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Detailed morphology of the tensor tympani muscle of the rat. An integrated light microscopical, morphometrical, histochemical, immunohistochemical and electron microscopical study in relation to function*

H. VAN DEN BERGE AND P. WIRTZ†

Department of Otorhinolaryngology, University of Maastricht, The Netherlands and †formerly Department of Cell Biology and Histology, University of Nijmegen, The Netherlands

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INTRODUCTION

Despite extensive research throughout centuries the precise function of the smallest striated muscles in mammals, the stapedius muscle and the tensor tympani muscle, remains rather obscure. Several speculations about their function have been proposed (see Guthrie, 1940; Borg, Counter & Rösler, 1984). One of the most attractive theories is that they protect the inner ear from noise damage. This theory, however, has become contested on the basis of assumptions that (i) contraction, especially of the tensor tympani muscle, takes place too slowly to protect the ear from loud impact noise and that (ii) a process of fatigue of the muscle reflex occurs over longer periods of noise exposure (Borg & Nilsson, 1984).

Detailed integrated knowledge of the morphological, enzyme histochemical and immunohistochemical characteristics would be of help in shedding light on the properties of the fibres of the tensor tympani muscle and hence help to understand their role in sound transmission in the middle ear. Most anatomical studies so far, however, have been focused on one, or at most only a few, of the characteristics mentioned above (Zuckerkandl, 1884; Krebs, 1905; Spiesman, 1927; Malan, 1934; Kobayashi, 1956; Malmfors & Wersäll, 1960; Brzezinski, 1962; Blevins, 1963; David, Gerhardt & Uerlings, 1966; Mascarello *et al.* 1982; Gerhardt, David & Marx, 1966; Fernand & Hess, 1969; Hirayama, Davidowitz & Daly, 1974; Anderson, 1975; Haugsten, Bendiksen, Dahl & Teig, 1982; Veggetti, Mascarello & Carpenè, 1982; Mascarello, Veggetti, Carpenè & Rowlerson, 1983; Haugsten, Dahl & Teig, 1984; Jong *et al.* 1988). In addition most reports have dealt with different animal species showing major interspecies differences (Kobayashi, 1956; Mascarello *et al.* 1982; Veggetti *et al.* 1982; Mascarello *et al.* 1983). As a result, the integrated knowledge of the exact characteristics of middle ear muscles in a single species is not, at present, available. We therefore set out to study the gross anatomy, microscopic anatomy, muscle fibre morphometry, enzyme histochemistry, immunohistochemistry and electron microscopy of the tensor tympani muscle in the rat, in an attempt to gain an insight into its possible role in sound transmission.

* Reprint requests to H. van den Berge, University Hospital of Maastricht, Department of Otorhinolaryngology, Postbox 1918, 6201 BX Maastricht, The Netherlands.

MATERIALS AND METHODS

After studying the topographical anatomy, ten tensor tympani muscles of twelve weeks old male Lewis rats were removed during pentobarbitone anaesthesia. Immediately after removal the muscles were frozen in liquid nitrogen-cooled isopentane. Serial cryosections of 10 μm were made and the following staining techniques were applied: (*a*) Sirius red (Sweat, Puchtler & Rosenthal, 1964, modified by Lubbers, Loermans & Wirtz, 1988); (*b*) myofibrillar ATPase (ATPase, EC 3 . 6 . 1 . 3, Dubowitz & Brooke, 1973; Padykula & Herman, 1955), preincubation pH 3·9, 4·1, 4·35, 6·4, 9·3 and 10·3; (*c*) succinic dehydrogenase (SDH, EC 1 . 3 . 99 . 1, Nachlas *et al.* 1957); (*d*) alpha glycerophosphate dehydrogenase (GPox EC 1 . 1 . 99 . 5, Wattenberg & Leong, 1960, modified for GPox by Meijer & De Vries, 1978); (*e*) immunohistochemical reactions with type-specific heavy chain myosin antibodies using the indirect peroxidase method (anti-embryonic, anti-neonatal, anti-slow, anti-slow plus IIA, anti-IIB and anti-slow tonic). As a second antibody RAMPO (rabbit anti-mouse peroxidase) was used.

Histochemical characterisation of the fibres was done using serial cross-sections of four muscles stained for ATPase, SDH and GPox according to Wirtz, Loermans, Peer & Reintjes (1983).

For the immunohistochemical stainings, reference sections of the soleus muscle and the extensor digitorum longus (EDL) muscle of the rat were included in the same series of incubation of tensor tympani muscle sections. This was done since the enzyme histochemical fibre type composition of the soleus and EDL is well documented (Zuurveld, Wirtz, Loermans & Veerkamp, 1985), and a good correlation with the immunohistochemical reactions was found (Reggiani *et al.* in preparation). For the anti-slow tonic stainings, sections of the tensor tympani muscle of a dog served as a reference, since positively reacting slow tonic fibres are found in this species (Mascarello *et al.* 1982). For the anti-embryonic and anti-neonatal staining, sections of one day old mouse hind limb muscle served as a reference, since positively reacting fibres were found in these sections (Reggiani *et al.* in preparation). The immunohistochemical characterisation of the muscle fibre types was compared to the enzyme histochemical characterisation.

The cross-sectional area (in μm^2) of the individual fibres was determined with a MOPP Videoplan 2000 digitiser. GPox-stained cross-sections from the mid-belly of the muscle were used for this purpose. About 400 muscle fibres in a representative area of a cross-section were measured. In addition measurements were made of groups of fibres at successive levels from tendon to tendon. Measurements were made in three different muscles.

For a reconstruction of the anatomical structure of the muscle, 10 μm serial frozen cross-sections from the origin to the insertion tendon were collected from three different muscles and stained with Sirius red.

For electron microscopy, three muscles were dissected out following perfusion fixation through the heart. Thin sections were double contrasted with uranyl acetate and lead citrate and examined in a Philips electron microscope EM 300.

RESULTS

Gross anatomy

The rat tensor tympani muscle is a unipennate muscle with a length of about 3 mm, including the tendons, and a maximal diameter of about 1 mm. Figure 1 shows

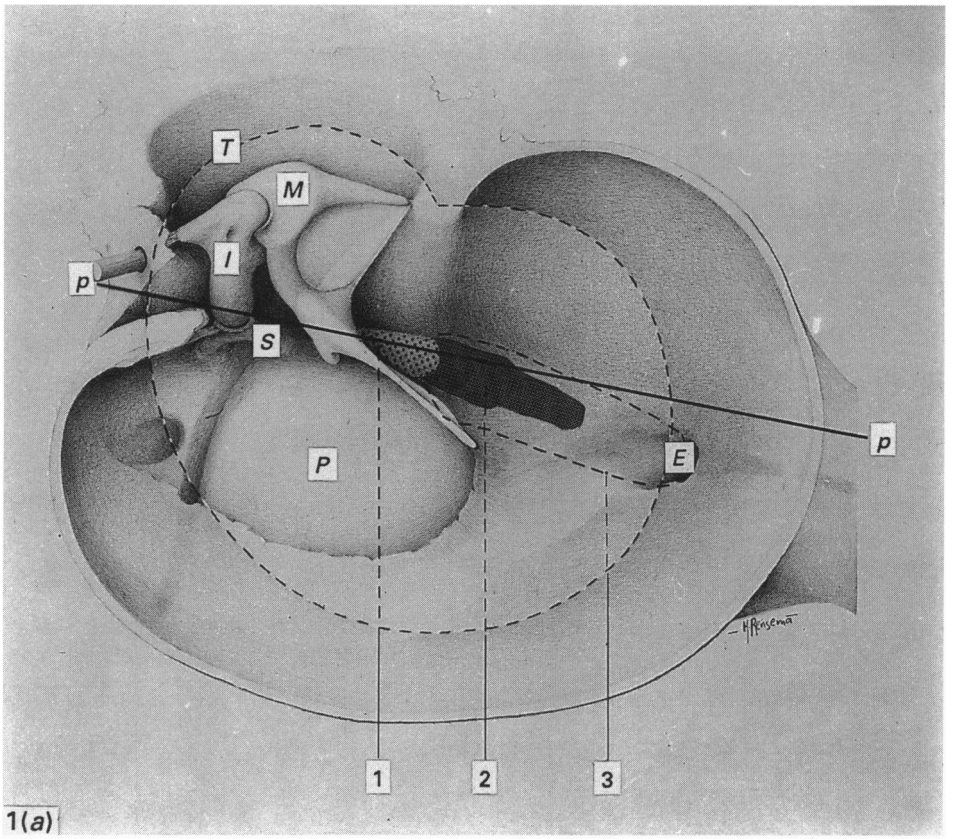


Fig. 1 (*a–b*). Schematic representation of the middle ear bulla of the rat. (*a*) Lateral view. (*b*) Section in the plane of line *p–p* in (*a*). *T*, tympanic membrane; *M*, malleus; *I*, incus; *S*, stapes, *P*, promontory; *E*, orifice of Eustachian tube; *L*, loose connective tissue; *C*, connective tissue; *B*, bone; 1–3, tensor tympani muscle; 1, tendon; 2, part of the muscle exposed in bulla; 3, projection of the whole muscle.

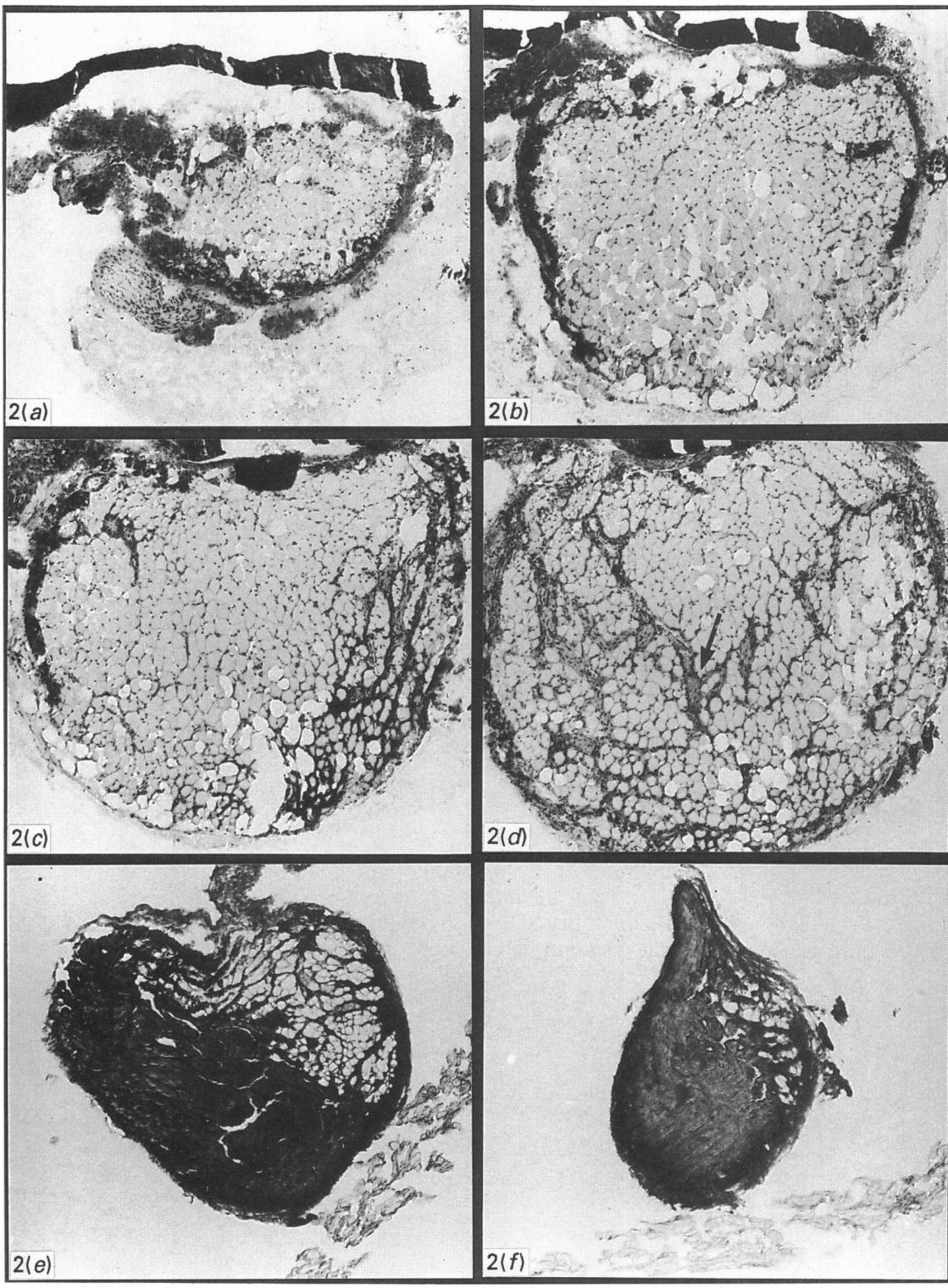


Fig. 2(*a–f*). Frozen cross-sections of an excised tensor tympani muscle of the rat. Sirius red stain. From (*a*) to (*f*) sections at successive levels from origin to insertion. Note the attachment of the tendons to the muscle compartment. Fat cells are present as small empty holes in between the muscle fibres. There is extensive nerve branching (arrow). × 73.

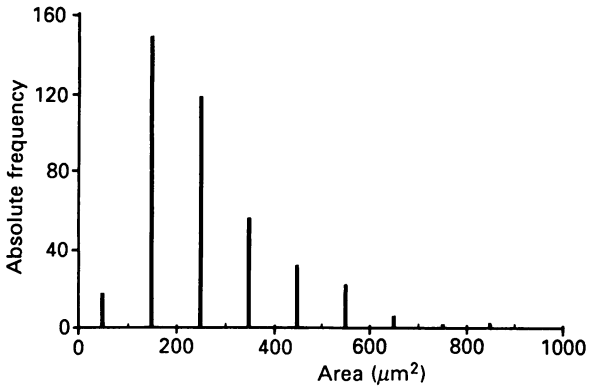


Fig. 3. Histogram of the cross-sectional area of single muscle fibres of the rat. N = 406. Mean single fibre cross-sectional area 259 μm^2; median 226 μm^2; S.D. 140.

schematically the anatomical relations. The muscle is situated in the medial wall of the middle ear bulla and has a somewhat C-shaped curve. A tympanic part and a small tubal part, along the Eustachian tube, can be distinguished. The tympanic part of the muscle is only partially covered by bone near the Eustachian tube. The small tubal part is situated medial to the Eustachian tube and appears to be in contact with the muscles of the nasopharynx. The insertion tendon is relatively thick and has an angle of 135° to the belly of the muscle and is attached to the medial process of the malleus.

Microscopic anatomy

Figure 2 shows cross-sections stained with Sirius red, at successive levels from origin to insertion.

The muscle is surrounded by a thick epimysium, that can be distinguished microscopically from the connective tissue of the tendons and from the endomysial connective tissue. A loose connective tissue compartment connects the muscle with the periosteum and the bone (Fig. 1*b*).

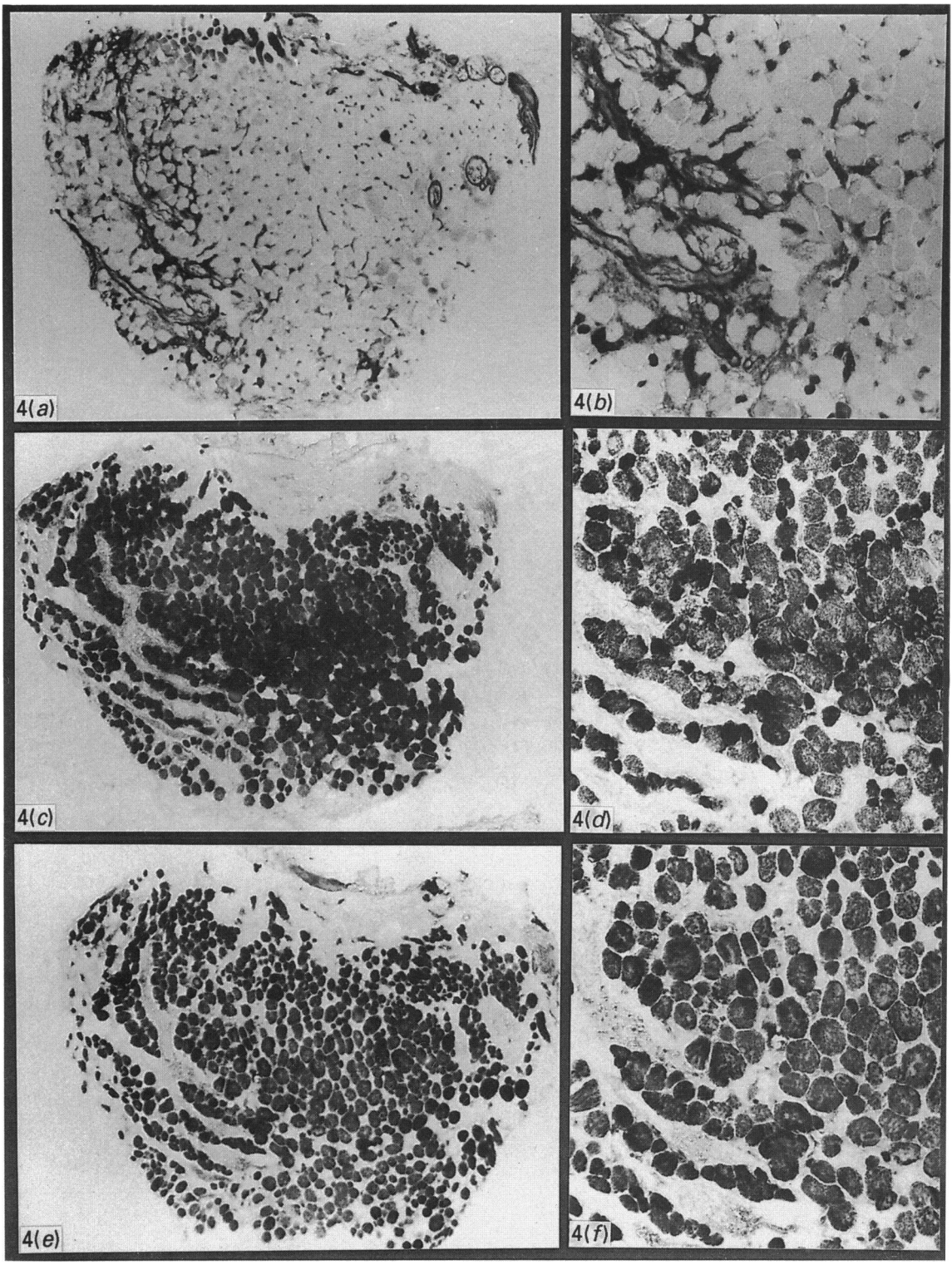
The muscle fibres are arranged in parallel but not in a very regular fashion. This is mainly due to the extensive, irregular strands of connective tissue that separate the muscle fibres. Scattered between the muscle fibres are fat cells (Fig. 2).

Each tendon forms a tendon plate to which the fibres are attached obliquely. This is most pronounced at the insertion side. The attachment site at the tendon of origin is more or less cup-shaped and encloses the muscle fibres, whereas the insertion tendon tapers alongside the muscle fibres over some distance.

Conspicuous features are the elaborate nerve branches that are contained within the connective tissue strands (Fig. 2*d*).

Morphometry

At the site of the largest cross-section, the muscle consists of about 1000 muscle fibres. The mean cross-sectional area of a single fibre is 259 μm^2, but the cross-sectional area of the individual muscle fibres shows considerable variation (S.D. 140). Relatively large fibres form the central part of the muscle, whereas smaller fibres dominate at the periphery (Fig. 2). The mean single fibre cross-sectional area of the enzyme histochemically typed slow twitch fibres, as measured in various rat tensor tympani muscles, is small (109 μm^2) related to the fast twitch fibres. This results in the histogram (Fig. 3) showing a somewhat skewed fibre distribution. Compared to the fibre areas in the belly of the muscle, the fibre areas at the tendon sites are about 45 %


4(a)
4(b)
4(c)
4(d)
4(e)
4(f)

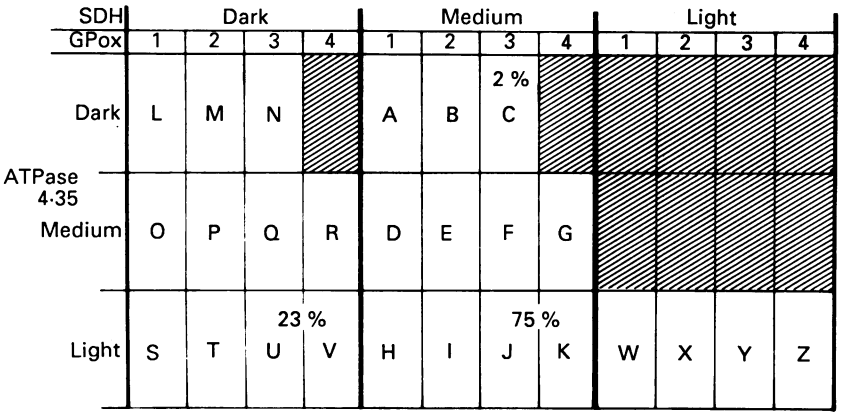


Fig. 5. Combination scheme of histochemical reactions (ATPase pH 4·35, SDH and GPox) indicating the different fibre types (Wirtz *et al.* 1983). A–C, slow fibres, equivalent to slow oxidative fibres* (SO); D–G, transitional fibres; L–N and O–R, rarely occurring fibres; S–V, fast-red fibres; of these U and V are equivalent to fast oxidative glycolytic* (FOG); H–K, intermediate fibres; W–Z, fast white fibres; of these Y and Z are equivalent to fast glycolytic fibres* (FG). The shaded blocks are non-occurring combinations. The figures 1–4 for GPox indicate weak–very strong staining intensity. (*, According to Peter *et al.* 1972.) The percentages of fibre types occurring in the tensor tympani muscle of the rat are indicated in the figures.

smaller (Fig. 2*e*). This indicates tapering of the fibres near the tendons. Qualitatively, no major inter-individual differences were found.

Enzyme histochemical characterisation

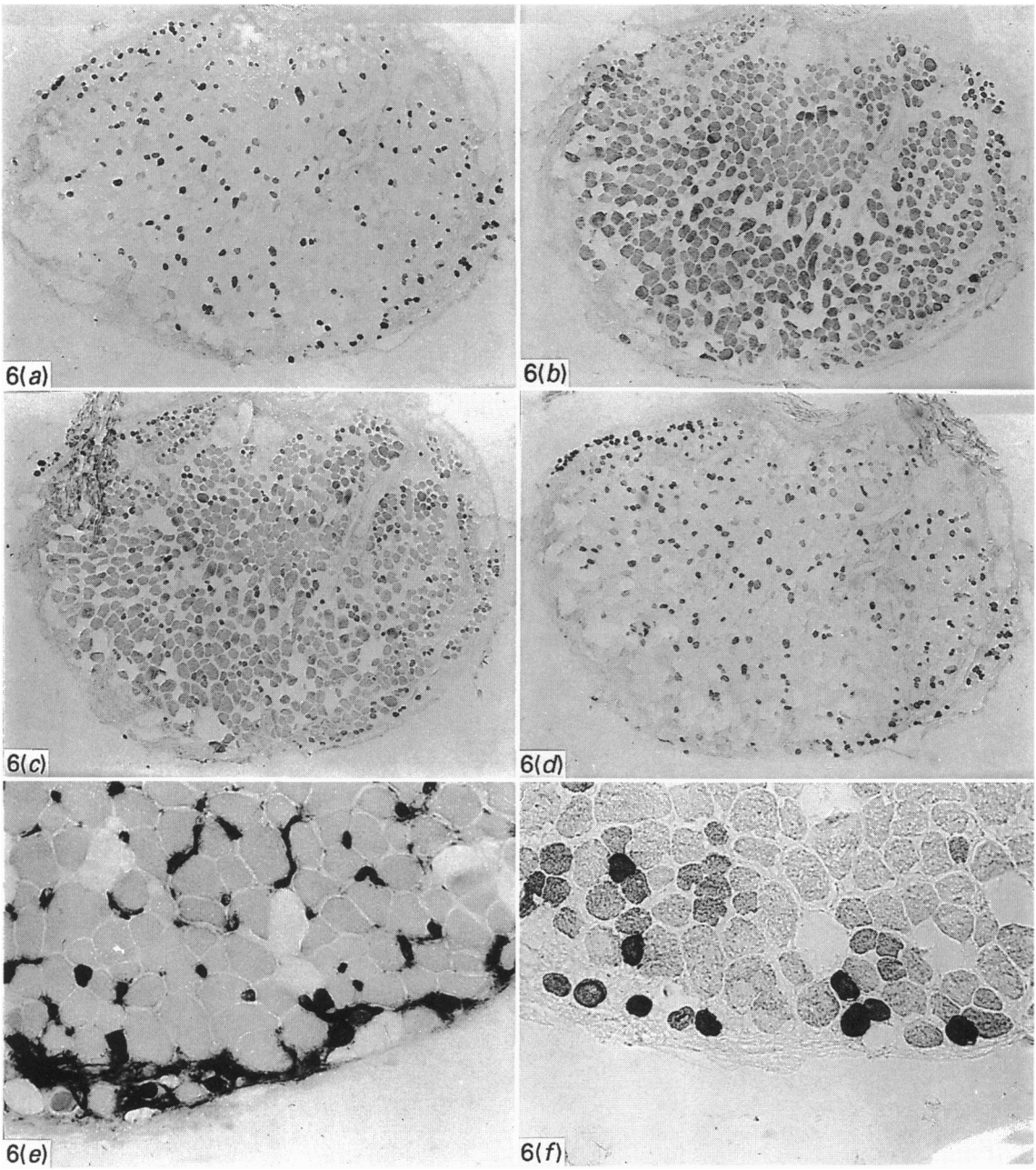
Figure 4 shows serial cross-sections stained for, respectively, ATPase (pH 4·35), SDH and GPox, while in Figure 5 the occurrence of the various fibre types is indicated schematically. The majority of the fibres reacted weakly with an acid-preincubated (pH 4·35) ATPase reaction (98 %). The staining intensity of these fibres for SDH was moderate to strong (Fig. 4*c*), and strong to very strong for GPox (fig. 4*e*). In many fibres the distribution of the formazan stain for SDH and GPox was atypically patchy (Fig. 4*d,f*). Electron microscopy revealed the mitochondria to be clustered in these fibres (see below). Based on enzyme histochemical criteria the fibres can be classified as fast oxidative glycolytic fibres, most closely resembling IIA fibres. Two per cent of the fibres showed a strong reactivity with the ATPase staining (pH 4·35). These fibres also revealed a moderate to strong staining intensity with SDH staining and a strong reactivity with GPox staining. In the more acid-preincubated ATPase stainings (pH 4·1 and 3·9), these fibres reacted negatively, as they did after alkaline ATPase (pH 10·3). Consequently these fibres should be called slow oxidative glycolytic fibres and not IIC fibres.

Immunohistochemistry

Figure 6 shows serial cross-sections stained with the various anti-heavy chain myosin antibodies.

With the anti-slow antibody (Fig. 6*d,j*), 31 % of the fibres were found to react positively when compared with the reference sections (26 % stained moderately, 5 %

Fig. 4(*a–f*). Frozen cross-sections of an excised tensor tympani muscle of the rat. On the left serial sections of respectively ATPase (*a*), SDH (*c*) and GPox (*e*). On the right (*b,d,f*) details of the corresponding sections. With the ATPase staining (pH 4·35) most fibres show a weak reaction (fast twitch fibres), only a few fibres being stained dark (slow twitch fibres). Note the strong activity and the atypical (patchy) distribution following SDH and GPox staining. (*a,c,e*) × 91; (*b,d,f*) × 185.



stained darkly). Not all fibres staining darkly with the anti-slow antibody were dark in the ATPase staining, but all the dark fibres in the ATPase staining (pH 4·35) also showed a strong staining with the anti-slow antibody (Fig. 6*e*, *f*).

All fibres reacted positively with the anti-slow plus IIA antibody (Fig. 6*c*, *i*). Some small fibres corresponding to the dark fibres in the anti-slow staining showed a more intense staining. Compared to the reference staining, the majority of the fibres showed a positive reaction after incubation with anti-IIB antibody (Fig. 6*d*, *h*). They could be clearly distinguished from the background level of the connective tissue. Thus these

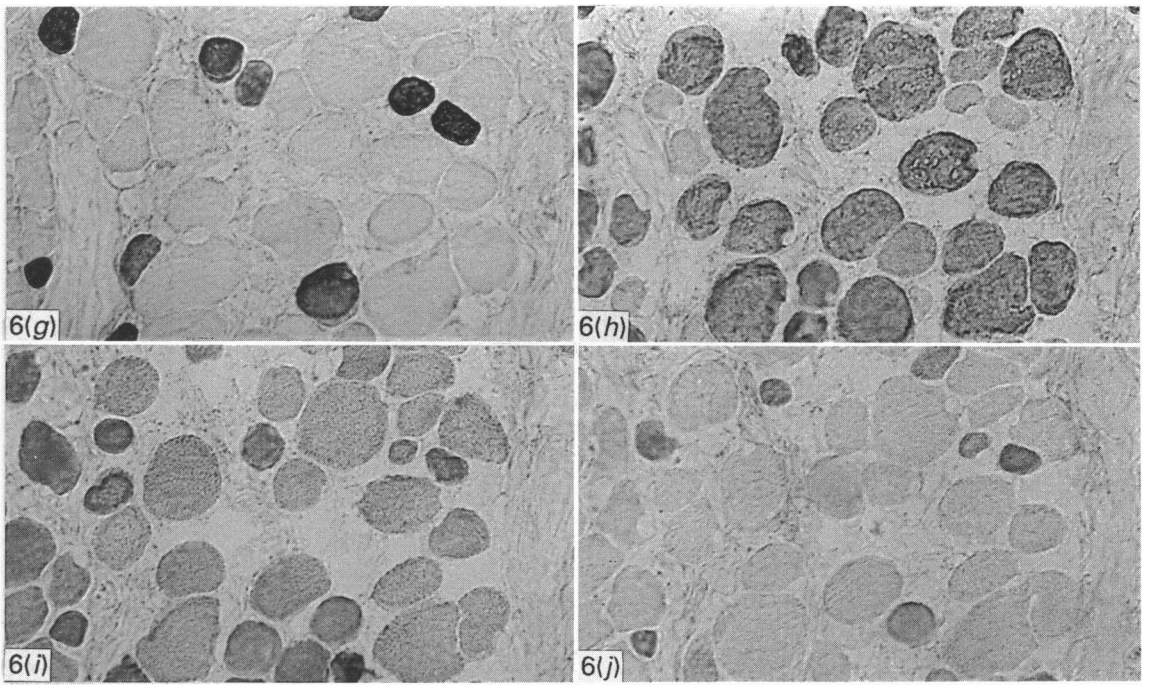


Fig. 6(*a–j*). Anti-heavy chain myosin stainings of serial cross-sections of the tensor tympani muscle of the rat. (*a–d*) Serial cross-sections; (*e–j*) details. (*a*) anti-neonatal; (*b*) anti-IIb; (*c*) anti-slow plus IIA; (*d*) anti-slow; (*e–f*) detail of respectively ATPase staining (pH 4·35) and the corresponding anti-slow staining. (*g–h*) details of (*a–d*). See text. (*a–d*) ×60; (*e–f*) ×236; (*g–j*) ×475.

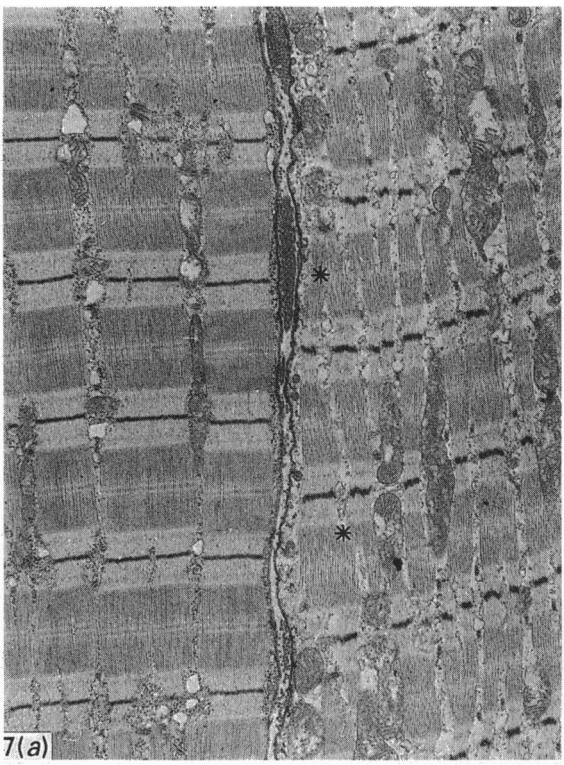
fibres, too, should be classified as anti-IIB positive. Some small fibres, partly corresponding with the dark fibres in the anti-slow staining, showed quite a weak reaction (compare Fig. 6*h, j*).

No fibres reacted positively with the anti-embryonal antibody. With the anti-neonatal antibody (Fig. 6*a, g*) 29% of the fibres showed a positive reaction. It appeared that many anti-slow positive fibres also reacted positively for the anti-neonatal antibody (compare Fig. 6*g, j*).

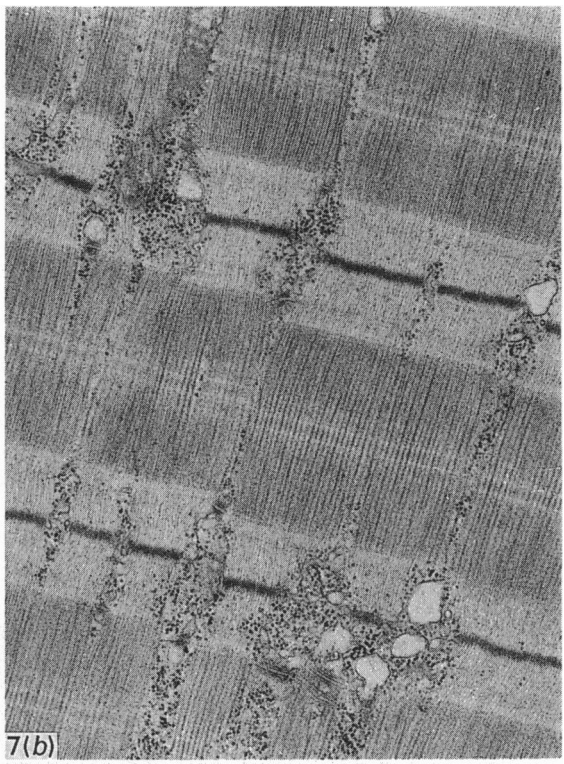
No fibres reacted positively with the anti-slow tonic antibody, but neither were positive fibres observed in reference sections from a dog.

Electron microscopy

Electron microscopy confirmed the abundant collagen fibres in the intercellular compartment, combined with a fair number of fibroblasts. The fibre population could be roughly divided into two groups of fibres according to the arrangement of their organelles. One group was formed by fibres that more or less resembled the fast glycolytic fibres in skeletal muscles of the extremities. The other group, however, was composed of fibres that possessed a rather atypical ultrastructural organisation of bundles of myofilaments and distribution of mitochondria (Fig. 7*a*). Bundles of myofilaments appeared to be branched and interconnected with other bundles of myofilaments (Fig. 7*d*). In several fibres large subsarcolemmal mitochondria were often distributed in clusters. Also long and elaborate interfilamentous mitochondria were found that easily spanned the length of a sarcomere (Fig. 7*a, c*). Other regions of the fibres were almost lacking in mitochondria. Figure 7 shows that the

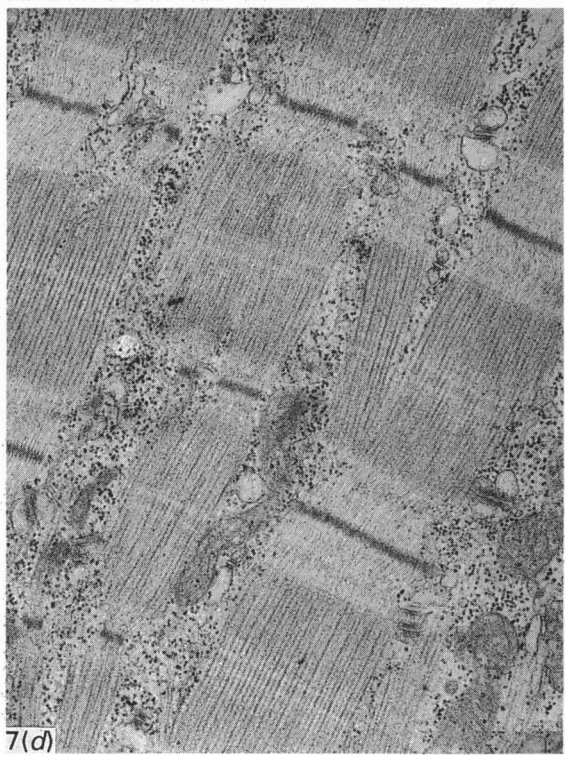


7(a)



7(b)

7(c)



7(d)

mitochondria of these fibres were distributed in a rather random fashion in contrast to, for instance, the regular pattern of localisation in the I-band region in fast glycolytic fibres from skeletal muscle of the extremities. This, together with the branched course of the bundles of myofilaments, resulted in an atypical, i.e. rather disorderly, ultrastructural arrangement of some of the fibres.

DISCUSSION

From our data it appears that, in comparison with other muscles of the rat, the tensor tympani muscle demonstrates several atypical features giving rise to interesting speculations about its function. The data collected in this study strongly suggest that the tensor tympani muscle displays an almost isometric contraction pattern.

In situ the muscle is C-shaped; the tendon of insertion is connected to the muscle fibres at an obtuse angle. Because the tubal part of the muscle is hardly covered by bone and because a loose connective tissue forms a sleeve between the attachment of the muscle and the bone, one would expect a relatively large range of motion to be possible at the site of attachment on the malleus when the muscle contracts. However, several factors may be considered to restrict the range of movement. First of all, the oblique attachment of the muscle fibres to the tendon of insertion diminishes the effective contraction force, and thereby the amplitude of motion (Close, 1964; Mascarello *et al.* 1982). Secondly, since the main part of the muscle is not covered by bone and a large part of the muscle is connected to the bone by a loose connective tissue, the angle between the muscle fibres and the bone becomes more obtuse upon contraction, a feature observed *in vivo*, resulting in less motion at the insertion site. Furthermore, the low compliance of the chain of ossicles and the tympanic membrane diminishes the possible amplitude of motion.

The muscle is also likely to exert an important influence on the dynamics of the chain of ossicles in a static situation: the tendon of insertion is relatively thick as in many mammals (Kobayashi, 1956), which undoubtedly influences the dynamic characteristics of the chain, and thereby the transmission of sound, by a damping effect. The role of the fat cells remains uncertain. In tensor tympani muscles of various mammals fat cells have been described (Kobayashi, 1956). Theories about a function related to the dynamics of the muscle and also about a metabolic function have been postulated (see, for references, Anderson, 1976). In view of our metabolic observations we favour the latter opinion.

With electron microscopy we observed branching of bundles of myofilaments which interconnect with other bundles, a feature not found in normal skeletal muscle. In heart muscle, branching of bundles of myofilaments does occur, but they do not interconnect with each other. It seems therefore that on contraction of the muscle, in addition to longitudinal forces, transverse forces are also being developed.

The above features would all fit in the concept of an isometric contraction pattern of the tensor tympani in the rat. Furthermore, the enzyme histochemical and immunohistochemical data point to other peculiar functional characteristics of this middle ear muscle. All fibres showed a high aerobic and a high glycolytic capacity. Thus the fast fibres (98 %) should be classified as fast oxidative glycolytic and the slow

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Fig. 7(*a–d*). Electron microscopy of longitudinal sections of the tensor tympani muscle of the rat. (*a*) Two muscle fibres. The fibre on the right has a larger number of more elaborate mitochondria than that on the left. Branching of myofilament bundles can be seen in the fibre on the right (*). (*b*) Detail of the fibre on the left in (*a*). (*c*) Detail of the fibre on the right in (*a*). Note large mitochondria with many cristae occurring in clusters. (*d*) Detail of a branching bundle of myofilaments. (*a*) ×6055; (*b–d*) ×18000.

fibres (2%) as slow oxidative glycolytic. The latter are not the classical Type I fibres, due to their high glycolytic capacity.

Contrary to skeletal muscles of the extremities, classification of the muscle fibres of the tensor tympani muscle according to immunochemistry was difficult. First of all, far more Type I fibres were found with anti-slow antibodies than with ATPase as a marker (respectively 31% and 2%).

There is no disagreement about the majority of the fibres being of a fast twitch type, but unlike normal adult skeletal muscles, e.g. of the extremities, many of the fibres reacted positively with both the anti-IIA and anti-IIB antibodies. Moreover, about one third of the fibres reacted positively with the anti-neonatal antibody. In adult skeletal muscle this myosin is absent (Schiaffino *et al.* 1988). Fibres positive for the anti-slow tonic antibody were not found, but our experiments are not conclusive, since reference sections of a dog tensor tympani muscle did not reveal positive fibres either, in contrast to the findings of Mascarello *et al.* (1982).

The above-mentioned immunohistochemical findings show that several heavy chain myosins can be present within one fibre, even in what would appear to be a rather well-differentiated striated muscle fibre of the tensor tympani. The fibres do contain the adult myosin types but, in contrast to normal skeletal muscle, they are found together. Supposedly, genes coding for the various myosin heavy chains continue to be activated, and this possibly occurs in different nuclei that are contained in the syncytium. During normal postnatal development gene activation and inactivation is a regular feature that, among other factors, regulates the successive production of myosin isotypes such as embryonic, neonatal, slow and fast. But in this case the co-occurrence of several myosin iso-types in a muscle fibre merely indicates a transitional situation. There are no indications that such a situation exists in the middle ear muscle we studied. The biological significance of the co-expression of different myosin fibres in well-differentiated muscle fibres, therefore, remains enigmatic.

When translating the enzyme and immunohistochemical characteristics into terms of function, the concept of a muscle with the potentials of a fast contraction pattern and a high fatigue resistance becomes apparent. From our data the tensor tympani muscle of the rat emerges as a muscle with an almost isometric contraction pattern, the fibres of which are fast to contract and at the same time fatigue-resistant. These characteristics, in our opinion, endow the muscle with ideal properties to serve a function in protecting the ear from loud noise over long periods of time. Electrophysiological studies, necessary to confirm this concept, are in progress.

SUMMARY

The gross anatomy, microscopical anatomy, morphometry, enzyme histochemistry, immunohistochemistry and electron microscopy of the tensor tympani muscle of the rat was studied. The aim of the study was to create an integrated insight into the morphology of the muscle and to discuss functional implications.

The tensor tympani muscle of the rat is an atypical muscle. It is a small muscle composed of very small muscle fibres with a complex microscopical and sub-microscopical architecture. Histochemically the muscle consists mainly of fast oxidative glycolytic fibres, but discrepancies are found when anti-heavy chain myosin antibodies are used for fibre typing. Different adult heavy chain myosin isotypes coexist in one single muscle fibre. The electron microscopical study shows that bundles of myofilaments branch and interconnect with other bundles of myofilaments.

The findings suggest that the muscle is able to contract fast and is fatigue-resistant.

Both features seem to suggest that the muscle has a function in protecting the inner ear against noise damage.

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