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Neonatal myosin in bovine and pig tensor tympani muscle fibres*

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INTRODUCTION

In previous studies of middle ear muscles we found that tensor tympani (TT) varies both in morphology and fibre type composition in different species. Histochemical classification of fibre types was particularly difficult in the bovine and porcine TT, in which the muscle fibre component is very much reduced in relation to the connective tissue. The very small diameter of TT fibres (mean $10 \pm 1{\cdot}6$ μm), combined with the anomalous myosin ATPase (mATPase) staining displayed by many of them (varying degrees of alkali and acid stability, which did not correspond to the classical adult types) suggested to us that they were possibly immature fibres (Veggetti, Mascarello & Carpenè, 1982; Mascarello, Veggetti, Carpenè & Rowlerson, 1983; Rowlerson, Mascarello, Veggetti & Carpenè, 1983).

During normal muscle development, fibres initially contain an 'embryonic' isoform of myosin, which in most fibres is soon replaced by a 'neonatal' isoform, and then later still by the normal adult isoforms (e.g. IIA, IIB, etc.) (Whalen *et al.* 1981). The embryonic and neonatal isoforms do not confer unique histochemical mATPase profiles on the fibres in which they are present, which means they have to be identified instead by appropriate isoform-specific antibodies. Furthermore, tonic fibres, which are present in tensor tympani in some species, are also difficult to identify by mATPase activity (Veggetti *et al.* 1982).

Here we re-examine TT in individuals of various ages in these 2 species, using immunohistochemical methods to identify the fibre types present.

METHODS

Samples and immunohistochemistry

TT muscles were removed from female, male and castrated male pigs aged 0, 2, 8, 24 and 62 days, 6, 7, 8 and 13 months and from calves aged 1 day and steers aged 18 and 24 months. Each TT muscle was wrapped in a piece of another skeletal muscle from the same subject immediately after its removal, and these were than frozen as a single block in isopentane cooled by liquid nitrogen or dry ice. These blocks were sectioned through the whole length of the TT muscles and serial 10 μm sections were

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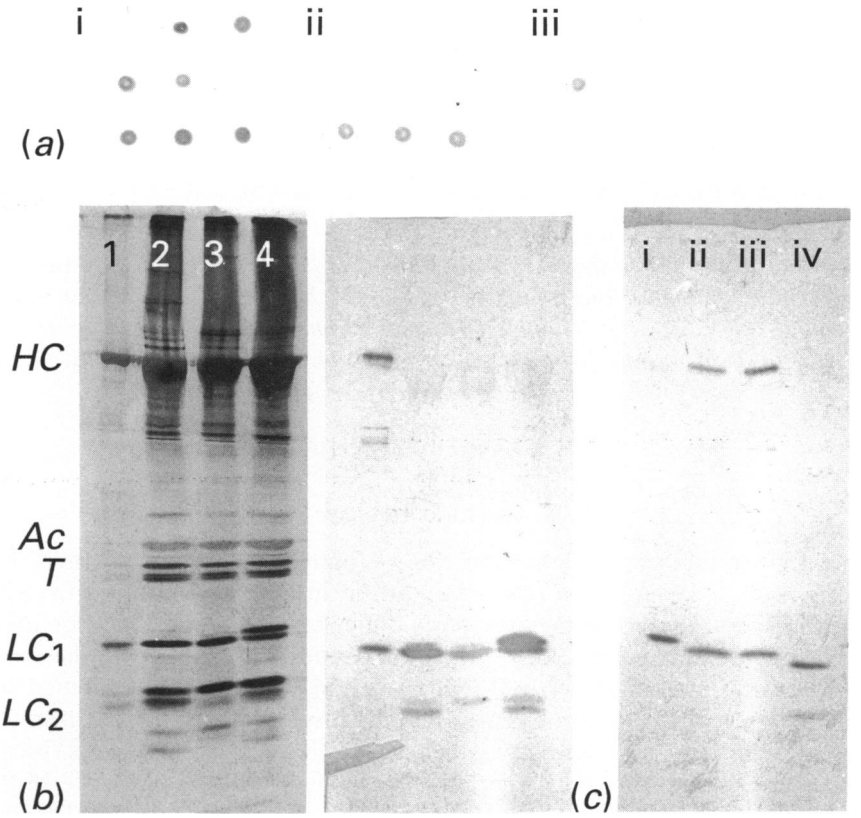

Fig. 1. Specificity of anti-NE. (*a*) Dot blots of myofibrillar samples of predominant fibre type composition as follows (same for (i), (ii) and (iii)). Top row: IIM, IIA, IIB; middle row: I, IIB; bottom row: embryonic and neonatal at 3 different sample loads. Antibodies used: (i) serum II-8 (an internal method control; reacts with most myosins except IIM (top left sample) and tonic (not shown here): (ii) anti-NE, which reacts only with samples containing embryonic and neonatal myosins (bottom row); (iii) anti-I, which reacts only with type I myosin. Note there is no cross-reaction between anti-I and embryonic/neonatal myosins or vice versa. (*b*) Reference gel (left) and corresponding immunoblot reacted with anti-NE. Gel consists of 2 parts: upper third, 8% acrylamide; lower two-thirds, 15% acrylamide. The samples were, for lane 1, myosin from bovine fetal muscle, and for lanes 2–4, myofibrils from adult fast (2), mixed (3) and slow (4) muscle. The major protein bands are labelled: *HC*, myosin heavy chains; *Ac* actin; *T*, tropomyosin and troponin-T; LC_1, LC_2, myosin light chains 1 and 2. Anti-NE reacts strongly both with fast and slow forms of LC_1, and very weakly with LC_2. It gives no reaction with adult fast and slow myosin heavy chains (lanes 2–4) but reacts strongly with heavy chains in the fetal muscle (lane 1) which contains embryonic and neonatal myosins. The relative strength of reaction with myosin heavy chains is greatly under-represented on this blot, since transfer of light chains to the blot was almost 100%, but heavy chain transfer was less than 30%. (*c*) Immunoblot of other myofibrillar samples against anti-NE (gel not shown). Samples were: lane (i), adult slow (type I); lane (ii), bovine fetus aged 14 weeks; lane (iii), bovine fetus aged 22 weeks; lane (iv), adult fast (types IIA and IIB). Anti-NE reacts with all forms of LC1, but is highly specific for the myosin heavy chains from fetal muscle.

tested for the following reactions: myosin ATPase (m-ATPase) (method A in Snow *et al.* 1982); indirect immunoperoxidase staining with polyclonal antibodies showing heavy chain isoform specificity for slow twitch fibre myosin (anti-I), fast twitch IIA myosin (anti-IIA), slow tonic fibre myosin (anti-ALD, kindly provided by Professor Schiaffino) and embryonic/neonatal myosins (anti-NE). Serial 30 μm sections of TT from male pigs aged 0 days and 13 months were stained for acetylcholinesterase

(AChEase) activity according to Gerebtzoff (1959) or were incubated for AChEase activity and stained to reveal axons as described by Ashmore, Vigneron, Marger & Doerr (1978).

The specificity of anti-I, anti-ALD and anti-IIA has been described previously (Mascarello *et al.* 1982). As anti-NE is critical for the identification of immature fibres but has not been used before, its preparation and specificity are described below.

Anti-NE antibody

Myosin was extracted from thigh muscles of a bovine fetus aged 22 weeks, when the predominant myosins present were neonatal and embryonic. This myosin was used to immunise New Zealand White rabbits, and its composition is shown in Figure 1*b*, lane 1.

The specificity of the antisera was tested by immunohistochemistry on composite blocks of a wide variety of muscle samples containing known fibre types, and by dot and Western blotting against myofibrillar samples of various origins. Dot-blot and immunohistochemical screening of the specificity of the anti-NE serum used in this study showed that at optimum dilutions it reacted only with samples or fibres from immature muscle (bovine fetuses aged 14 and 22 weeks – see Fig. 1 – and late fetal and young rats up to about 3 weeks after birth). Anti-NE gave no reaction with adult muscle samples (mammalian slow, fast IIA, fast IIB, fast IIM – see Fig. 1*a* – and chicken tonic fibres). At much higher concentrations a weak cross-reaction with adult fast fibres was observed. Blots of myofibrillar samples analysed by SDS-PAGE revealed that anti-NE reacted with light chains 1 (LC1) from all myosins (i.e. nonselectively) but was highly selective for the heavy chains (HC) of fetal muscle over all other heavy chains even when the other HC types were present in much higher amounts, as shown in Figure 1*b*. In the molecular weight range between actin and myosin HC there was again no reaction in the adult muscle samples; the weak reaction seen in the fetal muscle sample is probably with degradation products of the myosin HC (cf. Gambke & Rubinstein, 1984). When the reaction with samples from fetuses of 2 different ages was compared (see Fig. 1*c*), the reaction with myosin HC was stronger in the older sample (lane iii) in which the ratio of 2° myotubes to 1° myotubes was known to be greater, and therefore presumably a higher ratio of neonatal to embryonic myosin was present.

Anti-NE is thus highly selective for the myosin HC present early in muscle development. Our results do not exclude the possibility of a significant antiembryonic myosin component, but the principal selectivity of anti-NE is certainly for neonatal myosin.

RESULTS

Pig tensor tympani

In the adult pig, the muscle fibres occupy only a small fraction of TT, and mean fibre diameter is only $10 \pm 1{\cdot}6$ μm. Nonetheless, various fibre types could be identified, although some were different from the classical types found in adult skeletal muscle (Fig. 2). Immunohistochemically, fibre types giving the following reactions could be identified: anti-I positive only, anti-IIA positive only, anti-NE positive only, and positive with 2 or more of the antibodies used. In control skeletal muscle from same individuals, no fibres reacted with anti-NE (Fig. 2*a*). In tensor tympani, there were no fibres which reacted with anti-ALD.

Classical type I fibres (alkali-labile, acid-stable mATPase) were anti-I positive

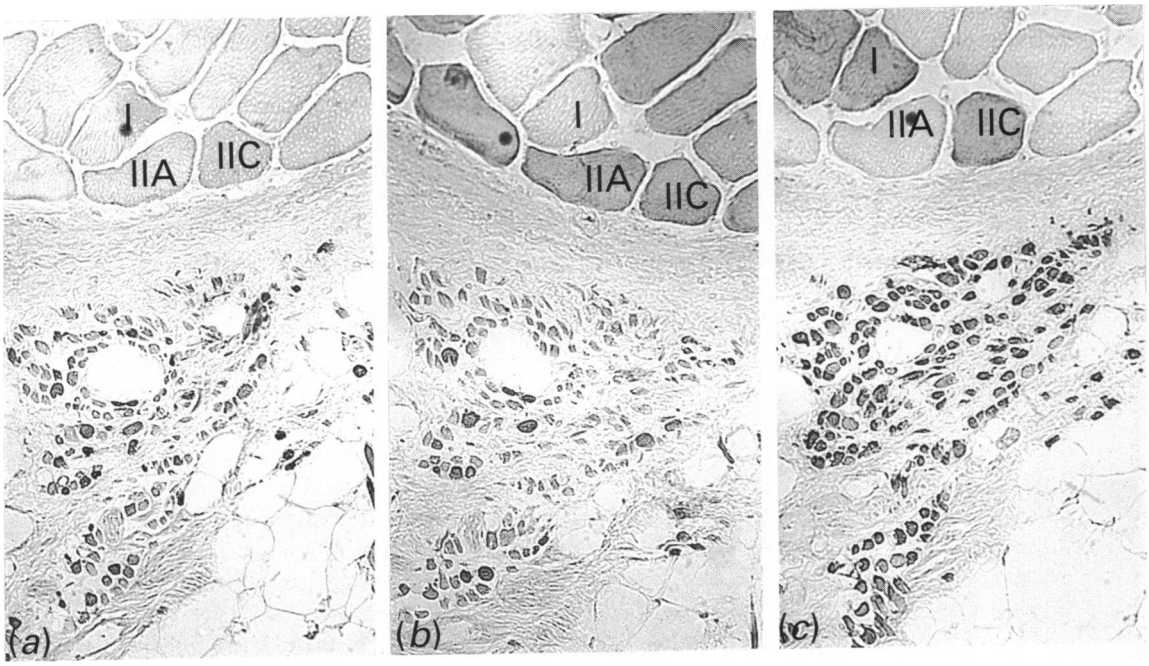

Fig. 2. Adult pig. Skeletal control muscle (top) and tensor tympani muscle (bottom). Immunoperoxidase with anti-NE (*a*), anti-IIA (*b*), and anti-I (*c*) sera. × 162. All the fibres of control muscle are negative to anti-NE serum but, in contrast, some fibres of TT are positive.

only, and classical type IIA fibres (alkali-stable, acid-labile mATPase) were anti-IIA positive only, as expected (Fig. 3*a–e*). Some fibres with both alkali- and acid-stable mATPase were both anti-I and anti-IIA positive, as expected for type IIC fibres. However, others (Fig. 3*a–e*, asterisk) with this mATPase profile reacted with only one or the other of these 2 antibodies. Furthermore, some anti-I positive only (Fig. 3*a–e*, star) and anti-IIA positive only fibres showed degrees of alkali- and acid-stable mATPase activity, respectively, which are not seen in classical type I and IIA fibres.

Some other fibres were strongly anti-NE positive but only a small minority of them reacted exclusively with this antibody; some also reacted with anti-I, and the majority with anti-IIA (Fig. 3*a–e*, arrow). This reaction with anti-IIA could not be attributed simply to a cross-reactivity of anti-IIA with neonatal or embryonic myosins, because the staining with anti-IIA and anti-NE did not always coincide (e.g. some fibres reacted with anti-NE but not with anti-IIA). As expected for fibres containing neonatal myosin, all anti-NE positive fibres had an mATPase activity which was alkali-stable but relatively acid-labile.

Both morphologically and in its fibre type composition, TT in young pigs was indistinguishable from adult TT. Even the proportion of anti-NE positive fibres, which might have been expected to be higher in young animals, was not obviously different.

In pigs aged 0 days (Fig. 4) and 13 months the fibres were focally innervated; no instances of distributed (multiple end-plate) innervation could be found. This pattern of innervation corresponds to that found previously in bovine, horse (Giannotti and Veggetti, 1976) and carnivore TT (Mascarello *et al.* 1982).

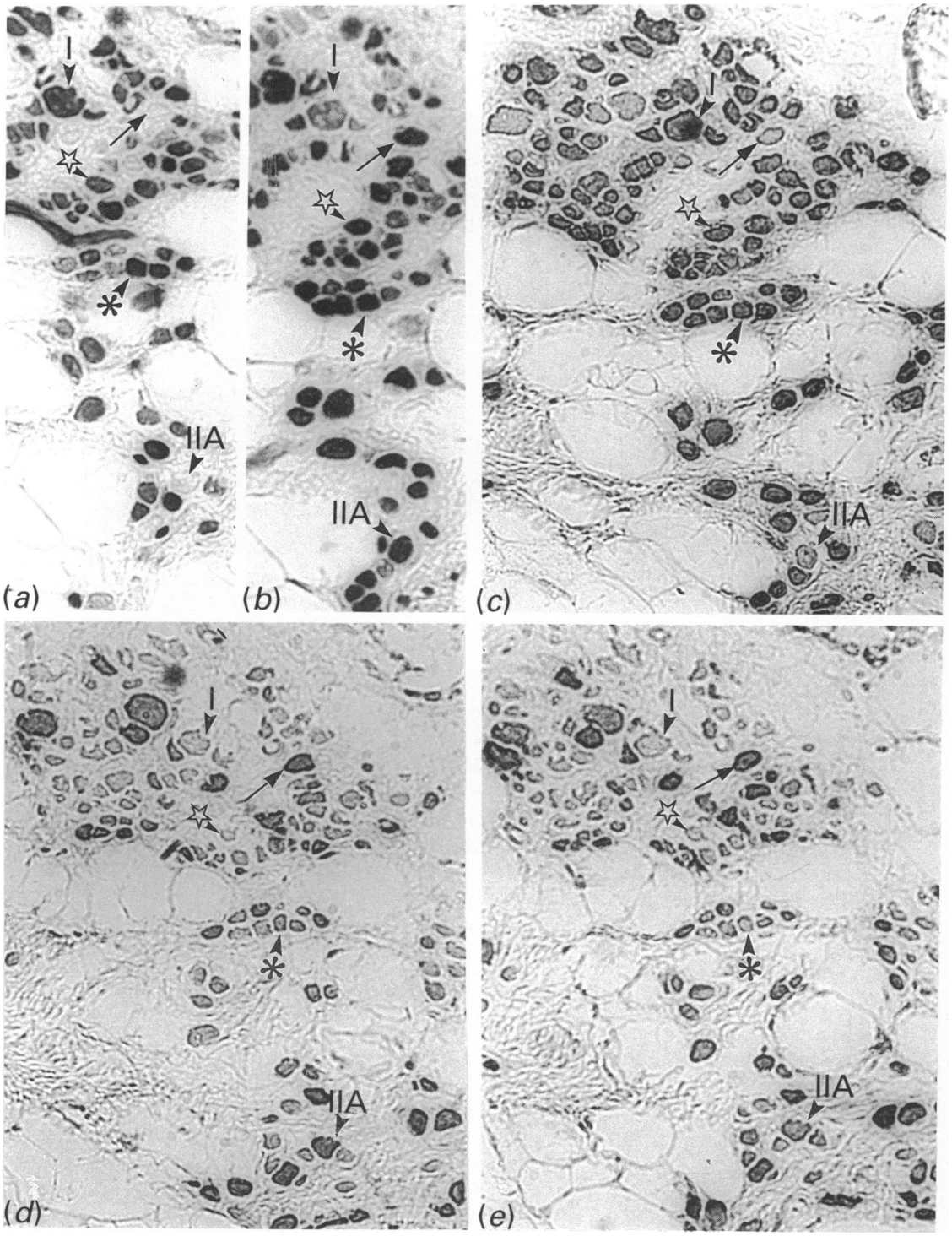

Fig. 3. Tensor tympani muscle of adult pig. m-ATPase activity after pH 4·38 (*a*) and pH 10·25 (*b*) preincubation. Immunoperoxidase with anti-I (*c*), anti-IIA (*d*) and anti-NE (*e*) sera. × 306. Most but not all anti-NE positive fibres also react with anti-IIA serum (arrow). Some fibres showing a mismatch between histochemical and immunohistochemical profiles (star, asterisk) are indicated.

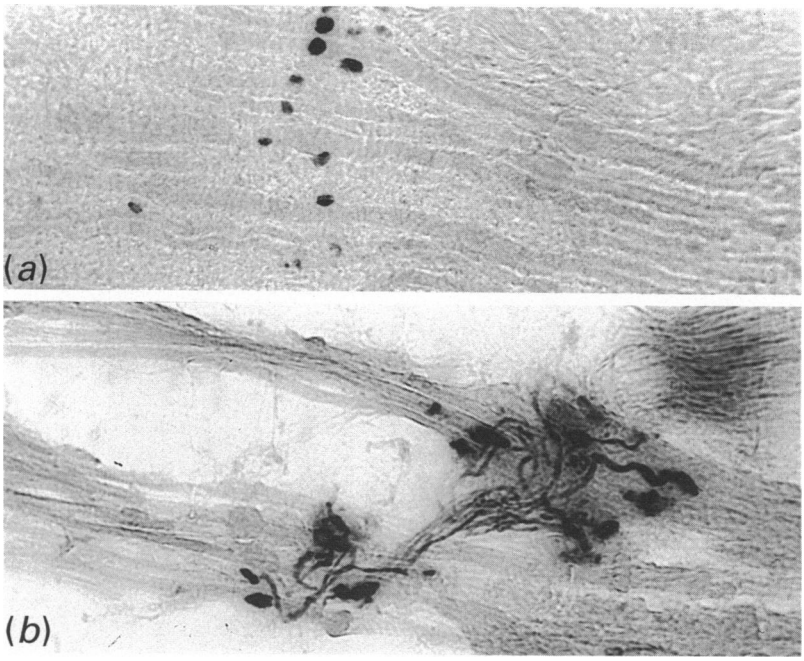

Fig. 4. Day 0 pig tensor tympani. Longitudinal sections showing AChEase staining (*a*) and fibre bundles stained for AChEase and axons (*b*). Note the arrangement of the single end-plates in single band (*a*) ×570, (*b*) ×710.

Bovine tensor tympani (all ages)

Anti-I positive only, anti-IIA positive only and anti-NE positive fibres could be identified. Again, no anti-NE positive fibres were present in 'control' skeletal muscle fibres of the same individuals (Fig. 5*a*).

As in the pig, some anti-I positive and anti-IIA positive fibres had the corresponding classical mATPase profiles, but there were many fibres with intermediate degrees of acid and alkali mATPase stability, which in many cases could be attributed to the presence of neonatal/embryonic myosins (Fig. 5*b–d*). As in the pig, no anti-ALD positive fibres could be found.

DISCUSSION

The fibre type composition of TT in both pigs and cattle was found to be highly unusual. Although classical type I and IIA fibres are present, the majority of fibres have a histochemical and immunohistochemical profile which is not found in most skeletal muscles (see Table 1). Thus some fibres which appear to be type I or IIA immunohistochemically, do not have the appropriate histochemical mATPase profile. Strictly, the type IIC histochemical profile (acid and alkali stable mATPase) is indicative of fibres which contain both fast (IIA) and slow myosins, and hence such fibres should react with both anti-I and anti-IIA (Billeter *et al.* 1980). Some 'IIC' fibres in TT did indeed react with both these antibodies, but others reacted with only one or the other.

A similar mismatch between histochemical and immunohistochemical profiles was observed by van den Berge & Wirtz (1989) in fibres of both TT and stapedius (ST) in

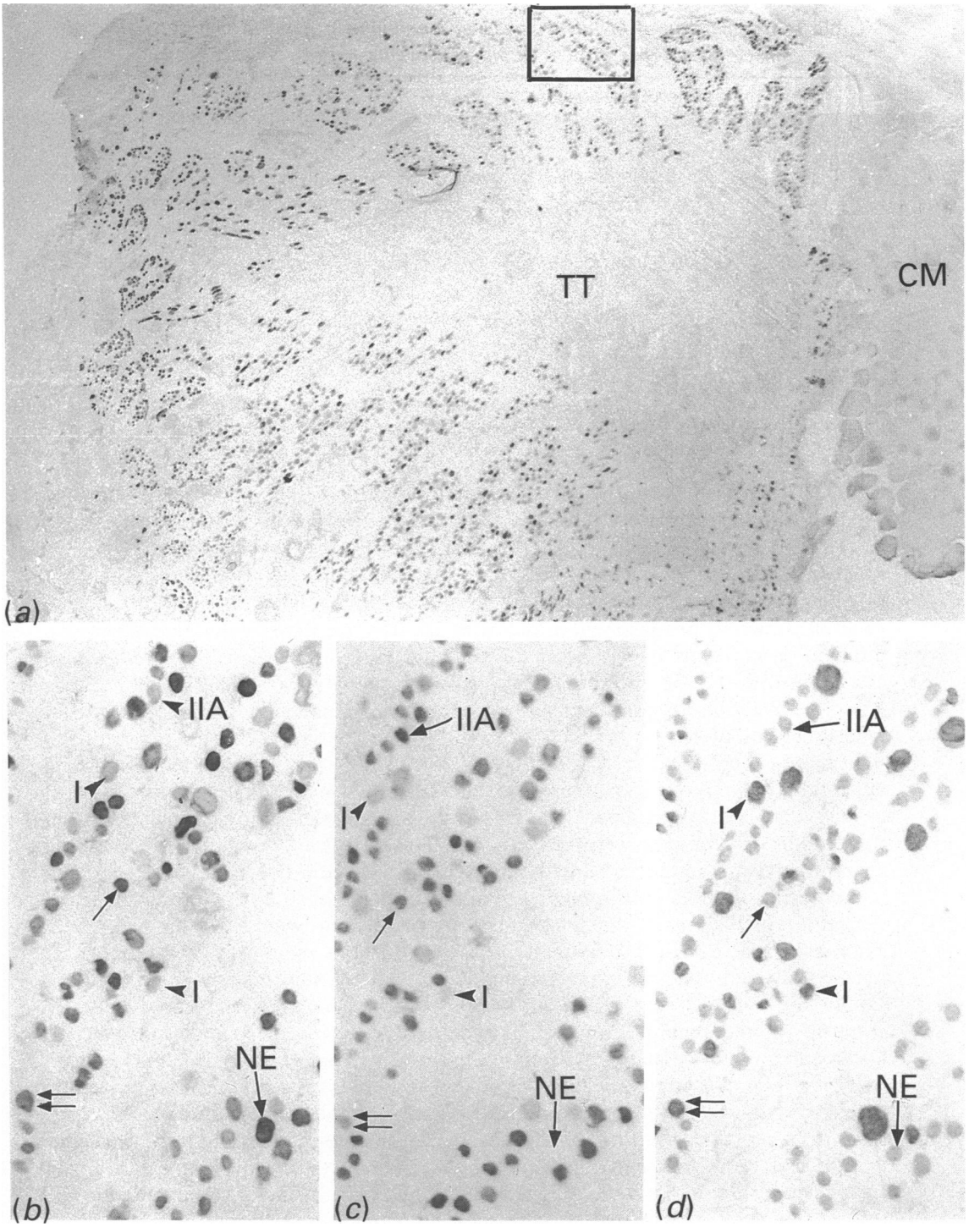

Fig. 5. Adult bovine tensor tympani muscle. Immunoperoxidase with anti-NE (*a*, *b*), anti-IIA (*c*), and anti-I (*d*) sera. (*b–d*) Higher magnification (× 256) of area shown in *a* (× 41). (*a*) A large number of anti-NE positive fibres are present in the TT but there are no anti-NE positive fibres in the control muscle (CM). (*b–d*) Some fibres are positive with only 1 of 3 antisera, e.g. those indicated: I, IIA, NE. However, most of the other anti-NE positive fibres react also with anti-IIA serum (arrows) or (few) with anti-I serum (double arrow).

Table 1. *Comparison of histochemical and immunohistochemical characteristics of muscle fibre types present in pig and bovine tensor tympani muscle*

Fibre types	m-ATPase activity after preincubation by		Reaction with antisera			
	acid	alkali	anti-I	anti-IIA	anti-NE	anti-ALD
I	+	−	+	−	−	−
IIA	−	+	−	+	−	−
IIC	+	+	+	+	−	−
NE	−/(+)	+	−	−	+	−
*	+	+	±	∓	−	−
☆	(+)/+	(+)/+	±	∓	−	−
↑,↑↑	−/(+)	+	∓	±	+	−

+, Strong positive reaction; (+), moderate or weak reaction; −, no reaction. The symbols in fibre types column refer to the corresponding fibre types quoted in the figures.

the rat. In their case, the mismatch concerned a minority of fibres which gave a positive reaction with an antibody specific for slow myosin, but no corresponding acid stable mATPase activity.

Most of the anti-NE positive fibres in the pig and bovine TT also reacted with anti-I (a few) or anti-IIA (the majority). As the staining intensity with anti-NE was generally inversely proportional to that with anti-IIA, we suppose that this reflects the different proportions of the neonatal and IIA myosins in these fibres. It is also consistent with the observation that acid mATPase activity was higher in fibres giving a stronger reaction with anti-NE, since neonatal myosin is weakly acid-stable but IIA myosin is acid-labile.

In the rat TT and ST, van den Berge & Wirtz (1989) also found fibres containing neonatal myosin, but most of those also contained slow myosin, rather than fast (IIA) as in the muscles we examined. The reason for the persistence of neonatal myosin in these adult TT muscles were unclear. Other examples of adult fibres containing neonatal myosin are the very small-diameter fibres in the orbital layer of extraocular muscles (Sartore *et al.* 1987; Jacoby, Ko, Weiss & Rushbrook, 1990) and the small diameter type II fibres of the human masseter (Buttler-Browne, Eriksson, Laurent & Thornell, 1988; Soussi-Yanicostas *et al.* 1990). In both these cases the presence of neonatal myosin was attributed to the reduced load-bearing function of these fibres, supposedly equivalent to the situation in new-born limb muscle when neonatal myosin is normally predominant. However, it is noticeable that in the human masseter the fibres which retain neonatal myosin are clearly larger than those of TT and occur in well organised normal muscle fascicles, not as scattered isolated fibres surrounded by connective tissue. The morphological appearance of the pig and bovine TT could perhaps be explained on the basis that the final stage of muscle development (neonatal to adult myosin) was not completed, although growth of the connective tissue continued, resulting in a 'muscle' which is morphologically much better suited to the role of ligament. This would be consistent with our observation that many fibres contained both neonatal and IIA myosins, and that this was the case even from birth. The cause of such an arrested development (if this is what it is) is of course unknown, but denervation of the muscle can be excluded as a cause since both AChEase and silver staining of these muscles show that motor innervation of fibres is retained.

SUMMARY

In previous studies of middle ear muscles, the classification of fibre types by histochemical methods was particularly difficult in the bovine and porcine tensor tympani muscle, suggesting the presence of immature fibres. We therefore reexamined the tensor tympani from pigs and cattle of various ages immunohistochemically, using a panel of antimyosin antibodies, including one (anti-NE) specific for neonatal and embryonic myosins. Fibres positive to anti-NE were found in tensor tympani in both species in all ages examined; only a few of these fibres reacted exclusively with this antibody; some also contained slow myosin and the majority also contained adult fast (type IIA) myosin. Furthermore, although the remaining fibres included some of the classical types I and IIA, the majority of them showed a mismatch between their histochemical and immunohistochemical profiles. The morphological appearance of the muscle, the widespread presence of neonatal myosin (often together with another myosin in the same fibre) and the persistence of this composition from birth to adulthood, could be explained by an incomplete development of the muscle fibres, resulting in a 'muscle' much better suited to the role of a ligament.

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