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Three myosin heavy chain isoforms in type 2 skeletal muscle fibres

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Summary

Mammalian skeletal muscles consist of three main fibre types, type 1, 2A and 2B fibres, with different myosin heavy chain (MHC) composition. We have now identified another fibre type, called type 2X fibre, characterized by a specific MHC isoform. Type 2X fibres, which are widely distributed in rat skeletal muscles, can be distinguished from 2A and 2B fibres by histochemical ATPase activity and by their unique staining pattern with seven anti-MHC monoclonal antibodies. The existence of the 2X-MHC isoform was confirmed by immunoblotting analysis using muscles containing 2X fibres as a major component, such as the normal and hyperthyroid diaphragm, and the soleus muscle after high frequency chronic stimulation. 2X-MHC contains one determinant common to 2B-MHC and another common to all type 2-MHCs, but lacks epitopes specific for 2A- and 2B-MHCs, as well as an epitope present on all other MHCs. By SDS-polyacrylamide gel electrophoresis 2X-MHC shows a lower mobility compared to 2B-MHC and appears to comigrate with 2A-MHC. Muscles containing predominantly 2X-MHC display a velocity of shortening intermediate between that of slow muscles and that of fast muscles composed predominantly of 2B fibres.

Introduction

Three main fibre types, designated type 1, 2A and 2B, can be identified in mammalian skeletal muscle using histochemical procedures for the demonstration of myosin ATPase activity (Brooke & Kaiser, 1970). These fibres belong to motor units having distinct functional properties: type 1 fibres correspond to slow-contracting motor units, type 2A fibres to fast-contracting fatigue-resistant motor units, type 2B fibres to fast-contracting fatigue-sensitive motor units (Edstrom & Kugelberg, 1968; Burke *et al.*, 1973). Type 2A and 2B fibres contain different myosin heavy chains (MHC) (Dall Libera *et al.*, 1980; Pierobon-Bormioli *et al.*, 1981; Billeter *et al.*, 1981) encoded by distinct genes (Wieczorek *et al.*, 1985; Izumo *et al.*, 1986). Type 2A and 2B fibres also differ with regard to the enzyme pattern of energy metabolism, 2A fibres being generally more oxidative than 2B fibres. However, histochemical staining for oxidative enzymes, as well as microchemical studies on isolated single fibres show an apparently continuous spectrum of enzyme activities in the type 2 fibre population (Schiaffino *et al.*, 1970; Kugelberg & Lindegren, 1979; Nemeth & Pette, 1981; Hintz *et al.*, 1984). Recent studies concerning the distribution of α-actinin, troponin and C-protein isoforms in mammalian skeletal muscle indicate that the simple classification of two discrete categories of 2A and 2B fibres is inadequate (Dhoot *et al.*, 1985; Moore & Schachat, 1985; Schachat *et al.*, 1985a; Schachat *et al.*, 1985b; Starr *et al.*, 1985).

Using electrophoretic procedures that permit the separation of MHCs and immunoblotting analysis with several monoclonal antibodies (mAbs), we have identified a novel MHC isoform, called 2X-MHC, in a specific subpopulation of type 2 fibres which are widely distributed in rat skeletal muscles. We report here that 2X-MHC is the major MHC in the soleus muscle transformed by high frequency chronic stimulation and correlates with a velocity of shortening intermediate between that of slow muscles and that of fast muscles containing predominantly 2B-MHC. The results clarify previous discrepancies in the classification of mammalian fibre types, with particular reference to the relation between myosin

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isoforms, energy metabolism and contractile properties. Some of these data have been presented elsewhere in preliminary reports (Schiaffino *et al.*, 1985; Schiaffino 1986b).

Methods

Animals and physiological studies

Most studies were performed on muscles from normal adult Wistar rats weighing 250–350 g. Two groups of experimental animals were also used. One group of rats was given daily intraperitoneal injection with thyroid hormone (T_3, 5 μg/100 g) for 10 days. Another group of animals was used for chronic stimulation experiments, which are described in detail elsewhere (Gorza *et al.*, 1988). In brief, the soleus muscle was denervated by cutting and reflecting the sciatic nerve in the thigh. Steel electrodes were implanted across the soleus muscle, one on each side, run under the skin, fixed on the skull and attached to a stimulator above the rat. Muscles were stimulated for 56 to 60 days by short pulse trains at high frequency (60 pulses at 100 Hz every 60 s, day and night).

Myosin preparation and gel electrophoresis

Purified myosin preparations were obtained from different rat muscles (soleus, red portion of tibialis anterior, white portion of tibialis anterior, diaphragm) using previously described procedures (Sartore *et al.*, 1987). Myosin subfragment 1(S-1) rod, heavy meromyosin (HMM) and light meromyosin (LMM) were prepared as described in Margossian and Lowey (1982). Proteins were separated in SDS-10% or 7.5% polyacrylamide gels (Laemmli, 1970) and stained with Coomassie Blue. For estimation of molecular weights the high molecular mass protein standard kit (Bio-Rad) containing myosin heavy chain (200 kDa) β-galactosidase (116 kDa) phosphorylase B(97 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa) was used.

The following rapid procedure was used to obtain myosin preparations for separation of MHC isoforms. Muscle samples were homogenized in 10 vol of 20 mM KCl, 2 mM K_2HPO_4, 1 mM EGTA, pH 6.8, and allowed to remain on ice for 15 min. After a second wash with the same medium the pellets were extracted with 40 mM $Na_4P_2O_7$, 1 mM $MgCl_2$, 1 mM EGTA, pH 9.5, for 15 min. The samples were centrifuged at 12 000 *g* for 15 min and the supernatant fractions containing myosin were processed for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 6% polyacrylamide gels. A critical factor affecting the resolution of MHC isoforms was the addition of glycerol to the gel matrix to a final concentration of 37.5% (v/v) (Danieli Betto *et al.*, 1986). The gels were stained with Coomassie Blue or silver (Morrissey, 1981).

Antibodies, immunoblotting and immunohistochemistry

Monoclonal antibodies were produced as described previously (Schiaffino *et al.*, 1986a). Native myosin preparations from bovine skeletal muscle were used as immunogen, except in one fusion experiment that yielded mAb RT-D9, when SDS-denatured HMM from rat tibialis anterior muscle was used. Selected hybridomas were cloned by limiting dilution and grown as ascites in pristane primed mice. Immunoglobulin (Ig) classes were determined by enzyme immunoassay with specific antisera (Miles). The monoclonal antibodies used in this study were either IgG_1 (BF-35, BA-D5, RT-D9, SC-71) or IgM (BF-F3, BF-32, SC-75). IgG_1 antibodies were purified from the ascites fluid by caprilic acid precipitation (Russo *et al.*, 1983) and IgM antibodies by chromatography on protamine sulphate column (Hudson & Hay, 1980).

Immunoblot analysis was performed on duplicate 10% or 6% gels which were electrophoretically transferred to nitrocellulose sheets as described by Towbin and coworkers (1979). The blots were first incubated with purified mAbs at concentrations of 0.1–10 μg ml^{-1}, then with peroxidase-conjugated rabbit anti-mouse Ig-antibodies (Dakopatts) and developed with diaminobenzidine in the presence of imidazole (Trojanowski *et al.*, 1983).

For immunohistochemistry, cryosections of skeletal muscles were incubated in the appropriate monoclonal antibody for 30 min at room temperature. Bound antibody was visualized by immunoperoxidase staining as previously described (Gorza *et al.*, 1986). Serial sections were stained for succinate dehydrogenase activity (Nachlas *et al.*, 1957) and for myosin ATPase activity after preincubation at pH 4.3 and 4.6 (Brooke & Kaiser, 1970) or after pretreatment in formaldehyde at pH 10.4 (Guth & Samaha, 1969).

Results

Specificity of antibodies

The seven antibodies used in this study were selected from a collection of over 50 hybridoma clones whose supernatants stained rat skeletal muscle and reacted with MCHs in enzyme immunoassays and immunoblots. Screening assays were performed on three myosin preparations, isolated from the rat soleus, the superficial white portion of the tibialis anterior and the deep red portion of the tibialis anterior muscles. These muscles have a different fibre type composition as determined by the histochemical reaction for myosin ATPase. The soleus muscle contains predominantly type 1 fibres with a minor type 2A component, the superficial white tibialis contains almost exclusively type 2B fibres, the deep red tibialis contains both type 2B and 2A fibres.

On immunoblots the mAbs showed a different pattern of reactivity with the three myosin preparations (Fig. 1). SC-75 reacted strongly with MHCs from the two tibialis myosin preparations and weakly with soleus MHC; BA-D5 reacted with soleus MHC but not with white and red tibialis MHCs; BF-32 reacted with soleus and, less strongly, with red tibialis but not with white tibialis MHC; BF-F3 and RT-D9 reacted with both white and red tibialis but not with soleus MHC; BF-35 reacted strongly with soleus MHC, moderately with red tibialis MHC and weakly with white tibialis MHC. A similar pattern of reactivity was obtained on enzyme immunoassay (not shown).

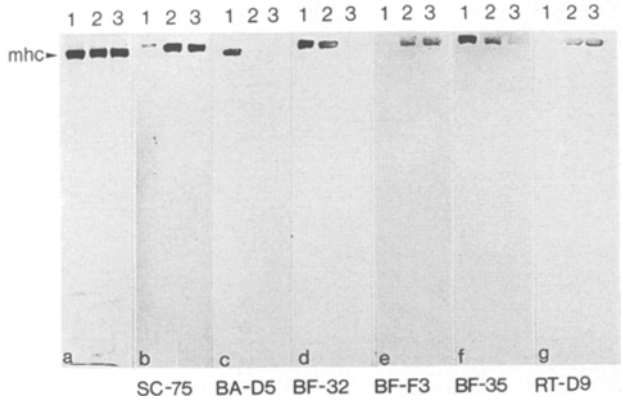


Fig. 1. Specificity of six anti-myosin mAbs determined by immunoblotting analysis. Purified myosins from the soleus (1), red tibialis (2) and white tibialis (3) muscles were separated by SDS-PAGE on 10% gels and either stained with Coomassie Blue (*a*), or transferred to nitrocellulose filters and reacted with mAbs SC-75 (*b*), BA-D5 (*c*), BF-32 (*d*), BF-F3 (*e*), BF-35 (*f*), and RT-D9 (*g*). Bound antibody was revealed by anti-mouse IgG conjugated with peroxidase.

The specificity of mAb SC-71 was tested using a myosin preparation of the rat diaphragm and its subfragments. As shown in Fig. 2, SC-71 reacted with undigested MHC, the rod subfragment and light meromyosin, whereas it was unreactive with HMM and S-1. This pattern of reactivity contrasted with that shown by mAb SC-75, that reacted with HMM and S-1 but not with LMM or rod (Fig. 2c). All the other mAbs were reactive with the rod and unreactive with S-1 when tested with the two fragments obtained from diaphragm or leg muscle myosins (not shown).

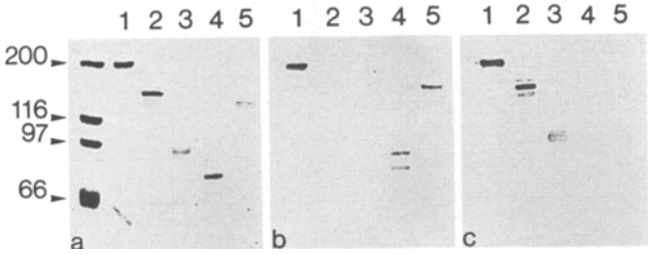


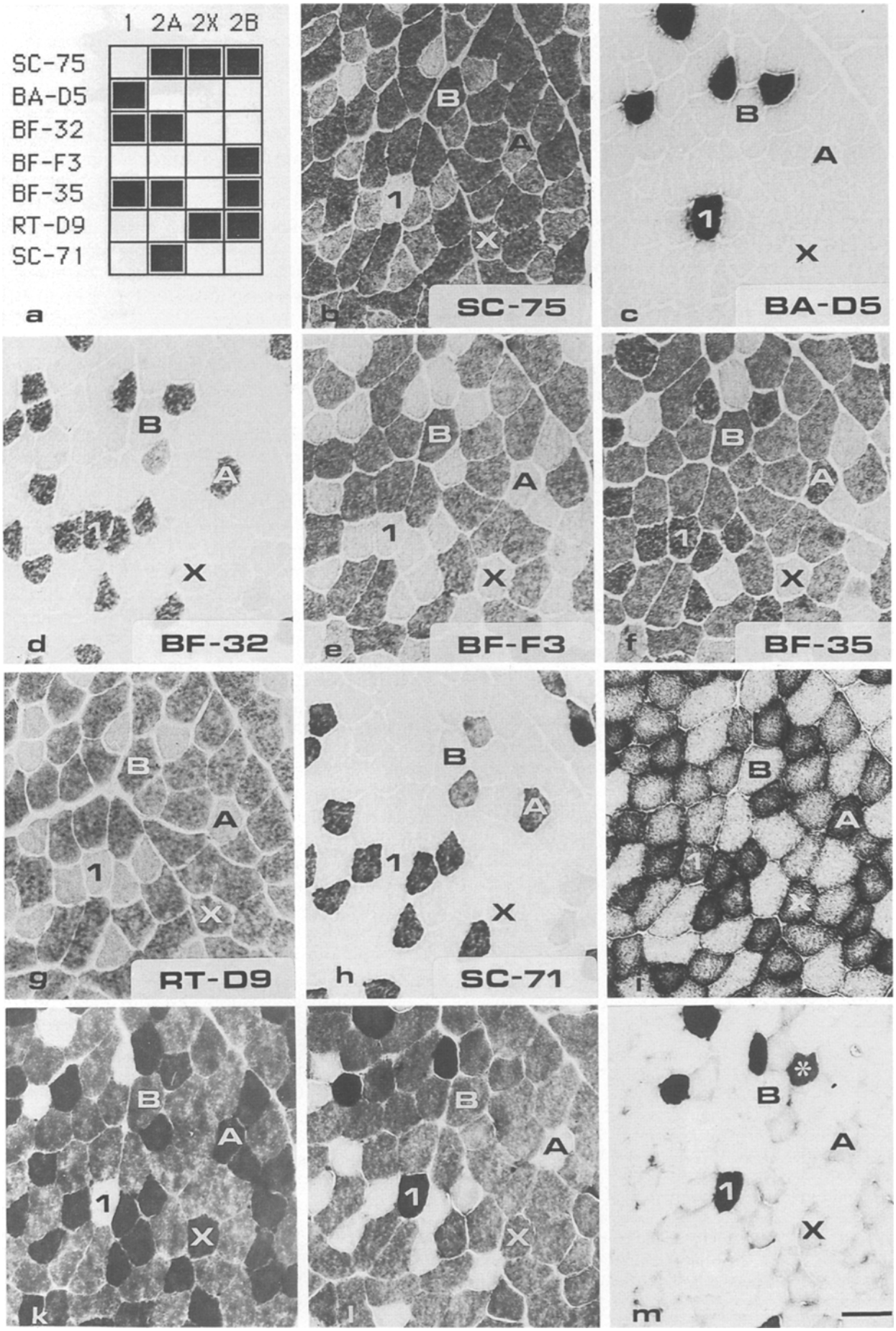
Fig. 2. Specificity of mAbs SC-71 and SC-75 for myosin and its subfragments. Myosin from rat diaphragm (1) and its subfragments heavy meromyosin (2), S-1 (3), light meromyosin (4) and rod (5) were separated by SDS-PAGE on 7.5% gels and either stained with Coomassie Blue (*a*), or transferred onto nitrocellulose and reacted with mAbs SC-71 (*b*) and SC-75 (*c*). Molecular weight markers were also loaded in the Coomassie Blue stained gel in the first lane on the left.

Identification of type 2X fibres

The immunohistochemical staining pattern of seven mAbs in rat skeletal muscle is schematically summarized in Fig. 3a and illustrated in Fig. 3b-h. Three subpopulations of type 2 fibres, referred to as type 2A, 2B and 2X, were identified. All these fibres were reactive with mAb SC-75 and were unreactive with mAb BA-D5 which stains specifically type 1 fibres. Type 2X fibres were stained by mAb RT-D9, that stains also 2B fibres, but were unlabelled by a mAb (BF-F3) specific for type 2B fibres and by two mAbs (BF-32 and SC-71) reactive with type 2A fibres, and, in addition, they were unlabelled by mAb BF-35 that stains all other fibre types. They were also unreactive with antibodies specific for embryonic and neonatal MHC (Sartore *et al.*, 1982; Schiaffino *et al.*, 1988b) and with antibodies that stain exclusively type 2 fibres in extrinsic eye muscles (Sartore *et al.*, 1987) (not shown). Type 2X fibres were also atypical as regards histochemical myosin ATPase staining, in that they stained like type 2A fibres after formaldehyde-alkali pretreatment but reacted like type 2B fibres after preincubation at pH 4.6 (Fig. 3k–m). As myosin ATPase staining after preincubation at pH 4.6 is frequently used for fibre typing, it appears that 2X fibres have been included into the type 2B class in many histochemical studies. On the other hand, type 2X fibres display moderate to strong succinate dehydrogenase activity (Fig. 3i) and would be lumped together with type 2A fibres in studies using histochemical methods for oxidative enzymes.

Evidence for a distinct 2X-MHC isoform

Type 2X fibres were present in variable proportion in most rat skeletal muscles as well as in mouse and guinea-pig muscles. The nature of the myosin present in type 2X fibres was investigated further using normal or experimental muscles in which type 2X fibres were found to represent a major component. Muscles selected for this study were the normal and hyperthyroid rat diaphragms, which were found to contain more than 35% 2X fibres, and soleus muscles chronically stimulated with a high frequency pattern of impulses, which contain more than 90% 2X fibres. An electrophoretic system that permits the separation of MHC isoforms was used to compare the MHC composition of the diaphragm with that of the normal soleus muscle and the white portion of the tibialis anterior muscle. Type 1- and type 2-MHCs are clearly separated by SDS-PAGE in 6% gels, type 1-MHC showing a relatively greater mobility under these conditions (Fig. 4A). Immunoblotting analysis showed that while the soleus contains essentially type 1- and 2A-MHC and the white tibialis 2B-MHC, the predominant type 2-MHC component in the diaphragm is not 2A- nor 2B-MHC, as it shows only a


1 2A 2X 2B
SC-75
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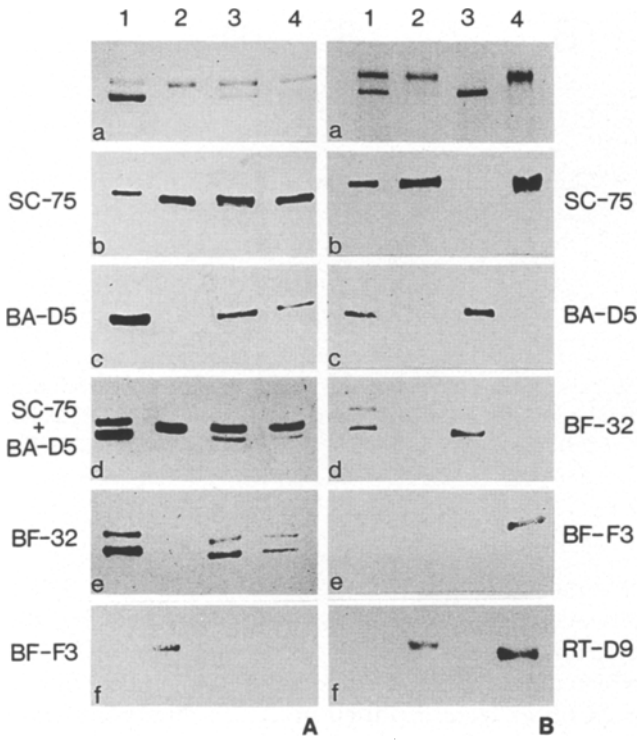

Fig. 4. Identification of 2X-MHC by immunoblotting analysis. **A**, immunoblotting analysis of myosins from soleus (lane 1), white tibialis anterior (lane 2), normal diaphragm (lane 3) and hyperthyroid diaphragm (lane 4) after SDS-PAGE in 6% gels. *a* Gel stained with Coomassie Blue; *b–f*, corresponding blots reacted with SC-75 (*b*), BA-D5 (*c*), SC-75 and BA-D5 applied together (*d*), BF-32 (*e*) and BF-F3 (*f*). **B**, immunoblotting analysis of soleus muscle myosin after denervation and implantation of sham electrodes (lane 1), denervation and stimulation at 100 Hz (lane 2), implantation of sham electrodes (lane 3). Myosin from white tibialis anterior muscle in lane 4. *a* Gel stained with silver; *b–f* corresponding blots reacted with SC-75 (*b*), BA-D5 (c), BF-32 (*d*) and BF-F3 (*e*) and RT-D9 (*f*). Only the portions of the blots corresponding to the MHCs are shown.

weak reaction with BF-32 and is unreactive with BF-F3.

The rat soleus muscle was denervated and stimulated chronically with a high frequency pattern of impulses that induces many properties typical of fast muscles (Gorza *et al.*, 1988). The electrophoretic pattern and corresponding immunoblots of myosins from stimulated and control muscles are shown in Fig. 4B. The stimulated soleus consists almost exclusively of type 2-MHC, with a small trace of type 1-MHC band. In contrast, the control soleus contains exclusively type 1-MHC and the denervated muscle contains similar amounts of the two components. On immunoblots the MHC present in the stimulated soleus is reactive for type 2- but unreactive for type 2A- and 2B-MHC. Our interpretation is that the stimulated soleus contains almost exclusively type 2X-MHC. This interpretation is consistent with the finding that the MHC band in the stimulated soleus is recognized by mAb RT-D9, that reacts with 2X- and 2B-MHC.

The electrophoretic mobility of the different type 2 MHC isoforms was further compared in 6% gels. As shown in Fig. 5, three MHC bands of increasing mobility can be resolved: the first corresponds to the 2A- and 2X-MHCs which appear to comigrate under these conditions; the second to 2B-MHC; the third to type 1-MHC. Co-electrophoresis experiments show that the 2A-MHC of soleus has a slightly slower mobility than the 2B-MHC of white tibialis and that the 2X-MHC present in diaphragm and stimulated soleus comigrates with the 2A-MHC band.

Discussion

Using seven different mAbs and myosin ATPase histochemistry it is possible to identify, in addition to 2A and 2B fibres, a third, type 2X, fibre population in rat skeletal muscles. This fibre type contains a novel 2X-MHC isoform which differs from 2B-MHC by not being recognized by mAbs BF-F3 or BF-35, and from 2A-MHC by not being recognized by BF-32, SC-71 or BF-35. Type 2X-MHC has an electrophoretic mobility different from that of 2B-MHC but similar to that of 2A-MHC. This MHC isoform contains an epitope common to all type 2-MHCs (recognized by mAb SC-75) and another epitope common to 2B-MHC (recognized by mAb RT-D9). The reaction with the latter antibody is particularly important for immunoblotting, as 2X- and 2B-MHC show different electrophoretic mobilities on immunoblots. RT-D9 thus permits specific positive identification of 2X-MHC.

Fig. 3. Identification of type 2X fibres by immunocytochemistry and enzyme histochemistry. Serial transverse sections of rat tibialis anterior muscle were stained with mAbs against MHCs or processed for the histochemical demonstration of succinate dehydrogenase and myosin ATPase activity. (*a*), scheme showing the pattern of reactivity of seven anti-MHC mAbs with different fibre types, as determined by immunoperoxidase staining. The filled and open boxes indicate positive and negative reaction, respectively. (*b–h*), Immunoperoxidase staining with mAbs SC-75 (*b*), BA-D5 (*c*), BF-32 (*d*), BF-F3 (*e*), BF-35 (*f*), RT-D9 (*g*) and SC-71 (*h*). Type 1 (1), type 2A(A), type 2B(B) and type 2X(X) fibres are indicated. (*i*), Histochemical staining for succinate dehydrogenase activity. (*k–m*), Myosin ATPase staining after alkali-formaldehyde pretreatment (*k*), and after acid pretreatment at pH 4.6 (*l*) or 4.3 (*m*). Asterisk in (*m*) marks a hybrid type 2C fibre that contains both type 2 (see *b* and *h*) and type 1 (see *c*) myosin. Scale bar, 50 μm.

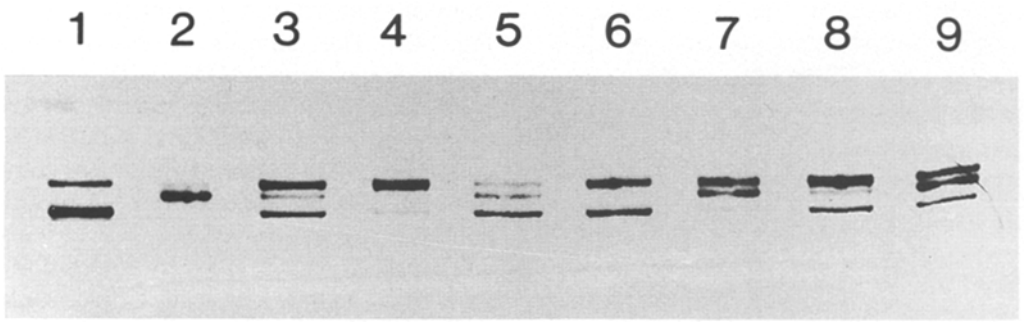

Fig. 5. Separation of type 2 MHC isoforms by SDS-PAGE on 6% gels. MHC isoforms were visualized by silver staining. Lanes 1 to 4 contain myosin preparations from soleus (1), white tibialis anterior (2), diaphragm (3) and stimulated soleus (4). Lanes 5 to 9 contain mixtures of 1+2(5), 1+4(6), 2+4(7), 3+4(8), 2+3(9). Only the portions of the gels corresponding to the MHCs are shown.

It remains to be determined whether 2X-MHC is the product of a distinct gene or results from differential RNA splicing or from post-translational modification of either 2A- or 2B-MHC. The last possibility appears unlikely and there is no evidence so far for differential splicing of MHC genes in mammals. Circumstantial evidence suggests that 2X-MHC may be the product of a specific mRNA. In a recent study cDNA probes specific for type 1-, 2A- and 2B-MHC genes were used to measure the relative amount of the corresponding MHC mRNAs in different muscles of normal, hypothyroid and hyperthyroid rats (Izumo *et al.*, 1986). In the normal diaphragm the 2B-MHC gene was expressed at low levels and its expression remained essentially unchanged during hyperthyroidism, in spite of the concomitant marked decrease in the expression of both type 1- and 2A-MHC genes. These results are consistent with our finding of decreased type 1- and 2A-MHC in hyperthyroid diaphragm without significant increase of type 2B-MHC (Fig. 4, and S. S., in preparation). The discrepancy between the low level of 2B-MHC mRNA and the apparently large proportion of histochemically defined 2B fibres in the normal diaphragm, as well as the paradoxical finding of a low expression of both type 1-, 2A- and 2B-MHC genes in the hyperthyroid diaphragm, could be explained assuming the existence of a distinct 2X-MHC mRNA which does not hybridize with the 2B-specific probe and which represents a major component in this muscle.

Fibres containing 2X-MHC are present in substantial numbers in most rat skeletal muscles, as well as in mouse and guinea-pig muscles (L. G., in preparation). In histochemical preparations these fibres display a variable, but generally intermediate to high SDH activity, whereas all fibres identified as type 2B by BF-F3 show a very weak SDH activity. Others have reported that 2B fibres may be rich in oxidative enzymes (Nemeth & Pette, 1981; Hintz *et al.*, 1984): however, type 2B fibres were identified by the myosin ATPase reaction after acid pretreatment, which does not distinguish between 2X and 2B fibres. If 2X fibres were included in the 2B fibre population this could explain a high oxidative enzyme activity in many of the fibres. The apparent heterogeneity of type 2B fibres with respect to the distribution of troponin T and α-actinin isoforms could likewise reflect the presence of two fibre populations (Moore & Schachat, 1985; Schachat *et al.*, 1985a, 1985b).

The identification of a novel MHC isoform in type 2 muscle fibres is relevant to the current debate concerning the functional significance of MHC heterogeneity and the relative role of MHCs and myosin light chains (MLCs) in controlling the maximum velocity of shortening of skeletal muscle. The observed close correlation between the maximum velocity of contraction and the specific activity of myosin ATPase (Barany, 1967) and the finding that the actin-activated ATPase of myosin is affected by the MHC but not MLC composition (Wagner, 1981) suggest that MHCs are the major determinant of shortening velocity. This interpretation is consistent with the finding that in cardiac muscle, changes in maximum velocity of shortening induced by thyroid hormone or haemodynamic overload in the ventricular myocardium are correlated with shifts between the high ATPase α-MHC and the low ATPase β-MHC without any change in the MLC composition (Hoh *et al.*, 1978; Schwartz *et al.*, 1981; Pagani & Julian, 1984).

In skeletal muscle the issue is more controversial. A number of studies on single fibres from mammalian and amphibian skeletal muscle showed that differences in MHCs but not MLCs correlate with shortening velocity (Julian *et al.*, 1981; Reiser *et al.*, 1985; Lannergren, 1987). For instance, in the fast fibres of *Xenopus* skeletal muscle no significant correlation has been found between shortening

velocity and the relative proportions of the two alkali light chains (Lannergren, 1987). On the other hand, variations in shortening velocity between type 2A and 2B fibres in the rat and rabbit appear to correlate with the relative proportion of the two alkali light chains (Sweeney *et al.*, 1986; Eddinger & Moss, 1987; Sweeney *et al.*, 1988). In one study, two groups of 2B fibres with different velocities of shortening were also identified in rabbit skeletal muscle (Sweeney *et al.*, 1988). This difference was correlated with a difference in the ratio of the two alkali light chains, whereas no difference in MHC composition was detected by one-dimensional peptide mapping. However, previous evidence of myosin heavy chain microheterogeneity in fast-white rabbit muscles (Zweig, 1981) and a recent preliminary report (Mabuchi *et al.*, 1988) suggest that a third type 2-MHC isoform may be present in the rabbit.

Physiological studies on whole rat muscles indicate that 2X-MHC is correlated with a lower velocity of shortening than 2B-MHC. We found that the maximum velocity of shortening of chronically stimulated soleus muscles, which contained about 85% 2X-MHC, was intermediate between that of normal extensor digitorum longus muscles, which contained about 75% 2B-MHC and that of soleus (Schiaffino *et al.*, 1988a). This result can explain previous discrepancies in studies relating physiological properties and fibre type composition of skeletal muscles. Luff (1981) reported that the mouse diaphragm, which contains more than 90% histochemically type 2 fibres, has a shortening velocity intermediate between that of the extensor digitorum longus and the soleus. We found that 2X-MHC is a major MHC isoform in the mouse diaphragm, while 2B-MHC is especially abundant in the extensor (Schiaffino *et al.*, in preparation). Physiological studies on skinned single fibres combined with MHC typing with specific mAbs should clarify the relationship between shortening velocity and MHC composition in type 2 fibre subpopulations.

Other groups have recently reported results concerning the existence of a third MHC type in fast muscle fibres. Anti-MHC monoclonal antibodies that distinguish three populations of type 2 fibres in rat and rabbit skeletal muscles have been described by Leung and co-workers (1987) and Mabuchi and co-workers (1988). In addition, Bär and Pette (1988) reported that a new MHC band showing lower mobility than the 2A and 2B bands can be resolved by SDS-PAGE in rat muscles. It is possible that this corresponds to the 2X-MHC described here, which could be separated from the 2A-MHC in an appropriate electrophoretic system. However, the criteria for distinguishing the new isoform and the 2A-MHC were not specified. Their finding that soleus muscle may contain a significant proportion of the new isoform but no 2A-MHC is in contrast with our finding that when a type 2-MHC component is present in this muscle it consists essentially of 2A-MHC (Fig. 4). This raises the possibility that the band indicated by Bär and Pette as the new isoform corresponds to 2A-MHC and the band labelled as 2A corresponds in fact to the new isoform.

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