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Differences in myosin composition between human oro-facial, masticatory and limb muscles: enzyme-, immunohisto- and biochemical studies

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Summary

Immunohistochemistry was used to determine the myosin composition of defined fibre types of three embryologically different adult muscles, the oro-facial, masseter and limb muscles. In addition, the myosin composition in whole muscle specimens was analysed with biochemical methods. Both similarities and differences between muscles in the content of myosin heavy chains and myosin light chains were found. Nevertheless, each muscle had its own distinct identity. Our results indicated the presence of a previously undetected fast myosin heavy chain isoform in the oro-facial type II fibre population, tentatively termed 'fast F'. The masseter contained aberrant myosin isoforms, such as foetal myosin heavy chain and α-cardiac myosin heavy chain and unique combinations of myosin heavy chain isoforms which were not found in the limb or oro-facial muscles. The type IM and IIC fibres coexpressed slow and fast A myosin heavy chains in the oro-facial and limb muscles but slow and a fast B like myosin heavy chain in the masseter. While single oro-facial and limb muscle fibres contained one or two myosin heavy chain types, single masseter fibres coexpressed up to four different myosin heavy chain isoforms. Describing the fibres according to their expression of myosin heavy chain isozymes, up to five fibre types could be distinguished in the oro-facial and limb muscles and eight in the masseter. Oro-facial and limb muscles expressed five myosin light chains, MLC_{1S}, MLC_{2S}, MLC_{1F}, MLC_{2F} and MLC_{3F}, and the masseter four, MLC_{1S}, MLC_{2S}, MLC_{1F}, and, in addition, an embryonic myosin light chain, MLC_{1emb}, which is usually not present in normal adult skeletal muscle. These results probably reflect the way the muscles have evolved to meet the specialized functional requirements imposed upon them and are in agreement with the previously proposed concept that jaw and limb muscles belong to two distinct allotypes.

Introduction

Human limb muscle fibres are usually classified into different types on the basis of differences in their stability for myofibrillar ATPase reaction after alkaline and acid preincubations. At alkaline preincubation, fibres with low myofibrillar ATPase reactivity are lightly stained and termed type I, whereas fibres with high ATPase activity are darkly stained and termed type II. The type II fibres can be further subdivided into types IIA, IIB and IIC based on their sensitivity to acid preincubations (Dubowitz, 1985).

In adult human limb muscles, slow twitch myosin heavy chain (MHC) is present in type I fibres and is coexpressed with fast twitch MHC in type IIC fibres. Two types of fast twitch MHC, fast A MHC and fast B MHC or mixtures of both, have been identified in the type II fibre population in man (Billeter *et al.*, 1981; Schantz & Dhoot, 1987; Klitgaard *et al.*, 1990; Staron, 1991). Furthermore, two developmental MHC isoforms, embryonic and foetal, are expressed in developing muscles. As the muscle fibres differentiate and mature these developmental myosin isoforms are gradually replaced by the adult fast and slow MHC isoforms (Schiaffino *et al.*, 1986; Butler-Browne *et al.*, 1988, 1990; Barbet *et al.*, 1991). In addition, three fast myosin light chains (MLC) are associated with the adult fast MHCs and two slow type MLCs are associated with the adult

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slow MHC (Pette & Staron, 1990). There is also a MLC which is developmentally regulated (MLC_{1emb}) (Whalen *et al.*, 1978). The essential role of MLCs for physiological speeds of muscle contraction has recently been reported (Lowey *et al.*, 1993).

Human limb muscles, originating from the somites and supplied by the spinal nerves, are in general composed of a mixture of type I, IIA and type IIB fibres (Dubowitz, 1985). The human oro-facial muscles, which originate from the second branchial arch and are facial nerve innervated, differ in several aspects from limb muscles (Stål *et al.*, 1987, 1990). The major and minor zygomatic muscles have a marked predominance of type II fibres, the majority of which have a staining intensity in between that of type IIA and type IIB (pH 4.6) and have been called type IIAB (Stål *et al.*, 1987). The orbicularis oris muscle has a predominance of type IIA and IIAB fibres, whereas the buccinator muscle contains about equal proportions of type I and type IIA fibres. Typically, both these muscles lack type IIB fibres (Stål *et al.*, 1990). The human masseter muscle, which originates from the first branchial arch and is innervated by the trigeminal nerve, differs in fibre type composition from both the oro-facial and the limb muscles. It is characterized by a predominance of type I fibres, small type IIB fibres and fibres with an intermediate ATPase staining at alkaline pH, type ATPase-IM (IM) and type IIC fibres (Ringqvist, 1974; Eriksson & Thornell, 1983). Using enzyme- and immunohistochemical techniques we have previously shown that the human masseter type IM and IIC fibres contain a mixture of slow and fast MHC isoforms in variable amounts (Thornell *et al.*, 1984). We have also found in the human masseter, that developmental isoforms of both myosin heavy and light chains are normally present in the adult muscle (Butler-Browne *et al.*, 1988; Eriksson *et al.*, 1994). The special isomyosin composition of the human masseter is further supported by the recent observation that an α-cardiac MHC is also present in this muscle (Bredman *et al.*, 1991; Pedrosa-Domellöf *et al.*, 1992). These findings indicate that unusual combinations of myosins are characteristic of this muscle. However, the fibres containing these myosin isoforms can not be identified by their ATPase staining pattern, indicating that muscle fibres which are defined enzyme-histochemically as the same type may differ in myosin composition. For the oro-facial muscles the myosin composition is at present unknown.

The aim of the present investigation was to analyse in further detail the expression of the various myosin isoforms in enzyme-histochemically defined muscle fibre types in four oro-facial and the masseter muscles. Enzyme- and immunohistochemical techniques were used to determine which MHC isoforms were present in the different fibre types, and electrophoretic methods were used to confirm the MHC and MLC compositions of the different muscles. For comparison we have also analysed a large arm and a small hand muscle.

Materials and methods

Muscle specimens

Specimens from the middle part of the major and minor zygomatic, from the superior and inferior parts of the orbicularis oris and the buccinator muscles and from the posterior and anterior portions of the masseter muscle, were obtained from the left side of five previously physically healthy subjects aged between 15–41 years (mean age 28.2) with normal cranio-facial morphology and a complete or nearly complete dentition in a normal occlusion. Samples from the biceps brachii and the first dorsal interosseus (FDI) muscles were taken for comparison. The specimens were obtained 1–3 days post mortem, a delay which does not hamper reliable fibre typing (Eriksson *et al.*, 1980). All samples, except for the masseter specimens, were identical to those used in previous studies by Stål and colleagues (1987, 1990). The investigation was approved by Socialstyrelsen, The National Board of Health and Welfare, Stockholm, Sweden.

Enzymehistochemical method for fibre typing

The muscle specimens were mounted in OTC compound (Tissue Tek®, Miles laboratories Naperville, IL, USA), frozen in liquid propane chilled with liquid nitrogen and cut in a cryostat microtome at −20°C into 5–10 μm thick serial cross-sections. The sections were stained for the demonstration of myofibrillar ATPase activity (EC 3.6.1.3) after either alkaline (pH 10.3) or acid (pH 4.6 and 4.3) preincubations (Dubowitz, 1985; Stål *et al.*, 1990). The classification of muscle fibres into different types based on their staining intensity for myofibrillar ATPase at alkaline and acid pH, is shown in Fig. 1.

Data on the fibre type composition of the different muscles have been taken from previous studies (Eriksson & Thornell, 1983; Stål *et al.*, 1987, 1990). On the basis of the present muscle fibre type classification, the original data for the biceps, FDI, buccinator and orbicularis have been modified to include the type IIAB fibres.

Antibodies

Previously characterized monoclonal (mAb) and polyclonal antibodies (Ab) against different MHC isoforms were used (Table 1).

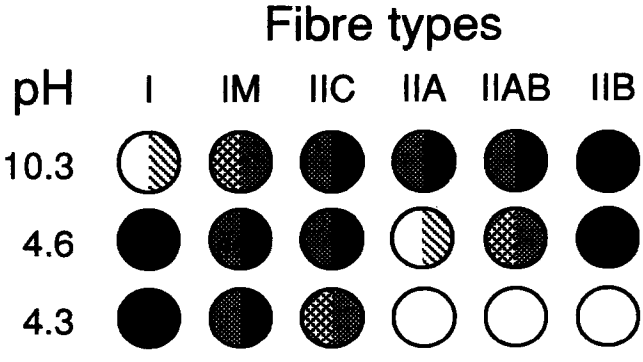

Fig. 1. Enzyme-histochemically defined fibre types in human limb, oro-facial and masseter muscles based on the staining reactions for myofibrillar ATPase at alkaline and acid pH. The various degrees of shading illustrate different degrees of weak (light), moderate, and strong (dark) staining.

Table 1. Reactivity of the different antibodies with different isoforms of MHC

Ab	*Referred to in the text*	*MHC isoform detected by the Ab*	*Reference*
BA-D5	anti-S	slow	Schiaffino and colleagues, 1989
X_1H_{10}	anti-S	slow	Schiaffino and colleagues, in prep.
SC-75	anti-F	fast	Schiaffino and colleagues, 1989
SC-71	anti-FA	fast A	Schiaffino and colleagues, 1989
BF-32	anti-SFA	slow and fast A	Schiaffino and colleagues, 1989
VII_1H_3	anti-SFB	slow and fast B	Schiaffino and colleagues, in prep.
NN5	anti-foetal	foetal	Butler-Browne and Whalen, 1984, 1990
2B6	anti-emb	embryonic	Gambke and Rubinstein, 1984
F88112F8	anti-α-cardiac	α-cardiac	Leger and colleagues, 1985/Sera-Lab, Sussex, England
ALD 19	anti-ST	slow tonic	Sawchak and colleagues, 1985 Thornell and colleagues, 1989

All antibodies are monoclonal except for the NN5 which is polyclonal.

Immunohistochemical methods

Immunohistochemical detection of bound antibody was performed either by indirect immunofluorescence as previously described by Butler-Browne and Whalen (1984) or with the immunoperoxidase staining technique of Sternberger (1979).

Sections serial to those used for enzyme-histochemical fibre typing, were mounted on glass slides, air dried, and incubated with 5% normal rabbit serum (Dakopatts, Glostrup, Denmark) for 15 min to inhibit unspecific staining. The sections prepared with the polyclonal antibody against foetal MHC were incubated with 5% normal swine serum (Dakopatts). All sections were then incubated with primary Abs diluted to appropriate concentrations with 0.01 M phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) for 60 min in a humid chamber at 37°C. After washing in 0.01 M PBS for 15 min, the sections were incubated with fluorescine conjugated rabbit anti-mouse IgG or mouse peroxidase antiperoxidase (PAP) (Dakopatts) for 30 min at room temperature. The sections prepared with the polyclonal anti-body against foetal MHC were incubated with fluoresceine conjugated swine anti-rabbit IgG or rabbit peroxidase antiperoxidase (Dakopatts). After repeated washes, the immunofluoresceine treated sections were mounted with Mowiol 4-88 (Hoechst, Frankfurt, Germany). Peroxidase binding was revealed by applying a solution containing diamino-benzidine (Sigma D5905) and hydrogen peroxidase (H_2O_2) for 5–10 min. Finally, the sections were rinsed with running water for 5 min, dehydrated in graded ethanol's followed by xylene treatment and mounted with DPX (BDH, Limited Poole, UK). Control sections were treated as described except that the incubation with the primary antibody was omitted.

The sections were examined using a Leitz Dialux (Leitz Italiana, Milano, Italy) or an Olympus Vanox, model AH-2 microscope (Olympus Optical Co. Ltd, Tokyo, Japan). The microscopes were equipped with epifluorescence for the examination of the antibody staining activity. Each muscle section was photographed in 2–4 areas. The classification of fibres and the evaluation of the relative frequency of fibres containing different MHC isoforms were performed at a magnification of 160×. Between 100 to 500 fibres were examined in each muscle of each subject. In all, more than 10000 fibres were evaluated in this study.

Biochemical analysis

The MLCs were analysed by two-dimensional (2-D) gel electrophoresis. Muscle extracts were prepared as described previously (Butler-Browne *et al.*, 1990). Electrophoresis was carried out according to O'Farrel (1975). Isofocusing in the first dimension was carried out between pH 4 and 6. Proteins were separated in the second dimension on 13% SDS polyacrylamide slab gels. The myosin heavy chains were analysed on 6% SDS-glycerol gels according to the technique of Carraro and Catani (1983). The separating gel contained 35% glycerol. Gels were migrated for 20–24 h at 120 V. The different native isoforms of myosin were identified by non-denaturing pyrophosphate gel electrophoresis (Butler-Browne *et al.*, 1990). Gels were stained in Coomassie Brilliant Blue prior to being photographed.

Results

ENZYMEHISTOCHEMISTRY

Type I, IM, IIC, IIA, IIAB and IIB fibres were detected in all limb and oro-facial muscles (Figs 2A, B, D, E, G, H, 3A–C), except for the buccinator and orbicularis oris muscles which lacked type IIB fibres (Fig. 2B, E, H). In the limb and oro-facial muscles the type IM and IIC fibres were rare. In the masseter muscle, type I, IM, IIC and IIB were generally detected, whereas type IIA were rare (Figs 2C, F, I, 4A–C).

IMMUNOHISTOCHEMISTRY

The pattern of antibody staining intensity of the different fibre types and muscles are summarized in Figs 5 and 6. Type IIA, IIAB and IIB fibres showed two different patterns of staining in the limb and oro-facial muscles and type IM, IIC and IIB fibres showed three different staining patterns in the masseter.

Anti-S (BA-D5, X_1H_{10})

The two mAbs gave identical results and stained the type I fibres strongly, the type IM moderately to strongly and the type IIC fibres weakly to strongly (Figs 2J–L, 3D). In general, the type IM fibres showed stronger staining than the type IIC fibres. In one subject a very small number

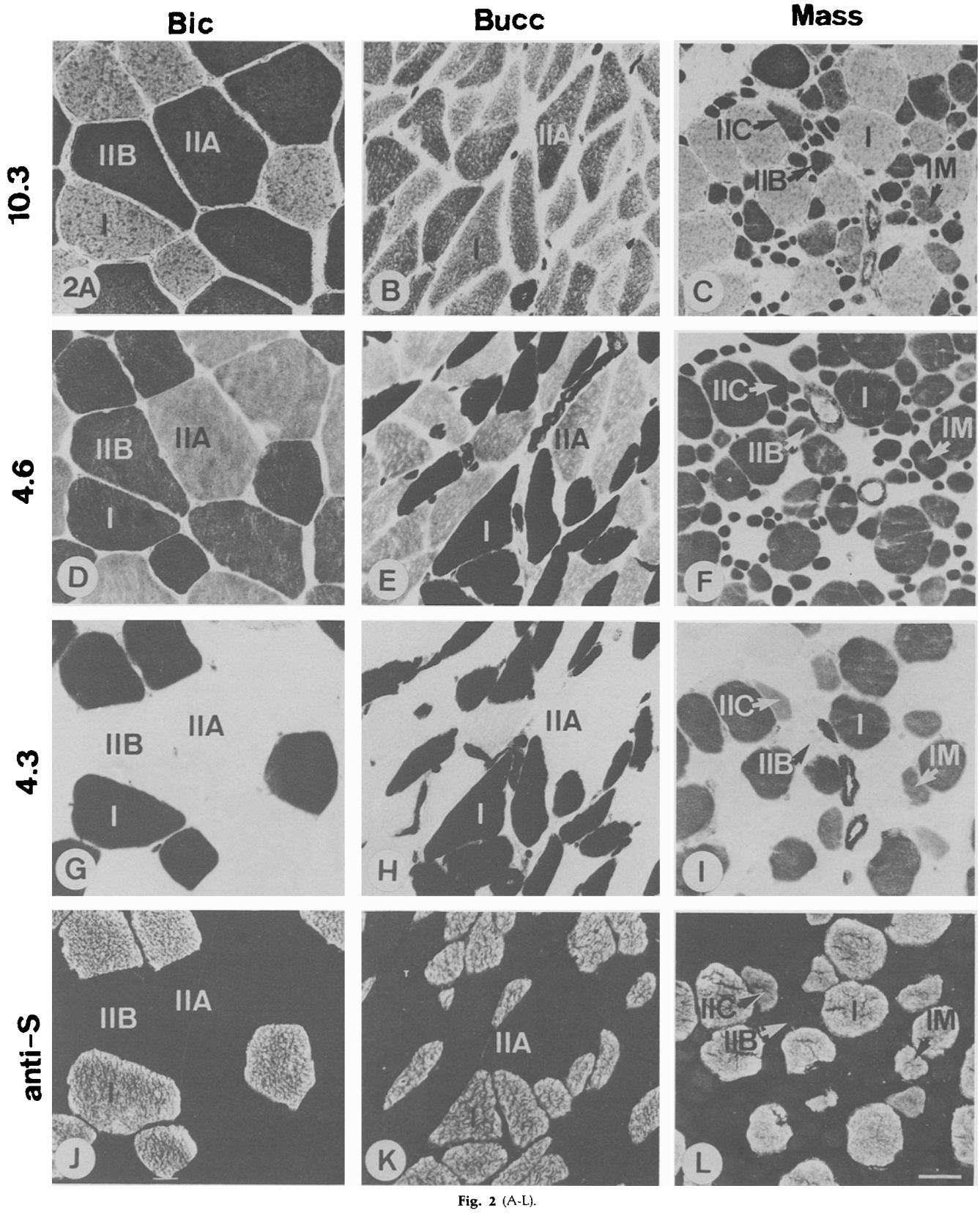

Fig. 2 (A-L).

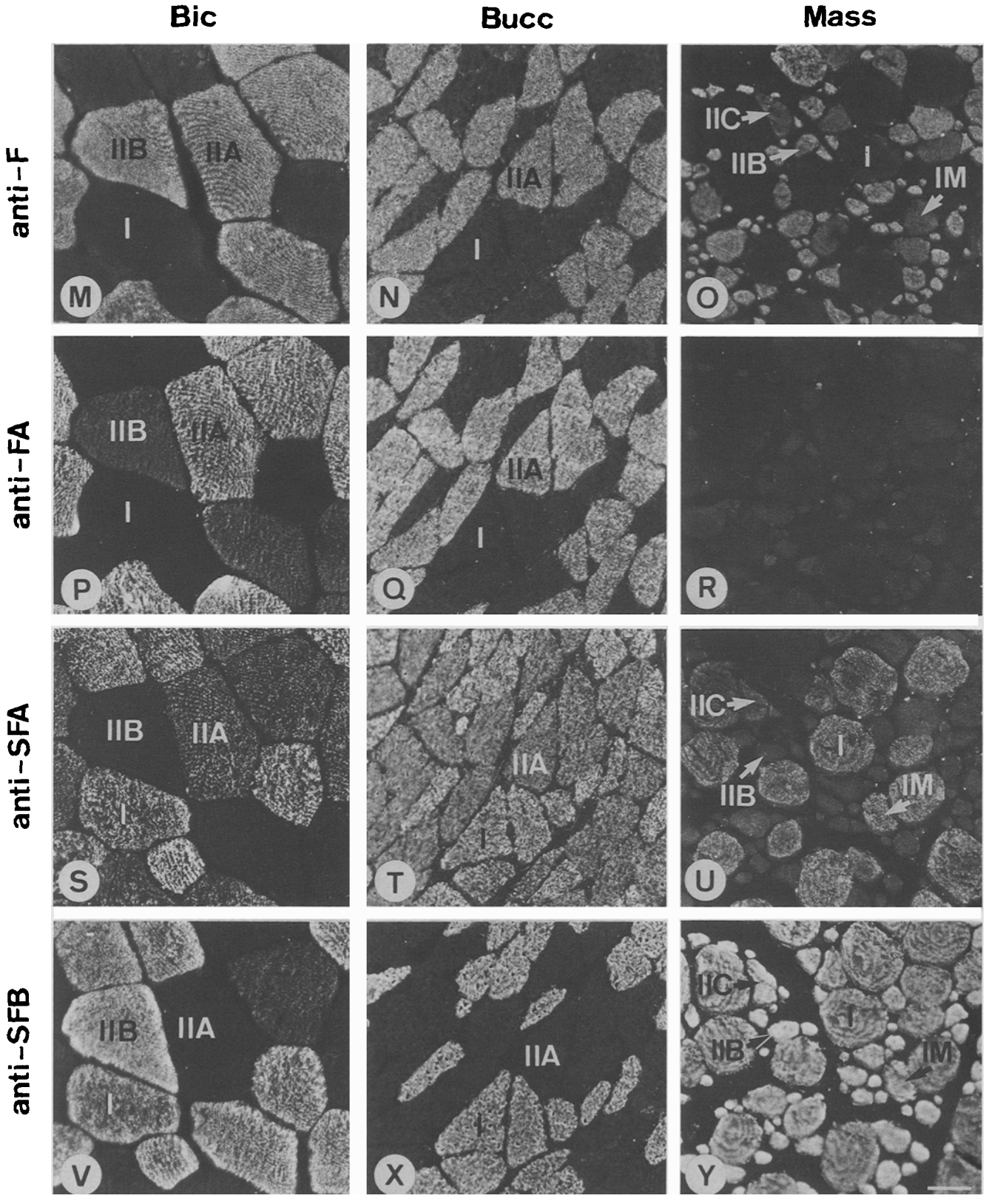

Fig. 2. Serial muscle cross-sections from the biceps brachii (Bic, left column), buccinator (Bucc, middle column) and posterior superficial portion of the masseter (Mass, right column) stained for the demonstration of myofibrillar ATPase at pH 10.3 (A–C), 4.6 (D–F), 4.3 (G–I), and for indirect immunofluorescense with anti-S (J–L), anti-F (M–O), anti-FA (P–R), anti-SFA (S–U) and anti-SFB (V–Y) mAbs. Fibre types I, IM, IIC, IIA and IIB have been labelled. Note that the masseter type IM and IIC fibres are unstained with the anti-FA mAb (R). ×160. Scale bars = 50 μm.

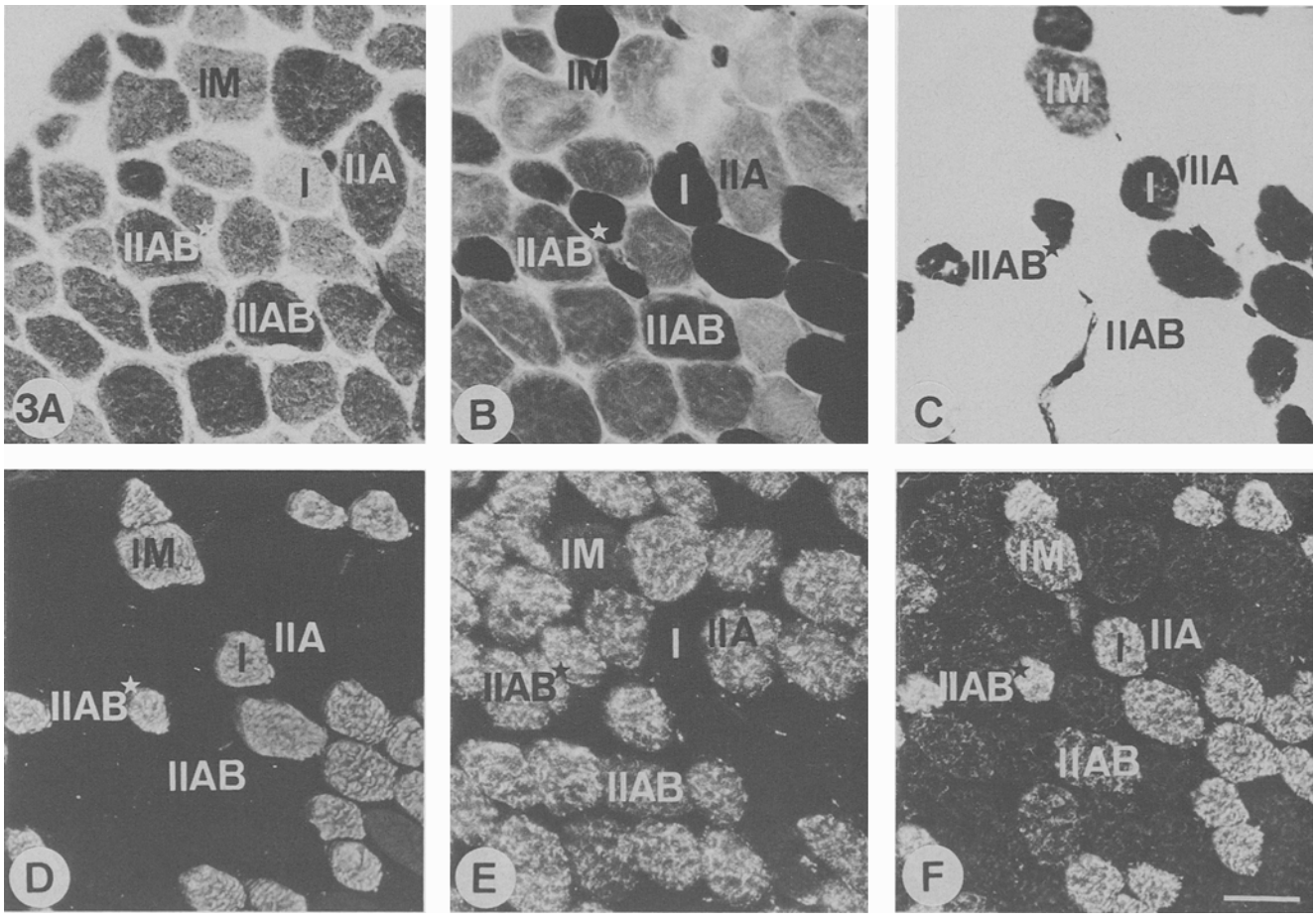

Fig. 3. Serial cross-sections from the major zygomatic muscle stained for mATPase at pH 10.3 (A), 4.6 (B) and 4.3 (C) and with indirect immunofluorescence with anti-S (D), anti-FA (E) and anti-SFB (F) mAbs. Fibre types I, IM, IIA, and IIAB have been labelled. Note two different patterns of antibody staining for IIAB fibres, one with staining for both the anti-FA and the anti-SFB and another with staining exclusively with the anti-FA mAb (IIAB*). Note also the staining of the type IM fibre with all three mAbs. ×200. Scale bar = 50 μm.

of the masseter type IIC fibres (<1%) were not stained with these antibodies (Fig. 4D, not shown in Fig. 6).

Anti-F (SC-75)

The type IIA and IIAB fibres stained strongly and type IIB fibres moderately to strongly with this mAb. The type IM fibres were weakly to moderately stained. The type IIC fibres were stained moderately to strongly in the limb and oro-facial muscles and moderately in the masseter (Fig. 2M–O).

Anti-FA (SC-71)

Type IIA fibres stained strongly and type IIAB weakly to strongly. In the limb and oro-facial muscles, 60–100% of the type IIB fibres were weakly stained and in the masseter 10% were weakly stained. The type IM and IIC fibres were moderately to strongly stained in the limb and oro-facial muscles and unstained in the masseter (Figs 2P–R, 3E, 4E).

Anti-SFA (BF-32)

Type I, IM, IIA and the limb and oro-facial type IIC fibres were stained moderately to strongly, and the masseter type IIC fibres moderately. Type IIAB fibres were weakly to moderately stained. A majority, 60–100%, of the limb and oro-facial type IIB fibres and 10% of the masseter IIB fibres were weakly stained (Fig. 2S–U).

Anti-SFB ($V_{11}H_3$)

The Type I, IM and IIB fibres were stained moderately to strongly. The strongest staining intensity was found in the small diameter (~10 μm) type IIB fibres in the masseter. The type IIC fibres were stained moderately in the limb and oro-facial muscle fibres, and weakly to moderately in the masseter. The type IIA fibres were weakly stained in 5–15% of the limb muscles, 20% of the minor zygomatic and 25% of the orbicularis oris. Type IIAB fibres were weakly to strongly stained in the limb muscles. In the oro-facial muscles, 20–65% of the type

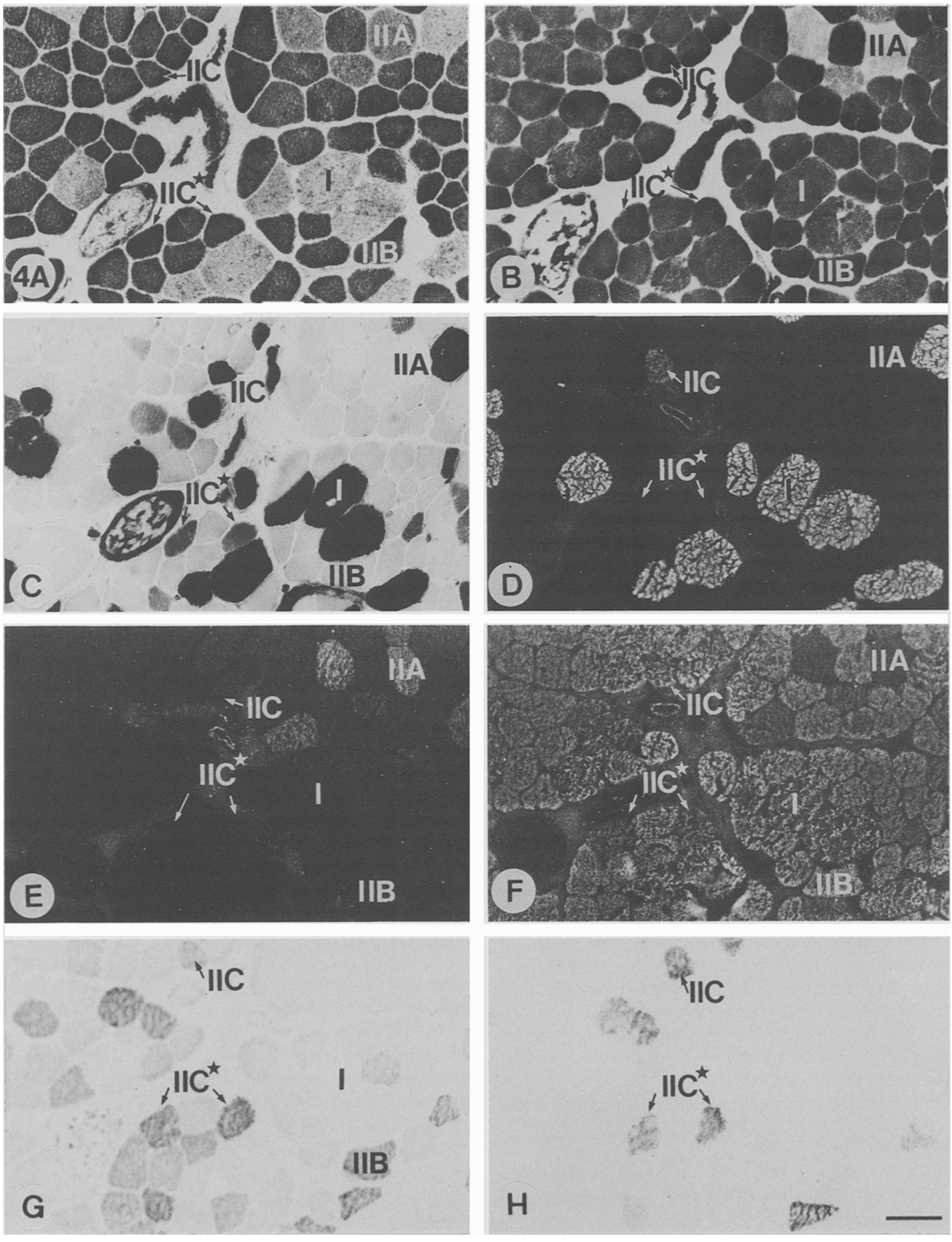

Fig. 4. Serial cross-sections from the anterior superficial portion of the masseter stained for mATPase at pH 10.3 (A), 4.6 (B) and 4.3 (C), indirect immunofluorescence with anti-S (D), anti-FA (E) and anti-SFB (F) mAbs, and indirect immunoperoxidase with anti-foetal (G) and anti-α-cardiac (H) Abs. Types I, IM, IIC and IIA fibres have been labelled. Note the two type IIC fibres (IIC) unstained with the anti-S mAb. ×200. Scale bar = 50 μm.

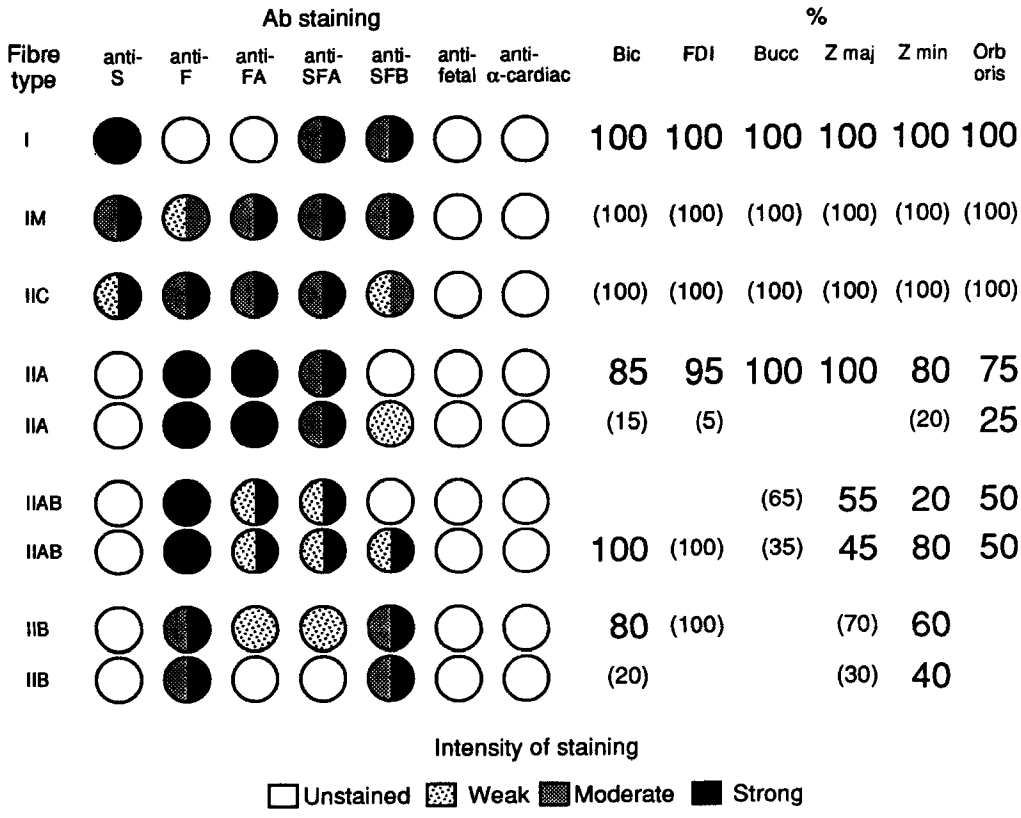

Fig. 5. Scheme on the staining intensity patterns of muscle fibre types I, IM, IIC, IIA, IIAB and IIB with anti-S, anti-F, anti-FA, anti-SFA, anti-SFB, anti-foetal and anti-α-cardiac antibodies, in the biceps brachii (Bic), first dorsal interosseus (FDI), buccinator (Bucc), major zygomatic (Z maj), minor zygomatic (Z min) and orbicularis oris (Orb oris) muscles. Note two different patterns for type IIA, IIAB and IIB fibres. The relative proportion (%) of each fibre type and in each muscle displaying the different staining patterns is shown to the right in the figure. Antibody staining patterns found in less than 5% of the total of the population fibre type are shown in parenthesis.

IIAB fibres were weakly to strongly stained, the rest was unstained (Figs 2V–Y, 3F, 4F).

Anti-foetal (NN5)

This Ab reacted almost exclusively with the masseter muscle, 65% of the type IIC and IM and 40% of the type IIB fibres. The staining was weak to moderate in the type IM fibres and moderate to strong in the type IIC and IIB fibres (Fig. 4G). In some muscle samples, the anti-foetal Ab reactive fibres were evenly distributed throughout the muscle cross-section, whereas in others, clusters or only a few fibres were found. No extrafusal fibre staining was found in any of the limb and oro-facial muscles except for some rare type IIC and IIAB fibres (<0.5% of the total fibre population) in three out of the ten zygomatic muscles.

Anti-emb (2B6)

This mAb did not stain any extrafusal muscle fibres, except for occasional type II fibres in eight out of the ten orbicularis oris muscles.

Anti-α-cardiac (F88112F8)

The anti-α-cardiac mAb reactivity was present in about 15% of the masseter type IM and IIC fibres, and was not found in any of the other muscles examined. The type IM fibres were stained weakly and the type IIC weakly to strongly (Fig. 4H). In general, the stained fibres were unevenly distributed throughout the muscle cross-section and in some areas up to 50% of the type IIC and IM fibres were anti-α-cardiac mAb positive. All anti-α-cardiac mAb reactive fibres were also reactive with the anti-foetal Ab.

Anti-ST (ALD 19)

No extrafusal fibres were stained with the anti-ST mAb.

Myosin heavy chain expression of the different muscles and fibre types, as evaluated from the antibody staining patterns.

Figure 7 and Table 2 show for different muscles the content of MHC isoforms, in comparison to muscle fibre type compositions. Figure 8 shows the relative distribution of MHC isoforms in the fibre types of the different muscles.

BIOCHEMISTRY

Table 3 summarizes the results showing the content of MLC, MHC and native isoforms in the different muscles.

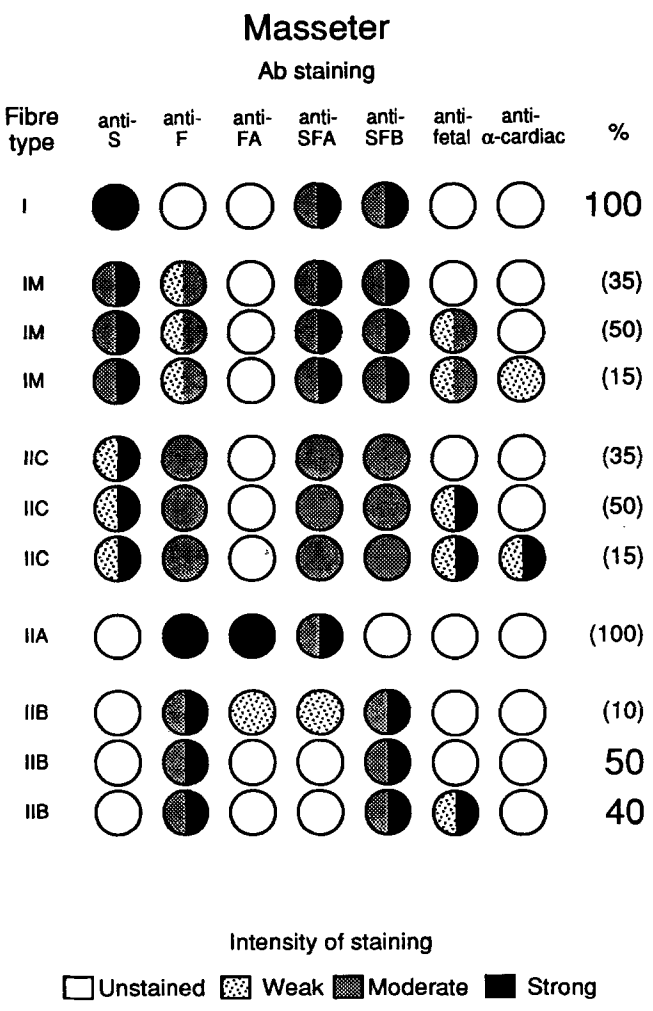

Fig. 6. Scheme on the staining intensity patterns of muscle fibre types I, IM, IIC, IIA, IIAB and IIB with anti-S, anti-F, anti-FA, anti-SFA, anti-SFB, anti-foetal and anti-α-cardiac antibodies in the masseter muscle. Note three different patterns for type IM, IIC and IIB fibres. The proportion (%) of each fibre type group displaying the different staining patterns is given to the right. Antibody staining patterns found in less than 5% of the total population of the fibre type are shown in parenthesis.

Myosin light chain isoforms

All muscles, except the masseter, had a full complement of three fast (MLC_{1F}, MLC_{2F}, MLC_{3F}) and two slow (MLC_{1S}, MLC_{2S}) myosin light chains (Fig. 9A–F). The biceps, FDI and buccinator contained roughly equal amounts of fast and slow MLCs. The zygomatic major contained slightly more fast than slow MLCs, whereas the orbicularis oris and zygomatic minor contained predominantly fast MLCs. The masseter expressed a characteristic pattern, different from that of the other muscles, containing MLC_{1F}, MLC_{1S}, MLC_{2S} and the embryonic MLC (MLC_{1emb}) and lacking MLC_{2F} and MLC_{3F} (Fig. 9G).

Myosin heavy chain isoforms

Four MHC isoforms could be identified by migration on 6% polyacrylamide-SDS-glycerol gels. Three bands were identified according to Klitgaard and colleagues (1990b) and Staron (1991) as corresponding to fast A, fast B and slow MHCs. A fourth band which was detected only in the masseter muscle was identified as corresponding to foetal MHC (Edom *et al.*, 1994). This band migrated just underneath the fast A MHC. The gel pattern is shown in Fig. 10. Each muscle had a characteristic profile of MHCs. All biceps muscles contained three bands with approximately 50% slow MHC, 25% fast A MHC and 25% fast B MHC. The FDI, buccinator, zygomatic major and orbicularis oris contained only two bands, corresponding to slow and fast A MHC. In the FDI, the slow MHC was the predominant species, whereas in the buccinator, there was almost equal amounts of slow MHC and fast A MHC. In the zygomatic major and orbicularis oris, the fast A MHC band predominated. In the zygomatic minor three bands were present, the slow MHC was the weakest band and fast A and fast B MHC were present in amounts which varied from subject to subject. In the masseter four bands could be detected. The slow band was present in the largest amount and fast B MHC and foetal MHC in variable amounts. A small band at the level of fast A MHC was also detected in one subject.

Native myosin isoforms

The separation of the native myosin isoforms present in extracts of the seven different muscles is shown in Fig. 11. Analysis of the two limb muscles (Bic and FDI) using this technique showed that the myosin from these muscle extracts could be resolved into three fast twitch isoforms (FM1, FM2, FM3) and one or two slow isoforms (SM1, SM2). In the oro-facial muscle group each muscle had a characteristic myosin pattern. The buccinator had a pattern very similar to that found in the limb muscles with one slow band (SM2) and three fast twitch bands (FM1, FM2, FM3). In the zygomatic major, zygomatic minor and orbicularis oris again both fast and slow twitch bands could be identified but the band at the FM3 level was resolved as a doublet. The masseter contained predominantly slow myosin (SM2) although small amounts of fast (FM3) and foetal myosin (f3, f2) were also detected.

In order to visualize the bands more clearly, gels were photographed and printed at a higher magnification (Fig. 12). At this resolution six bands could be distinguished in the major and minor zygomatic and orbicularis oris muscles. The two slowest migrating bands corresponded to SM1 and SM2. Four fast twitch bands could be resolved in these oro-facial muscles. The three fastest migrating bands corresponded to FM1, FM2 and FM3. The fourth band, here termed FM4, was of equal intensity to FM3 and migrated close above this band. The presence of FM4 as a unique band was confirmed by comigration with extracts from the different muscles. The biceps, which contains about equal amounts of type IIA and IIB

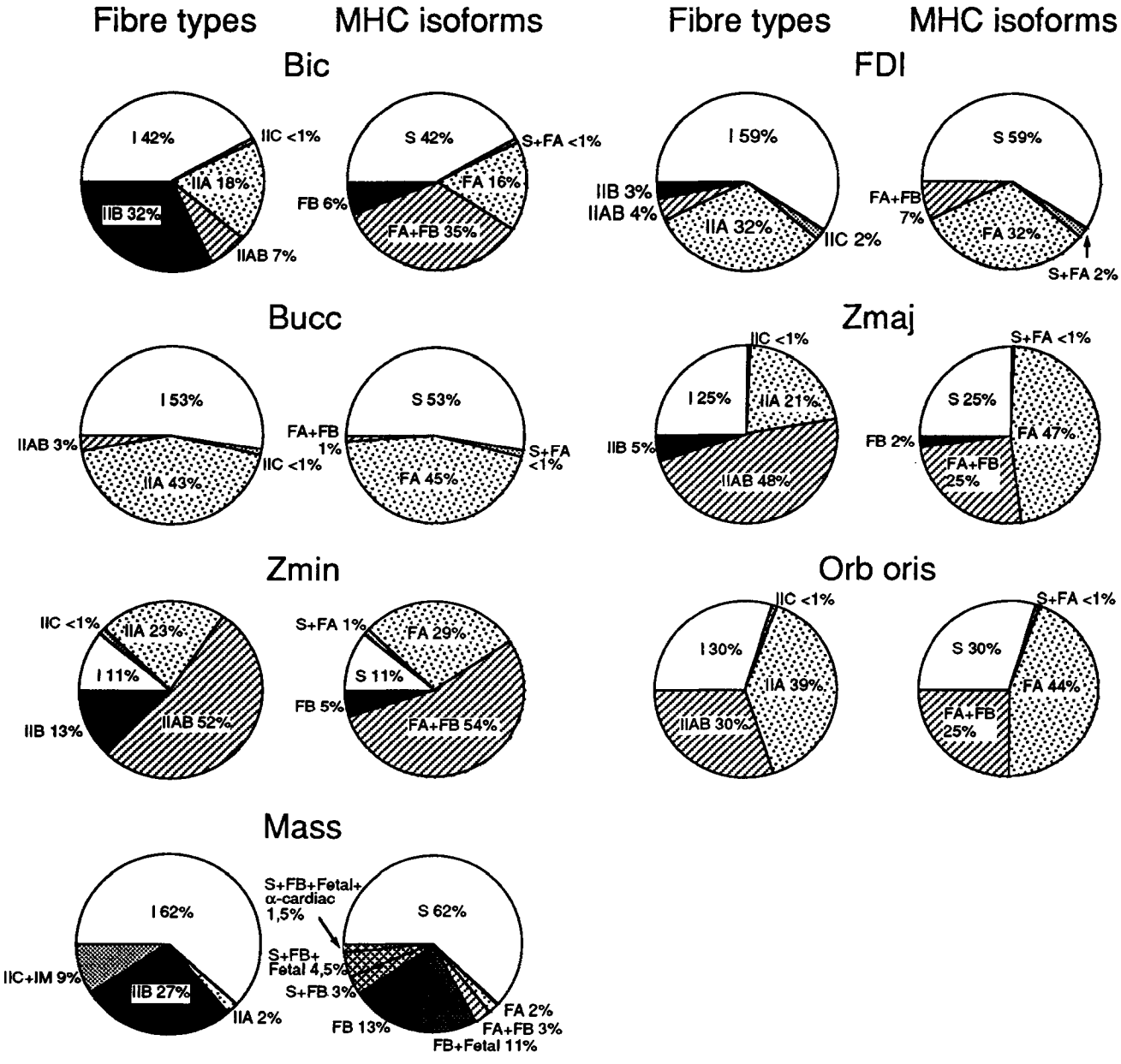

Fig. 7. Proportion (%) of fibre types I, IM, IIC, IIA, IIAB and IIB, according to Eriksson and Thornell (1983) and Stål and colleagues, (1987, 1990), and proportion (%) of the total muscle fibre population containing slow (S), fast A (FA), fast B (FB), foetal and α-cardiac MHC isoforms, as evaluated from the antibody staining pattern, in the biceps brachii (Bic), first dorsal interosseus (FDI), buccinator (Bucc), major zygomatic (Z maj), minor zygomatic (Z min), orbicularis oris (Orb oris) and masseter (Mass) muscles. In the limb and oro-facial muscles the few type IM fibres have been included in the type IIC fibre group. Note combinations of MHC isoforms, especially prominent in the masseter muscle.

fibres, and the buccinator, which contains only type IIA fibres, were always resolved as three fast bands, FM1, FM2 and FM3. Comigration of these two muscle extracts (data not shown) gave also three fast bands FM1, FM2 and FM3. However, comigration of these samples with the zygomatic or orbicularis oris muscles always revealed a doublet at the level of FM3 due to the presence of the extra band, FM4 (Fig. 12).

Discussion

Several approaches were used to analyse the myosin composition in five cranial nerve and two spinal nerve innervated muscles. We show that although these muscles do have certain features in common, each muscle has its own distinct identity. Our observations of intermuscular diversities in MHC composition are in agreement with the concept introduced by Hoh and colleagues (1989)

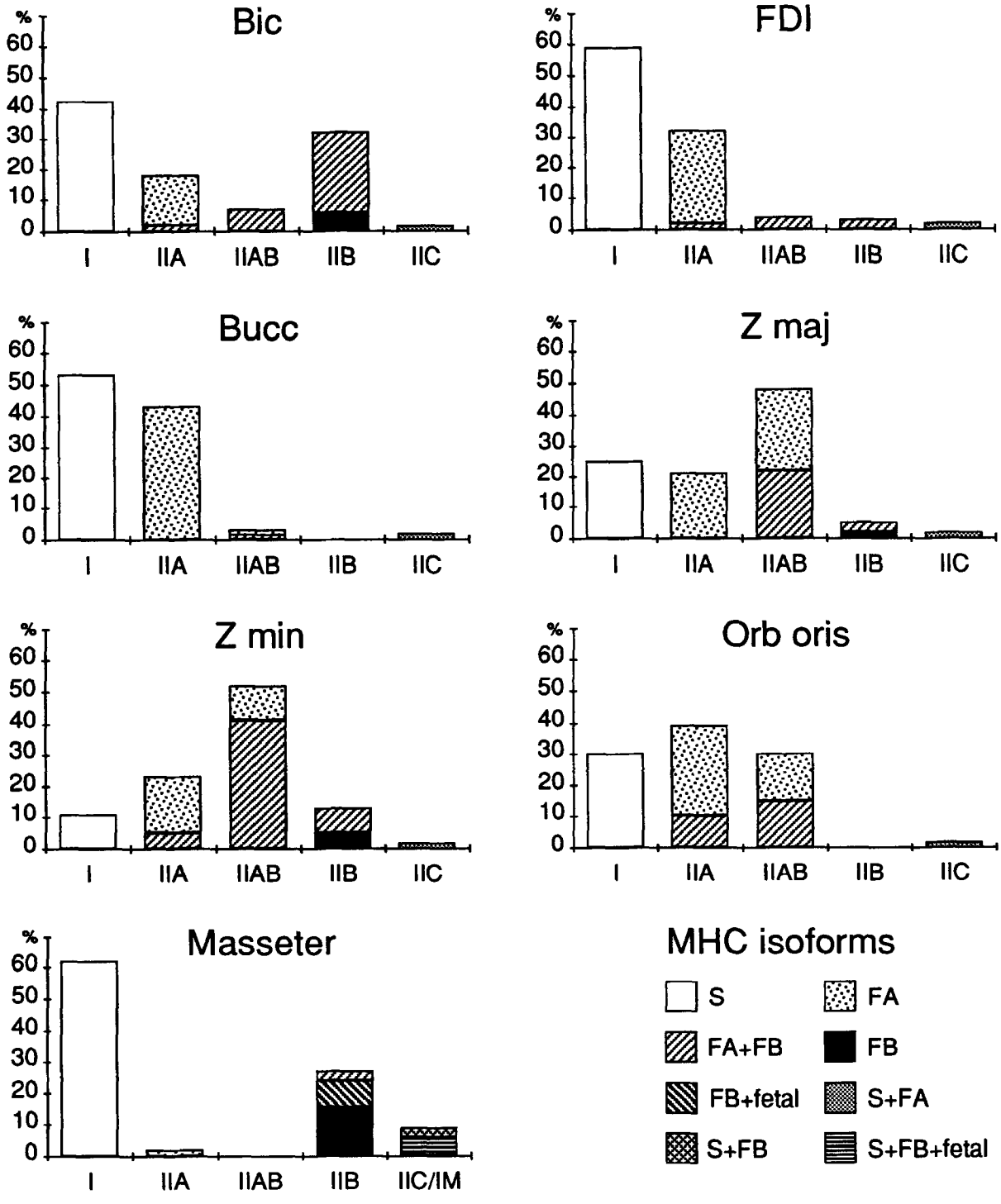

Fig. 8. Histograms on the content (%) of MHC isoforms, and combinations of MHCs, in the different muscle fibre types in the biceps brachii (Bic), first dorsal interosseus (FDI), buccinator (Bucc), major zygomatic (Z maj), minor zygomatic (Z min), orbicularis oris (Orb oris) and masseter (Mass) muscles, as evaluated from the antibody stainings. Fibre type compositions of the muscles are according to Eriksson and Thornell (1983) and Stål and colleagues (1987, 1990). In the limb and oro-facial muscles the type IM fibres have been included in the type IIC fibre group. Of the masseter type IM/IIC fibres, 1.5% also showed immunoreactivity for α-cardiac MHC (not shown in Fig.).

that jaw and limb muscles belong to two separate distinct allotypes. Based on experimental studies in cat jaw and limb muscles, Hoh and colleagues postulated that each muscle allotype has a specific repertoire of myosin gene expression and that the ability to express different myosin isoforms is intrinsic to the muscle type and

Table 2. The relative frequency (%) of muscle fibres expressing different MHC isoforms in the biceps (Bic), first dorsal interosseus (FDI), buccinator (Bucc), major zygomatic (Z maj), minor zygomatic (Z min), orbicularis oris (Orb oris) and masseter (Mass) muscles, as evaluated from the antibody staining pattern

	MHC isoform							
Muscle	*Slow MHC*	*Fast MHC*	(*)	*Fast A MHC*	*Fast B MHC*	(**)	*Foetal MHC*	*α-cardiac MHC*
Bic	43	58	(1)	52	41	(35)		
FDI	61	41	(2)	41	7	(7)		
Bucc	54	47	(1)	47	1	(1)		
Z maj	26	75	(1)	73	27	(25)		
Z min	12	89	(1)	84	59	(54)		
Orb oris	31	70	(1)	70	25	(25)		
Mass	71	38	(9)	5	36	(3)	17	1.5

Figures in parenthesis show proportion of fibres that coexpress fast and slow MHC(*) and fast A and fast B MHCs(**).

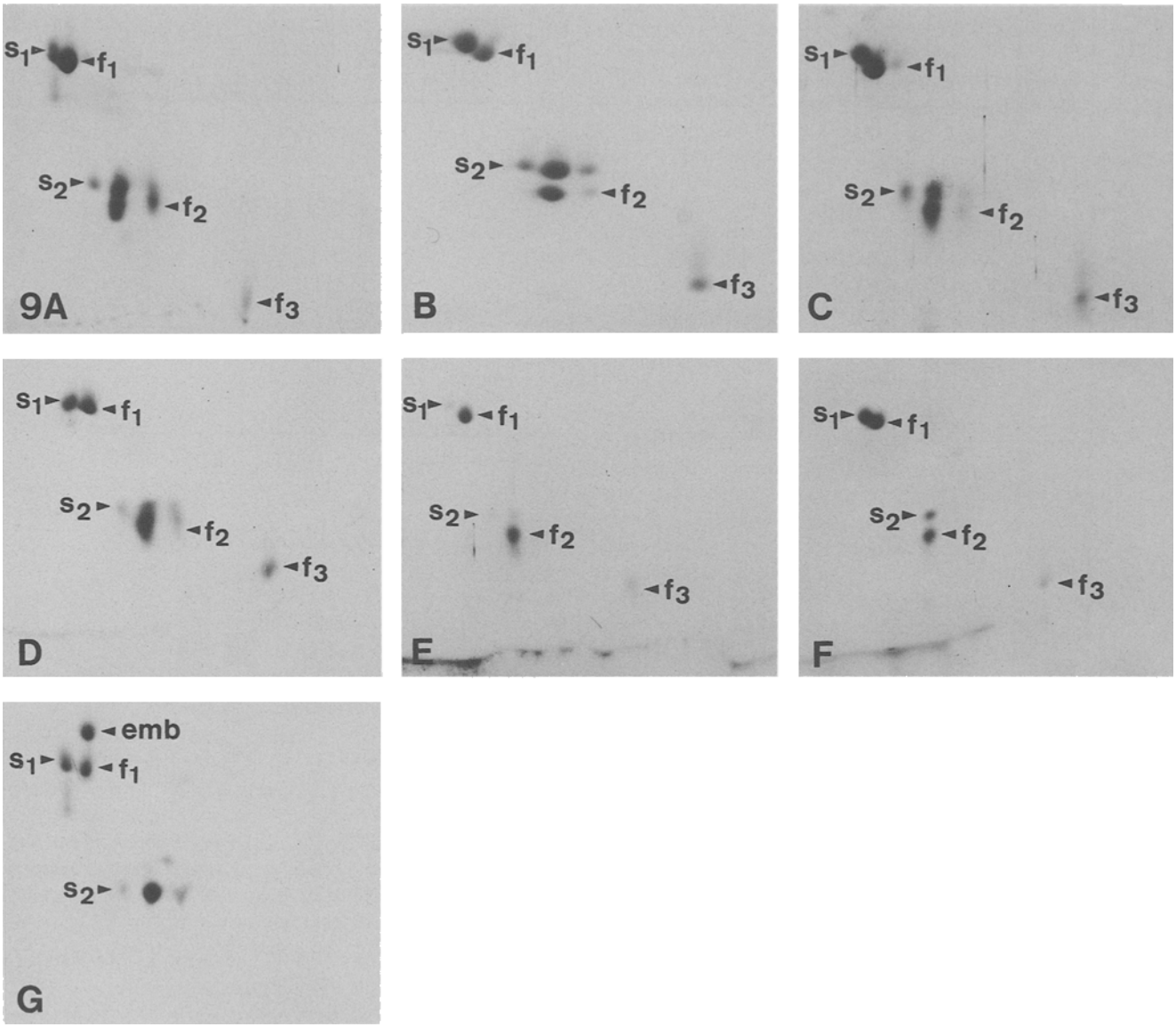

Fig. 9. Two dimensional gel electrophoresis of the biceps brachii (A), first dorsal interosseus (B), buccinator (C), major zygomatic (D), minor zygomatic (E), orbicularis oris (F) and masseter (G) muscles. The myosin light chains have been labelled as follows: $f_1 = LC_{1fast}$; $f_2 = LC_{2fast}$; $f_3 = LC_{3fast}$; $s_1 = LC_{1slow}$; $s_2 = LC_{2slow}$; emb = LC_{1emb}.

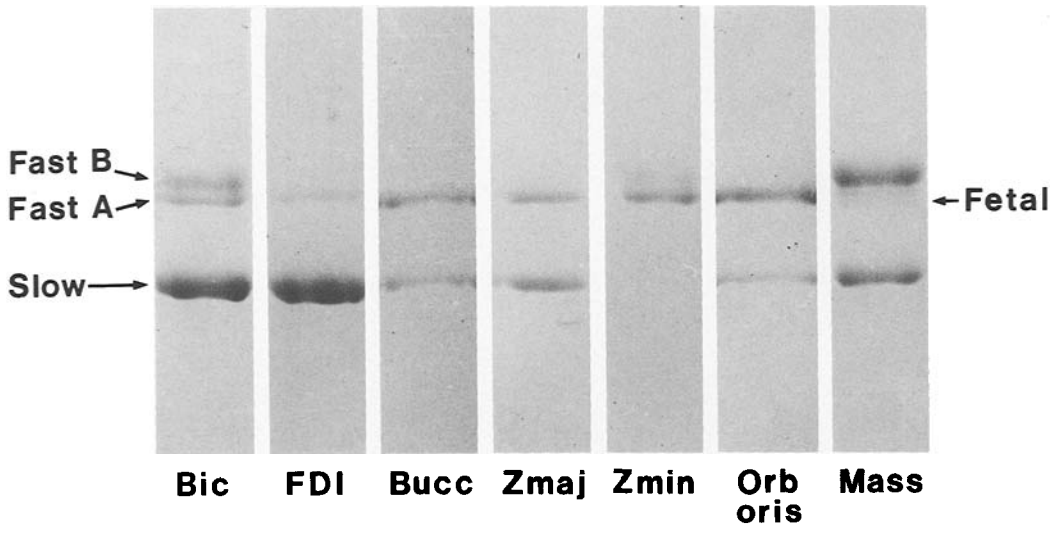

Fig. 10. MHC content on 6% polyacrylamide-SDS-glycerol gels of the biceps brachii (Bic), first dorsal interosseus (FDI), buccinator (Bucc), major zygomatic (Z maj), minor zygomatic (Z min), orbicularis oris (Orb oris) and masseter (Mass) muscles. The bands corresponding to slow, fast A, fast B and foetal MHC are indicated.

independent of the type of innervation. The results also show that although enzyme-histochemical fibre typing reflects the predominant type of myosin present in muscle fibres, immunohistochemical and biochemical techniques are needed to gain a more precise knowledge of the MHC composition in single muscle fibres, whole muscles and muscle groups. While single oro-facial and limb muscle fibres contained one or two MHC types, single masseter fibres were found to coexpress up to four different MHC isoforms. Moreover, describing the fibres according to their MHC isozyme content, up to five different fibre types could be distinguished in the oro-facial and limb muscles, and eight in the masseter.

IMMUNOHISTOCHEMICAL CHARACTERISTICS OF ENZYME-HISTOCHEMICAL FIBRE TYPES

In all muscles, type I fibres contained exclusively slow MHC and almost all type IIA fibres exclusively fast A MHC only. This is in agreement with observations made on the human vastus lateralis muscle (Staron, 1991), except that in our muscles a small population of the limb and oro-facial type IIA fibres also contained fast B MHC.

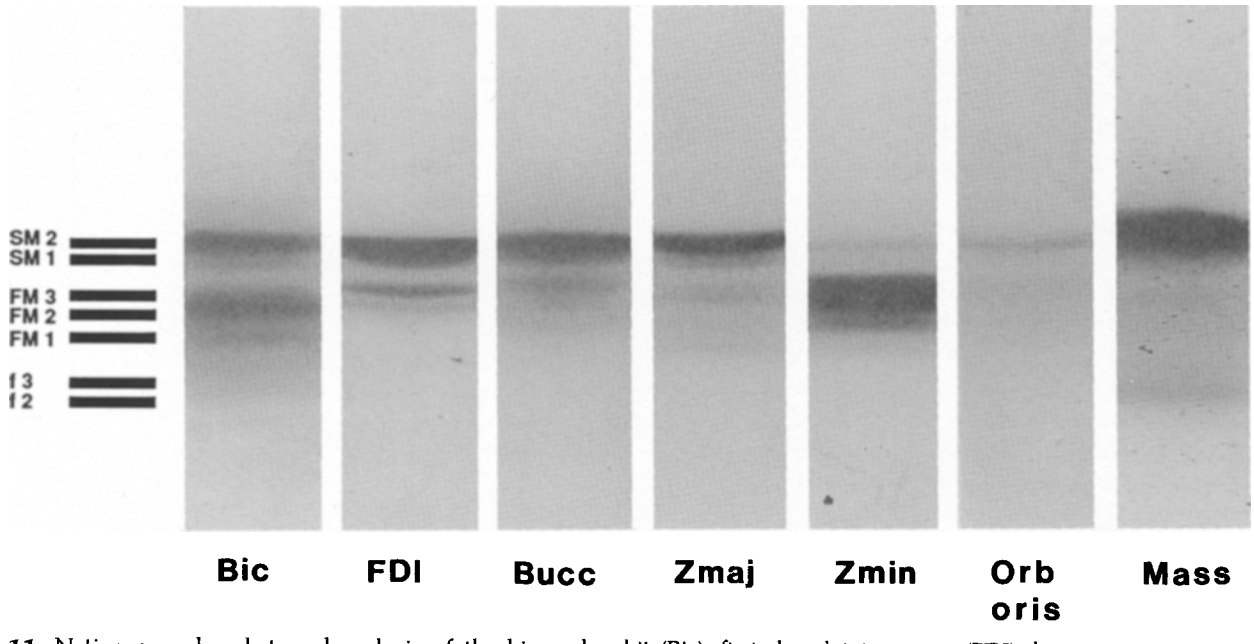

Fig. 11. Native pyrophosphate gel analysis of the biceps brachii (Bic), first dorsal interosseus (FDI), buccinator (Bucc), major zygomatic (Z maj), minor zygomatic (Z min), orbicularis oris (Orb oris) and masseter (Mass) muscles. Schematic illustration of the electrophoretic mobilities of human foetal (f2, f3), adult fast (FM1, FM2, FM3) and slow (SM1, SM2) myosins, according to Fitzsimons and Hoh (1981), are given to the left in the figure.

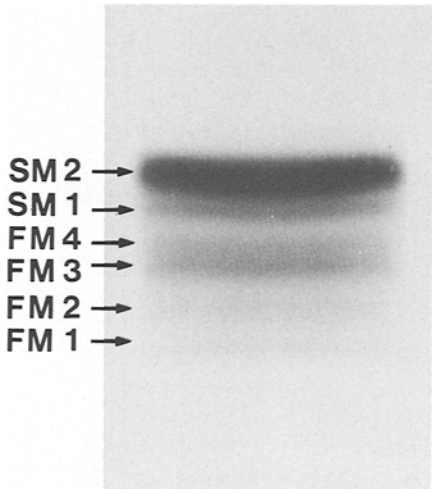

Fig. 12. Native pyrophosphate gel of the major zygomatic muscle, at a higher magnification. Note the presence of a unique band above the FM3, here termed FM4.

An obvious difference in MHC expression between the limb and the oro-facial muscles was observed in the type IIAB fibres which showed one MHC pattern in the limb muscles and another in the oro-facial muscles. In the limb muscles all type IIAB fibres showed immunoreactivity for fast A and fast B MHCs, whereas in the oro-facial muscles a type IIAB subpopulation showed immunoreactivity exclusively for fast A MHC. This reaction pattern was particularly prominent in the major zygomatic muscle in which more than half of the type IIAB fibre population reacted solely with the mAbs against fast A MHC.

The coexpression of fast A MHC and fast B MHC in the majority of the oro-facial and limb type IIB fibres is in agreement with previous findings from electrophoretic studies of single human limb muscle fibres (Biral *et al.*, 1988; Staron, 1991), that type IIB fibres contain a mixture of fast A and fast B MHC isoforms. However, in the masseter type IIB fibres the MHC expression was different from the other muscles and three combinations of MHC could be distinguished. Half of the masseter type IIB fibres showed immunoreactivity exclusively to fast B MHC, whereas a mixture of fast B and foetal MHCs predominated in the other half. In half of the muscles a small number of the masseter type IIB fibres also showed immunoreactivity for fast A MHC. In addition, the finding that the smallest type IIB fibres also showed the strongest immunoreactivity for fast B MHC suggests a difference between small and large diameter type IIB fibres in the composition of the myofibrils.

In all muscle groups we found differences in immunoreactivity between the type IM and IIC fibres. The intense reactivity of the type IM fibres with the anti-S MHC mAbs suggests a relatively high content of slow MHC in this fibre group. In contrast, the type IIC fibres were generally more reactive with the anti-F mAb than with the anti-S mAbs, suggesting a relatively larger amount of fast twitch MHC in the type IIC fibres.

Table 3. Expression of MLC and MHC and native myosin isoforms, as evaluated from gel electrophoresis, in the human biceps (Bic), first dorsal interosseus (FDI), buccinator (Bucc), major zygomatic (Z maj), minor zygomatic (Z min), orbicularis oris (Orb oris) and masseter (Mass) muscles

	Myosin light chain isoform						*Myosin heavy chain isoform*				*Native myosin isoform*						
Muscle	MLC_{1S}	MLC_{2S}	MLC_{1F}	MLC_{2F}	MLC_{3F}	MLC_{1emb}	*Slow MHC*	*Fast A MHC*	*Fast B MHC*	*Foetal MHC*	*SM1*	*SM2*	*FM1*	*FM2*	*FM3*	*FM4*	*f2*	*f3*
Bic	x	x	x	x	x		x	x	x		x	x	x	x	x			
FDI	x	x	x	x	x		x	x			x	x	x	x	x			
Bucc	x	x	x	x	x		x	x				x	x	x	x			
Z maj	x	x	x	x	x		x	x			x	x	x	x	x	x		
Z min	x	x	x	x	x		x	x	x		x	x	x	x	x	x		
Orb oris	x	x	x	x	x		x	x			x	x	x	x	x	x		
Mass	x	x	x			x	x		x	x		x			x		x	x

We found also three muscle dependent differences between the type IM and type IIC fibres. Firstly, in the limb and oro-facial muscles, the few IM and IIC fibres showed immunoreactivity for both slow MHC and fast A MHC, whereas in the masseter, in which IM and IIC fibres are consistently present (Eriksson and Thornell, 1983), these fibres showed immunoreactivity for slow MHC and fast B MHC. To our knowledge this study is the first to indicate the coexistence of slow MHC and fast B MHC within the same fibre type. As we have used a mAb directed to slow and fast B MHCs, we can only indirectly conclude the presence of fast B MHC. However, the reactivity with the anti-fast mAb and the lack of reactivity with the anti-fast A mAbs, exclude the possibility of fast A MHC in these fibres. It remains to be established whether the fast B MHC immunoreactivity in the masseter type IM and IIC fibres reflects the presence of a fast B MHC or a fast B like MHC. These alternatives are now under investigation in our laboratory. Previous observations in human limb muscles indicate that the frequency of fibres with combinations of slow and fast myosin increases during physically training (Billeter *et al.*, 1980; Schantz & Dhoot, 1987; Staron & Hikida, 1992) and ageing (Klitgaard *et al.*, 1990) and that these fibres are always composed of slow and fast A MHCs. It has also been proposed that the MHC isoforms in adult limb muscles follow a fixed sequence of transition in response to changes in functional demands; slow ⇔ slow/fast A ⇔ fast A ⇔ fast A/fast B ⇔ fast B MHC or vice versa (Billeter *et al.*, 1980; Pierobon-Bormioli *et al.*, 1981; Biral *et al.*, 1988; Staron, 1991). However, in the light of our present results this MHC transition pattern does not seem to be fully valid for the human masseter.

Another difference between the limb and oro-facial versus the masseter type IM and IIC fibres was the reaction of the masseter IIC and IM fibres with the anti-foetal Ab. It has previously been shown that during postnatal muscle development, embryonic and foetal myosin isoforms disappear completely in limb muscles (Whalen *et al.*, 1981; Butler-Browne *et al.*, 1988, 1990; Barbet *et al.*, 1991) but persist in the human masseter (Eriksson *et al.*, 1988; 1994) and extraocular muscle fibres (Wieczorek *et al.*, 1985; Sartore *et al.*, 1987). Finally, a third particularity of the masseter IIC and IM fibres was the immunoreactivity of these fibres with the anti-α-cardiac mAb. Bredman and colleagues (1991) reported that 20–45% of all the human masseter fibres contained α-cardiac MHC and that the immunoreactivity was region and subject dependent. In their study the anti-α-cardiac MHC positive fibres coexpressed either fast MHC or both slow and fast MHCs. In our study, the total amount of α-cardiac MHC positive fibres was 1.5%, all of which were IM and IIC fibres which showed immunoreactivity also for slow, fast B and foetal MHCs. Regional variation in the location of α-cardiac positive fibres were also observed in our muscles. The discrepancy between our findings and those reported by Bredman and colleagues (1991) may be related to differences in the sensitivity and/or specificity of the antibodies used and/or intra- and intermuscular differences in muscle fibre type composition (Eriksson & Thornell, 1983). Furthermore, the tissue specimens used in the study by Bredman and colleagues (1991) were chemically fixed whereas our specimens were cryo-fixed.

The findings of a persistence of foetal MHC, expression of α-cardiac MHC and coexpression of slow MHC and a fast B or a fast B like MHC in the adult masseter fibres, illustrate that special and muscle specific combinations of MHCs are expressed in the human masseter muscle. This would suggest that the mechanism which controls the pattern of MHC gene expression is different for the masseter as compared to the limb and oro-facial muscles.

CORRELATION BETWEEN IMMUNOHISTOCHEMICAL AND BIOCHEMICAL FINDINGS

Myosin light chains

The 2-D gel electrophoresis revealed that each muscle had a distinct phenotype. A good correlation was found between the proportions of the different MLCs and the quantity of MHCs detected by gel electrophoresis. The FDI and masseter muscles, which contain predominantly type I fibres (Eriksson & Thornell, 1983; Stål *et al.*, 1987), expressed mainly slow MLCs whereas the major and minor zygomatic and the orbicularis oris muscles, all characterized by a predominance of type II fibres (Stål *et al.*, 1987, 1990), expressed predominantly the three fast MLCs (MLC_{1F}, MLC_{2F}, MLC_{3F}). This observation was independent of the differences in the relative content of fast A and fast B MHCs that we found in these muscles. The biceps and buccinator muscles, both characterized by about equal proportions of type I and type II fibres, also contained stoichiometric amounts of all fast and slow MLCs. Our result is in agreement with studies carried out on single limb muscle fibres (Billeter *et al.*, 1981).

The masseter muscle differed, again, from the other muscles. Firstly, embryonic myosin light chain, MLC_{1emb}, was consistently present and secondly the MLC_{2F} and MLC_{3F} were absent. The MLC_{1emb} has previously been shown to be preferentially localized in the small diameter type IIB fibres of the masseter (Butler-Browne *et al.*, 1988). Thus, the fast myosin molecule in the masseter type IIB fibres seems to have an unusual composition of myosin light chains, which further underlines the unique structure of the human masseter and the possibility that a special fast B MHC might be expressed in this muscle.

Myosin heavy chains

A good correlation was also found between the immunohistochemical and the biochemical analyses of the MHC composition. However, in the oro-facial muscles we found some diverging results. In spite of the unusually

high proportion of type IIAB fibres in these muscles (Stål *et al.*, 1987, 1990), very little or no fast B MHC could be detected on 6% SDS-glycerol polyacrylamide gels. This result is supported by our current immunohistochemical finding which showed a lack of immunoreactivity for fast B MHC in a subpopulation of the oro-facial type IIAB fibres. These facts, as well as the presence of a fourth fast twitch band (FM4) exclusively in these muscles, revealed by the non-denaturating pyrophosphate gel, might suggest that part of the oro-facial type II fibre population contains a MHC which is not fast B MHC but an unidentified oro-facial MHC, which we have called 'fast F' (fast Facial) MHC. In rodent limb and masseter muscles a fourth fast band termed 'IM' has been found to migrate above the FM3 band (Fitzsimons & Hoh, 1983; Gregory *et al.*, 1987; Tsika *et al.*, 1987a, b). This IM band has been associated with the type IIA fibres, whereas the FM1, FM2 and FM3 bands have been correlated to the type IIB fibres (Fitzsimons & Hoh, 1983; Tsika *et al.*, 1987a). However, the presence of a fourth band in rodents is unlike the situation in human muscles, as shown here and elsewhere (Fitzsimons & Hoh, 1981). In the human biceps, which contains approximately equal numbers of type IIA and type IIB fibres, we found three fast bands (FM1, FM2, FM3), not four as would be the case in rodents. This pattern of three fast bands was further confirmed by comigrating extracts from different muscles with different proportions of type IIA and IIB fibres. Three fast bands were also present in the buccinator muscle which contains only type IIA fibres, and comigration of the buccinator and biceps muscle extracts revealed the same three fast band pattern (FM1, FM2, FM3), suggesting that human fast A and fast B myosin comigrate. Thus, the fourth band (FM4) in the oro-facial muscles seems to be unique and correlated with the presence of type IIAB fibres. Moreover, since only one fast band was identified when the muscles were analysed under denaturating conditions, it seems most likely that 'fast F' and fast A MHCs comigrate in this gel system. A further indication of a special molecular composition of the oro-facial type II fibres is their comparatively high activity of oxidative enzymes (Stål *et al.*, 1987, 1990).

A novel MHC isoform called 2X (Schiaffino *et al.*, 1989; Gorza, 1990) or 2D (Bär & Pette, 1988) has been demonstrated in a large subset of rodent type II fibres which also show a high activity of oxidative enzymes. This new MHC isoform has also been recognized as a separate band by gel electrophoresis (Bär & Pette, 1988; Schiaffino *et al.*, 1989). Several cDNA clones selectively expressing this MHC have now been cloned from rat diaphragm (Constanza *et al.*, 1992). By comparing these rat clones with an adult human skeletal cDNA library (Saez & Leinwand, 1986), a clone identical to that expressing rat 2X myosin was identified for human skeletal muscle (Schiaffino, unpublished observation). So far it is not clear in which fibre type and muscle the human 2X MHC is expressed. Future studies are needed to show whether the 'fast F' MHC, which we have observed in the oro-facial muscles, corresponds to the 2X MHC or is still another MHC isoform expressed exclusively in the human oro-facial muscles.

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