USE OF TYPE-SPECIFIC ANTIMYOSINS TO DEMONSTRATE THE TRANSFORMATION OF INDIVIDUAL FIBERS IN CHRONICALLY STIMULATED RABBIT FAST MUSCLES

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ABSTRACT

Continuous stimulation of a rabbit fast muscle at 10 Hz changes its physiological and biochemical parameters to those of a slow muscle. These transformations include the replacement of myosin of one type by myosin of another type. Two hypotheses could explain the cellular basis of these changes. First, if fibers were permanently programmed to be fast or slow, but not both, a change from one muscle type to another would involve atrophy of one fiber type accompanied by de novo appearance of the other type. Alternatively, preexisting muscle fibers could be changing from the expression of one set of genes to the expression of another. Fluorescein-labeled antibodies against fast (AF) and slow (AS) muscle myosins of rabbits have been prepared by procedures originally applied to chicken muscle. In the unstimulated fast peroneus longus muscle, most fibers stained only with AF; a small percentage stained only with AS; and no fibers stained with both antibodies. In stimulated muscles, most fibers stained with both AF and AS; with increasing time of stimulation, there was a progressive decrease in staining intensity with AF and a progressive increase in staining intensity with AS within the same fibers. These results are consistent with a theory that individual preexisting muscle fibers can actually switch from the synthesis of fast myosin to the synthesis of slow myosin.

KEY WORDS myosin • chronic stimulation • fast muscle • fiber types • antimyosin antibodies

A typical fast or slow muscle can be transformed into the opposite muscle type by cross reinnervation (7, 10, 19, 34, 37) or, in the case of a fast muscle, by chronic stimulation of the intact nerve at a rate corresponding to the frequency of stimuli to a slow muscle (10 Hz = 10 impulses per second) (28, 29, 33). These transformations change not only the physiological parameters of the muscle, such as the speed of contraction, half time of relaxation, twitch and tetanus tensions, and resistance to fatigue, but also the biochemical and ultrastructural properties. For example, switches occur in the type of myosin and troponin found in slow and fast muscles, in the enzymes of...
energy metabolism characteristic of the muscle, in the Ca\(^{2+}\) transporting activity of the sarcoplasmic reticulum, and in the Z-disc thickness (1, 25, 27, 32–35, 37).

Within a given muscle, certain fiber types can be distinguished, chiefly on the basis of the alkali-stable myofibrillar adenosine triphosphatase (ATPase) reaction and the relative proportions of oxidative or glycolytic enzymes (11). Type I fibers, which have a low, alkali-labile ATPase activity, also have high oxidative and low glycolytic enzyme activities; among the type II fibers, which have a high, alkali-stable ATPase activity, two subtypes with high and low oxidative enzyme activities can be distinguished. Cross reinnervation produces a complete type II → type I or a partial type I → type II change, depending on whether a fast → slow or slow → fast nerve switch has taken place (7). When the intact nerve to a fast muscle is chronically stimulated at 10 Hz, a complete transformation of type II → type I fibers occurs (28, 29, 34).

The changes in myosin types produced by cross reinnervation and chronic stimulation have been extensively studied. The myosin of a slow muscle changed into a fast one exhibits the high, alkali-stable ATPase activity and the fast myosin light chains found in normal fast muscles; conversely, the myosin from a fast muscle transformed into a slow one has the low, alkali-labile ATPase activity and the slow myosin light chains found in normal slow muscles (30, 32–34, 37). It should be noted that the extent of the changes in ATPase activity or light chain pattern depends on the animal species and the type of intervention (i.e., cross reinnervation or chronic stimulation).

Interest in the mechanism of these transformations is heightened by the occurrence of similar changes in metabolic, biochemical, and physiological properties of embryonic muscles during normal maturation (3, 6, 9, 20, 26, 31).

In all the situations described, the cellular basis for the transformation of muscles from one type to another is uncertain. Two mechanisms have been discussed (13, 19, 33). According to one, if the fibers were permanently programmed to be fast or slow, a change from one muscle type to another would imply atrophy of one fiber type accompanied by de novo formation of the other. Alternatively, preexisting muscle fibers themselves could switch from the expression of one set of genes to the expression of another.

Some circumstantial evidence favors the latter view. In earlier studies of the effects of chronic stimulation, no signs of selective atrophy were noted, and the changes in the various parameters showed a different time course: changes in the activity of the metabolic enzymes and sarcoplasmic reticulum Ca\(^{2+}\) uptake occurred well before the histochemical or in vitro myosin ATPase activity or the light chain pattern (24, 25, 35). This lack of coordination suggests a sequential transformation of preexisting fibers. This interpretation is also supported by the histochemical examination of partially transformed muscles which reveal fibers with a wide range of staining characteristics intermediate between type I and type II (24, 25). This evidence, however, is indirect. Because of the importance of resolving this question, we have looked for direct evidence bearing on the mechanism underlying muscle transformations.

Since earlier work suggested that individual fibers in normal muscles may contain only fast or slow myosin (2, 36), we used fluorescein-labeled antibodies specific for fast or slow type myosins to examine the transformation of individual fibers in chronically stimulated fast muscles. The findings reported here show that (a) in normal muscles, each fiber does contain only fast myosin or slow myosin, but not both; and (b) during chronic stimulation of a fast muscle, slow myosin begins to appear in fibers already containing fast myosin and eventually totally replaces that fast myosin. This supports the theory that, during chronic stimulation, preexisting fast fibers themselves actually change to the opposite fiber type.

MATERIALS AND METHODS

**Preparation of myosin**

Fast twitch muscle myosin was prepared from the adductor magnus and the superficial portion of the vastus lateralis muscles of the rabbit, and slow twitch muscle myosin was isolated from the soleus, semitendinosus, and intertransversarius muscles (33). The myosin was further purified by repeated chromatography on diethylaminoethyl Sephadex A50 to remove C protein and other contaminants. The purity and light chain pattern of myosin were examined by electrophoresis on sodium dodecyl sulfate polyacrylamide gels as previously described (33).

**Stimulation of Muscles**

Continuous stimulation of the rabbit left common peroneal nerve, which supplies the tibialis anterior, extensor digitorum longus, and peroneal muscles, was
performed as described earlier (28, 33). After stimulation at 10 Hz for various periods of time, the animals were killed and both the stimulated muscles and their contralateral unstimulated counterparts were removed; the soleus muscles were taken as representative of slow twitch muscles. Frozen serial sections (8 μm) were prepared in a cryostat for histochemical enzyme assays as well as for reaction with fluorescent antibodies (see below). Histochemical assays were performed for ATPase at pH 9.5 and 4.3 (4), α-glycerophosphate dehydrogenase using menadione (5), and NADH-tetrazolium reductase (12, 14).

Preparation of Specific Antimyosins

Antibodies against rabbit fast and slow myosins were elicited in goats essentially according to Arndt and Pepe (2). Immunoglobulin G (IgG) was precipitated from serum by the addition of solid ammonium sulfate to 50% and was stored in a solution containing 0.1 M KCl, 1 mM MgCl₂, 10 mM phosphate, pH 7.0 (standard salt) at −20°C. IgG was coupled to fluorescein isothiocyanate (FITC) by the method of Fothergill (15) using 3 mg of FITC/20 mg of protein. Unbound FITC was removed by gel filtration through Sephadex G25, followed by exhaustive dialysis against standard salt.

To assure type specificity of the antimyosins, each antimyosin fraction (anti-fast or anti-slow) was absorbed with the myosin of the opposite type (slow or fast) coupled to p-aminobenzylcellulose, as described by Pepe (21) and Arndt and Pepe (2).

Immunodiffusion

Immunodiffusion was performed on microfilms manufactured by Sebia (Issy Les Moulineaux, France). Films were hydrated in deionized water for 1 h and then equilibrated with a solution containing 0.4 M KCl, 0.03 M phosphate, pH 7.3. Antibodies and myosins were dialyzed against this same solution. ~15 μl of solution containing 30 μg of myosin were loaded into each well.

After diffusion for 48 h in a moist chamber at 4°C, the films were washed for 24 h with 0.4 M KCl, 0.03 M phosphate, pH 7.3, stained for 2 h in 1% thiazine red, and destained in 1% acetic acid. Destained films were air-dried and used as negatives for photographic prints.

Staining of Frozen Sections with Fluorescent Antibodies

After frozen sections of muscle were air dried onto cover slips, a drop of normal goat IgG in a 1:3 mixture of glycerine:standard salt was placed on each section in a moist chamber at 4°C. After 3 h, the normal IgG was replaced by a drop of specific antimyosin, also in glycerine:standard salt (1:3), for 24 h, washed for 24 h in glycerine:standard salt (1:3), and examined with a Zeiss Photomicroscope II, using epifluorescence illumination.

RESULTS

Specificity of Antibodies

The absorbed antibody fractions show specificity for the original antigens in double-diffusion experiments. The specific anti-fast myosin antibody (AF) reacts only with fast muscle myosin, while the specific anti-slow myosin antibody (AS) precipitates only slow muscle myosin (Fig. 1). This specificity is observed over a wide range of antibody and antigen concentrations. The experiments show the myosin type specificity of AS and AF, and also demonstrate that the original antigens contained only fast or only slow myosin.

To check whether AF and AS react only with the appropriate myosin and not with any other muscle proteins, AS and AF were allowed to diffuse against high salt extracts of fast and slow muscles. In each case, only one precipitin line was seen, and this line showed identity with the line

Figure 1  Double diffusion of specific anti-fast and anti-slow myosin antibodies against fast and slow myosins. (a) AF was placed in the central well and 15 μl of a solution containing 3 mg/ml of fast (f) or slow (s) myosin was placed in each peripheral well. AF reacts only with fast muscle myosin. (b) AS was placed in the central well and 15 μl of a solution containing 1 mg/ml (top well on each side) or 2 mg/ml (remaining wells) of fast or slow myosin was placed in each peripheral well. AS reacts only with slow myosin.
produced by diffusion of the specific antibody against the appropriate purified myosin (not shown).

**Staining of Normal Muscle Fibers**

The specific anti-fast and anti-slow myosin antibodies were applied to frozen sections of normal rabbit fast and slow muscles. As expected in a fast muscle such as the peroneus longus, most fibers react with AF (Fig. 2a). Only two of the fibers in Fig. 2a are unstained; in a serial section exposed to AS (Fig. 2b), these are the only ones that show reaction with the antibody. Examination of a large number of serial sections of normal fast and slow muscles failed to reveal any fibers that reacted with both antibodies; thus, in normal muscles each fiber contains either the fast or slow type myosin, but not both.

Serial sections incubated for conventional ATPase and metabolic enzyme activities enabled us to examine the antibody staining characteristics of fibers of identified type. Fibers staining with AF exhibit the alkali-stable ATPase characteristic of fast myosin (Fig. 2c), whereas fibers which stained with AS show the acid-stable ATPase activity characteristic of slow fibers (Fig. 2d).

Histochemical tests for α-glycerophosphate dehydrogenase with menadione and NADH-tetrazolium reductase demonstrate that the majority of fibers that stain with AF and have an alkali-stable ATPase also possess a strong glycolytic and a weak oxidative metabolic system (Fig. 2e). One of the fast fibers, however, contains high oxidative and low glycolytic enzyme activities and presumably corresponds to the fast-oxidative fiber type described by Burke et al. (8). All the fibers staining with AS and possessing acid-stable ATPase activity exhibit the high oxidative, low glycolytic activity pattern.

**Staining of Chronically Stimulated Muscles**

The fact that each fiber normally contains only fast or slow myosin makes it possible to determine whether the muscle transformation elicited by chronic stimulation is due to replacement of fibers or to alterations within preexisting fibers. For example, if each fiber were permanently committed to the synthesis of only one myosin type, transformation of a fast muscle to a slow muscle during chronic stimulation would have to result from some combination of atrophy and degeneration of fast fibers accompanied by de novo formation of slow fibers. Regardless of the stage of transformation of the muscle, each fiber would still stain with AF or AS, but not with both. Alternatively, if transformation involves a change in gene expression within preexisting fibers, single fibers staining with both AF and AS should be found at some time during the transition from fast to slow muscle.

Fig. 3 demonstrates the progression of the myosin light chain pattern from a muscle containing three fast light chains to one characterized by three slow chains with increasing periods of stimulation of the fast tibialis anterior muscle. At corresponding stages of stimulation, sections of peroneus longus muscles were treated with AF or AS to follow the process of transformation in single fibers. After 3 wk of stimulation, most fibers still stain with AF, while a small number are unstained (Fig. 4a). All fibers are smaller in diameter, but there is no selective atrophy of only one fiber type. Comparison of serial sections stained with AS (Fig. 4b) shows that all fibers—both those that stain with AF and those that do not stain with AF—now stain with AS. At this stage, the light chain pattern shows the presence of both fast and slow subunits (Fig. 3).

After 4.5 wk of stimulation, most fibers still stain with both AF and AS (Fig. 5a and b), although the intensity of the staining with AS has increased. By this time, however, the metabolic change from a predominantly glycolytic to a predominantly oxidative type muscle is almost complete (Fig. 5e and f). This agrees with a previous observation that the metabolic changes precede the changes in contractile proteins (25). ATPase activity after acid preincubation shows an increase in most fibers, whereas after alkali preincubation a decrease in activity is seen (Fig. 5c and d). It should be noted that four large fibers that did not stain with AF do show a small amount of alkali-stable ATPase activity; this phenomenon occurs in all our stimulated muscles and has been seen in a previous study (25).

At these intermediate stages of transformation (Figs. 4 and 5) two types of fibers are apparent: (a) those that stain only with AS and were probably slow fibers before stimulation began and (b) those that stain with both AS and AF and must be fast fibers in the process of transforming into slow fibers.

By 8 wk of stimulation, all the fibers stain uniformly with AS, but fail to react with AF (Fig.
Figure 2  Serial sections of an unstimulated rabbit fast muscle (m. peroneus longus). After exposure to normal, non-immune, nonfluoresceinated goat IgG, sections were stained with AF (a) or AS (b). Only the cells failing to react with AF stain with AS. ATPase activity after alkali (c) or acid (d) preincubation reveals that fibers staining with AF have an alkali-stable ATPase, whereas those reacting with AS have an acid-stable ATPase. Histochemical staining for α-glycerophosphate dehydrogenase using menadione (e) and for NADH-tetrazolium reductase (f) shows that all fibers staining with AS show the oxidative type metabolic pattern, while most cells reacting with AF have the glycolytic type metabolic pattern. One AF-positive fiber has a high oxidative enzyme content (see text). Note that with antibody staining, a light fiber is positive, while a dark fiber is negative. With the histochemical stains, a dark fiber is positive, while a light fiber is negative.
6a and b). The ATPase assays are also indicative of a homogeneous population of slow fibers (Fig. 6c and d), whereas the myosin light chains are those of a slow muscle except that a band corresponding to LC1r persists (Fig. 3; cf. reference 35). Similarly, the enzyme assays show a complete transformation to a muscle with high oxidative enzyme activity (Fig. 6e and f).

**DISCUSSION**

Chronic stimulation of a mammalian fast muscle at a frequency that imitates the firing rate of a motoneuron supplying a slow muscle transforms the fast muscle into a slow muscle. On the basis of staining of serial sections of a stimulated fast muscle with two antibodies specific for slow and fast myosins, we conclude that the transformation is the result of a switch from the expression of one set of genes to the expression of another within the same fiber. This conclusion rests on two facts: (a) normal muscle fibers contain either fast or slow myosin, but not both; and (b) in stimulated muscle, slow myosin appears in fibers containing fast myosin.

The first of these is established by our demonstration that in serial sections individual fibers stain with either anti-fast or anti-slow myosin antibody, but not with both. Groschel-Stewart and Doniach (17) showed that several myosin types could be distinguished immunologically. Arndt and Pepe (2) were the first to prepare antibodies specific to both fast and slow muscle myosins and to directly demonstrate the existence of two immunologically distinct myosin types within the same muscle. Since they did not use serial sections, they could make only an indirect case for the segregation of the two myosin types into different fibers. Subsequently, Gauthier and Lowey (16) showed that all white and some red fibers stained with an anti-fast myosin antibody. Since they used only an anti-fast myosin antibody, they could not deal directly with the question of...
FIGURE 5 Serial sections of a rabbit fast muscle (m. peroneus longus) after chronic stimulation in vivo for 4.5 wk. Sections were stained with AF (a) or AS (b), for ATPase activity after alkali (c) or acid (d) preincubation, for α-glycerophosphate dehydrogenase (e) and NADH-tetrazolium reductase (f). Most fibers still contain both fast and slow myosins, but the metabolic pattern is almost completely transformed to the oxidative type. This lack of coordination of metabolic pattern changes with myosin changes has been noted previously (24, 25, 35).
FIGURE 6 Serial sections of a rabbit fast muscle (m. peroneus longus) after chronic stimulation for 8 wk. Sections were stained with AF (a), AS (b), for ATPase activity after alkali (c) or acid (d) preincubation, for α-glycerophosphate dehydrogenase (e) and NADH-tetrazolium reductase (f). All fibers stain intensely with AS and fail to react with AF; thus, this muscle has been completely transformed from a fast to a slow muscle. ATPase activities confirm the total transformation.
whether fast and slow myosins coexist within the same fiber. Weeds et al. (36) were the first to use sodium dodecyl sulfate gel electrophoresis of protein from single fibers; and although their data show that a fast white fiber contains only the myosin light chains normally found in fast muscles, they did not deal with the possibility that fast-red or slow fibers could contain both types. Recently, using sodium dodecyl sulfate gel electrophoresis of single fibers, Pette and Schnez (22) reported the existence of two types of fibers: one containing only fast myosin light chains, the other only slow myosin light chains.

Having established the presence of only one type (fast or slow) of myosin in a fiber, we could look for the appearance of slow myosin in chronically stimulated muscle fibers and investigate whether slow myosin replaced fast myosin within a single fiber or whether fibers synthesizing slow myosin replaced other fibers synthesizing fast myosin. Our results indicate that, during chronic stimulation of a rabbit fast muscle, there is a decrease in the amount of fast myosin and a concomitant increase in the slow myosin content within the same fibers. These results rule out the hypothesis that muscle fibers are permanently programmed for the synthesis of only one myosin. This is a direct confirmation of the theory that individual, preexisting muscle fibers actually can undergo reprogramming and switch from the synthesis of fast myosin to the synthesis of slow myosin. While this paper was being prepared for publication, a report by Pette and Schnez (23) reported similar conclusions based on gel electrophoretic studies of total protein from single fibers of stimulated muscles.

As discussed in the Introduction, the noncoordinated transformation of different muscle properties during chronic stimulation or cross reinnervation suggested that the changes were occurring within individual fibers and not by replacement of existing fibers. Some experiments suggest that the same type of mechanism may account for fiber changes during development. Kugelberg (18), for example, found a smooth gradation of increasing contraction times and decreasing ATPase activity among motor units in the developing rat soleus and concluded that fast fibers present in embryonic soleus muscle were gradually transformed into slow fibers during maturation, as a result of the differentiation of the motoneurons. Our finding that stimulation of a fast muscle through the intact nerve leads to a reprogramming of preexisting cells makes it quite likely that the transformation of a cross reinnervated muscle rests on the same mechanism. However, until experimental verification has been obtained, the question is open.

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