity in the soleus was much lower than in the four fast muscles. Considering the reproducibility of the data on times-to-peak tension and ATPase activities (very small se), it is reasonable to state that myosin, not actomyosin ATPase activity, is best correlated with the contraction...
times. In addition the data of Table 1 suggest that the AT\textsubscript{P}asc of natural actomyosin extracted from red fibers is enhanced less by alkali than that of white fibers.

From the data obtained in this study, one might conclude that kinetically there are only two populations of fiber types, i.e., fast and slow twitch. Fast-twitch fibers might further be classified as red or white according to the mitochondrial content and other features described by Gauthier (10). Studies comparing the yield and biochemical characteristics of sarcotubular vesicles (fragmented sarcoplasmic reticulum) also support the view that kinetically there are two populations of fiber types. Vesicles prepared from red and white fibered muscles show identical characteristics which correlate with the half relaxation times. Vesicles from the soleus show a slower rate of Ca\textsuperscript{2+} accumulation, which corresponds to the slower relaxation time (8a).

Although skeletal muscle fibers can easily be divided into slow or fast twitch according to their histochemical myosin AT\textsubscript{P}asc activity, Yellin (19) has reported that muscle fibers in the eye contain a mixture of filaments with both ATPase activity, and white fibered muscles show identical characteristics of sarcotubular vesicles (fragmented sarcoplasmic reticulum) also support the view that kinetically there are two populations of fiber types. Vesicles prepared from red and white fibered muscles show identical characteristics which correlate with the half relaxation times. Vesicles from the soleus show a slower rate of Ca\textsuperscript{2+} accumulation, which corresponds to the slower relaxation time (8a).

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We would emphasize that conclusions concerning the isometric contraction kinetics in individual fiber types are derived from data on whole muscles, only one of which is homogeneous by histochemical techniques. Variability in the contraction times of individual fibers of one morphological type is not precluded. Indeed, other investigators have reported ranges of contraction times for individual motor units (4, 6, 12). These ranges, however, may be a reflection of different elastic components associated with different size motor units. Burke (1) has studied individual motor units in the medial gastrocnemius of the cat (a mixed muscle) and has suggested that there are three types of motor units. It is not known if his motor units are composed of our specific fiber types; however, his data suggest that this is the case. Henneman and Olson (12) have also described three fiber types in the medial gastrocnemius of the cat. Their classification was based on histochemical analysis of mitochondrial AT\textsubscript{P}asc and individual fiber diameters. However, their intermediate fiber ("intermediate in size and AT\textsubscript{P}asc activity are B fibers with peripheral accumulations of mitochondria") appears to fit the description of our red fibers with regard to the peripheral accumulation of diformazan granules. It has also been our experience that the relative size of our red and intermediate fibers is not consistent from one muscle to another. This is just one of the many examples of confusion which may result when mitochondrial enzyme staining is employed as the sole basis for the histochemical classification of skeletal muscle fibers.

In view of these findings we suggest that fibers be classified as fast-twitch red, fast-twitch white, and slow-twitch intermediate. Although the biochemical and contractile data reported in the present study have been obtained from only guinea pig skeletal muscle, we have found that the histochemical classification of red, white, and intermediate fibers can be demonstrated in other laboratory animals including the rat, cat, and mouse. We recommend that future biochemical studies with skeletal muscle should confirm the types of fibers present in the muscles under investigation to avoid much of the confusion which has existed in the past due to the generalizations about visually red or white muscle.

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