Is there an Explicit Correspondence Between Physiological and Morphological Features in Amphibian Twitch Skeletal Muscle Fibres?

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Abstract
The contractile characteristics, fatiguability, and ultrastructure were examined in individual twitch skeletal muscle fibres dissected from the iliofibularis muscle of frog *Rana temporaria* in order to define if an explicit correspondence exists between physiological and morphological features in different types of muscle fibres. Three groups of twitch fibres were distinguished in the study according to their fatiguability: fatigue resistant (FR), moderately fatigued (MF) and easily fatigued (EF). The ultrastructural features such as regularity of striation, the density of myofibrils, Z-band morphology, mitochondrial and lipid droplet contents, which are commonly used to distinguish skeletal muscle fibre types, are shown to correlate with physiological properties verifying FR, MF and EF fibre groups only in a statistical way. In individual fibres, however, there is no strong correlation between contraction speeds, fatiguability, and ultrastructural features. The conclusion is made that ultrastructural features of individual fibres may not be used to predict the physiological properties attributed to particular fibre type.
Key words: amphibian skeletal muscle, twitch fibres, fatiguability, ultrastructure.

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There are two main types of muscle fibres in amphibian skeletal muscles: twitch (tetanic) and tonic (slow) fibres which form large-nerve and small-nerve motor units respectively. The tonic fibres constitute a homogeneous (at least in the *Rana* species) fibre group easily distinguished from twitch fibres by certain combinations of physiological and morphological features, the most important of which include multiple innervation; inability to produce action potentials and twitches; capacity to maintain contracture upon membrane depolarization; lack of M-lines in sarcomeres; less numerous mitochondria; low contents of dehydrogenase enzymes [3, 9, 11, 12, 13, 15].

In contrast to tonic fibres the group of twitch fibres is heterogenous in its physiological, histochemical and morphological characteristics. For the first time non-uniformity of twitch fibres was described by Zhukov and Leushina [22] who identified a population of slowlier contracting and more fatigue resistant twitch fibres. Later, at least three twitch fibre types were distinguished in amphibian twitch muscles on the basis of the contraction speed, fatiguability, metabolic capacity, sarcomere appearance, and development of the intracellular membrane system [5, 10, 15, 16, 17].

Attempts have been made to define correlations between the properties of different muscle fibres to picture morphological-histochemical-physiological relationship in twitch fibre types [1, 4, 5, 7, 19]. As a result, the general conclusion has been arrived at that each twitch fibre type may be described by distinct characteristics in almost every respect: contractility, structure, biochemistry. Meanwhile, combined studies have kept appearing, though in limited numbers, which tested selected physiological characteristics of twitch fibres to provide more evidence for the above conclusion.

In addition to other physiological characteristics most important of which is a contraction speed, resistance to fatigue appeared to be the best physiological criterion to distinguish twitch fibre types. As most twitch fibre parameters, contraction speed smoothly varies between both ends of the range, and this limits the capacity of this parameter to help in determining the fibre type when single fibres are concerned. When fatiguability is taken as the criterion, each single twitch fibre can be determined as belonging to the particular fibre type [14, 20]. The fibre types identified by the fatiguability criterion have been shown to correlate well with particular histochemical properties and the speed of contraction [14, 19, 20].

The present combined physiological-morphological study was aimed at determining if there is any correspondence between resistance to fatigue and morphological properties of certain types of twitch fibres as suggested by the generally accepted idea of a relationship between physiological and morphological properties. The specific approach of the study was that single fibres were first tested individually to define their contractile characteristics, then the fibres were fixed and examined under electron microscope. Parts of this study have been published in abstract form [6].

Materials and Methods

The results of the present study are based on the investigations of single fibres dissected from different parts of the iliofibularis muscle of adult frog *Rana temporaria*. The frogs were killed by decapitation followed by destruction of the spinal cord. Close attention was paid to define the part of the muscle where each fibre was isolated from. For that purpose, the iliofibularis muscle was zoned using the modified scheme of Rowlerson and Spurway [15].

The dissection, experimental setup and stimulation procedure were as described in detail in previous papers [11, 14]. Briefly, the fibres were transferred to an experimental bath with facilities for direct electrical stimulation with platinum plate electrodes and rapid exchange of solution. One tendon was fixed by a clamp, the other one was attached by a fine silver-wire hook to the arm of a tensometer [8]. The fibres were stretched up to 20 % of their slack length. Isometric contractions were elicited by single 1.3x threshold amplitude electrical shocks 2 ms duration for twitches or 70 Hz trains 0.5 s duration for tetani. The contractions were displayed on a digital transient recorder (SE 561, BBC GOERS METRAWATT, Austria).

To standardize the fatiguability repeated tetanic stimulation was applied to the fibres at 70 Hz trains for 0.5 s every 3 s during 2-7 min. According to fatiguability, twitch fibres were divided into three groups: fatigue resistant (FR, around 90% of initial tension after 7 min stimulation), moderately fatigued (MF, around 55% of initial tension after 4 min stimulation), and easily fatigued (EF, around 55% of initial tension after 2 min stimulation). The fibres were allowed to rest for not less than 40 min before being fixed for following morphological study. At the end of this period the fibres were stimulated to produce single twitch and tetanic tensions. Only fibres which fully recovered were used for the following treatment.

The Ringer solution contained (in mmol/l): 115 NaCl; 2.5 KCl; 1.8 $CaCl_2$; 3.0 sodium phosphate buffer; pH 7.0-7.2 at room temperature. Potassium contractures were induced by rapid application of a high potassium (80 mmol/l K^+) solution keeping the $[K]_o x [Cl]_o$ product constant. Sodium was substituted with potassium, and chloride was replaced with propionate.

The tonic fibres were identified by their inability to produce twitches and ability to sustained contractures in high potassium environment.

For electron microscopic study, muscle fibres were fixed in experimental bath with 2% glutaraldehyde in cacodylate buffer for 30 min. After postfixation in 1% OsO_4 for 30 min and staining with 2% aqueous uranyl acetate the fibres were dehydrated in ethanol series and embedded in Durcupan. Ultrathin longitudinal sections were cut with a Porter-Blum MT 2 ultramicrotome, stained with lead citrate, and examined by a JEM 1200 (JEOL, Japan) electron microscope at 80 kV. Longitudinal sections of the central part of each fibre were evaluated.

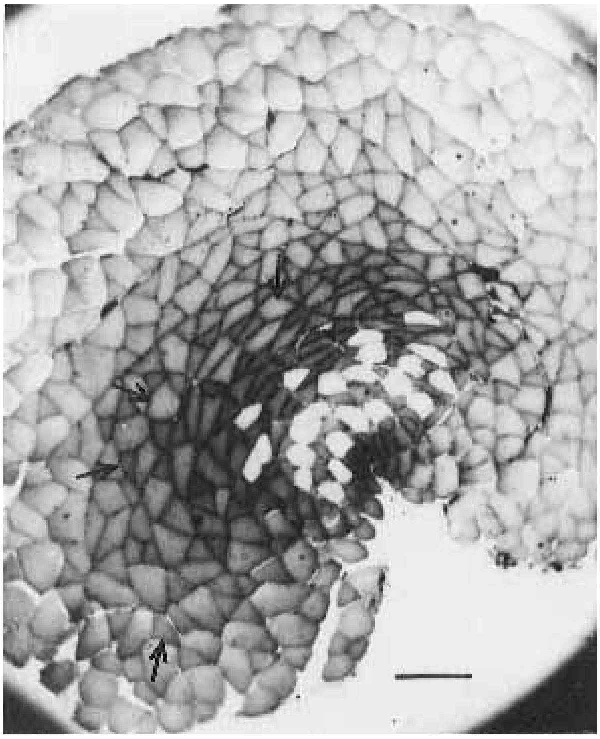
This investigation was approved by the Ethical Commission of the Institute of Molecular Physiology and Genetics of the Academy of Sciences, Bratislava, Slovakia.

Results

The distribution of fibre types in the muscle

M. iliofibularis is a very convenient object to study different types of skeletal muscle fibres. This muscle contains all fibre types described for *Rana* species; the various types are distributed in this muscle in a special way. The distribution of the muscle fibres allows a researcher to go to an area of the muscle and get better chance to dissect the desirable type of the fibre. The distribution of fibre types in m.iliofibularis of *Rana temporaria* has been shown in a number of early studies which used different techniques to identify the fibre types (see Introduction). As an example, Figure 1*a* shows a cross section of m.iliofibularis stained for lipids. The central part of this muscle, so-called tonic bundle, contains tonic (no lipids) and twitch (high contents of lipids) fibres. Other parts of the muscle include twitch fibres with varying lipid contents. There are large diameter pale fibres on the periphery of the muscle. In the middle part some pale fibres can be seen among smaller dark fibres and *vice versa* (arrows).

The positions of 64 single muscle fibres (8 tonic and 56 twitch) isolated from different parts of the iliofibularis muscles for this study are shown on the diagram in Figure 1*b*. The diagram presents zones of a cross section of the m. iliofibularis according to the distribution of different types of muscle fibres in this muscle after staining for lipids. All twitch fibres represented in the diagram were tested for fatiguability.



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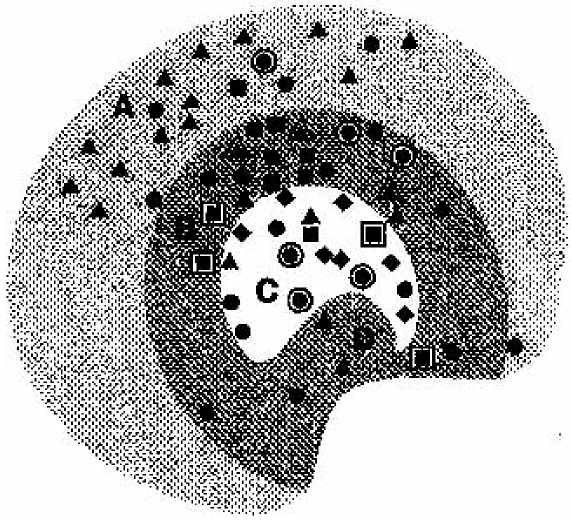

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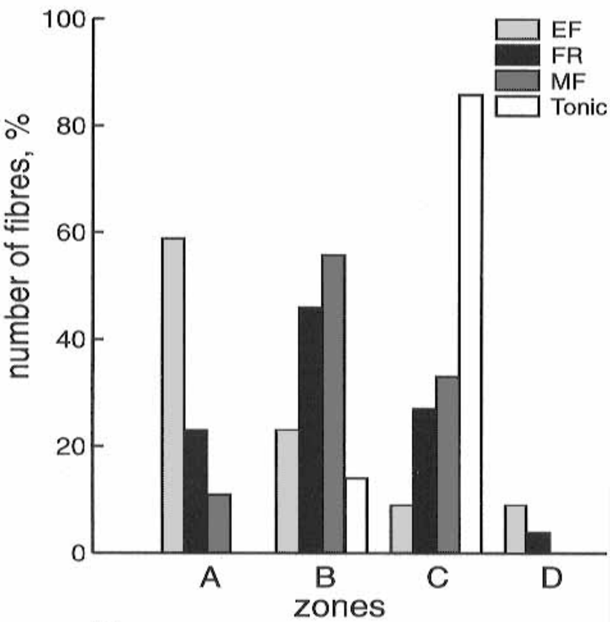

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Figure 1. The distribution of muscle fibre types in the m. iliofibularis of Rana temporaria*;* a*, cross section stained for lipids by Sudan B; large pale fibres among small dark fibres and vice versa (arrows), scale mark 200 µm;* b*, diagrammatic representation of the zonal distribution of twitch fibres with different resistance to fatigue. Symbols: circles, fatigue resistant (FR); squares, moderately fatigued (MF); triangles, easily fatigued (EF), rhombs, tonic; the circled symbols represent the twitch fibres subjected to subsequent electron microscopic examination;* c*, a histogram from data in* b *showing the zonal distribution of muscle fibres with differences in fatiguability. y axis, percentage of the number of fibres of each group (FR, MF, EF, or tonic).*

The histogram (Fig.1*c*) based on the data of Figure 1*b* illustrates the preferential distribution of twitch fibres with differences in fatiguability throughout zones A-B-C-D of m.iliofibularis. EF fibres were mostly found in the A and B zones though some EF fibres were located in zones C and D. FR fibres were found throughout all zones but most of them were located in zones B and C. MF fibres were localized more or less equally throughout zones A-B-C though more MF fibres were found in zone B. Some MF fibres were also found in zone D. Most tonic fibres identified by maintaining high potassium contractures and absence of twitches were located in C zone, and no one of them found in zones A and B. These data show that there is no strong zone correlation of fibres inside the muscle as distinguished by the fatiguability criterion, but a preferential position can be seen of each muscle fibre type. Tonic and EF fibres have a stronger tendency to be located in distinct zones, whereas FR and MF fibres have a tendency to be spread among different parts of the muscle.

Fatiguability of fibres: correlation with electron microscopic examination

Thirteen of the 56 single twitch fibres with defined contractile characteristics were taken for electron microscopical examination. The position of each fibre in the m.iliofibularis is shown in Figure 1*b* (circled symbols). As can be seen, the fibres were isolated from different parts of the muscle.

The diagram in Figure 2 summarizes the data obtained after physiological tests for fatiguability were applied to the fibres. Significant differences can be seen between those fibres after already 2 min stimulation. Six of thirteen fibres displayed the characteristics of FR fibres. They showed around 10% decline of initial tension after 7 min stimulation (140 tetanic contractions). Four fibres were indentified as MF fibres (around 55% decline of initial tension after 4 min stimulation, 80 tetani). Three fibres that showed around 55% decline of initial tetanic tension after only 2 min stimulation (40 tetani) were identified as EF fibres. These results correspond well to those described earlier for larger numbers of twitch fibres [14].

Contractile and ultrastructural characteristics of the individual fibres

Tables 1-3 (each table represents data on one fibre group) include data on twitch contraction characteristics, 5 times tetanus/twitch ratio, and a description of the ultrastructure of thirteen individual fibres. The zones of the iliofibubilaris muscles where the fibres were isolated from are also indicated in the tables. There were no uniform speed characteristics or tetanus/twitch ratios for fibres within a group. Some FR fibres contracted fast (fibre V_5), while slow contracting fibres were found among the EF group (fibre P_6).

Each of the thirteen fibres was examined for ultrastractural characteristics which are commonly used to distinguish fibre types in amphibian muscles, such as regularity of striation, the presence of the M-line, morphology of Z-band, mitochondrial and lipid droplet contents. All fibres were characterised by the presence of M-line, which is typical of twitch fibres distinguishing them from tonic fibres [12], and by regular striation, except for EF fibre P_6. This fibre had

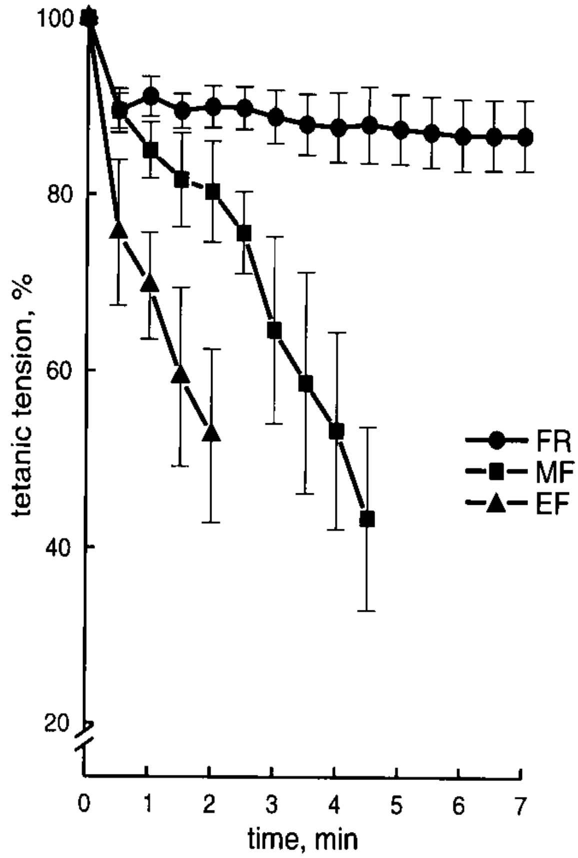

Figure 2. Tetanic tension plotted against the time of repeated stimulation of single muscle fibres (means and standard errors): squares, fatigue resistant; circles, moderately fatigued; triangles, easily fatigued.

irregular striation which is unusual for this fibre group, however the low contents of lipid droplets of this fibre is typical. The fibres showed differences in Z-band morphology. Most fibres had straight Z-bands although there were slight differences in Z-band

Table 1. Contractile and ultrastructural characteristics of the fatigue-resistant frog iliofibularis muscle fibres.

Fibre code	Zone of the muscle	Tetanic tension after 2 min stimulation, %	Twitch, rate of rise, mg/s	Twitch, half decay rate mg/s	Tetanus / twitch ratio	Z-band look	Mito-chondrial content*	Lipid droplet content*
D_6	C	98	2.8	1.1	5.95	jagged	+	+
W_5	C	94	0.6	0.8	13.3	straight	✚	✚
V_5	C	91	0.5	0.3	5.74	straight	✚	✚
W_6	B	90	1.1	0.5	5.56	jagged	✚	✚
I_{11}	A	84	2.2	1.1	4.36	straight	✚	+
B_{11}	B	83	3.7	0.9	2.57	straight	+	+
Mean		90	1.8	0.8	6.25			

* ✚ denotes mitochondria packed in double- or/and multirows, high lipid content;
+ denotes mitochondria packed in single rows, low lipid content.

Table 2. Contractile and ultrastructural characteristics of the moderately fatigued frog iliofibularis muscle fibres.

Fibre code	Zone of the muscle	Tetanic tension after 2 min stimulation, %	Twitch, rate of rise, mg/s	Twitch, half decay rate mg/s	Tetanus / twitch ratio	Z-band look	Mito-chondrial content*	Lipid droplet content*
V_6	B	89	9.9	4.9	2.14	straight	**+**	**+**
Z_6	B	84	6.8	3.1	2.66	jagged	**+**	+
L_6	B	80	2.0	0.8	6.93	straight	+	+
O_6	C	73	4.3	2.1	2.69	straight	+	**+**
Mean		82	5.8	2.7	3.61			

* symbols as in Table 1.

Table 3. Contractile and ultrastructural characteristics of the easily fatigued frog iliofbularis muscle fibres.

Fibre code	Zone of the muscle	Tetanic tension after 2 min stimulation, %	Twitch, rate of rise, mg/s	Twitch, half decay rate mg/s	Tetanus / twitch ratio	Z-band look	Mito-chondrial content*	Lipid droplet content*
C_{11}	B	71	3.2	1.1	4.25	straight	+	+
P_6	B	52	9.1	4.5	5.45	jagged	**+**	+
A_6	B	42	2.7	0.9	5.45	jagged	+	+
Mean		55	5.0	2.2	5.05			

* symbols as in Table 1

morphlogy (see below), and there was no preference for the fibre group distinguishable. There was a single fibre (P_6) with jagged Z-line in combination with irregular striation. The fibres contained different numbers of mitochondria. In some fibres, the mitochondria rarely occurred in single rows between myofibrills (low mitochondria content), whereas in other fibres mitochondria formed thick rows, not infrequently across the fibre (high mitochondria content). It is evident from Tables 1-3 that most FR fibres have more mitochondria than fibres of other groups, although two FR fibres (D_6 and B_{11}) showed low contents of mitochondria. One of three EF fibres showed high contents of mitochondria. The contents of lipid droplets varied from fibre to fibre for FR and MF fibres. Low contents of lipid droplets was typical for EF fibres.

Thus, no strong correlation could be observed between the contractile characteristics and the ultrastructural appearance of individual fibres, although a correlation between fatiguability and features that characterise the metabolic capacity, such as lipid droplets and mitochondria, was visible. Most FR fibres have higher metabolic capacity than EF fibres.

Examples of ultrastructure of extreme FR and EF groups of fibres

Figures 3-6 illustrate ultrastructure of two fibres of each of extreme (FR and EF) fibre groups. One fibre illustrates ultrastructure typical of the given group of fibres, as it arises from the common point of view. The other fibre is to illustrate ultrastructure which seems non-typical or differs from what is thought of this type of twitch fibres. Figures 3 and 4 compare the ultrastructure of two fibres which showed high fatigue resistance. There are clear-cut differences in ultrastructure of the two fibres. The FR fibre (V_5) in Figure 3 has relatively straight Z-bands and high contents of mitochondria and lipid droplets. The FR fibre (B_{11}) in Figure 4 has straight Z-bands, and low contents of mitochondria and lipid droplets.

Figures 5 and 6 represent longitudinal sections of two EF fibres. One of the fibres (C_{11}) showed moderate speed characteristics as it is seen from Table 3. There are straight Z-bands in this fibre (Fig. 5). Mitochondria in rare thin rows are situated in some areas of the sarcoplasm. There are but few lipid droplets. The features of the ultrastructure of this fibre are close to those of the FR fibre illustrated in Figure 4. Another EF fibre (P_6) with a different ultrastructure is shown in Figure 6. Z-bands different from those of the EF fibre in

Figure 5 characterise this particular fibre. The fibre has a high content of mitochondria arranged in thick rows. A low content of lipid droplets seen in this fibre is typical of the group of EF fibres. The ultrastructure of this fibre is close to that of the FR fibre illustrated in Figure 3.

Discussion

The role which every segment of the muscle plays in the performance of the motion program in the organism depends on its physiological characteristics, most important among which are contraction speed and fatiguability of muscle fibres making up the given segment. This means that contraction speed and fatiguability must be considered key features for any single muscle fibre. The values of these characteristics are determinated by the location of the fibre within the muscle according to the functional anatomy of the muscle to enable it to perform mechanically as needed. Other features such as some structural and molecular peculiarities, must conform to these two general characteristics in order to provide for appropriate values.

In amphibians, two main types of muscle fibres are easily distinguished: twitch and tonic fibres, with dictinct physiological, histological and morphological characteristics. Various methodological approches have proved the existence of few twitch fibre types. Rowlerson and Spurway [15], from their elaborate histochemical and immunohistochemical study, distinguished F1, F2 and F3 fibre types that supposed to be twitch fibres with different oxidative and glycolytic capacities. A conclusion was made that contraction speed decreases in the order from F1 to F3, according to mATPase activity. A direct relationship was found to exist between velocity of shortening and the myofibrillar ATPase activity based on differences in myosin heavy-chain composition for single fibres, as well as between velocity of shortening and the cross-sectional area [1, 21]. The fibre types F1, F2 and F3 described by Rowlerson and Spurway [15] must

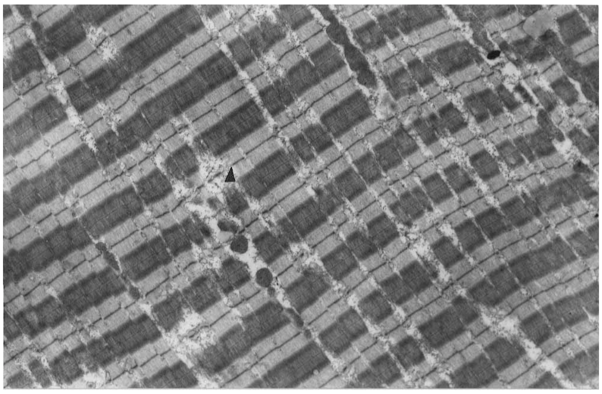

Figure 4. An electron micrograph of the longitudinal section of the fatigue resistant fibre (B_{11}; see Table 1) with straight Z-band (arrowhead), low contents of mitochondria that form thin rows in the myofibrillar space, very low contents of lipid droplets. Magnification x8,000.

correspond to fibre types 1, 2 and 3 reported by Smith and Ovalle [17] who showed that, besides differences in myosin ATPase and SDH activity, fibre types 1, 2 and 3 also differred in myofibril size and organization, morphology of the M- and Z-bands, glycogen and mitochondrial contents, and organization of the sarcotubular system.

Though there are few studies showing direct correlation between ultrastructural and physiological properties [4, 7] it is generally accepted that in amphibian skeletal muscles large fibres with high contraction speeds and low resistance to fatigue must have regular striation, straight Z-band, a high occurrence of triads. The smaller fibres contracting slowlier and less readily fatigable with high metabolic capacity have been supposed to have irregular striation, jagged Z-bands, rear triads.

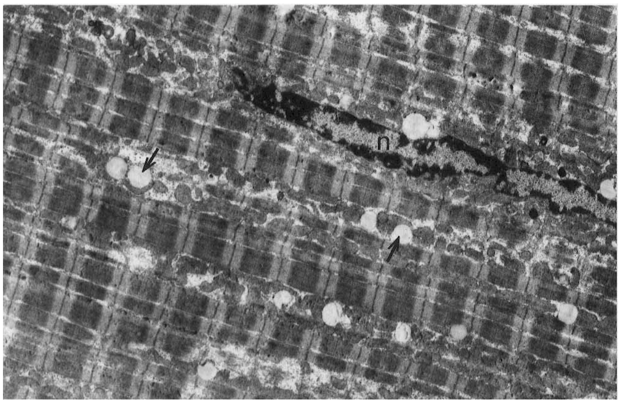

Figure 3. An electron micrograph of the longitudinal section of the fatigue resistant fibre (V_5; see Table 1) with large rows of mitochondria and lipid droplets (arrows) between the mito-chondria. n, nucleus. Magnification x8,000.

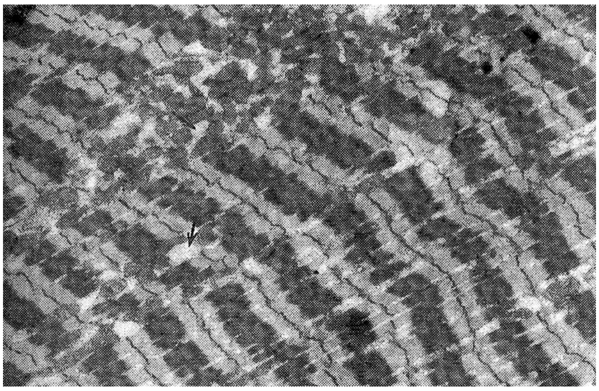

Figure 5. An electron micrograph of the longitudinal section of easily fatigued fibre (C_{11}; see Table 3) with straight Z-band and low contents of mitochondria forming thin rows (arrows) in the intramyofibrillar spaces; there is a low content of lipid droplets in this fibre. Magnification x8,000.

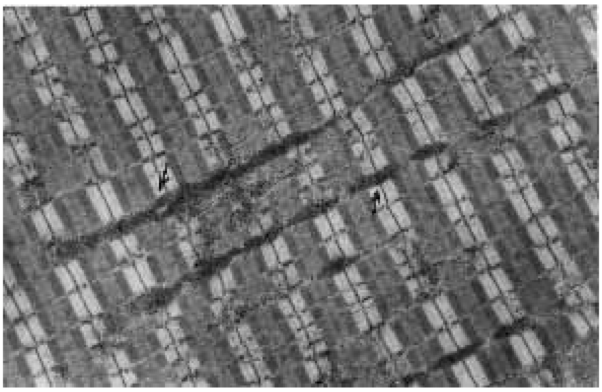

Figure 6. An electron micrograph of the longitudinal section of easily fatigued fibre (P_6; see Table 3) with thick rows of mitochondria and low contents of lipid droplets (arrows), Z-band is jagged; the striations are not regular between the myofibrils. Magnification x8,000.

The three types of twitch muscle fibres have been shown to have specific distribution pattern within the frog iliofibularis muscle. More readily fatiguable and fast contracting fibres with response to fast myosins and acid stable myofibrillar ATP-ase activity occupy the perifery of the muscle whereas fibres contracting slowlier and more fatigue resistant with high metabolic capacity, nonregular striation, jagged Z-bands and rare triads are mostly found in the vicinity of the tonic fibres in the muscle centre [5, 10, 15, 17, 22]. The results of our investigation have shown that it is difficult to categorize individual muscle fibres as a certain fibre type using their dynamic properties as a criterion. The same conclusion may be made when based on differences in histochemical and immunohistochemical properties, because their values are often continuous (e.g., see Spurway Rowlerson [18]) and do not allow arbitrary classification, especially when dealing with a limited number of features.

In the present study, the parameter of fatiguability was used to distinguish between various types of twitch fibres. Single fibres were investigated to show whether some physiological characteristics would correspond well to ultrastructural features, according to the generally accepted idea. The fibres were dissected from different parts of the iliofibularis muscle, and special attention was paid to which muscle zone the fibre was isolated from. The fibres belonged to the twitch fibre type because they produced twitches and unsustained high potassium contractures.

All fibres examined using physiological methods showed a specific distribution within the iliofibularis muscle which corresponded to the general concept: fibres that fatigued fast preferentially occupied the periferal zone of the iliofibularis muscle whereas fatigue resistant fibres were mostly located in the central bundle that contains tonic fibres. Our previous results showed that there is significant direct relationship between fatiguability and twitch contraction speed for twitch fibres of the iliofibularis muscle of *Rana temporaria* [14].

Thirteen individual fibres first characterized with respect to fatiguability and twitch contraction speed were subsequently subjected to electron microscopic investigation to study the relationship between the main physiological characteristics and the ultrastructure. Those fibres were FR, MF or EF fibres, i.e. they came from each of the fibre groups which show significant differences in fatiguability. Ultrastractural investigation confirmed that they were twitch fibres because M-line presented in the middle of the I-band. Ultrastructural characteristics that are commonly used to distinguish between skeletal muscle fibre types such as myofibrillar organization, Z-band morphology, mitochondrial and lipid contents, were examined. The thirteen fibres were found to be located in different zones of the m.iliofibularis, and there was no preferential distribution distinguishable in the muscle zones for these fibres, even when the fibres could clearly be classified into one of the fibre group based on their fatiguability.

We expected significant differences to appear in contraction speed among the thirteen fibres examined under electron microscope, i.e. easily fatigued fibres to contract fast and fatigue resistant fibres to contract slowly. Instead, a range of twitch contraction speeds was observed from fibre to fibre in each fibre group with different fatiguability though the mean values of contraction speeds (see Tables) correspond expected order.

Ultrastructural investigations also showed a range of features within each group of fibres with differences in fatiguability so that some FR fibres had clear similarity in ultrastructure with one being typical for EF fibres, and *vice versa*.

The results showed that FR fibres as a whole had higher contents of lipids and mitochondria than EF fibres. This confirmed the data about higher metabolic capacity of fibres with high resistance to fatigue though, again, some exceptions could also be observed, e.g. that concerning the FR fibre B_{11} listed in Table 1.

In conclusion, the present results showed that the features of individual skeletal muscle fibres are unpredictable; this prevents estimates of physiological characteristics for muscle fibres being done based on ultrastructural description. In addition, it supports the notion that no simple explanation can be given of mutual relationships between morphology, histochemistry and physiology of muscle fibres [2].

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