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AN ARTHROPOD MUSCLE INNERVATED BY NINE EXCITATORY MOTOR NEURONES

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SUMMARY

The anatomical and physiological organization of the locust metathoracic flexor tibiae was examined by a combination of intracellular recording and electron microscopy. Nine excitatory motor neurones, three fast, three intermediate and three slow innervate the muscle; each is uniquely identifiable using a combination of physiological response and soma location. A simple spatial distribution of inputs to the muscle from the individual motor neurones was not found. Individual muscle fibres responded to as many as seven of the motor neurones in various combinations.

The muscle fibres are heterogeneous, ranging from slow (tonic) to fast (phasic) in a continuum from predominantly phasic proximally to tonic distally. This is demonstrated by contraction and relaxation rates to direct and indirect stimulation, as well as contraction elicited by action potentials in a single flexor motor neurone. The fast and slow contractile properties of the muscle fibres are matched by appropriate ultrastructures.

Such a high degree of complexity of neuromuscular innervation as that found in the metathoracic flexor tibiae has not previously been described for an arthropod muscle.

INTRODUCTION

Most insect muscles studied in detail to date can be divided into two general groups based upon their innervation: those in which one or two excitatory motor neurones divide to innervate all the fibres, and those in which separate bundles are innervated separately. Many limb muscles belong to the first group with one or two excitatory motor neurones innervating muscle fibres in many bundles (e.g. Hoyle, 1978), whereas dorsal longitudinal flight muscles are classic examples of the second category (e.g. Neville, 1963; Hoyle, 1974). The innervation patterns of limb flexors have not yet been described, but they are suspected of being highly complex and are known to receive input from as many as eight motor neurones (cockroach flexor tibiae: Dresden & Nijenhuis, 1958) and nine in the locust (metathoracic flexor tibiae: Burrows & Hoyle, 1973; Burrows & Horridge, 1974; Phillips, 1977). In an earlier study (Hoyle, 1964), the complex innervation of the locust metathoracic flexor tibiae was demonstrated by electromyography. However, the techniques available at that time did not allow correlation of identified motor neurones with the activity recorded in the muscle.

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The present study was undertaken to determine the details of the locust flexor tibiae muscle and its pattern of innervation.

The methathoracic legs of the locust are always much larger than the first two pairs of walking legs, being specialized to provide power for jumping, kicking and swimming (Heitler, 1974, 1977; Bennet-Clark, 1975; Pflüger & Burrows, 1978). The flexor tibiae is a long, thin muscle which lies under the ventral cuticle of the femur (Snodgrass, 1929). It tapers, following the contour of the femur, and is about three times as wide at its proximal end as it is distally. There are 24 bundles of muscle fibres which arise from cuticular origins equally divided between the anterior and posterior parts. The fibre bundles attach to a central apodeme at an angle of less than 10°, and vary in length between 6 and 7 mm. In the hind leg, the flexor tibiae is much smaller than its antagonist (cross sectional area 2·2 mm^2 and 17·5 mm^2 respectively; Bennet-Clark, 1975), whereas in the first two pairs of legs it is larger than the extensor. The pro- and meso-thoracic flexors also differ from the flexor of the hind leg in having the cuticular origin of the most proximal fibre bundle attached in the trochanter. In the metathoracic leg, the whole of the flexor tibiae is contained within the femur (Snodgrass, 1929).

This paper describes the innervation, physiology and anatomy of the locust metathoratic flexor tibiae. Physiological and anatomical evidence will be given to show that there is an overlapping innervation rather than discrete spatial segregation. It will be shown, both by direct and indirect depolarization of the muscle fibres and by their ultrastructure, that each muscle fibre bundle of the flexor tibiae comprises a mixture of muscle fibre types which cannot be separated into distinct classes, and which form a continuum. The fibres in the proximal portion are predominantly fast-type or phasic, while those in the distal portion are predominantly slow-type or tonic. Individual muscle fibres of the locust flexor tibiae are always innervated by more than two excitatory motor neurones. The innervation pattern of the flexor is therefore unique, unlike that described for any other type of insect muscle.

MATERIALS AND METHODS

Adults of the African desert locust, *Schistocerca americana gregaria* (Forskål) (see Dirsh, 1974) were obtained from cultures at the Zoology departments at the University of British Columbia and the University of Cambridge. Some of the muscle physiology experiments were repeated on the American locust, *S. nitens* (Thurnberg) (previously known as *S. vaga*; see Dirsh, 1974) from a colony at the Biology department at the University of Oregon.

Physiology

The dissection and recording procedure for intracellular studies on the motor neurones was similar to that described by Burrows & Hoyle (1973). In most of the experiments the ganglia were initially exposed to a dilute solution of protease (~ 1%) in saline. During the course of the experiment, the animal was continuously perfused with an insect saline (Hoyle, 1953).

For intracellular recording the cell bodies were impaled with glass microelectrodes containing either an 18% solution of cobalt nitrate in saline, a 4% solution of Procion

bine, black, or brown, or 2 M potassium acetate. Intracellular muscle recordings were made with 2 M potassium acetate filled electrodes. Muscle activity was recorded extracellularly, with differential pairs of 50 μm diameter silver or stainless steel wire. Tension was measured directly by an RCA 5734 mechanoelectronic transducer. The response of the transducer was linear over the force range of the flexor muscle. The axons of the motor neurones were sometimes stimulated with platinum hook electrodes placed under nerve 5 (terminology, Campbell, 1961) to evoke contraction of the muscle. In these experiments nerve 5 was always cut just after leaving the ganglion to prevent re-excitation via central pathways. Suction electrodes were used to record the response of the fine nerve branches of N5B2 to the flexor muscle during whole nerve stimulation.

The effects of directly depolarizing the muscle were studied in the isolated femur by bathing the muscle in saline in which all the sodium chloride had been replaced by potassium chloride (150 mM KCl). Tension was measured as above. Data were recorded in the following ways: directly on a two-channel Brush recorder, filmed directly from the oscilloscope with a Grass camera, or recorded on a four-channel FM tape recorder and filmed later.

Flexor motor neurone somata were identified by a combination of standard physiological and anatomical criteria (Hoyle & Burrows, 1973; Hoyle, 1978). Action potentials recorded from the somata were correlated with excitatory junctional potentials (EJPs) seen in intracellular muscle recordings and myograms, and with tension for physiological identification. Secondary classification of the motor neurone was as anterior, posterior or lateral, according to the position of the cell body within the ganglion. For this purpose, 46 of the motor neurones identified were stained with cobalt or Procion dyes according to standard methods (Wilson, 1979*a*).

Anatomy

Flexor muscles from adult animals one week post-moult were fixed for light and electron microscopy in the following way. The ventral cuticle was removed from the femur, exposing the flexor tibiae, but leaving its origins on the cuticle intact. Part of the extensor tibiae with its overlying cuticle was also removed. The animal was then caused to autotomize the hind leg at the trochanterofemoral joint, and the femur and tibiae were rapidly pinned out at rest length, and flooded with cold glutaraldehyde (6·25%, 0·1 M phosphate buffer). When the muscle had turned a light straw or salmon colour ($\sim \frac{1}{2}$h) it was dissected free from the cuticle and transferred to fresh fixative at 4 °C without shrinkage. Following buffer wash and post fixation in osmium (1%), the tissue was dehydrated through a series of ethanols, embedded in Epon 812, and sections cut on a Porter-Blum MT-2 ultramicrotome. For light microscopy 1·5 μm sections were stained with 1% paraphenylenediamine in methanol. Sections were viewed and photographed using a Zeiss microscope with a 25× phase objective and polarizing filters. For electron microscopy, sections were stained with lead citrate and uranyl acetate, and viewed in a Philips 300 EM.

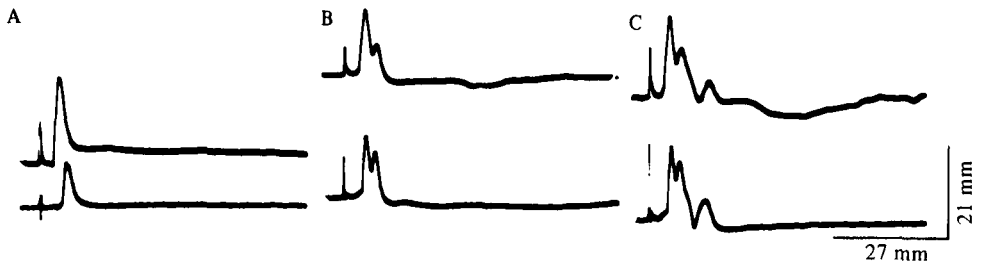

Fig. 1. EJPs from two widely separated muscle fibres (A) occur at the same current strength of extracellular depolarization to nerve 5. The similar threshold is evidence the EJPs were caused by spikes in the same neurone. In another pair of fibres (B), current strength was increased past threshold for the first EJP, eliciting another EJP in both fibres. Further current increase elicits a third common EJP (C). Scale mark, vertical 25 mV, time 25 ms.

RESULTS

Innervation of the flexor tibiae

Since all the motor neurones travel to the muscle in the same nerve, the only way that the nerve terminals on the muscle could be mapped was to record the EJPs in the muscle with one, or sometimes two, microelectrodes during intracellular stimulation of an identified flexor soma. When a flexor motor neurone had been penetrated and identified, muscle fibres were impaled and sampled in as many of the fibre bundles as possible. If a single microelectrode was being used for sampling muscle fibres, EJP sampling was begun in the proximal bundles and progressed distally with alternation between the two sides of the tendon. When two muscle electrodes were used, each was moved independently in the proximal and distal half of the muscle. In 60 successful penetrations, each of the identified, fast, intermediate and slow flexor motor neurones made terminals in both the proximal and distal halves of the muscle. No focally distinct distributions of terminals were discerned, each of the 9 flexor motor neurones making terminals throughout the length of the muscle. This basic observation is compatible with the following observations. (1) When muscle fibres were sampled to determine how many different neurones innervated them by giving maximal single shocks to nerve 5, an average of five inflexion points was found. No fibre had fewer than three EJPs and some had as many as seven. (2) Similar junctional potentials were observed in widely separated fibres at the moment of exceeding threshold (Fig. 1). (3) Counts were made of the number of common action potentials recorded with suction electrodes from pairs of primary twigs of nerve 5B2 in response to stimulation of nerve 5. The numbers of common inflexion points between any two pairs ranged from 3 to 9 (average 6, $n = 17$ pairs, S.D. = 1·5). (4) Examination of these primary twigs in the electron microscope revealed between 7 and 13 axon profiles. In addition to the nine excitatory flexor motor neurones, there are two specific inhibitors (Burrows & Horridge, 1974) and two dorsal unpaired medial (DUM) neurones appear to innervate the muscle, which together account for the maximum number of 13 profiles observed.

Mechanical responses

Two mechanical response characteristics were measured: the time to 95% peak tension and the time from peak tension to 50% relaxation, for whole muscle, anteri

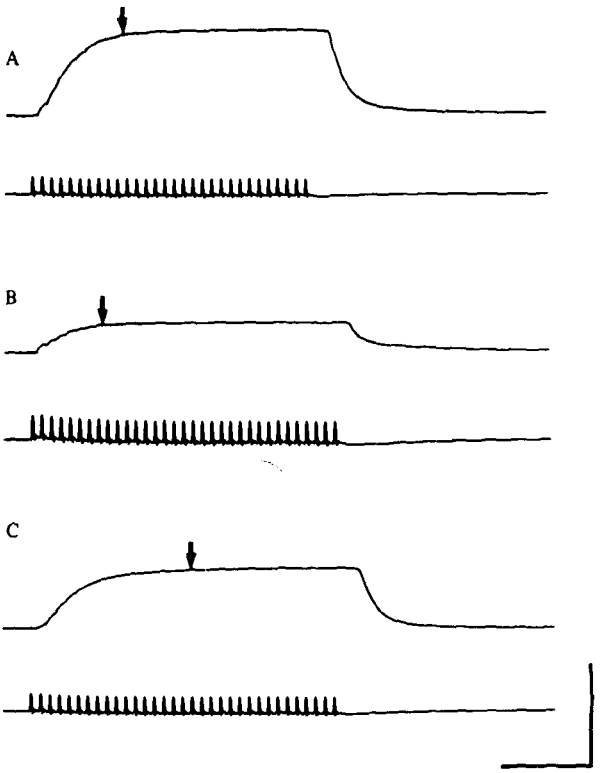

Fig. 2. Response of the flexor tibiae to stimulation of nerve 5. Tension was measured from the whole muscle in A, the proximal eight fibre bundles in B and the distal 12 bundles in C. Arrows denote rise times to 95% peak tension. See text for details. Scale mark vertical, 4 g; time, 200 ms.

and posterior, and proximal and distal, muscle halves. It was found that the rates of contraction and relaxation of the proximal half were different from those of the distal half. Initially tension was measured from the whole muscle and then the cuticular origins were cut away from the anterior or posterior aspect of the leg. Contraction was again elicited and tension from the remaining bundles was recorded. When the muscle was divided in this way, the rates of contraction and relaxation were found to be similar to the rates for the whole muscle. Responses of the proximal and distal halves of the muscle were then compared with each other and with the response of the whole muscle (Fig. 2). After recording tension from the whole muscle, the tendon was cut near its midpoint and the response of only the distal fibre bundles recorded. Next, a few muscle fibre bundles were removed from the cut end of the tendon to allow for transducer placement, so that tension/time characteristics of the proximal fibres could be measured. These were significantly different from corresponding responses of the distal bundles (raise time to 95% peak, 125 ms proximal, 348 ms distal). The response in the distal fibres continued to increase throughout the 650 ms duration of the stimulus. A long-continued rise in tension is a distinguishing characteristic of arthropod slow or tonic muscle (Hoyle, 1978). These data show that the flexor tibiae can be

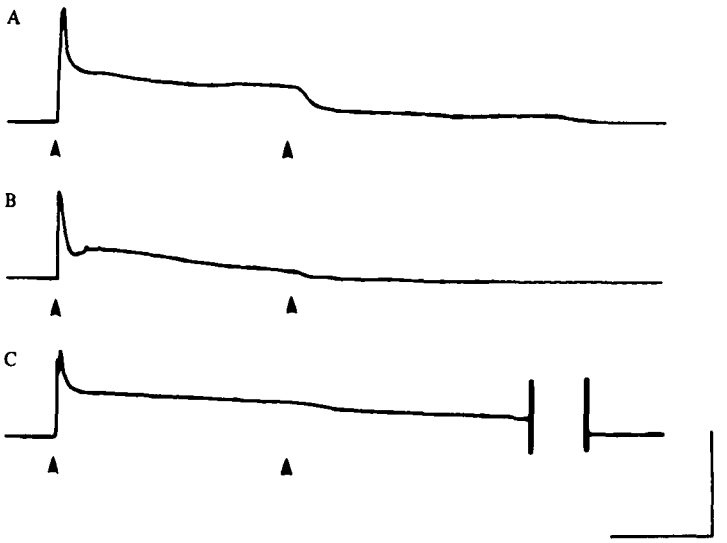

Fig. 3. Contraction was elicited in the flexor tibiae by bathing the muscle in a saline where all the sodium was replaced by potassium. Arrows denote time in depolarizing saline. The response of the whole muscle is shown in A, the proximal fibre bundles in B, and the distal fibre bundles in C. See text for details. Scale mark vertical, 10 g; time 4 min. The break in C is 30 min.

regarded as comprising two parts, proximal and distal, approximately equal in size, containing principally fast-type and slow-type fibres respectively.

To further demonstrate that the differences in rise and fall of tension were due to different properties of the muscle fibres and not to differences in neuromuscular transmission, the muscle was also directly depolarized by high potassium saline, and the tension responses recorded. By this technique insect fast-type muscle fibres give first a brisk contraction, then relex fairly rapidly, while slow-type fibres develop tension slowly, but tension, once developed, is maintained (Aidley, 1967; Hoyle, 1961). Fig. 3 shows the responses of the parts of the flexor, compared with the whole, as in Fig. 2, with arrows denoting the times in high potassium saline. The response of the whole muscle (Fig. 3A) reflects the mixed fibre composition. The size of the maintained tonic contraction is a third that of the phasic one. The response of the proximal fibres is also mixed (Fig. 3B), but the phasic response is the predominant one. The tonic response declines rapidly and averages one fifth of the phasic one and is not maintained even while the muscle is in the depolarizing saline. The tonic response of the distal bundles is more than half of the phasic and it is maintained long after return to normal ringer (Fig. 3C).

The results of direct depolarization are in agreement with the phasic/tonic tension differences seen in the response to whole nerve stimulation. However, since neither muscle half gives a purely phasic or tonic response to direct depolarization, this indicates there is some mixing of fibre types. The muscle may therefore be described as a mixed population of fibres arranged in a continuum, from the proximal bundles which are mostly of fast-type to the distal bundles which are predominantly slow. Since the fast/slow separation is not associated with a similar distribution of fast/slow mot

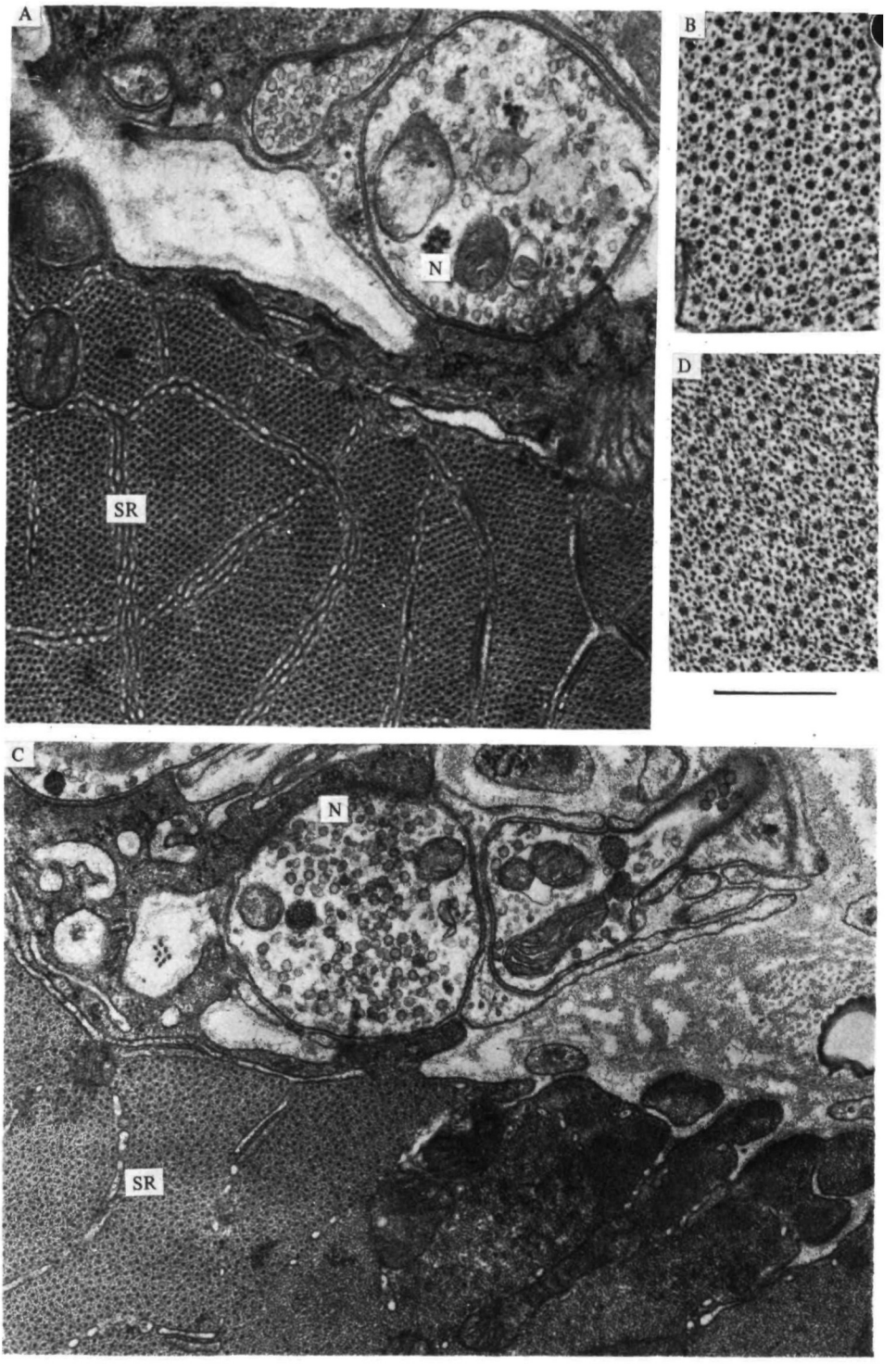

Fig. 4. Cross sections through a proximal (A) and a distal (C) fibre. Note the difference in the amounts of sarcoplasmic reticulum (SR) in the two regions. The insets show the filament arrangements in the proximal (B) and distal (D) fibres at a higher magnification. Nerve terminals (N) make similar contacts with the muscle fibres in both sections. Scale mark, A, C, 0·5 μm; B, D, 0·2 μm.

neurone terminals, differences in neuromuscular junctional transmissions cannot be responsible. There must instead be differences in the intrinsic properties of the muscle fibres which can be expected to reflect ultrastructural differences.

Ultrastructure of the flexor tibiae muscle fibres

In the light microscope, cross sections of flexor fibres showed closely packed fibrils, of a fairly uniform shape. The sarcomeres were not in perfect register. The average diameter of a fibre was somewhat smaller distally, averaging 60 μm, compared with 80–85 μm in the proximal and medial regions. The size difference appears to be associated with the tapering of the femur as the fibril size was similar throughout the muscle ($\sim$ 0·5 μm). The Z-bands were noticeably thicker in the distal part of the muscle, due in part to higher concentrations of mitochondria.

Sarcomere lengths were measured by drawing 11 serial Z-bands, measuring the length and dividing by 10. In this way 400 sarcomeres were measured from two animals. The muscles were cut into four pieces of approximately equal length. Mean sarcomere lengths were determined for each of the four regions: they increased progressively by 1·6 μm from the proximal to distal end, or 30% (5·3 to 6·9 μm, S.D. 0·5 μm). A t-test between two means for each of the two most proximal regions, compared with the most distal (t = 6·38; 5·26), showed the means to be significantly different at the 0·001 level.

The general features of the ultrastructure of the flexor tibiae are similar to previously described locust leg muscles (Elder, 1975). The fibres are multinucleate, with the majority of nuclei located at the periphery and a few centrally. Neuromuscular junctions are also of the typical form (Hoyle, 1974). The muscle sends up a pillar with rete synapticum to meet the nerve (Fig. 4A, C), and it is on these processes that synaptic contacts are made. Also, as is common for insect limb muscles, the thick filaments do not show a regular hexagonal array. The invaginated tubules appear to be equally developed throughout the muscle.

Nevertheless there were significant differences between proximally and distally located fibres. The sarcoplasmic reticulum (SR) was well developed in the proximal fibres, sparse in the distal ones (Fig. 4A, C). The thin filaments are irregularly arrayed about the thick filaments and varied in number from fibre to fibre. The thin to thick filament ratio counted at the area of A–I overlap increased progressively from the proximal to distal ends of the muscle (Fig. 4B, D), being never more than 3:1 in the most proximal fibres but greater than 5:1 in the distal ones.

Contractions evoked by single flexor motor neurones

A major question concerns the possible behavioural significance of the differences in mechanical responses that could be exploited by the insect using different motor neurones or combinations thereof. A preliminary question to this one is, are the differences in mechanical response observable when only a single motor neurone is active? To test this, flexor motor neurones were stimulated directly with an intracellular electrode and the ensuing pattern of contractions compared. Times to peak tension were measured as well as times to 50% relaxation.

The results from one of these experiments are shown in Fig. 5. The anterior fast flexor motor neurone was intracellularly depolarized and the times to peak tension

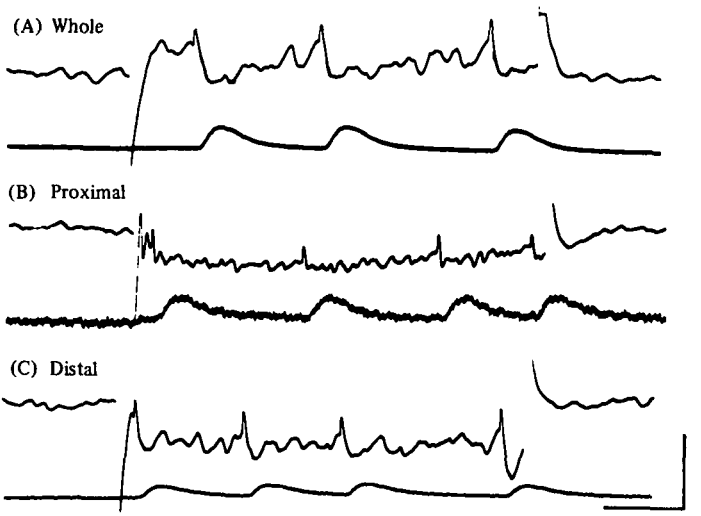

Fig. 5. Intrasomatic injection of current to the anterior fast flexor elicited action potentials (top traces) which cause muscle twitches (bottom traces). Tension is measured from the whole muscle in A. The tendon was then cut near its midpoint and tension in the proximal eight bundles recorded (B). In C the distal 12 fibre bundles have a longer rise time to peak tension. Scale mark, vertical 8 mV soma; 1 g/A, C, 200 mg B: time 100 ms.

were 18, 14 and 22 ms for the whole muscle, proximal and distal bundles. Corresponding times to 50% relaxation were 60, 50 and 70 ms respectively. Similar results were found with other flexor motor neurones. This and the other demonstrations of differences in mechanical response, together with the different ultrastructures are evidence that the differences in response are caused at least in part by differences in properties of the muscle fibres themselves, rather than to differences in neuromuscular transmission.

DISCUSSION

Innervation fields in the muscle

Mapping of the EJPs evoked by activity in each of the flexor motor neurones failed to reveal any specific spatial distribution for endings of the nine motor neurones that innervate the flexor tibiae. This was in contradiction to the suggestion made earlier that the flexor comprises a few independently innervated muscle units in parallel (Hoyle, 1957). In the metathoracic extensor tibiae, fast and slow motor neurones exclusively innervate some bundles of muscle fibres comprising only fast-type or slow-type muscle fibres respectively (Hoyle, 1978). In the flexor tibiae, by contrast, fast motor neurones synapse on slow-type fibres, slow motor neurones synapse on fast-type fibres, and fast, intermediate, and slow motor neurones may all synapse upon the same fibre.

The distribution of neuromuscular junctions of the flexor tibiae motor neurones appears to be different in each of the three pairs of legs. There is some overlap of innervation fields in the mesothoracic leg (Wilson, 1979*b*), but in the prothoracic leg this was not found. The extensive overlapping of terminals in the metathoracic leg may be related to the need for maximum tension in cocking the leg for jumping. T

Overlapping innervation also allows for tension to be more precisely regulated, because the number of neurones active, as well as the individual firing frequencies, will modulate the tension output.

Muscle anatomy

The fibres of the flexor tibiae do not form a structurally homogeneous population. When cross sections from proximal and distal muscle fibres are compared, two differences in their ultrastructure are apparent. First, the double and triple layers of sarcoplasmic reticulum dividing the fibrils of the proximal fibres are in contrast to the limited single layer of SR in the distal fibres with their poorly defined fibrils. The second difference is the proximal to distal increase in thin to thick filament ratios from about 3:1 to greater than 5:1. A high thin to thick filament ratio, reduced amounts of SR, and poorly defined fibrils have long been recognized as consistent features of slow fibres (Hoyle, 1967; Elder, 1975). Based on these criteria the distal portion of the muscle may be classified as slow or tonic.

There is, however, no sharp line of demarcation between either the structural features or the different physiological properties of the two sets of fibres. Thin to thick filament ratios and sarcomere lengths increase progressively from the proximal to distal ends of the muscle. The mixed response to direct depolarization with high potassium saline in the proximal and distal muscle halves also reflects the non-uniformity of muscle fibre composition. The fibres are better thought of as being arranged in a continuum from predominantly fast proximally to predominantly slow distally. Fibres of intermediate type also occur in the extensor tibiae (Hoyle, 1978).

Muscle physiology

Tension measurements from muscle contractions elicited by whole nerve stimulation, by direct depolarization of the muscle fibres, and by action potentials in a single identified flexor motor neurone, all support the anatomical finding that the muscle fibres are arranged in a proximal to distal, phasic to tonic continuum.

Preliminary ultrastructural investigations of the pro- and meso-thoracic flexor tibiae indicate a distribution of sarcoplasmic reticulum similar to that found in the metathoracic leg. This suggests a phasic to tonic arrangement of muscle fibre properties in these legs. A proximal-phasic/distal-tonic mechanical response difference has been found in stick insect metathoracic extensor tibiae. Differential innervation by the fast and slow motor neurones is thought to be responsible in this case (U. Bässler, personal communication). In the pro- and meso-thoracic extensor tibiae of the locust, a phasic/tonic division in response exists based on fibre innervation. Intracellular muscle recordings found terminals of the fast extensor tibiae motor neurone on 80% of the proximal fibres but on only 30% of the distal fibres (Burns & Usherwood, 1979). It may be that this arrangement of muscle fibre properties will prove to be a common feature of insect limb muscles. There are, however, exceptions known. In both locust and cockroach metathoracic extensor tibiae, many extreme proximal fibres have exclusively slow innervation (Hoyle, 1978; Atwood, Smyth & Johnston, 1969).

The proximal to distal response differences might have their origins in the intrinsic properties of the muscle fibres. In dually innervated muscles like the extensor, fibres exclusively innervated by the fast motor neurone may have their intrinsic response properties either enhanced or reversed by neural trophic influences. In the flexor, on

the other hand, all of the fibres are multiply innervated. Therefore no individual neurone can specify the intrinsic mechanical properties of the muscle fibres.

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