Slow Myosin Heavy Chains in Cat Jaw and Limb Muscles are Phenotypically Distinct: Expression of Jaw-specific Slow Myosin Phenotype in Regenerated and Chronically Stimulated Jaw Muscles

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Abstract

The jaw-closing muscles of the cat are intrinsically distinct from limb muscles in their ability to express superfast but not fast myosin during development or regeneration. Jaw and limb muscles contain slow fibres, both types of which react with an anti-slow myosin heavy chain (MHC) antibody. Slow fibres in cat jaw muscles, however, react with another monoclonal antibody (1A10) which stains limb fast, but not limb slow fibres. Slow fibres with the same immunocytochemical reactivities as jaw slow fibres are also found in: [1] regenerates of jaw muscle (temporalis) reinnervated by the nerve normally innervating the limb slow soleus muscle, and [2] temporalis muscle subject to chronic, electrical stimulation at low frequency. SDS gel electrophoresis failed to resolve a difference in mobility between jaw and limb slow MHCs. However, cyanogen bromide (CNBr) peptide maps of electrophoretically purified slow MHCs revealed differences between jaw-slow and limb-slow myosins. The data suggest that slow MHCs in jaw and limb muscle fibres are structurally distinct isoforms, and that the competence of muscle cells to express the jaw isoform is restricted to jaw muscle fibres. The jaw-specific slow MHC may be the same as the variant of β-MHC reported by others in mammalian hearts.

Key words: Myosin heavy chain, masticatory muscle, muscle fibre allotype, peptide mapping, immunocytochemistry.

BAM 1 (4): 285-294, 1991

The jaw-closing muscles of the cat are faster and more powerful than limb fast muscles; these properties are associated with their ability to express jaw-specific isoforms of myofibrillar proteins: superfast myosin [35] and jaw-specific tropomyosin [21, 33]. During myogenesis, presumptive superfast fibres in jaw muscles express these jaw-specific proteins, but their fast limb muscle counterparts do not [1, 14, 17]. Jaw and limb muscles, however, express some myofibrillar proteins in common, e.g., developmental (embryonic and foetal) isoforms of myosin during myogenesis. The myosin expressed in slow fibres of jaw muscles and that in slow fibres in limb muscles bind the same monoclonal antibody, and have hitherto been considered to be the same protein [14, 18].

Constraints on the pattern of myosin gene expression in jaw and limb muscles are seen during regeneration. Regenerating jaw muscle will express slow and superfast myosins as well as developmental myosin isoforms even without innervation [16]. However, when innervated by nerves normally innervating limb fast and slow muscles, the outcome is different. Jaw muscle regenerates eventually express superfast myosin when innervated by the nerve to a limb fast muscle, but tend to express slow myosin when innervated by a nerve normally innervating a limb slow muscle [13]. Regenerates of limb muscles do not express superfast myosin [19].

The basal lamina of jaw and limb muscles apparently play no role in specifying the type of myosin expressed during regeneration (15). Jaw muscle cells in culture, however, strongly express jaw-specific myofibrillar proteins [24]. Jaw and limb muscles are therefore intrinsically different from each other, and belong to two distinct allotypes [11, 19]. Myoblasts derived from these allotypes belong to different lineages, and have restricted potentials for expressing myofibrillar proteins.

Slow muscle fibres are virtually absent in the posterior temporalis muscle of the cat, but may be found in the anterior temporalis and masseter muscles [14, 17, 35].

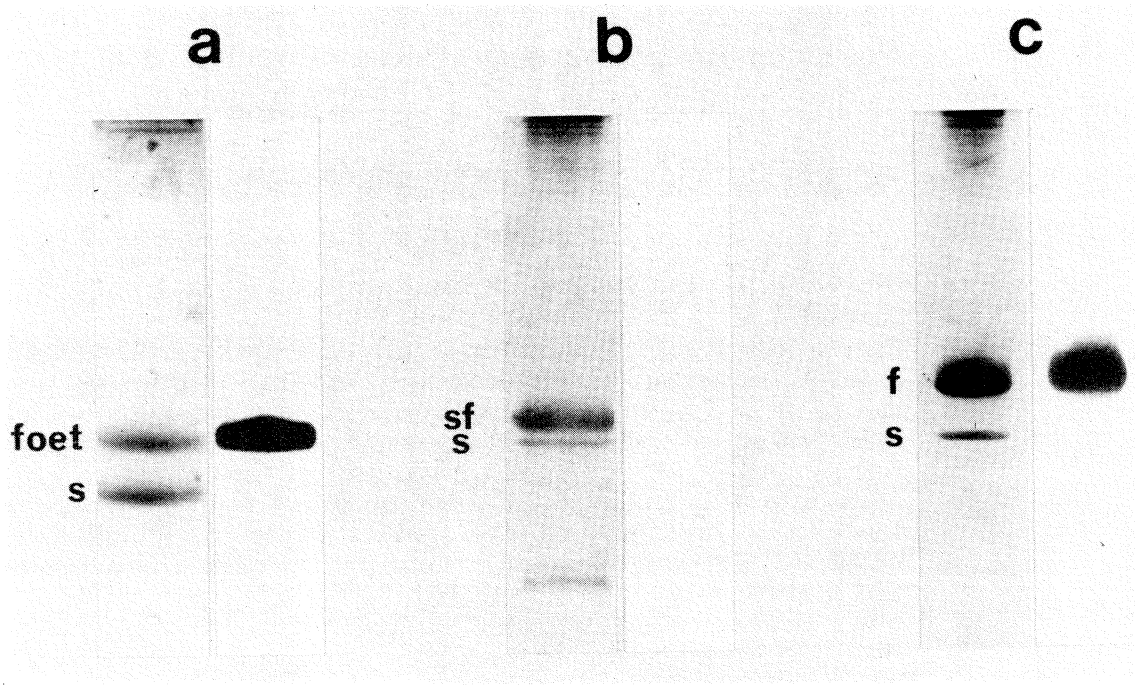

Figure 1. SDS gels (left) and corresponding Western blots stained by antibody 1A10 (right) of MHCs from 4-day-old kitten soleus (a), adult cat anterior masseter (b) and adult cat anterior tibialis (c) muscles. "s" = slow MHC, "sf" = superfast MHC, "foet" = foetal MHC, and "f" = fast MHC.

They are also found in regenerates of cat temporalis muscle reinnervated by the nerve to the slow soleus muscle [13], and in temporalis muscle subject to chronic, electrical stimulation at low frequency [12].

In this work, we provide evidence that the slow MHCs of cat jaw and limb muscle fibres are immunochemically and structurally distinct, and that the slow MHCs present in regenerated and chronically stimulated jaw muscle are phenotypically the same as the form in normal jaw slow fibres. A brief account of this work has been presented [20].

Materials and Methods

Muscles

Muscles were dissected from normal adult cats, kittens or experimental cats killed with an overdose of anaesthetic. The tissues were either frozen in melting isopentane chilled in liquid nitrogen for immunocytochemistry or used for myosin extraction. The masseter was taken as representative of jaw-closing muscle with both superfast and slow fibres, the tibialis anterior and extensor digitorum longus as representatives of limb fast muscles with minor components of slow fibres, and the soleus muscle as a homogeneously slow limb muscle. The soleus muscle of the 4-day-old kitten was used as a source of foetal and slow MHCs.

Muscle transplantation and stimulation

Under general anaesthesia (30 mg/kg sodium pentobarbitone administered intraperitoneally), a biopsy of the jaw-closing muscle posterior temporalis (200 mg) was taken from a cat and heterotopically transplanted into the bed of the soleus muscle after the complete removal of the soleus muscle as described previously [13]. As a control, in the contralateral leg, the soleus muscle was removed and a piece of the same muscle homotopically transplanted. The regenerated transplants were removed for immunocytochemical analysis after 7 months.

Under general anaesthesia, a specially constructed stimulator measuring 2.5 cm^3 was attached onto the skull of an adult cat. Platinum electrodes were attached to the homogeneously superfast posterior temporalis muscle, and the muscle electrically stimulated continuously with 1 ms pulses at 10 Hz for 7 weeks. Stimulus strength (8 V maximum) was adjusted to produce palpable local contractions of the muscle without stressing the animal. The stimulated muscle was removed under general anaesthesia for study after 7 weeks of continuous stimulation. Details

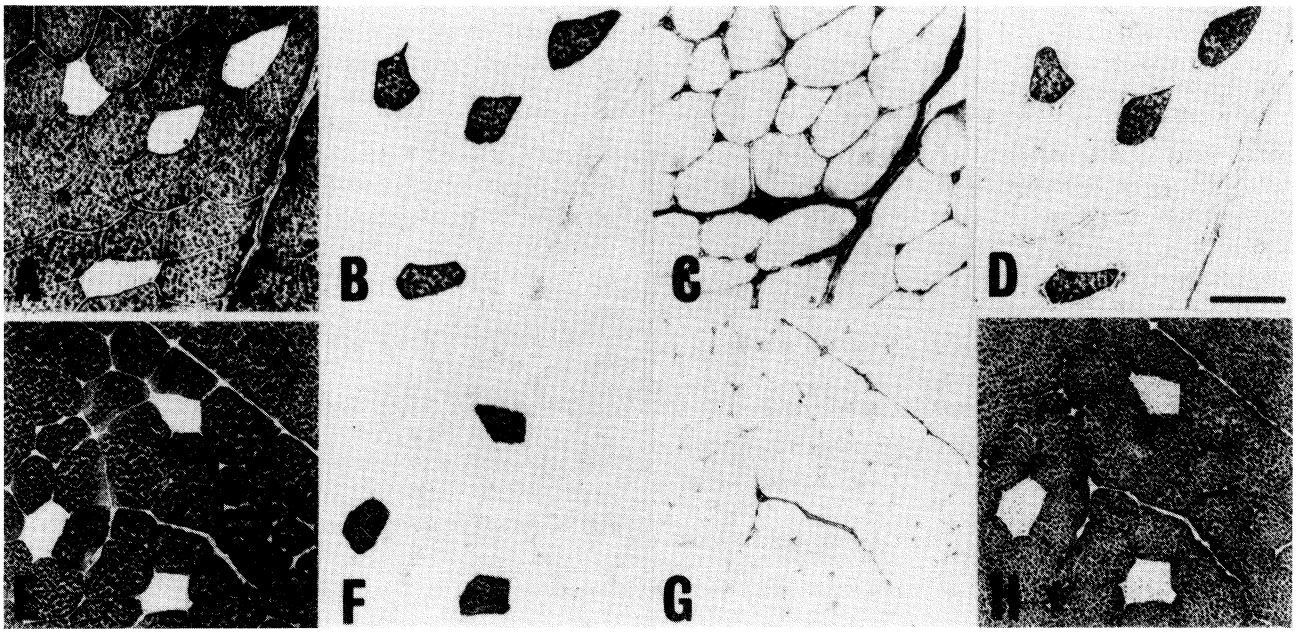

Figure 2. Serial sections of adult cat masseter (jaw) muscle (A-D) and the principally fast-twitch extensor digitorum longus (EDL) limb muscle (E-H) stained with anti-superfast myosin (A), anti-fast myosin (E), anti-slow myosin (B, F), anti-foetal myosin (C, G) and 1A10 (D, H) antibodies.

of the methods will be fully described in a subsequent publication.

Immunocytochemistry

Cryostat sections of extensor digitorum longus, soleus and masseter muscles removed from anaesthetized adult cat, as well as the experimental muscles described above, were studied by indirect immunocytochemistry using specific anti-MHC primary antibodies. The antibodies used were specific for the MHCs of slow (monoclonal NOQ-7-5-4D), fast (monoclonal NOQ-7-5-2B) and foetal (polyclonal) myosins as described previously [17, 18]. A new monoclonal antibody (1A10) raised against chicken fast MHC [8] but having reactivity against foetal and fast mammalian limb muscles, was also used. Secondary antibodies used were HRP-labelled rabbit anti-mouse immunoglobulin and rabbit anti-sheep immunoglobulin (Dako Corporation, Denmark).

Biochemical analysis of MHCs

Muscles were homogenized in neutral phosphate buffer and washed in the same buffer three times to remove soluble proteins. The washed myofibrils were collected by low speed centrifugation and extracted with 100 mM sodium pyrophosphate, pH 8.3, containing 0.5 mM phenylmethysulphonylfluoride, 0.5 mg/ml antipain, 0.5 mg/ml chymostatin, 0.5 mg/ml pepstatin A, 2 mg/ml leupeptin, and 100 U/ml aprotinin as anti-proteolytic agents.

Electrophoretic separation of MHCs was performed on mini-slab gels (8 cm x 10 cm, 0.75 mm thick) according to a modification of the procedure of Danieli-Betto *et al.* [6]. The composition of the resolving gel was 6% acrylamide (0.8% bisacrylamide), 40% glycerol (v/v), 0.375 M Tris (pH 8.8), 0.1% SDS, 0.05% TEMED, 0.05% ammonium persulphate. The stacking gel contained 4% acrylamide, 40% glycerol, 0.125 M Tris (pH 6.8), 0.1% SDS, 0.1% TEMED, and 0.1% ammonium persulphate. The running buffer contained 25 mM Tris, 129 mM glycine (pH 8.3) and 0.1% SDS. Prior to electrophoresis, protein samples were suspended in sample buffer containing 0.625 M Tris, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0013% (w/v) bromophenol blue and boiled for 4 minutes. Gels were loaded with 1 to 5 μg of crude myosin and were run at 4°C with a driving voltage of 120 V for a period of about 16 hours. The gels were stained with Coomassie Brilliant Blue R-250 (0.1% in 40 methanol, 10% acetic acid) and destained in 40% methanol, 10% acetic acid.

SDS gel electrophoresis of MHC of stimulated muscle was done using extracts of the cryostat sections in sample buffer. Tissue sections (10-15 x 10 μm thick) were boiled in 100 ml sample buffer in an Eppendorf tube and cleared by centrifugation (bench microfuge) for 1 minute before application to the gel.

Western blotting was performed as described by Towbin *et al.* [37]. MHC samples were run in 6% polyacrylamide gels as described above. The primary antibody used was monoclonal 1A10, while the secondary antibody was HRP-coupled anti-mouse immunoglobulin raised in rabbits. Blotting buffer contained 25 mM Tris, 129 mM glycine, 20% (v/v) methanol, pH 8.3. Staining of the blotting

membrane for protein was performed as described by Kumar *et al.* [25].

CNBr mapping was performed by the method of Pepinsky (32). Peptide maps of MHCs were done by first separating the heavy chains in 6% SDS gels. The slow MHC bands were cut out and incubated in 35 mg/ml CNBr for 1 hour. The gel pieces containing peptides were incubated in sample buffer (without bromophenol blue), and were loaded directly onto 7.5% Laemmli gels [26] for electrophoretic analysis. The gels were silver-stained [30] using the Bio-Rad silver staining kit.

Results

Characterization of monoclonal antibody 1A10

Figure 1 shows SDS gels stained for protein (left) and 1A10 stained Western blots (right) of MHCs in (a) soleus muscle from a 4-day-old kitten, (b) adult masseter muscle and (c) adult tibialis anterior muscle.The 4-day-old soleus showed 2 MHC components, a faster migrating slow MHC and foetal (neonatal) MHC. Only the foetal component is stained in the transblot. Masseter MHCs showed a prominent superfast component and a minor slow component. Neither MHC bound 1A10. The adult tibialis anterior showed a thick fast MHC component and a minor slow MHC band; only the fast MHC reacted with 1A10 in the transblot. Thus, 1A10 recognizes a MHC epitope common to foetal and fast myosins and does not react with denatured slow MHC in jaw or limb muscle. This antibody does not react with the many other proteins from jaw-closing muscle seen in transblots after shorter electrophoretic runs (data not shown).

Immunocytochemically, however, 1A10 does react with slow fibres in the masseter, but not the slow fibres in the extensor digitorum longus (EDL), a predominantly fast limb muscle (See Fig. 2). Most fibres in the EDL are fast fibres; these are stained by the anti-fast myosin antibody

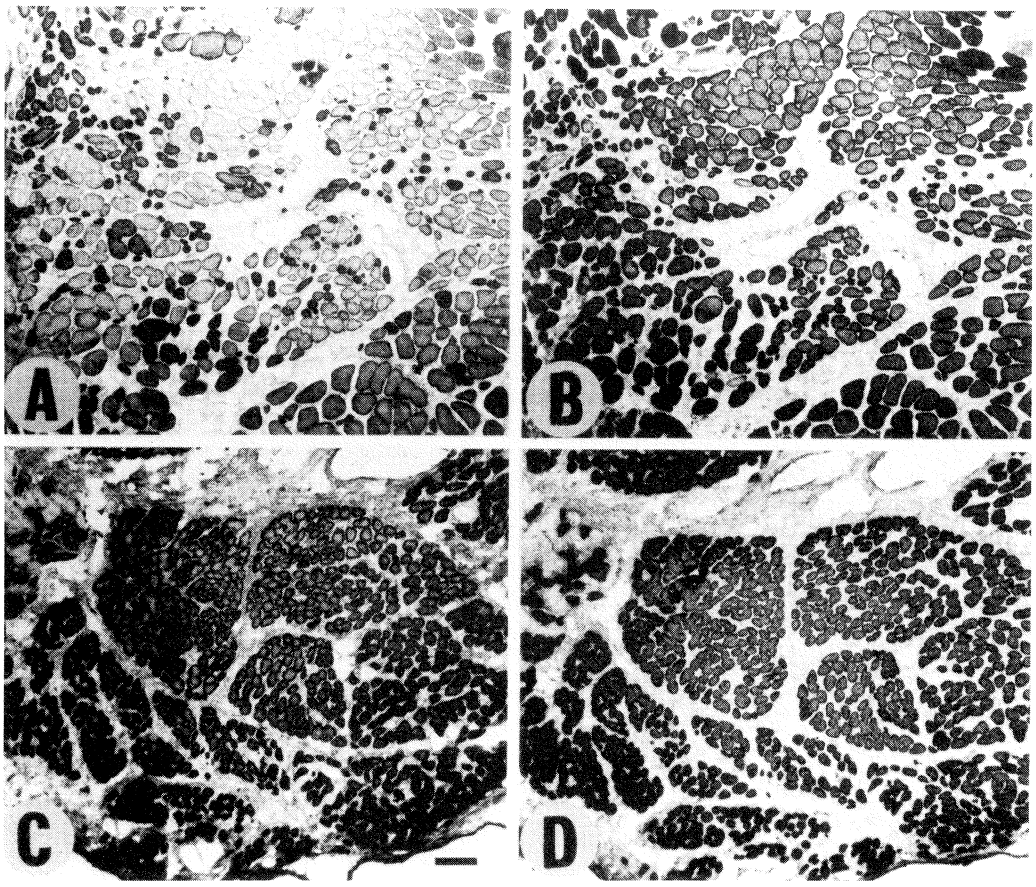

Figure 3. Immunoperoxidase staining of soleus (A & B) and the superfast temporalis muscle (C & D) regenerated in the soleus muscle bed and reinnervated by the nerve to this muscle. Serial sections of these muscle regenerates were stained using antibody 1A10 (A & C) and anti-slow myosin antibody (B & D).

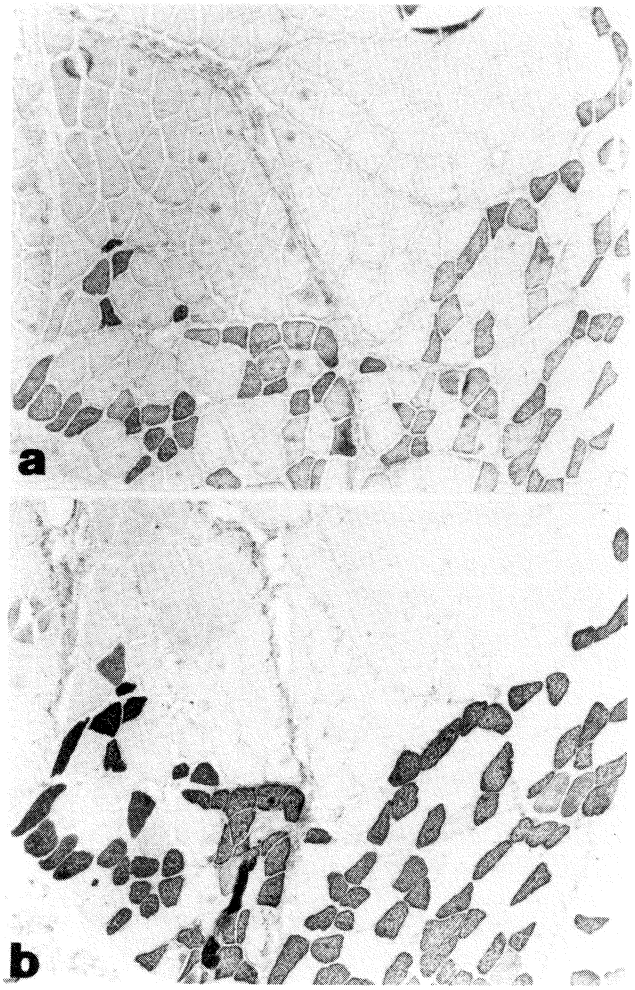

Figure 4. Immunoperoxidase staining of posterior temporalis muscle stimulated for 52 days continuously at 10 Hz. Serial sections were stained with antibody 1A10 (a) and anti-slow myosin antibody (b).

(Fig. 2E) as well as by antibody 1A10 (Fig. 2H). The 3 slow fibres in the EDL muscle are stained only by the anti-slow myosin antibody (Fig. 2F), and not by 1A10 (Fig. 2H). In contrast, the 4 slow fibres in the masseter are as strongly stained by 1A10 (Fig. 2D) and by the anti-slow myosin antibody (Fig. 2B). This reaction with 1A10 is not due to the presence of foetal or fast myosins in the slow fibres, as masseter slow fibres were not reactive with the anti-foetal (Fig. 2C) and anti-fast myosin antibodies [14], and also confirmed in the present study. Moreover, Fig. 1b, in showing that 1A10 does not react in Western blots with masseter superfast MHC, excludes the presence of a fast-like MHC in masseter which may co-migrate with superfast MHC in SDS gels.

Slow myosin in regenerated and stimulated jaw muscles

Superfast muscle fibres can be induced to express slow myosin in two situations: when a regenerating superfast

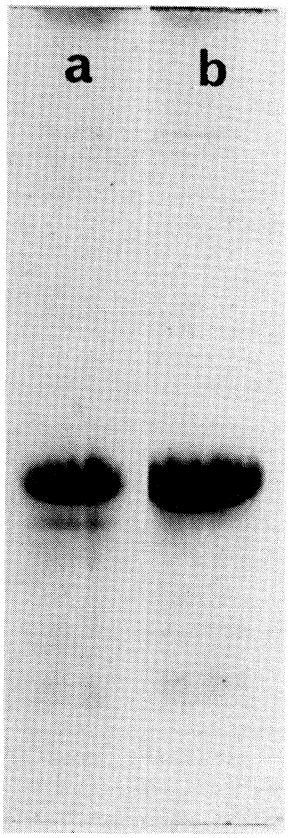

Figure 5. SDS gel electrophoresis of MHCs in 6% polyacrylamide gels. The samples are from cryostat sections of (a) cat temporalis muscle stimulated electrically at low frequency continuously for 52 days and (b) unstimulated, control temporalis muscle.

muscle is innervated by the nerve to a slow limb muscle [13] and when a superfast muscle is chronically stimulated at low frequency [12]. Figs. 3 and 4 show respectively that in each of the above situations, the transformed slow muscle fibres react with 1A10 immunocytochemically.

Fig. 3 shows immunocytochemistry of the slow soleus (A and B) and the superfast temporalis muscle (C and D) regenerated in the soleus muscle bed and reinnervated by the nerve to this muscle. Serial sections of these regenerates are stained using antibody 1A10 (A and C) and anti-slow myosin antibody (B and D). Virtually all fibres in both muscles stain for slow myosin (Fig. 3B and D). Antibody 1A10 stains virtually all fibres of the temporalis regenerate (Fig. 3C), but only scattered fibres in regenerated soleus muscle (Fig. 3A). This latter reaction is against fast myosin (data not shown), consistent with an earlier report showing that long-term soleus regenerates innervated by their own nerve express slow and fast myosins [13]. The slow fibres in regenerated temporalis muscle innervated by the soleus muscle nerve therefore behaved immunocytochemically as the normal slow fibres in the masseter (Fig. 2) in reacting with both anti-slow and 1A10.

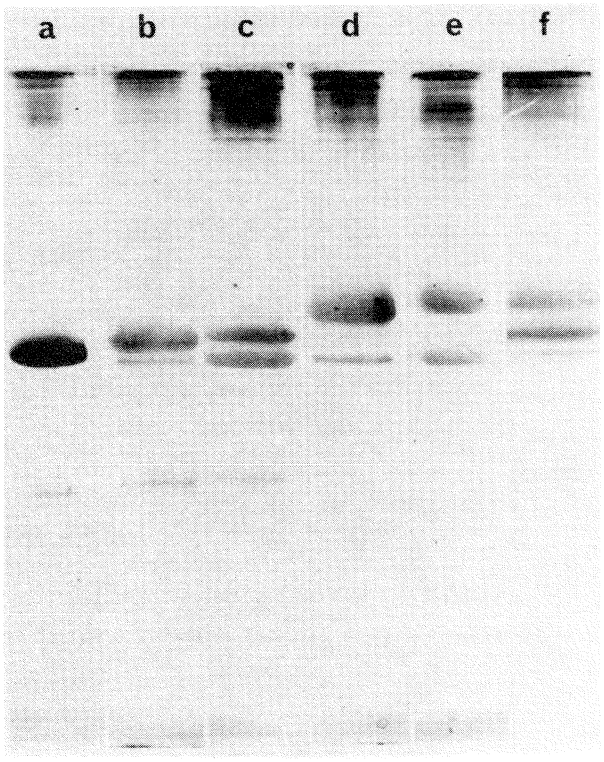

Figure 6. SDS gel electrophoresis of MHCs in 6% polyacrylamide gels. The samples are from cat soleus (a), masseter (b), a mixture of cat soleus and masseter (c), tibialis anterior (d), a mixture of soleus and tibialis anterior (e) and a mixture of masseter and tibialis anterior (f). Slow MHC from jaw and limb muscles failed to resolve into distinct components.

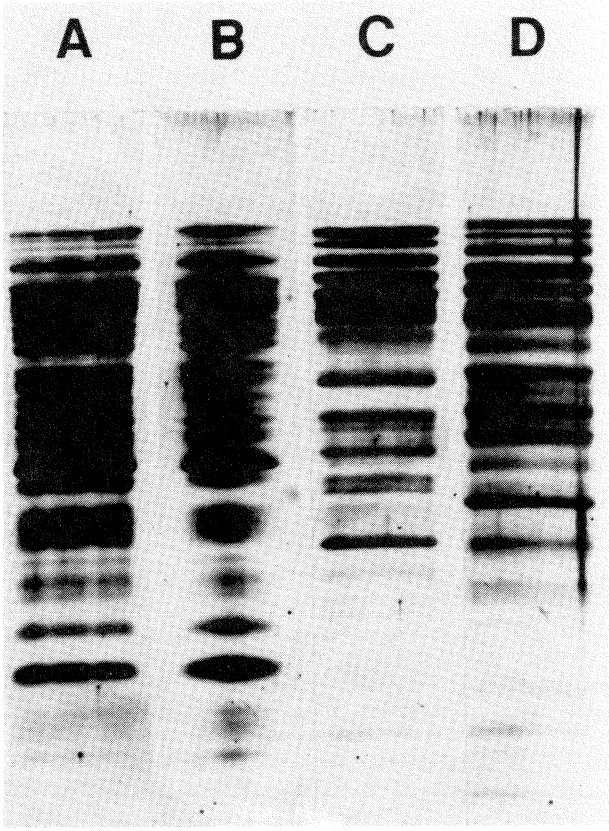

Figure 7. Silver stained CNBr peptide maps of limb slow MHC from the soleus muscle of neonatal kitten (A), adult cat soleus MHC (B), foetal MHC from neonatal kitten soleus (C) and fast MHC from adult tibialis anterior muscle (D).

Fig. 4 shows immunocytochemistry of posterior temporalis muscle which has been stimulated for 52 days continuously at 10 Hz. This muscle normally contains no slow fibres [17, 35]. The serial sections are stained with antibody 1A10 (Fig. 4a) and anti-slow myosin antibody (Fig. 4b). Of the fibres in Fig. 4a shown to be stained by the anti-slow antibody in Fig. 4b, 95% also stain with 1A10. Fig. 5 shows SDS gel electrophoresis of extracts of that portion of the stimulated muscle showing the presence of slow fibres (Fig. 5a) and the unstimulated control temporalis (Fig. 5b). The stimulated muscle shows a major superfast MHC component and a minor component of higher mobility, the slow MHC, but no fast MHC, which has a lower mobility than superfast MHC.The stimulated muscle fibres do not react with anti-fast MHC antibody (data not shown). These results suggest that the stimulated fibres reacting with 1A10 do not contain a mixture of slow and fast MHC.

Biochemical characterization of jaw-specific slow MHC

Attempts have been made to separate the jaw and limb slow MHC by SDS gel electrophoresis in 6% polyacrylamide gels. Fig. 6 shows the results of the analysis of MHCs from soleus, masseter and tibialis anterior muscles. Mixtures of jaw and limb slow MHCs (Fig. 6C, F) failed to resolve into distinct components.

In seeking evidence for a difference in primary structure between the slow MHCs of jaw and limb muscles, CNBr peptide maps of these and other MHCs were compared. In Fig. 7 the maps are for neonatal limb slow MHC (A), adult slow (B), foetal (C) and fast MHC (D). Maps for the MHCs from the two limb slow muscles (A and B) appear identical, but the technique readily reveals differences between slow, foetal and fast MHCs. Fig. 8 shows CNBr maps, from another run, of superfast MHC (a), slow MHC from a chronically stimulated temporalis muscle (b) and slow MHC from neonatal kitten soleus muscle (c). The map for the jaw slow MHC shows clear differences from that for the soleus muscle (arrows in Fig. 8), in that lacks a band (top arrow) present in the limb slow MHC map. A doublet can also be seen (lower arrow) in the stimulated jaw muscle MHC map while only a single band is seen in the corresponding region of the limb slow MHC. A comparison of Figs. 8a and 8b shows that these differences are unlikely

to be due to contamination of slow MHC from stimulated jaw muscle with superfast MHC.

Discussion

The results of this work show that the slow muscle fibres in the normal cat masseter react with a monoclonal antibody which binds to cat foetal and fast MHCs in Western blots. The antibody, however, does not bind to the superfast and slow MHCs of the masseter muscle in Western blots. The slow fibres in jaw muscles are unlikely to contain a mixture of slow and fast MHCs for the following reasons: 1) Slow fibres in jaw muscles do not react with an anti-fast MHC antibody which stains all limb fast muscle fibres (14, confirmed in this study). 2) Masseter muscle does not contain a fast MHC component which is electrophoretically distinct from superfast MHC (Fig. 1b, Fig. 6b), nor does it contain a fast-like (1A10 binding) MHC which has the same electrophoretic mobility as superfast MHC (negative Western blot, Fig. 1b). 3) Compared with the control, SDS gels of protein extracts from chronically stimulated temporalis muscle with fibres reactive against 1A10 and anti-slow antibodies reveal the presence of slow MHC but not fast MHC (Fig. 5). The protein that binds 1A10 is unlikely to be a non-myosin myofibrillar protein which shares a common epitope with slow MHC, since it does not bind any protein from the masseter separated on SDS gels. It is most likely that the antigen it binds in jaw tissue sections is a jaw-specific slow myosin isoform in its native conformation. Presumably this conformation is destroyed in SDS. In the course of raising monoclonal antibodies, our laboratory [1, 18] and others [38] have encountered monoclonal antibodies which reacted strongly on tissue sections, but failed to react with SDS denatured antigens in transblots.

The immunochemical difference between jaw-slow MHC and limb-slow MHC could be produced by one of three possible mechanisms: 1) alterations of antibody binding due to the presence of some protein bound to myosin, 2) posttranslational modification, and 3) the presence of jaw- and limb-specific slow MHC isoforms which differ in primary structure. The results of CNBr peptide mapping show a striking general similarity between the two slow MHCs. Specific differences in the pattern of peptides have been observed, however, and these exclude the first hypothesis.Posttranslational modifications of amino acid side chains in MHC that have been reported are the methylations of histidine [23] and lysine residues [22]. Phosphorylation could potentially affect serine and threonine residues. None of these reactions, however, are known to affect the CNBr cleavage of MHC, which occurs at methionine residues. Thus, a difference in CNBr peptide map between jaw- and limb-slow MHCs is very suggestive of a difference in primary structure of these slow MHCs. This problem can best be pursued using recombinant DNA methods. This laboratory has initiated work in this direction, and has isolated a cDNA clone from an expression library of a cat masseter muscle using an anti-slow MHC antibody. This clone shows considerable sequence homology with β-MHC genes in other species, but further work is necessary to ascertain whether it differs from the slow MHC gene expressed in cat limb muscles (Qin and Hoh, unpublished work).

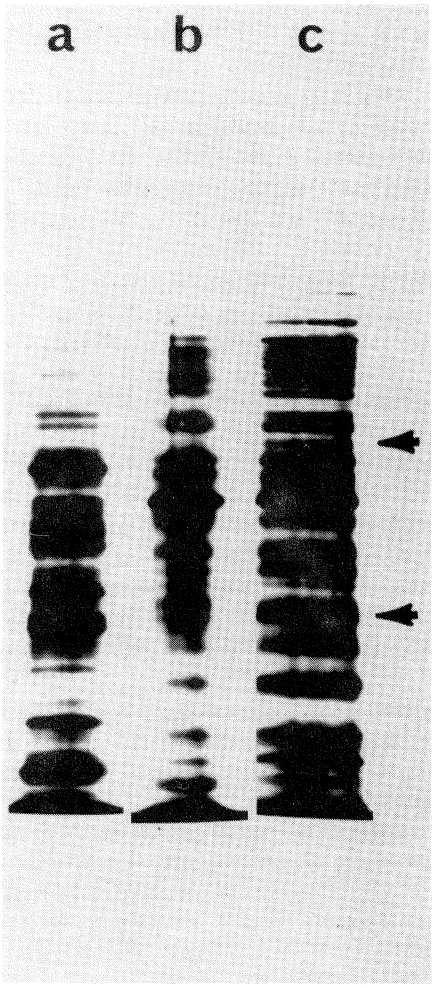

Figure 8. Silver stained CNBr peptide maps of superfast MHC (a), slow MHC from a chronically stimulated temporalis muscle (b) and slow MHC from neonatal kitten soleus muscle (c). The map for jaw slow MHC shows differences from that for soleus slow MHC in the regions indicated by the arrows.

Muscle fibres of the jaw allotype in the cat are known to differ from limb fibres in their ability to express superfast MHC, light chains, tropomyosin and several unidentified low molecular weight proteins [33-35]. These known jaw-specific myofibrillar proteins are all co-expressed in the same type of fibre, the superfast fibre [1]. The present work adds a jaw-specific slow MHC to this list of allotype-specific myofibrillar proteins which fibres of the jaw are competent to express. Further, this MHC is normally expressed in the minor population of slow fibres in cat jaw muscles, which hitherto could not be distinguished from

slow fibres of the limb. Thus, whatever the phenotypic option, fibres of the jaw allotype can now be distinguished fibres of the limb allotype.

When fibres in a jaw muscle regenerate innervated by a limb nerve express superfast myosin, it is easy to assert that alien innervation has not altered the fibre allotype. For regenerated jaw fibres expressing slow myosin, however, it has hitherto not been possible to rule out the hypothesis that reinnervation has transformed the allotype of these fibres. The discovery of a jaw-specific slow myosin phenotype has enabled us to refute this possibility. Our observations show that jaw fibres under the influence of the slow limb nerve have merely responded by expressing another jaw-specific phenotypic option. The effect of chronic electrical stimulation further shows that using another method known to perturb MHC gene expression in limb fast fibres, superfast fibres again respond in an allotype-specific manner by expressing jaw-specific slow MHC. These observations strongly reinforce the view [11], hitherto based on the expression of two jaw-specific markers, superfast MHC and tropomyosin, that the jaw muscle itself determines the subset of myofibrillar genes it is competent to express.

Superfast and slow fibres also occur in masticatory muscles of the dog and other carnivores [33, 34]. The structural difference between cat jaw- and limb-specific slow MHCs presumably applies to carnivores generally. This notion helps to explain the subtle difference in histochemical behaviour of canine jaw and limb slow fibres reported by Orvis and Cardinet [31]. Slow fibres of the jaw have a different pH sensitivity profile compared with slow fibres of the limb. Slow fibre ATPase is labile in alkali but stable in acid. For limb-slow fibres, preincubation of tissues in the pH range 4.6-4.3 leads to a stronger ATPase reaction compared with the histochemical reaction at pH 9.8. The staining of jaw-slow fibres were only mildly elevated with acid preincubation at pH 4.6-4.5 and was not enhanced with acid preincubation at pH 4.3. Further support for a jaw/limb difference in canine slow myosin is the observation that sera from dogs with masticatory myositis react with limb-slow fibres, but not with jaw-slow fibres [36]. It was not ascertained, however, whether the antisera reacted with MHC or some other limb slow fibre specific muscle protein.

The results of this work raise the question whether slow myosin in jaw-closing muscle in other mammals is generally different from slow myosin in limb muscle. There is some experimental evidence for a jaw/limb difference in slow MHC in the rabbit. Intact rabbit jaw-slow myosin is electrophoretically distinct from limb-slow myosin in pyrophosphate gels [29]. Further, peptide maps of these two slow myosins generated by enzymic digestion are also different [29]. These observations on a non-carnivore suggest that the jaw/limb difference in slow MHC may be applicable to all mammals generally.

There are also differences in non-slow fibres between jaw and limb muscles of non-carnivores. Superfast myosin also occurs in the jaw-closing muscles of primates, but curiously, it is apparently absent in man [34]. In non-human primates, superfast and slow fibres are present along with foetal myosin [34]. The expression of foetal myosin in adult jaw muscle is also characteristic of the several species of mammals [4]. The jaw muscles of the guinea-pig are sensitive to testosterone [28]. Furthermore, peptide maps of fast MHCs from the rabbit jaw have been shown to differ from those of the corresponding MHCs in the limb [29]. This is consistent with more recent evidence showing that rabbit jaw muscle expresses cardiac α-MHC [3, 5].

The slow skeletal MHC is encoded by the same gene as that which encodes cardiac β-MHC [27]. Several authors have described variants of ventricular β-MHC in various mammalian species. S1 nuclease mapping studies in the rabbit suggest that ventricular β-MHC mRNA is heterogeneous [7]. There is a microheterogeneity in the amino acid sequence of a fragment of the bovine β-MHC: at six positions in the sequence, two different amino acid residues are present, suggesting the presence of two distinct β-MHCs [9]. Immunochemical data also pointed to the presence of two β-MHC subtypes in the human ventricle [2]. Tsuchimochi *et al.* [38] also provided immunochemical evidence for β-MHC heterogeneity in the human heart. They further used monoclonal antibodies to isolate two subtypes of bovine β-MHCs and showed that they gave different peptide maps. Two forms of β-MHC can be resolved by SDS gel electrophoresis in the baboon [10] and the rat (RL Moss, personal communication). These studies strongly raise the possibility of variants of slow MHC in skeletal muscle.

We propose that the jaw-slow isoform of MHC described in this paper is the same as alternative form of β-MHC in the mammalian heart. This possibility is all the more intriguing in view of the recent observation that the rabbit masseter muscle expresses cardiac α-MHC [3, 5].

Acknowledgements

This work was supported by grants from the Muscular Dystrophy Association of the United States of America and the National Health and Medical Research Council of Australia. We thank Dr R. B. Fitzsimons for monoclonal antibodies NOQ-7-5-4D and NOQ-7-5-2B.

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