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A comparative study of the transverse tubular system of the rat extensor digitorum longus and soleus muscles

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INTRODUCTION

Activation of skeletal muscle involves the release of calcium from the terminal cisternae of the sarcoplasmic reticulum in response to depolarisation of the transverse (T) tubular membrane. In 1973 Schneider & Chandler measured a voltage-dependent charge movement in frog skeletal muscle fibres which was possibly involved in the coupling of calcium release to tubular depolarisation. The estimated tubular density of charge particles is similar to that of the electron-dense 'feet' which extend between the T system and sarcoplasmic reticulum membranes in frog skeletal muscle (Franzini-Armstrong, 1970) and Schneider & Chandler suggest that one charge particle may be associated with each foot.

In a comparative study of charge movement in rat skeletal muscles, Hollingworth & Marshall (1981) found that the tubular density of charge was three to five times greater in fibres from fast twitch (extensor digitorum longus) than from slow twitch (soleus) muscles. In order to examine whether this difference in charge movement is associated with a difference in the fine structure of the T system and in particular in the distribution of the feet, a quantitative electron microscope investigation has now been carried out of the T system in the extensor digitorum longus and soleus muscles.

There is detailed published data on the ultrastructure of the T system in fast and slow twitch guinea-pig muscle (Eisenberg, Kuda & Peter, 1974; Eisenberg & Kuda, 1975), in fast and slow twitch mouse muscle (Luff & Atwood, 1971) and in the frog sartorius (Peachey, 1965; Mobley & Eisenberg, 1975) but not in rat muscle.

MATERIALS AND METHODS

Six healthy Wistar rats weighing 200–250 g were used in this study. They were killed by cervical dislocation and the extensor digitorum longus and soleus muscles were rapidly removed. Thin slivers of the muscle were taken and pinned onto pieces of dental wax at approximately rest length before being placed in fixative. The primary fixative was 5% glutaraldehyde in 0·1 M phosphate buffer at pH 7·0–7·2. After 1 hour the muscle was cut into blocks $2 \times 1 \times 1$ mm and left in fixative for a further hour. The blocks were then washed three times in the buffer, post-fixed for 1 hour in 1% osmium tetroxide, dehydrated in graded alcohols and embedded in Araldite. Thin sections were cut on a Reichert OMU2 ultramicrotome and collected on uncoated grids. They were stained with uranyl acetate and lead citrate and examined in a Siemens EM102 electron microscope.

Selection of blocks and electron micrographs

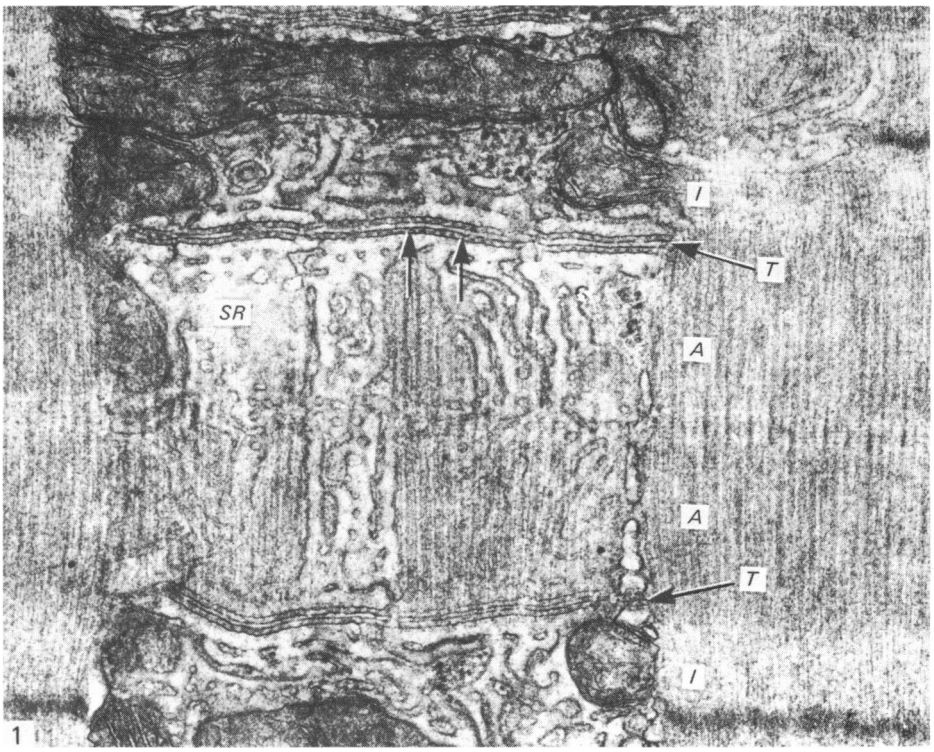
Blocks from each muscle were selected in which the orientation of the muscle fibres was either exactly longitudinal or exactly transverse. The criterion for good longitudinal orientation was that the A filaments could be followed from end to end in five successive sarcomeres. The criterion for good transverse orientation was that the A filaments were clearly defined and were approximately hexagonally spaced. The triads were photographed in longitudinal and transverse section at a nominal magnification of ×30000, subsequently enlarged to approximately ×90000. The true magnification was calculated using the length of the A filaments as a calibration standard. This length was considered not to vary from fibre to fibre and from muscle to muscle and measurements by various techniques yield values of 1·57 μm (Sjöström & Squire, 1977), 1·60 μm (Page & Huxley, 1963; Ishikawa, 1980) and 1·63 μm (Cullen & Weightman, 1975). A value of 1·6 μm has been used in the present calculations. Measurements of T tubule dimensions were made from the electron micrographs using a ×8 eyepiece magnifier with a graticule divided at 0·1 mm intervals (Polaron Ltd). For each tubule both the external and internal width (perpendicular to the longitudinal axis of the fibre) and depth (parallel to the longitudinal axis) were measured, thus allowing comparison of the cytoplasmic (external) and luminal (internal) surface areas. Where the outlines of the tubule membranes appeared wavy or irregular, the lines on the graticule were aligned along the long axis of the tubule so that they measured the average depth.

In most electron micrographs the orientation of the section was random with respect to the plane of the T tubules and only a small proportion of sections cut the tubules at 90° or near 90° to their longitudinal axes, when the measured width should have been a minimum. As the sections became more oblique to the axis the outer edges of the tubules became ill-defined. All such profiles were disregarded. Only T tubules which were clearly defined were measured (less than 2% of the tubules encountered). Where tubules were sectioned parallel to their axes (Figs. 1–3) the rows of feet projecting from the adjacent sarcoplasmic reticulum could be distinguished and measured.

Although the majority of myofibres in the rat extensor digitorum longus were of fast twitch type, about 2–5% were slow twitch. These were recognised by their thicker Z lines (Salmons, Gale & Sreter, 1978; Davey & Wong, 1980) and were not included in the sample of triads taken from the extensor digitorum longus. Similarly in the soleus where the majority of fibres were slow twitch, a variable minority were fast twitch, recognisable by their thinner Z lines, and these fibres were deliberately disregarded in sampling the triads. Thus the values given in the Tables below were for slow twitch fibres (Type 1) compared with fast twitch fibres (Types IIA and IIB) and not, strictly speaking, for a slow muscle compared with a fast muscle. For each

Fig. 1. Longitudinal section of part of a myofibre of the rat extensor digitorum longus muscle. Part of the section has passed along the axes of two tubules of the T system (*T*) which lie at the level of the junction of the A band (*A*) and I band (*I*). Regular densities or feet can be seen in the gap between the sarcoplasmic reticulum (*SR*) and the T tubules. (There are five feet between the two vertical arrows.) ×32500.

Fig. 2. Longitudinal section of part of a myofibre of the rat soleus muscle. The arrangement of the T system (*T*) and the sarcoplasmic reticulum (*SR*) is the same as in the extensor digitorum longus in Fig. 1. M, mitochondrion. ×32500.



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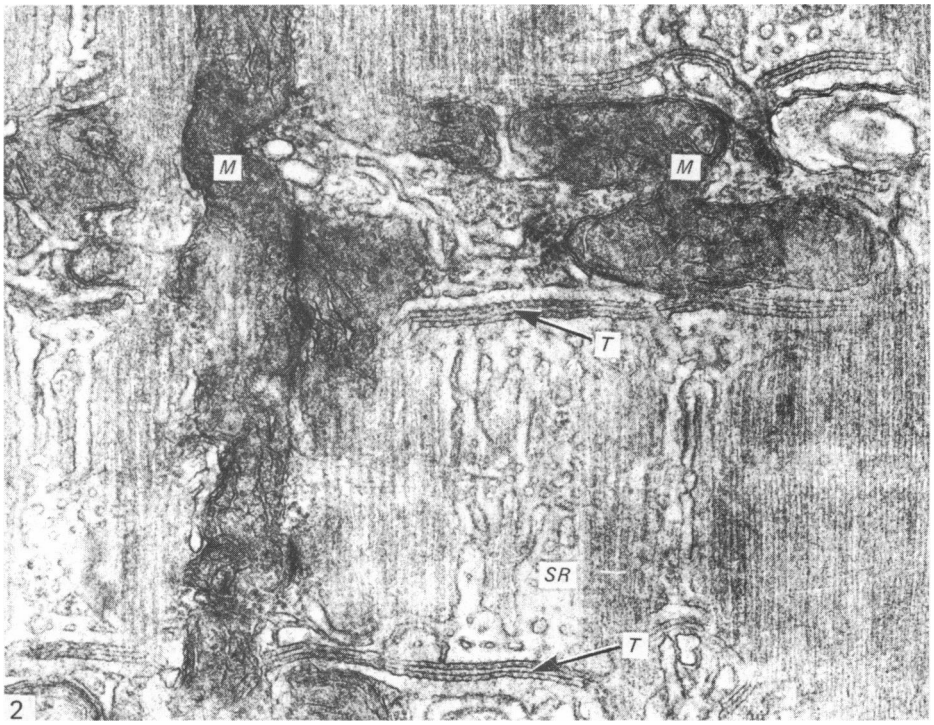

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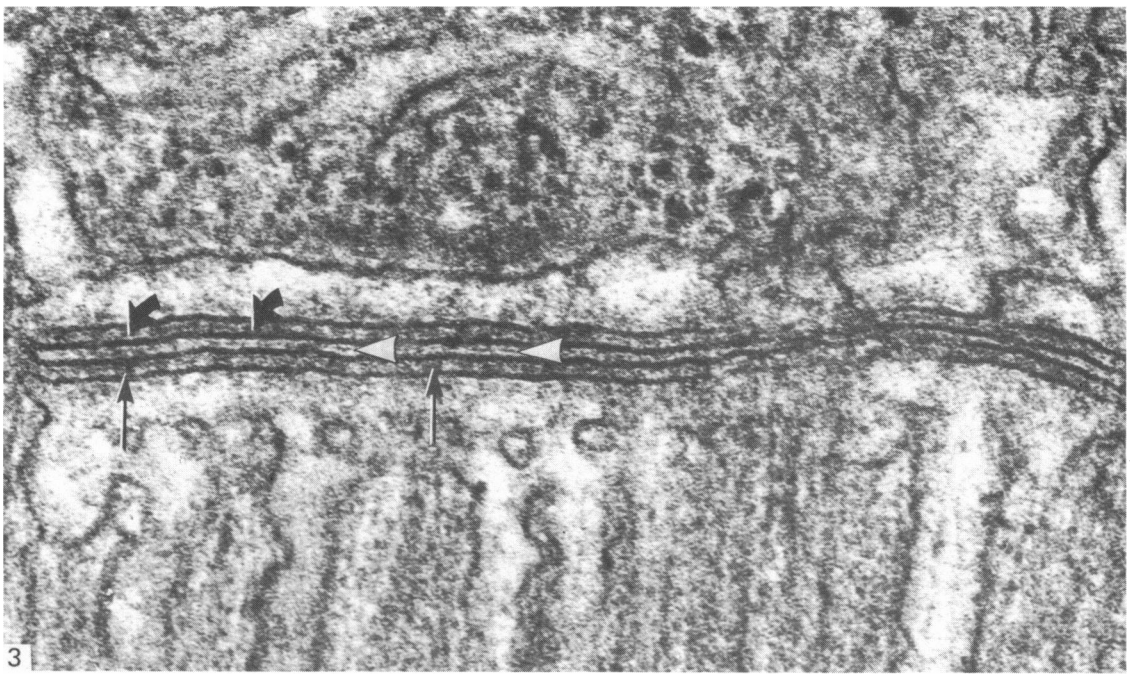

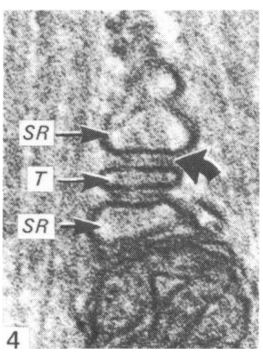

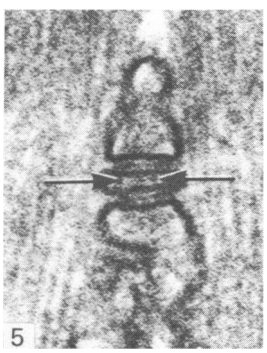

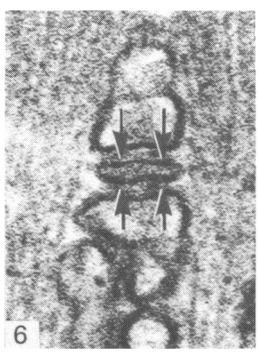

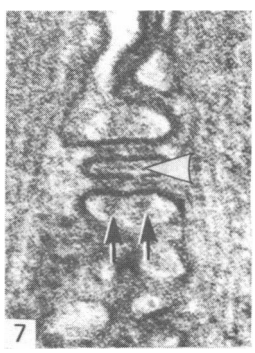

Fig. 3. A transverse tubule and the associated sarcoplasmic reticulum in the extensor digitorum longus muscle viewed at higher magnification. The feet situated in the junctional gap appear either as blocks (vertical arrows) or as interrupted laminae (curved arrows). There is also a lamina within the lumen of the T tubule (white arrowheads). ×90000.

Fig. 4. A triad composed of two terminal cisternae of the sarcoplasmic reticulum (*SR*) and a tubule of the T system (*T*). The feet appear as a poorly defined lamina (curved arrow). Soleus muscle. ×110000.

Fig. 5. In this triad the lumen of the T tubule contains particulate material (arrows). Extensor digitorum longus muscle. ×110000.

Fig. 6. In this triad the paired feet (arrows) are more solid structures than in Figs. 4 and 5. Extensor digitorum longus muscle. ×110000.

Fig. 7. A triad showing dense material (arrows) in the lumen of the terminal cisternae situated opposite the feet that are on the external face of the cisternal membrane. In the lumen of the T tubule there is a very clear lamina (white arrowhead). Extensor digitorum longus muscle. ×110000.

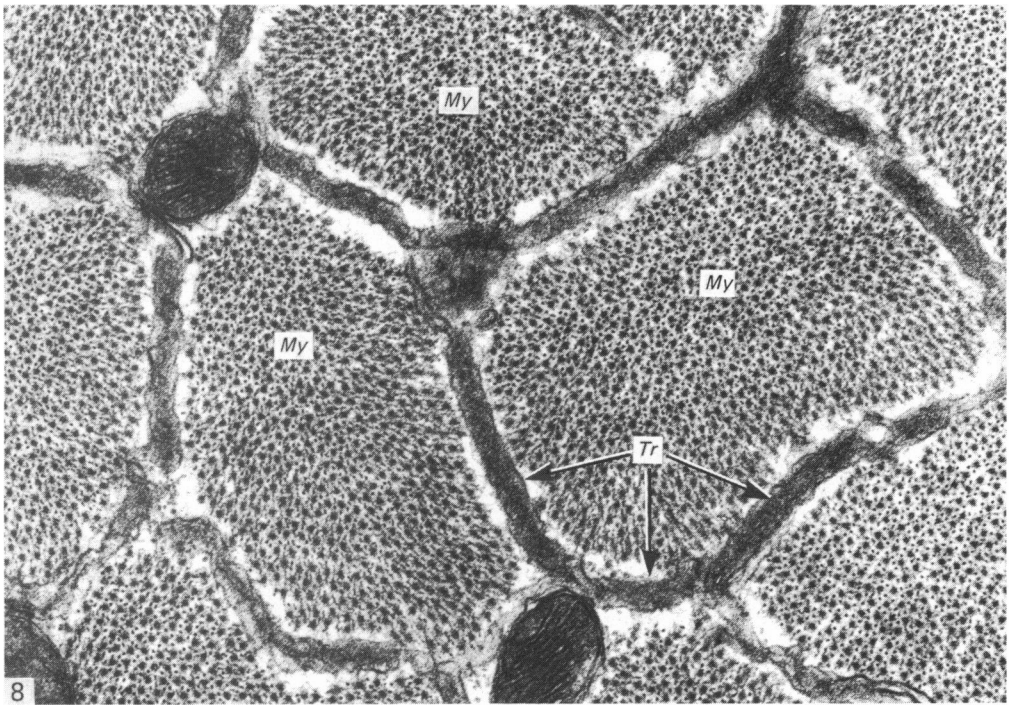

Fig. 8. A transverse section through part of a myofibre of the triads (*Tr*) which define the individual myofibrils (*My*). × 50000.

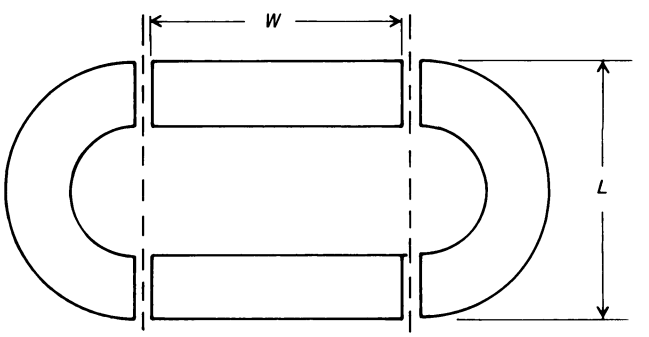

Fig. 9. Schematic diagram showing how the tubule profile was treated as consisting of a rectangle and two semicircles. The surface to volume ratio is equal to the perimeter to area ratio. Perimeter $=2W+\pi L$. Area $= WL+\pi(\frac{1}{2}L)^2$.

muscle the dimensions of eight triads were measured, i.e. sixteen measurements from each of the six animals.

In calculating the fractional area occupied by the T system in a transverse section passing through it, electron micrographs were selected in which the section had passed through the triad. Typically, the thickness of a section was about twice the depth of a transverse tubule so that the chance of a section passing through the T system alone without including any adjacent sarcoplasmic reticulum was extremely small. Fortunately the width of the terminal cisternae (perpendicular to the fibre axis) was very similar to that of the T tubules (Figs. 4–7) so that when the terminal cisternae were included in the transverse sections (Fig. 8) they could, as a close

approximation, be treated as T system in calculating its fraction area. This was done by standard point counting techniques (Weibel, Kistler & Scherle, 1966; Cullen & Weightman, 1975).

Measurements

Volume fraction

This was calculated from a method modified from Peachey (1965). The fraction occupied by the T tubules in transverse section through the triads was multiplied by the length (depth) of one tubule expressed as a fraction of the length of one half sarcomere (there being two triads per sarcomere length). Sarcomere length was measured in sections cut from longitudinally orientated blocks taken from the same region of the muscle as the transversely oriented blocks.

Surface to volume ratio of the tubules

To obtain the surface to volume ratio, the perimeter length to cross sectional area was calculated by treating the tubule profile as made up of a rectangle and two semicircles (Fig. 9).

Surface density

The surface density, or surface of T system per unit volume of myofibre, was calculated by multiplying the surface to volume ratio of the tubules by the volume fraction of muscle occupied by them.

Tubular density of the feet

The centre to centre spacing of the feet was measured where the triads had been sectioned longitudinally and the number per unit area of T tubule membrane calculated assuming a double row on both adjacent junctional membranes of the terminal cisternae of the sarcoplasmic reticulum.

Sources of error

The calculated values for the volume fraction and surface density were likely to be slightly underestimated for two reasons. Firstly, no estimate was included of the contribution made by longitudinal elements of the T system; these are perhaps best visualised in stereo pairs of semithin sections (Peachey, 1981). However segments of T tubules were followed along a total summed distance of 100 μm, and no junction to a longitudinal branch was encountered, so the longitudinal contribution, though present, must have been small.

The second potential source of inaccuracy lay in the methods of measurement employed which did not allow for the occasional tortuosity in the shape of the tubules but were based on an ideal tubule of uniform profile. Unfortunately it was not possible to estimate or even guess how much of the tortuosity was genuine and how much was introduced during fixation and subsequent processing of the tissue – the so-called 'familiar irreducible error of structural measurements' (Eisenberg & Eisenberg, 1982).

RESULTS

When viewed in longitudinal section both the extensor digitorum longus and soleus muscles showed the familiar myofibrillar array of alternating A and I bands. As in all mammalian skeletal muscles (Landon, 1982) the transverse tubules of the

Table 1. *Mean values with standard deviations of the parameters measured and used in calculating the data shown in Table 2*

Each dimension represents the overall mean of 48 measurements (8 from each of 6 animals).

	% of fibre volume occupied by T system in transverse section at triad	Sarcomere length (μm)	Length (depth) of tubule at triad (nm)	Width of tubule at triad
Extensor digitorum longus	15·69±0·81	2·38±0·06	32·12±2·93	118·90±14·0
Soleus	10·67±0·81	2·41±0·05	31·25±4·29	120·98±15·0

Table 2. *The volume fraction, surface to volume ratio and surface density of the T system in rat extensor digitorum longus and soleus muscles*

	Volume fraction (% fibre volume)	Surface to volume ratio of the tubules ($\mu m^2/\mu m^3$ tubule)	Surface density of T system ($\mu m^2/\mu m^3$ myofibre)
Extensor digitorum longus	0·424	76·32	0·32
Soleus	0·277	77·67	0·215

T system passed around the myofibrils at the level of the junctions of the A and I bands, two per sarcomeric unit (Figs. 1, 2). Segments of T tubule that could be followed for more than 1·0 μm across the muscle (Figs. 1–3) were more common in the extensor digitorum longus than in the soleus muscles. More usually, however, little of the tubule structure could be seen because the plane of section lay at an oblique angle to its long axis. When this angle approached 90°, the triad composed of a central T tubule profile between two terminal cisternae of the sarcoplasmic reticulum could be distinguished (Figs. 4–7). When the muscles were sectioned transversely at the level of the triads, the T tubule and terminal cisternae were observed to form a continuous or nearly continuous band around the myofibrils (Fig. 8).

While the basic structure of the triad was consistent, the ultrastructural appearance of the junctional gap was highly variable. More or less regularly spaced densities, the junctional feet (Franzini-Armstrong, 1970), could frequently be seen in the gap between the two membrane systems (Fig. 1). At one extreme, these appeared as electron-dense blocks, approximately 20 nm × 15 nm (Fig. 3), while at the other they were reduced to a narrow lamina running in the centre of the gap (Figs. 3–5). In favourable sections they could be seen to be arranged in pairs (of rows) on either side of the T tubule (Fig. 6).

In the soleus muscle the triads occasionally occurred as dyads, i.e. the terminal cisternae of the sarcoplasmic reticulum abutted one side only of the T tubule. In a sample of 469 triads examined in the soleus muscle, 44 or 9·4% were dyads so approximately 4·7% of the surface of the tubules was not associated with feet. In a similar sample of 487 triads in the extensor digitorum longus muscle, none were single sided.

Inside the lumen of the terminal cisternae, pairs of amorphous accumulations of electron-dense material could occasionally be observed located opposite the pairs of feet (Fig. 7). Moreover, lying within the lumen of the transverse tubules, there was

Table 3. *The measured centre to centre spacing of the feet* (n = 18) *and their density in rat extensor digitorum longus and soleus muscles*

The figure is adjusted for the soleus muscle because 9·38% of the triads were single sided, i.e. they were dyads.

	Centre to centre spacing of feet (nm)	Density of feet (number per μm^2 T system membrane)
Extensor digitorum longus	38·31 ± 1·77	381·15
Soleus	36·96 ± 1·36	390·16 372·06 adjusted

Table 4. *The dimensions of the inner (luminal) surface of the T tubules and the calculated surface to volume ratios of the T system (inner face) in the rat extensor digitorum longus and soleus muscles*

The values are means (n = 48) with standard deviations.

	Length (depth) of tubule inner surface (nm)	Width of tubule inner surface (nm)	Surface to volume ratio ($\mu m^2/\mu m^3$)
Extensor digitorum longus	12·64 ± 2·19	99·38 ± 13·65	174·48
Soleus	13·04 ± 3·27	102·57 ± 15·37	169·13

material which could be particulate (Fig. 5) or, more commonly, laminar in form (Figs. 3, 7).

Values for quantitative measurements made on the T tubules are listed in Tables 1–4. There was no difference in the dimensions of the tubules in the two muscles and the differences in volume fraction and surface density resulted from their closer spacing in the transverse plane in the extensor digitorum longus muscle. This was reflected in the values for the percentage of fibre volume occupied by the T system and for its surface density which were about 50% higher in the extensor digitorum longus muscle (Table 2).

There was no difference in the foot spacing on the terminal cisternae facing the T tubules in the two muscles (Table 3) and the density of feet relative to T system area was the same. The adjusted value for foot density in the soleus muscle (Table 3) allowed for the 4·7% of T tubule membrane surface which was not adjacent to feet.

The data listed in Tables 1–3 are based on measurements made on the outer, cytoplasmic, face of the tubule membranes. It follows, therefore, that a large part of the volume enclosed by this outer surface was occupied by the membrane itself which had a width of 9–10 nm. If measurements were made on the inner luminal face of the tubule (Table 4) the calculated surface to volume ratio, for both muscles, was more than doubled (Table 4). This was because, although the inner surface was clearly considerably smaller than the outer, the volume of the lumen alone was reduced proportionally far more when compared with the total volume enclosed by the outer face of the membrane.

Table 5. *Published values for the volume fraction, surface to volume ratio and surface density of the T system in mammalian muscles*

Animal	Muscle	Volume fraction (% fibre volume)	Surface to volume ratio of the tubules ($\mu m^2/\mu m^3$ tubule)	Surface density of T system ($\mu m^2/\mu m^3$ myofibre)	Reference
Mouse	Extensor digitorum longus	0·40	—	0·41*	Luff & Atwood (1971)
	Soleus	0·22	—	0·24*	Luff & Atwood (1971)
	Gastrocnemius	—	—	0·26	Silverman & Atwood (1980)
Guinea-pig	Soleus	0·14	46	0·064	Eisenberg, Kuda & Peter (1974)
	White vastus	0·27	54	0·146	Eisenberg & Kuda (1975)
	Red vastus	0·28	53	0·148	Eisenberg & Kuda (1976)
	Semimembranosus accessorius	0·16	48	0·076	Eisenberg (1983)
Human	Quadriceps fast	0·28	—	0·17	Eisenberg (1983)
	Quadriceps slow	0·13	—	0·09	Eisenberg (1983)
	Quadriceps mixed	—	33	—	Hoppeler *et al.* (1973)

* Calculated from Luff & Atwood's data by Eisenberg (1983).

DISCUSSION

Measured values for volume fraction, surface to volume ratio and surface density of the T system in rat extensor digitorum longus and soleus muscles (Table 2) are of the same order as those found in other mammalian fast and slow twitch muscles by other workers (Table 5). Both in the present study and in those of mouse, guinea-pig and human muscle, the fast twitch fibres contain a more extensive T system than do the slow twitch fibres. The difference appears to be less marked in the rat where both for volume fraction and for surface density the ratios in the fast to slow fibres are approximately 3:2. The differences occur because the T system has more branches in the fast twitch fibres and are not due to tubule calibre differences. This is shown by the similarity of the surface to volume ratios in the fast and slow fibres both in the rat muscles studied here and in the guinea-pig studied by Eisenberg and her colleagues (Table 5).

The presence of single sided triads (dyads) in rat soleus muscle has previously been remarked upon, but not quantified, by Davey & Wong (1980). In the present study, approximately 9% of the T tubule–sarcoplasmic reticulum junctions in the soleus muscle are single sided. However, even when this is taken into account, the overall density of feet facing the T tubule membrane in the soleus muscle is closely similar to that in the extensor digitorum longus muscle (Table 3).

In frog skeletal muscle there is a numerical similarity between the tubular density of feet (Franzini-Armstrong, 1970) and the tubular density of the intramembrane charge particles estimated by Schneider & Chandler (1973). These charge particles are thought to sense tubular membrane potential as a step in the voltage-dependent coupling of the tubular action potential and calcium release. In the model proposed by Chandler, Rakowski & Schneider (1976) it is suggested that each charge particle is associated with one foot.

In a recent study of charge movement in rat skeletal muscle, Hollingworth & Marshall (1981) find at least three to five times more charge in the fast twitch

extensor digitorum longus fibres compared with the slow twitch soleus fibres. Since, in this study, a similar density of feet in both fibre types has been found, the one to one association of charge particles and feet cannot apply in both fibre types. It appears, therefore, that the relationship between charge movement and foot density is more complex, at least in the rat, than Schneider & Chandler (1973) originally proposed.

Using frog muscle stained with tannic acid, Somlyo (1979) describes 'bridges' linking the cytoplasmic leaflets of the T and sarcoplasmic reticulum membranes. The bridges are considered to be located within the feet. No such sites of direct membrane contact have been identified in the present study. Eisenberg and her colleagues, also using frog muscle, have described 'pillars' which run between the T tubule and sarcoplasmic reticulum junctional membranes (Eisenberg & Gilai, 1979; Eisenberg, Eisenberg & Gilai, 1979; Eisenberg & Eisenberg, 1982). Eisenberg & Eisenberg (1982) equate their 'pillars' with Somlyo's 'bridges', and have shown that their number increases on stimulation of the muscle. They speculate that either the pillars might represent the rods that are postulated to link charge movement in the T tubule membrane to calcium release from the terminal cisternae of the sarcoplasmic reticulum (Chandler *et al.* 1976) or, in the alternative model of Mathias, Levis & Eisenberg (1980), they might be the structures that permit ionic current to flow from the T tubule membrane to the terminal cisternae.

A major problem in trying to correlate triad structure and function in excitation–contraction coupling in mammalian skeletal muscle is that much of our knowledge is based on work using frog muscle and, to a lesser extent, fish muscle (Franzini-Armstrong, 1975; Somlyo, 1979; Eisenberg & Eisenberg, 1982). In general, the clarity of the images of mammalian muscle has not yet matched that from the lower vertebrates. It is not known whether this is a technical problem or whether there are real differences, both qualitative and quantitative, between mammalian and frog triads, apart from their difference in position relative to the sarcomere bands.

Using freeze-fracture electron microscopy, Dulhunty, Gage, Valois & Hoh (1981) have shown that there is a strong correlation in rat triads between the number of indentations in the terminal cisternae and the amount of voltage-dependent charge movement in the extensor digitorum longus and soleus muscles. Dulhunty and her colleagues describe the indentations as having a diameter of 50 nm and a separation of 20–30 nm; this gives them a centre to centre spacing of approximately 75 nm which is twice the spacing of the feet measured in the present study using the same muscles. The structural relationship between the indentations and the feet is therefore unclear.

Despite a long history of interest and speculation the function of the feet at the vertebrate triad is still in doubt. It is possible that they have no role in excitation–contraction coupling and that their function is purely mechanical, holding the two membranes in apposition at a fixed distance apart, so countering the compressive and tensile forces encountered during the normal functioning of the muscle. The presence of blocks of amorphous material inside the terminal cisternae opposite the feet suggests that there may be a transmembranous connection to them which might support such a mechanical role.

As well as the paired dense structure inside the terminal cisternae, there is a generally amorphous electron density which has been identified as calsequestrin (Meissner, Conner & Fleischer, 1973). To the authors' knowledge, however, no attention has been paid in previous studies to material within the lumen of the T

tubules. In the present investigation, substructure within the lumen can be seen in the majority of tubule images, yet all published drawings of muscle membrane systems show empty T tubules (for example, Fig. 4 in Eisenberg, 1983). In the present study, the internal structure usually takes the form of a fine discontinuous lamina approximately 5 nm wide running along the centre of the tubule section. A similar structure has been observed in frog, mouse and human transverse tubules (authors' unpublished observations) and the authors look forward to other workers' descriptions of this structure and to their speculations as to its nature.

SUMMARY

An ultrastructural comparison was made between the transverse tubular systems of the extensor digitorum longus (fast twitch) and the soleus (slow twitch) muscles in the rat. Quantitative measurements were made of the volume fraction, surface to volume ratio, surface density and foot density of the T system membrane. The values for the volume fraction and surface density were approximately 50% higher in the fast twitch (extensor digitorum longus) than the slow twitch (soleus) muscle. The dimensions of the tubules and hence the surface to volume ratio was the same in the two muscles. There was no difference in the spacing of the feet, so the reported three to fivefold difference in charge movement cannot be associated with a difference in foot density.

Material lying within the lumen of the T tubules was observed in the majority of tubule profiles. This material, not hitherto described, was particulate or, more commonly, laminar in form.

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