The Journal of Experimental Biology 200, 677–691 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JEB0180

DIFFERENTIAL PHYSIOLOGY AND MORPHOLOGY OF PHASIC AND TONIC MOTOR AXONS IN A CRAYFISH LIMB EXTENSOR MUSCLE

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Accepted 25 November 1996

Summary

The main leg extensor muscle of the crayfish *Procambarus clarkii* is innervated by two excitatory motor axons (phasic and tonic) and by the common inhibitory axon. The axons and their terminals were investigated using vital fluorescent dyes, antibodies and electron microscopy. Correlative physiological observations were made using intracellular microelectrodes and focal 'macro-patch' electrodes. The excitatory axons innervate the muscle fibres more extensively than does the inhibitory axon. Striking morphological and physiological differences between the two excitatory axons were discerned. The tonic motor axon contains many mitochondria and has varicose junctional terminals; the phasic axon has fewer mitochondria and thin terminals. In freely moving animals, the tonic axon is very active, whereas the phasic axon is inactive but fires in brief bursts during walking. Throughout the muscle, tonic excitatory postsynaptic potentials (EPSPs) are very small at low frequencies and facilitate greatly at higher frequencies. The EPSPs of the phasic axon are larger at low frequencies and depress with maintained stimulation. At identified tonic terminals, quantal content at 1–10 Hz is very low, whereas at identified phasic terminals, quantal content is 50- to 200-fold greater. The results indicate that transmitter release is regulated differently at the synapses of the two axons.

Key words: motor axon, crayfish, *Procambarus clarkii*, mitochondria, synaptic transmission, confocal microscopy.

Introduction

Crustacean motoneurones have provided excellent experimental models for investigating synaptic transmission. Individual axons of phasic or tonic type can be individually identified and show many of the physiological features of synaptic plasticity observed in other less accessible neurones. Mechanisms of synaptic release, short- and long-term facilitation and depression have been described (Atwood and Wojtowicz, 1986). The fact that many of the crustacean motoneurones can be broadly assigned to 'phasic' or 'tonic' categories (Kennedy and Takeda, 1965*a*,*b*) permits investigation of the factors that underlie synaptic differentiation. Neuronal differences are related to the behavioural repertoire of the intact animal. The present study was undertaken to correlate physiological and morphological features of individual phasic and tonic motoneurones co-localized in a crayfish limb muscle, the extensor of the carpopodite (main leg extensor muscle). Although its muscle fibres have been used in previous studies of excitation–contraction coupling (Girardier *et al.* 1963), little is presently known about its synaptic physiology. This muscle was investigated because it offers a particularly favourable situation for investigating phasic–tonic neuronal differentiation, both morphologically and physiologically, and in addition shows physiological features that are somewhat different from those of other well-studied crustacean muscles.

The advent of new vital fluorescent dyes (Magrassi *et al.* 1987) and improved methods for marking sites of physiological recordings for further morphological analysis (Wojtowicz *et al.* 1994; Cooper *et al.* 1995*a*) have made possible a more comprehensive correlational study of different nerve endings within the same muscle. It is now easier to obtain a range of observations, both physiological and morphological, from selected, identifiable nerve terminals. We undertook such an analysis in the main extensor muscle of the crayfish leg, in which the two excitatory motor axons, which supply all of the fibres within the muscle with dual excitatory innervation, are well-differentiated into phasic and tonic phenotypes. These axons are also readily accessible for further detailed experimentation involving techniques such as intra-axonal injection of pharmacological agents, fluorescent dyes and Ca^{2+}

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indicators. The present paper reports observations on the axons and nerve endings obtained using several microscopical and physiological methods ranging from whole-animal observations to electron microscopy of individual nerve endings. These observations indicate that, although some of the physiological differences between phasic and tonic neurones can reasonably be accounted for on the basis of morphological differences, others cannot and must be further investigated. In addition, physiological differentiation of nerve endings of a single axon differs from what has been seen previously in the 'opener', 'stretcher', 'closer' and 'accessory flexor' muscles in limbs of crayfish and other crustacean species (Atwood and Bittner, 1971; Rathmayer and Erxleben, 1983; Walrond *et al.* 1993).

Materials and methods

Animals and solutions

Freshwater crayfish, *Procambarus clarkii* Girard, of intermediate size (6 cm from rostrum to telson, mass 5–6 g) were used for these experiments. The extensor muscle of the first (or occasionally the second) pair of walking legs was used. The medial surface of the muscle was used throughout this study. The muscle was exposed by removing the cuticle on the lateral aspect of the meropodite along with the entire flexor muscle and the main leg nerve. The motor nerve of the extensor muscle separates from the main leg nerve as it enters the meropodite and is left in place on the muscle after the main leg nerve has been removed. For fluorescence microscopy and electrophysiology, a modified van Harreveld's crayfish solution was employed, which contained (in mmol l^{-1}): NaCl, 205.3; KCl, 5.3; $CaCl_2 \cdot 2H_2O$, 13.5; $MgCl_2 \cdot 6H_2O$, 2.5; and Hepes buffer, 0.5 mmol l^{-1} adjusted to pH 7.4. Dyes used for fluorescent staining of the nerve were dissolved in this solution, as indicated below.

Muscle fibre histochemistry and sarcomere lengths

The histochemical approach that we used to differentiate fast and slow fibres within the extensor muscle is based upon myofibrillar ATPase activity. The method of Ogonowski and Lang (1979) developed for crustacean muscles was used. This procedure results in fast fibres staining more darkly than slow fibres. The muscle was clamped, so that the fibres were in a stretched position, and frozen by immersion in cold isopentane. Cross sections were made at various locations along the length of the meropodite and stained for myofibrillar ATPase activity.

Supplementary observations on sarcomere lengths of muscle fibres sampled from several locations within the muscle were also made after fixing a muscle (slightly stretched) in Bouin's fixative, isolating single fibres, and measuring their sarcomere lengths under the light microscope with an eyepiece micrometer. In crustacean muscles, sarcomere length is often correlated with physiological characteristics of the muscle fibre (Atwood, 1972).

Innervation patterns

For visualization of the total innervation pattern, fluorescence microscopy was used in conjunction with the aid of a vital fluorescent dye, 4-Di-2-Asp {4-[4-(diethylamino)styryl]-*N*-methylpyridinium iodide} (Magrassi *et al.* 1987), obtained from Molecular Probes (Eugene, Oregon). Concentrations of 2–5 μmol l^{-1} in crayfish solution were used; preparations were immersed for 2–5 min and rinsed twice with dye-free solution before viewing. In a previous study (Cooper *et al.* 1995*a*), it had been determined that synaptic transmission in preparations so treated continues normally for the duration of the experiment as in standard solution, provided that prolonged illumination with blue light is avoided. An upright epifluorescence microscope (Nikon Optiphot model) equipped with 20× (dry) and 40× (water immersion) objectives, appropriate filter blocks and a photomicrographic attachment was used for preliminary morphological work and for simultaneous electrophysiology. For more detailed morphological work, the fluorescence microscope was used in conjunction with a Bio-Rad 600 confocal laser microscope.

The details of the innervation of the muscle by phasic, tonic and inhibitory neurones were investigated using two different methods. In the first, Lucifer Yellow was selectively loaded by ionophoresis into either the tonic or the phasic axon and photographs of a defined region were taken using the confocal microscope. Afterwards, the preparation was stained with 4-Di-2-Asp to reveal all of the innervation, and the same terminal region was re-photographed for direct comparison.

A second approach was to follow the distribution of the inhibitory innervation using an anti-GABA antibody. We first stained the preparation with 4-Di-2-Asp (5 μmol l^{-1} for 3 min) and photographed terminals in a region of interest. The stained excitatory and inhibitory terminals were visualized using the confocal microscope with a 40× Nikon water-immersion objective lens. The visualized area was labelled by leaving behind fluorescent beads, to aid later identification. This was done by dipping the tip of a 'macro-patch' electrode (10–20 μm in diameter) into a slurry of fluorescent polystyrene beads (0.5 μm in diameter, Duke Scientific Co.) a few times, with air-drying between dips, and then touching the electrode to the muscle surface at the region of interest. The beads adhere to the muscle and remain after several washings (Wojtowicz *et al.* 1994; Cooper *et al.* 1995*a*). The preparations were immersed for fixation in 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.2) for 1 h. This was followed by a 30 min wash in 0.1 mol l^{-1} PBS. After rinsing, specimens were treated for 30 min in 0.1 mol l^{-1} Tris–glycine to reduce background fluorescence. The fixed tissue was washed overnight in PBS (4 °C), then permeabilized by incubation in 4% Triton X-100, 5% bovine serum albumin (BSA) and 2% normal goat serum (NGS) for 1 h. Subsequently, the specimens were incubated in the primary antibody, a polyclonal rabbit anti-GABA antiserum (a gift from Dr L. Brodin, Stockholm, Sweden; dilution 1:1000) for 48–50 h. This was followed by an overnight wash in PBS, after which specimens were incubated in fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (dilution 1:200, Sigma) for 18–24 h. The same area previously

visualized after staining with 4-Di-2-Asp was now identified with the aid of the fluorescent beads and photographed again to ascertain the distribution of both inhibitory and excitatory terminals.

Mitochondrial fluorescence

Individual mitochondria were visualized with confocal microscopy in living axons after staining with 4-Di-2-Asp or Rhodamine-123 at a concentration of 13 μmol l^{-1} for 10 min, as described by Nguyen and Atwood (1994). Both dyes are accumulated in mitochondria (Chen, 1988; Harrington and Atwood, 1995) and are released by metabolic inhibitors. Rhodamine has been more extensively used as a mitochondrial probe in previous work and is generally thought to provide a measure of the mitochondrial membrane potential, because the fluorescence of Rhodamine in mitochondria increases when their membrane potential is high (Chen *et al.* 1981; Emaus *et al.* 1986). 4-Di-2-Asp bleaches less rapidly and provides better images, so it was used for many of the axonal comparisons in the present study. The intensity of mitochondrial fluorescence was compared in phasic and tonic axons by measuring the percentage of area within the imaged axon fluorescing above a selected intensity. The available pixel intensities ranged in relative value from 0 to 255. Software from BioRad available with the confocal microscope was used. Samples of the axons were obtained by collecting 1–2 μm thick optical sections of the surface of the axon; such samples included regions where the mitochondria were concentrated within the axon. Individual optical sections were then combined to produce a 5–7 μm thick composite series which provided an image for analysis.

Electron microscopy

Samples of axons and neuromuscular junctions were obtained using standard fixation, embedding and sectioning procedures (Jahromi and Atwood, 1974). In the present study, representative locations were selected to illustrate general features of the phasic and tonic terminals.

Electrophysiology of synapses

Recording of excitatory postsynaptic potentials (EPSPs) using intracellular microelectrodes and measurement of focal synaptic currents at selected terminals followed standard procedures (Wojtowicz *et al.* 1994). EPSPs were measured in the muscle fibres of several preparations by stimulating selectively the phasic or tonic excitatory motoneurones at various frequencies. This stimulation was delivered by a macro-patch electrode with an inner diameter of 15–20 μm placed directly on the phasic or tonic axon. The axonal type is easily identifiable after staining the preparation with 4-Di-2-Asp because, as illustrated in the Results, this dye stains the tonic axon more brightly owing to the larger number and brighter fluorescence of its mitochondria.

Focal extracellular recordings of synaptic currents with macro-patch electrodes (Dudel, 1981) of 10–15 μm inside diameter were selectively made from either phasic or tonic terminals with the aid of an amplifier obtained from Zeitz Instruments Vertriebs GmbH (Augsburg, Germany). Lightly stained preparations support transmission without apparent adverse effects, provided that illumination is kept to a minimum (Cooper *et al.* 1995*a*). The evoked responses from each recording site met the requirements of stationarity in activity for the time analyzed; at least 1000 sweeps were collected for averaging. Estimations of quantal content at low frequencies were obtained by counting quanta (tonic axon) or by measuring the ratio of areas of evoked responses to unitary quantal events (Cooper *et al.* 1995*b*).

Electromyography

Myographic recordings were obtained from the extensor muscles of intact crayfish by carefully placing the ends of two insulated thin copper wires through small holes in the shell over the extensor muscle in its central region where there are no muscle fibre insertions. The wires were held in place with hard dental wax, which adhered to the shell. The techniques followed similar ones developed by Atwood and Walcott (1965), Lnenicka and Atwood, (1985, 1988) and others for crustacean limb muscles. The potentials were recorded differentially using a Grass AC preamplifier (P15) and acquired by computer or displayed with a Gould Brush pen recorder. The myographic potentials were counted for each second of the recording period to obtain the firing frequency. In previous work on the crayfish closer muscle, Lnenicka and Atwood (1985) had shown that myographic potentials of phasic and tonic axons can be readily distinguished owing to the large difference in their amplitude. We found that the same is true for the leg extensor muscle. In the intact animal, relatively large myographic potentials appeared infrequently, along with much smaller potentials at a relatively high frequency. When the leg was removed from the animal with the myographic electrodes in place and stimuli applied to the leg nerve to recruit the extensor axons, the large potential appeared at about half its original amplitude. A small window was made through the shell and a microelectrode inserted into an extensor muscle fibre. The large EPSP of the phasic axon appeared when the large myographic potential was recruited. The much smaller EPSP of the tonic axon was not accompanied by a measurable myographic potential at low to moderate frequencies in the isolated leg with the window made for intracellular recording; it appeared that the isolation procedures compromised the electromyographic recording of this small potential. Thus, the phasic and tonic myographic potentials are clearly very different in amplitude in the leg extensor muscle, as in the claw closer muscle, and can be easily distinguished in myographic recordings.

Results

Muscle fibre composition

The histochemical staining of the muscle fibres for myofibrillar ATPase revealed that most of the fibres in the muscle are lightly staining (‘slow’ to ‘intermediate’ in

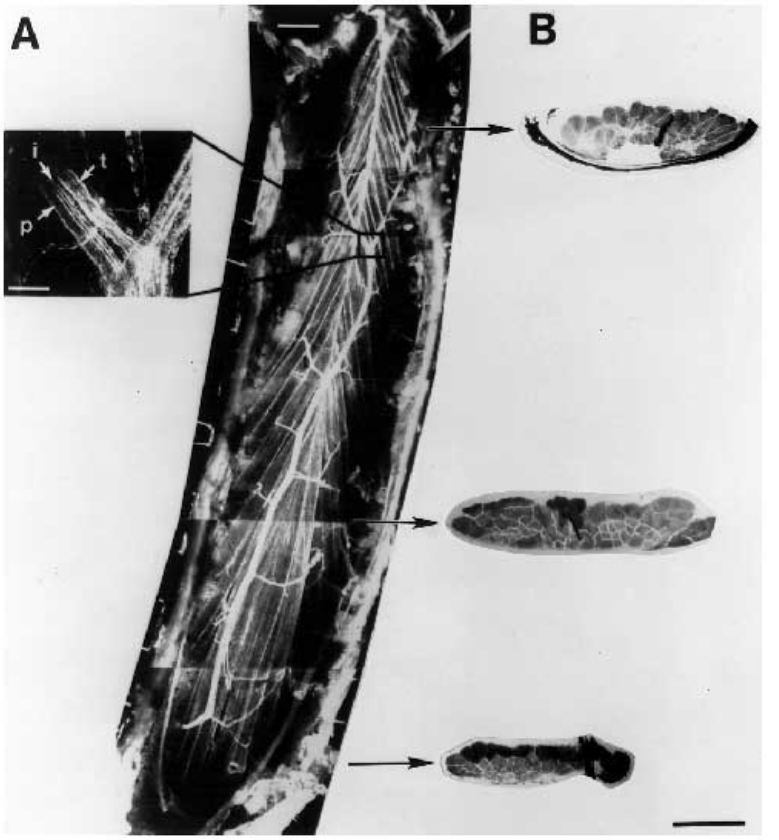

Fig. 1. (A) Montage to show the pattern of innervation of the exposed 'deep' surface of the carpopodite extensor muscle of the walking leg (confocal micrographs of 4-Di-2-Asp-stained preparations). The inset shows the three major axons at higher magnification. p, 'phasic' excitatory axon; t, 'tonic' excitatory axon; i, 'common' inhibitory axon. The distal end of the muscle is at the top of the figure. Scale bars, 500 μm and 50 μm. (B) Cross sections of the muscle taken at three different locations (indicated by lines and arrows), illustrating the ATPase reaction for muscle fibres. A small group of fibres in the proximal region stained more darkly than the rest. Scale bar, 500 μm.

contractile properties). However, there is a well-defined wedge-shaped region in the proximal end which stains more darkly and is thus predominantly made up of 'fast' fibres. This region appears in the sections in Fig. 1B. The bundle of fast fibres is found only on the inner (medial) surface of the proximal region.

Measurements of sarcomere lengths in fixed muscle fibres indicated that fibres with short sarcomeres occur in the superficial proximal bundle (coinciding in location with the fibres staining darkly for myofibrillar ATPase). Mean values (± S.D.) for sarcomere lengths sampled from a fixed muscle of an animal 4 cm in length were as follows: proximal superficial bundle, 3.4±0.3 μm; proximal deep bundle, 9.0±0.5 μm; central superficial bundle, 11.3±0.2 μm; central deep bundle, 9.9±0.5 μm (30–40 myofibrils sampled from each bundle of muscle fibres). These readings confirm that the superficial proximal region contains fast muscle fibres, while the rest of the muscle contains slow to intermediate fibres. Short-sarcomere fibres were also observed in this region in another species of crayfish (Law and Atwood, 1971). In confocal microscopy, both long- and short-sarcomere fibres were observed in the proximal region, indicating the possibility of specimen variation or age-related changes in muscle fibre composition.

Innervation

In decapod crustaceans, the main extensor muscle receives two excitatory axons and a branch of the 'common' inhibitory axon (Wiens, 1989; Wiens and Wolf, 1993). The two excitatory axons are physiologically differentiated: one is 'fast' (phasic) and the other is 'slow' (tonic), as in the claw closer muscle (Wiens, 1993).

The distribution of the axons can be readily visualized by removing the antagonistic flexor muscle and main leg nerve and staining the exposed inner surface of the extensor muscle briefly with 4-Di-2-Asp. Conventional fluorescence microscopy and confocal microscopy were employed to follow the distribution of the axons throughout the muscle (Fig. 1A).

The motor nerve progresses in a near-central location towards the distal end of the muscle, where it makes a prominent 'Y' by separating into two lateral divisions supplying the two sides of the distal end of the muscle. From the main central motor nerve trunk, approximately 15 prominent secondary lateral branches are given off to groups of muscle fibres. Several additional secondary lateral branches arise from the two distal primary divisions of the main nerve. Each secondary branch divides into smaller tertiary and quaternary branches, some of which run longitudinally on the muscle fibres for varying distances and form neuromuscular junctions.

Within the main nerve trunk and secondary branches, three prominent axons (two large, one smaller) can be easily resolved with the confocal microscope (Fig. 1A). As shown below, these axons differed substantially in their appearance when viewed after treatment with fluorescent probes; their identity was established through conjoint physiological and

morphological observations. The two large axons are excitatory, and the smallest is inhibitory.

To establish the physiological identity of these axons, we impaled each of the two larger axons with intracellular stimulating and recording microelectrodes or selectively stimulated them with focal extracellular macro-patch electrodes. The largest axon produced a very small (<1 mV) EPSP at a low frequency of stimulation, whereas the second-largest axon produced a large EPSP (5–30 mV) for a single impulse. Thus, the largest axon was identified as the 'slow' or 'tonic' excitatory axon, and the second largest as the 'fast' or 'phasic' axon. Previous work on inhibitory innervation in the crayfish (Wiens, 1993) has shown that the smallest of the prominent axons to this muscle is a branch of the 'common' inhibitory axon. The identities of the other much smaller axons in this nerve have not been established. In other crustacean species, axons of tension receptors are known to be associated with parts of this nerve (Macmillan and Dando, 1972).

Morphology and fluorescence of the axons

Transverse sections of the main nerve showed the three prominent axons and usually 1–4 smaller axons within a nucleated glial sheath (Fig. 2A). The largest (tonic) axon was richly endowed with mitochondria, mostly near the perimeter of the axon. The slightly smaller phasic axon had only 20–25 % of the number of mitochondria in these cross sections. The smallest of the prominent axons, subsequently confirmed as inhibitory, had variable numbers of mitochondria in different

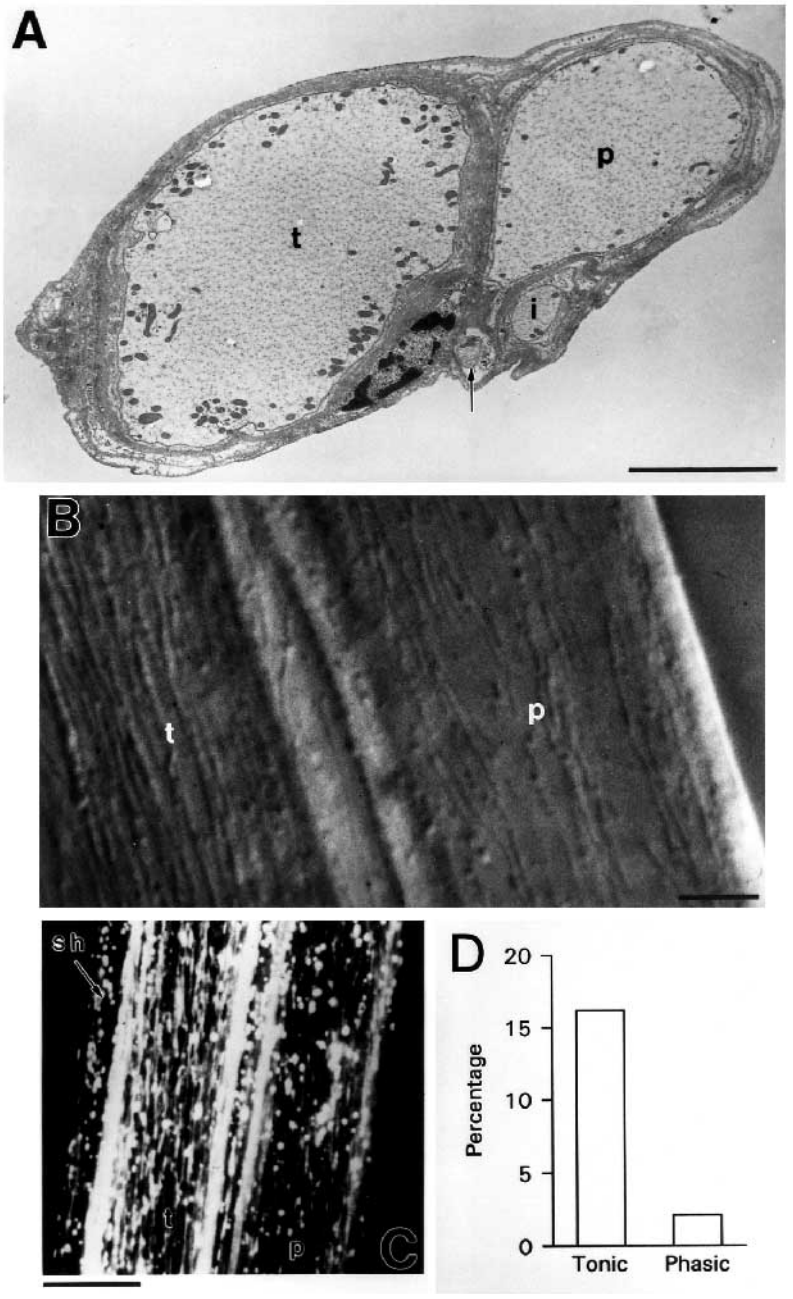

Fig. 2. Features of the axons in the major central nerve. (A) Electron micrograph to illustrate the difference in mitochondrial content in phasic (p) and tonic (t) excitatory axons, and the common inhibitory axon (i). An additional small unidentified axon is also present (arrow). (B) Nomarski view of mitochondria oriented longitudinally in phasic (p) and tonic (t) excitatory axons. The mitochondria are often very long and clustered just below the surface of the axon with few in the centre. The number of mitochondria is larger in the tonic axon. Scale bars, 5 μm. (C) Micrographs of the main axons stained with Rhodamine-123 (confocal microscopy) to illustrate the differences in number and brightness of the mitochondria in the phasic (p) and tonic (t) excitatory axons. sh, glial sheath. (D) Intensity measurement from the confocal microscopy image of C. The percentage of axon area occupied by structures with a relative fluorescence intensity of 215–255 is greater in the tonic axon (see Materials and methods). Scale bars, 25 μm.

locations, but comparison of mitochondrial counts per unit cross-sectional area showed that this small axon was more closely similar to the largest (tonic excitatory) axon in mitochondrial density. In the example of Fig. 2A, the numbers of mitochondria per μm^2 of axonal cross-sectional area were as follows: tonic axon, 1.21; phasic axon, 0.72; inhibitory axon, 2.02. Thus, the phasic axon has a lower mitochondrial count in relation to its cross-sectional area than the other two axons. This general result was consistently observed in all samples of the main axons and pre-terminal branches (see also Fig. 5) and was confirmed in observations made with Nomarski optics (Fig. 2B) and fluorescence optics (Fig. 2C).

Additional microscopic observations showed a difference in mitochondrial numbers in the two large excitatory axons. After fixation, individual mitochondria can be observed in these axons with Nomarski optics (Case and Lnenicka, 1992). The individual mitochondria were often very long and were observed mainly at the periphery, with few in the centre of the axon (Fig. 2B). The packing density of sub-surface mitochondria was clearly greater for the tonic axon than for the phasic axon.

After Rhodamine-123 staining, a greater number of elongated bright structures appeared within the tonic than within the phasic axon (Fig. 2C). The same result was obtained with 4-Di-2-Asp staining. Sections taken with the confocal microscope to include the sub-surface layer of mitochondria were compared by analyzing the percentage area of the axon occupied by brightly fluorescing structures, taken to be

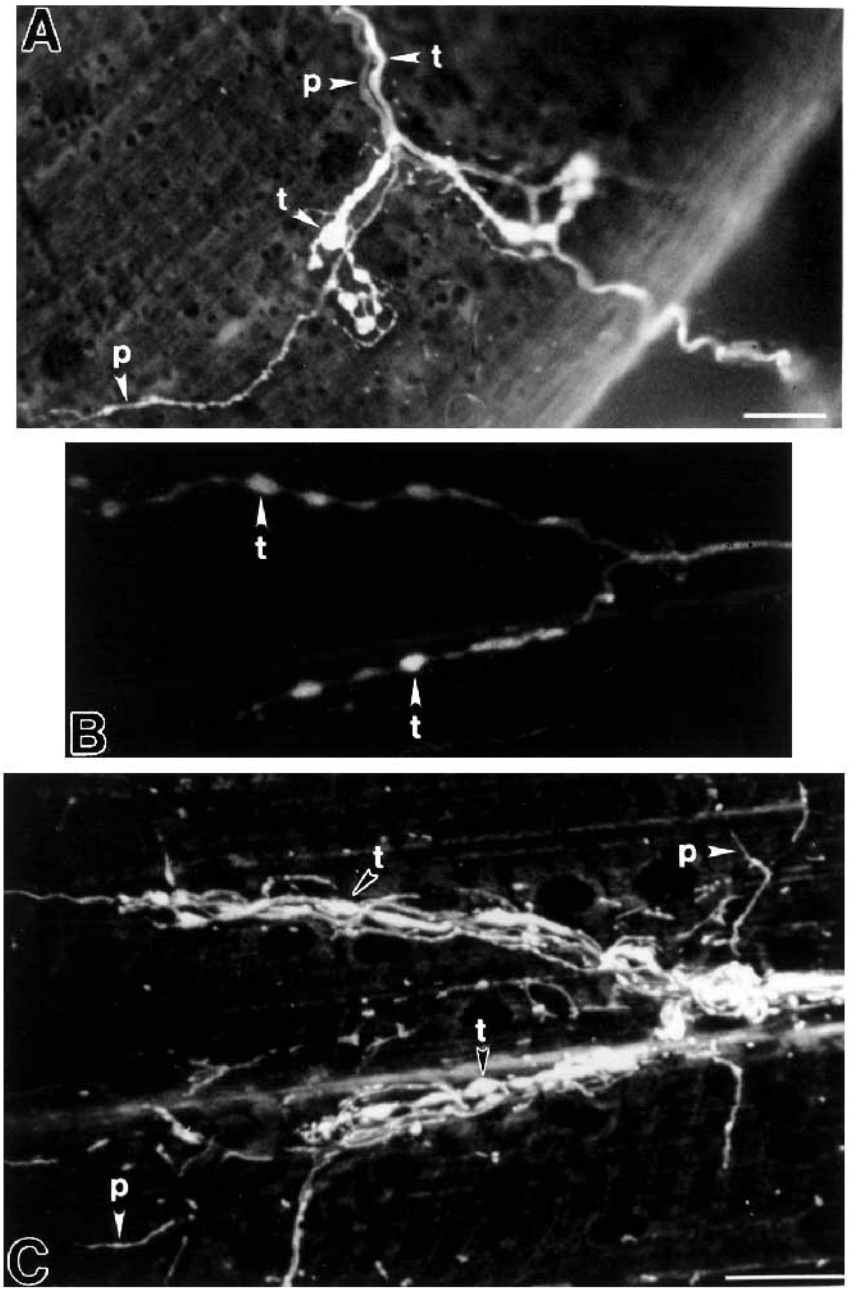

Fig. 3. Examples of neuromuscular junctions formed by phasic (p) and tonic (t) axons. (A) Tonic and phasic junctions are distinguished by size and shape. The endings of the phasic axon are thin and filiform; they often extend well beyond the tonic endings, which are larger in diameter, less extensive and more fluorescent. Note that phasic terminals sometimes 'wrap around' the tonic terminals. Scale bar, 25 μm. (B,C) Identification of axons by selective intracellular injection. (B) A tonic axon ending after intracellular injection of Lucifer Yellow into the main axon, showing terminal varicosities. (C) The same junctional region after exposure to 4-Di-2-Asp, showing additional extensive phasic (and possibly some inhibitory) terminals. Phasic (p) and tonic (t) terminals are indicated by arrowheads. Scale bar, 25 μm.

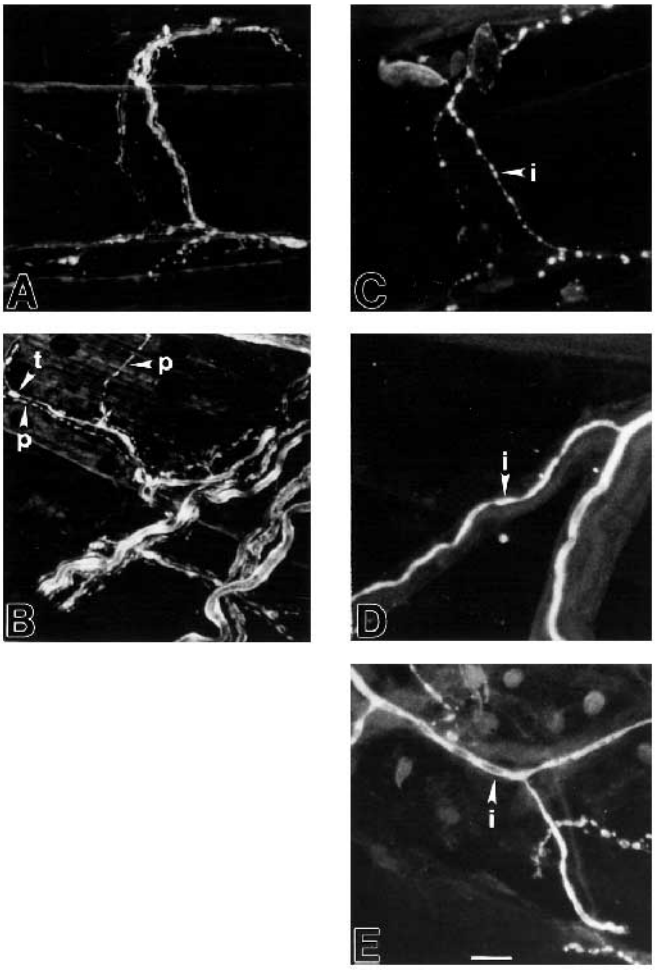

Fig. 4. Inhibitory innervation demonstrated with an anti-GABA antibody and a fluorescent secondary antibody; examples of inhibitory terminal distribution relative to that of the excitatory axons. (A,B) 4-Di-2-Asp-stained excitatory (phasic and tonic) and inhibitory axon branches and junctions. All three axons (two excitatory, one inhibitory) are stained. (C,D) The same regions as in A and B, respectively, seen after selective staining of inhibitory axons and junctions with the anti-GABA and fluorescent secondary antibodies. The 4-Di-2-Asp staining disappears with fixation. Note that the inhibitory axon (i) is not distributed to all of the regions supplied by the phasic (p) and tonic (t) excitatory axons (B,D). (E) Examples of inhibitory terminals more distal to the axon branches in B and D, showing varicose inhibitory junctional terminals stained with the anti-GABA antibody. Scale bar, 40 μm.

mitochondria with a relatively high membrane potential (Johnson *et al.* 1981; Chen *et al.* 1981; Chen, 1988). The tonic axon invariably produced a much higher reading in this assay (Fig. 2D). These results indicate a higher mitochondrial content for the tonic axon, and very probably also a larger proportion of mitochondria with a relatively high membrane potential, indicative of high metabolic capacity (Nguyen and Atwood, 1994; Atwood and Nguyen, 1995).

Comparisons of relative fluorescence values of individual mitochondria stained with 4-Di-2-Asp in four different pairs of axons visualized with the same settings of the confocal microscope were made. After subtraction of background fluorescence values from the measurements of mitochondrial fluorescence, overall mean values (± S.E.M.) of 64.2±2.5 (N=87) and 43.6±1.6 (N=93) were obtained for net fluorescence of mitochondria in tonic and phasic axons, respectively. Analysis of variance indicated a significant difference (P<0.001) for mitochondria of the two axon categories.

Nerve terminals

The secondary branches of the two major axons could readily be followed in 4-Di-2-Asp-stained preparations to terminals on the muscle fibres (Fig. 3). In many locations, the excitatory axons appeared to provide the only innervation. Two types of ending were seen in such locations: (a) large varicose terminals, with individual varicosities ranging widely in size; and (b) thin terminals of relatively uniform diameter and wider distribution. The former could be followed back to the tonic axon, the latter to the phasic axon.

The largest varicosities of the tonic axon (sometimes up to 25 μm in diameter) usually occurred more proximally in a string of boutons, and the smaller ones more distally. The thin terminals of the phasic axon often appeared to ‘wrap around’ the varicosities of the tonic axon (Fig. 3A). The terminals of the tonic axon fluoresced more brightly than those of the phasic axon, indicating a higher mitochondrial content.

Experiments were performed to confirm and demonstrate more clearly the identities of the two terminal types. In the example of Fig. 3B,C, injection of the tonic axon in the main nerve trunk with Lucifer Yellow led to the appearance of fluorescent varicose terminals on the muscle fibres (Fig. 3B). Subsequent staining of the same location with 4-Di-2-Asp revealed in addition a great profusion of thin, widely distributed terminals, typical of the phasic axon (Fig. 3C). Innervation from the inhibitory axon was also stained in this location, but was hard to separate visually from the other innervation.

To clarify the distribution and appearance of the inhibitory terminals, we treated preparations with an anti-GABA antibody after specific locations had been visualized with 4-Di-2-Asp. As illustrated in Fig. 4, the GABA-reactive axon was smaller than the two excitatory axons and more restricted in its distribution. It did not send branches to all locations innervated by the excitatory axons. The endings of the inhibitory axon were varicose in structure, but not as large as those of the tonic axon.

We noted that the main branches of the inhibitory axon were interrupted along the length of the muscle at 1–3 locations in many of our preparations. The common inhibitory axon probably leaves and rejoins the main leg nerve within the meropodite and, when the latter is removed, the ‘common’ inhibitory axon is often broken. This type of situation was noted for another crustacean species (the lobster *Homarus americanus*) by Wiens (1990). Thus, the method of preparation we used here, which entails removal of the main leg nerve from the meropodite, permits physiological investigation only of the

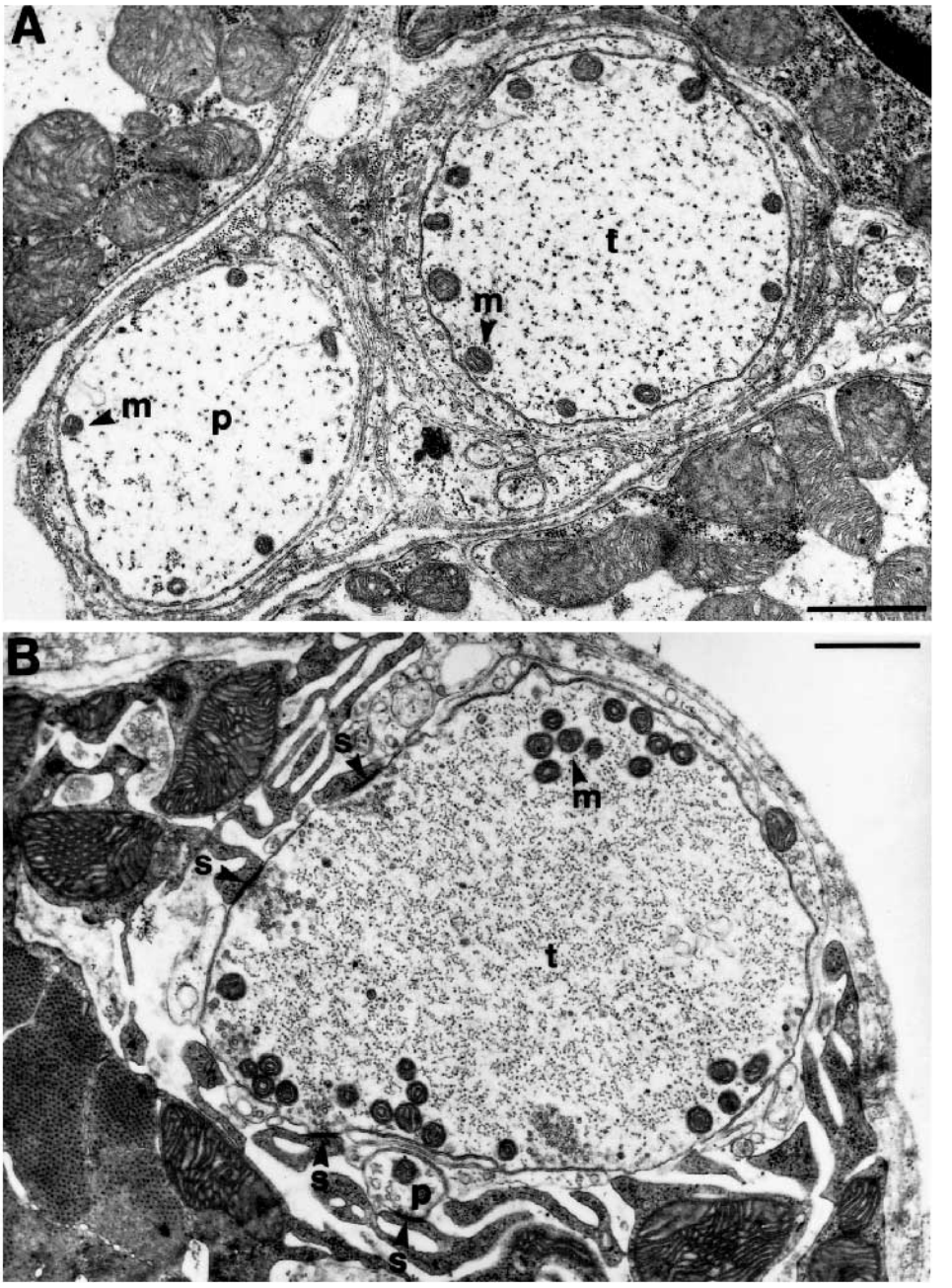

Fig. 5. Electron micrographs of pre-terminal axon branches (A) and terminal junctional processes (B) of phasic (p) and tonic (t) excitatory axons. Mitochondria (m) and synapses (s) are of more frequent occurrence in the large tonic endings. Scale bars, 1 μm.

excitatory axons, which were left intact in carefully treated preparations.

The two excitatory axons sent terminals to all fibres of the exposed inner surface of the muscle, including the fast muscle fibres identified by histochemistry. The characteristic phasic and tonic terminals could be seen together throughout the muscle. In the proximal region, the phasic terminals extended well past the tonic terminals, as in Fig. 3A. Although not quantified, this pattern may have provided a relatively greater length of phasic terminals to fibres of the proximal region, known to be of the fast type from histochemistry (Fig. 1).

Electron microscopy of representative muscle fibres (Fig. 5) showed pre-terminal branches of the phasic and tonic axons in many locations, distinguishable on the basis of their differing mitochondrial contents (Fig. 5A). Neuromuscular junctions could also be clearly identified by their relative size and mitochondrial content (Fig. 5B). In a quantitative study of these terminals based on eight serial sections, the percentage volume of mitochondria averaged 17 % in tonic terminals and 6 % in phasic terminals, a threefold difference that is statistically significant (King, 1995). Inhibitory terminals were often not present, but were identified in some locations on the basis of synaptic vesicle size and shape, as established in numerous previous studies (Atwood and Morin, 1970; Atwood *et al.* 1972; for a review, see Atwood and Tse, 1993). Individual synapses of the tonic and phasic endings were

generally similar in appearance and have been found from serial section reconstructions to be similar in contact area (King *et al.* 1996).

Impulse production of the excitatory axons

Surface electromyographic recordings were obtained from crayfish moving freely in a small aquarium (Fig. 6A,B). Activity of the extensor muscle during postural maintenance or slow walking was in the form of small, facilitating (usually biphasic) waveforms similar to those of the opener muscle (Wilson and Davis, 1965), but usually occurring at a higher average frequency (15–40 Hz). Frequency increased and the potentials facilitated when active extension of the limb was observed. Occasionally, isolated larger potentials occurred during slow locomotion (Fig. 6A). As indicated in the Materials and methods section, this large potential was clearly identified with the phasic axon, while the more numerous small potentials are those of the tonic axon. The large potentials also occurred during walking (Fig. 6B) or in response to imposed movement of the limb. At such times, overall electrical activity was often high and complicated by the occurrence of muscle action potentials, so that individual phasic synaptic potentials could not easily be counted. Nevertheless, it was evident that the phasic axon often provided a brief burst of a few impulses at the beginning of a leg extension movement (Fig. 6B). The observations are fully consistent with previous work on crustacean locomotion (Atwood and Walcott, 1965; Pahapill *et al.* 1985) in showing that one of the two excitatory motor axons of muscles receiving a dual excitatory motor supply is exclusively active during many low-level normal activities,

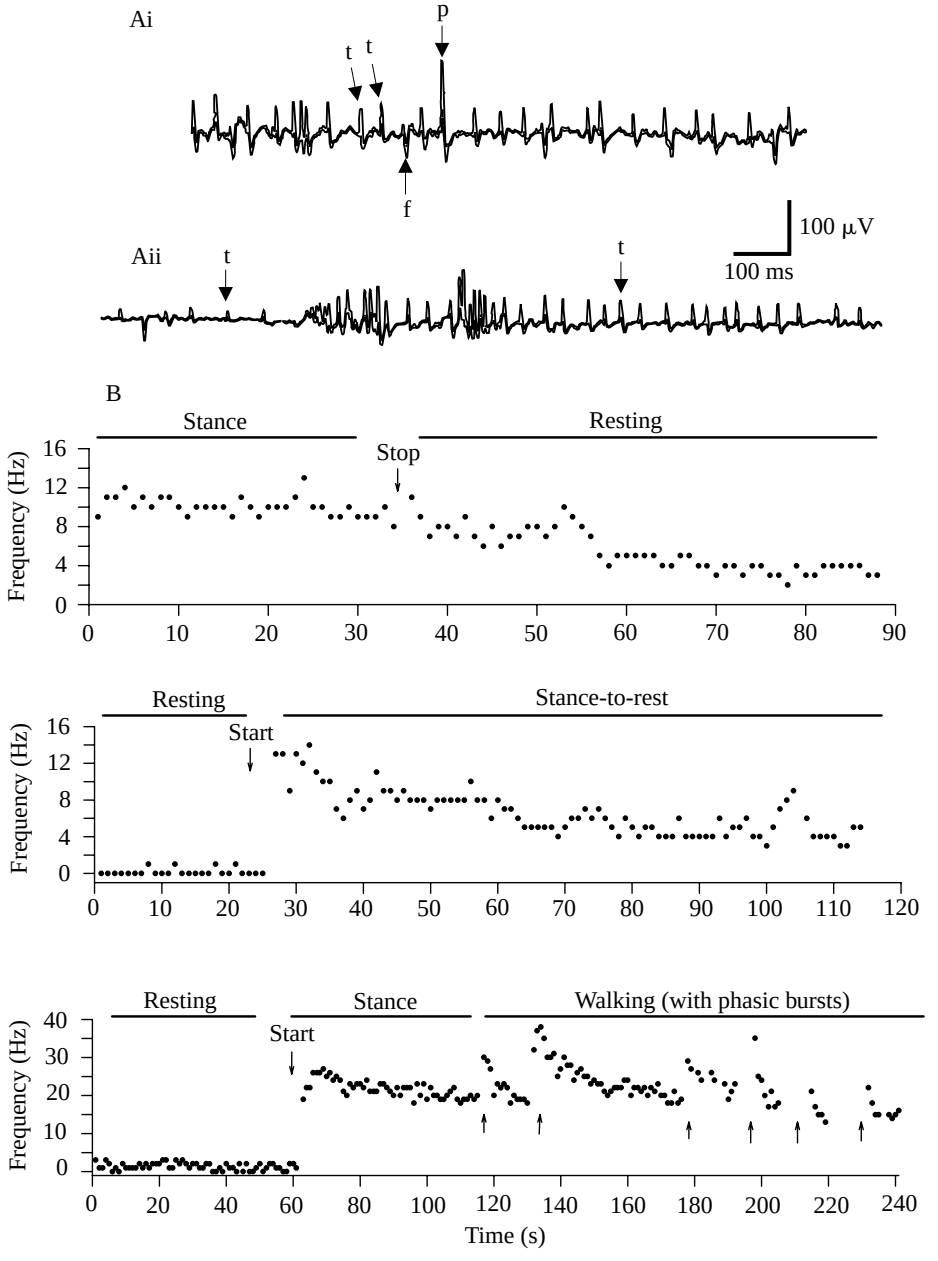

Fig. 6. (A) Myographic records from the superficial surface of the extensor muscle in a freely moving crayfish. (Ai) Maintained activity of the tonic axon (t), with one clear phasic impulse (p). Inverted myographic potentials (f) occur with movement of the antagonistic main flexor muscle. (Aii) Facilitating bursts of tonic potentials (t) during slow walking, with pronounced post-tetanic potentiation. (B) Frequency of tonic myographic potentials measured during several types of activity in one animal ('Stance', animal alert and standing but stationary; 'Resting', animal not using its legs to stand; 'Walking', slow forward locomotion). The phasic potentials (p) appeared in brief bursts during walking, usually at the start of an episode of increased tonic axon activity; their frequency was not measured because of interference from muscle action potentials.

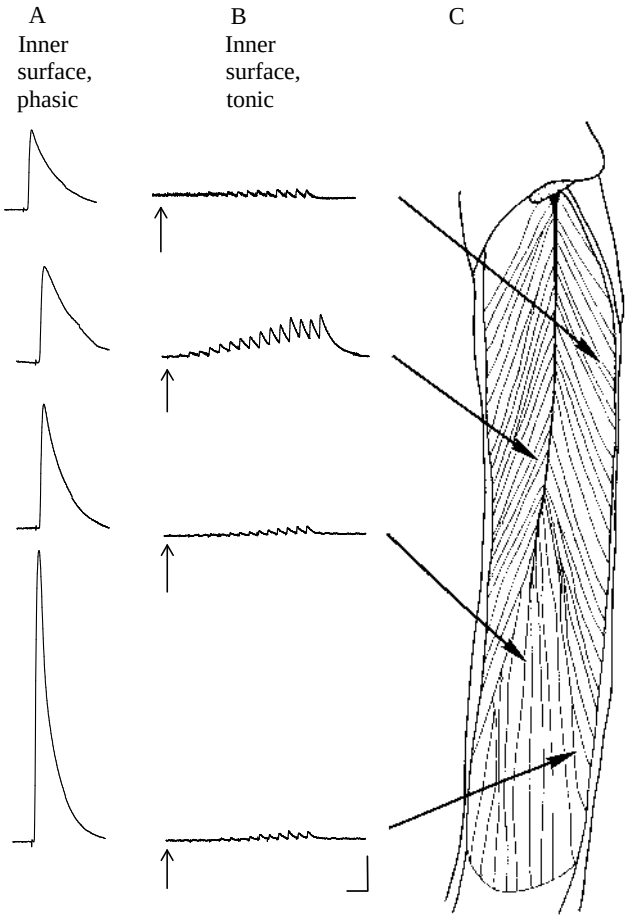

Fig. 7. Phasic and tonic excitatory postsynaptic potentials (EPSPs) recorded in representative individual fibres at various locations on the exposed inner surface of the muscle, showing the range of responses encountered. The distal end is at the top. (A) Phasic EPSPs generated by selective focal stimulation of the phasic axon. (B) Facilitation of tonic EPSPs with train durations of 300 ms and 50 Hz stimulation frequency. Note that the first responses in a train are usually too small to be readily seen or measured. (C) Drawing of the inner surface of the extensor muscle to show the locations at which the recordings were taken. Scale bars, time: A, 10 ms; B, 40 ms; voltage: A and B, 1 mV.

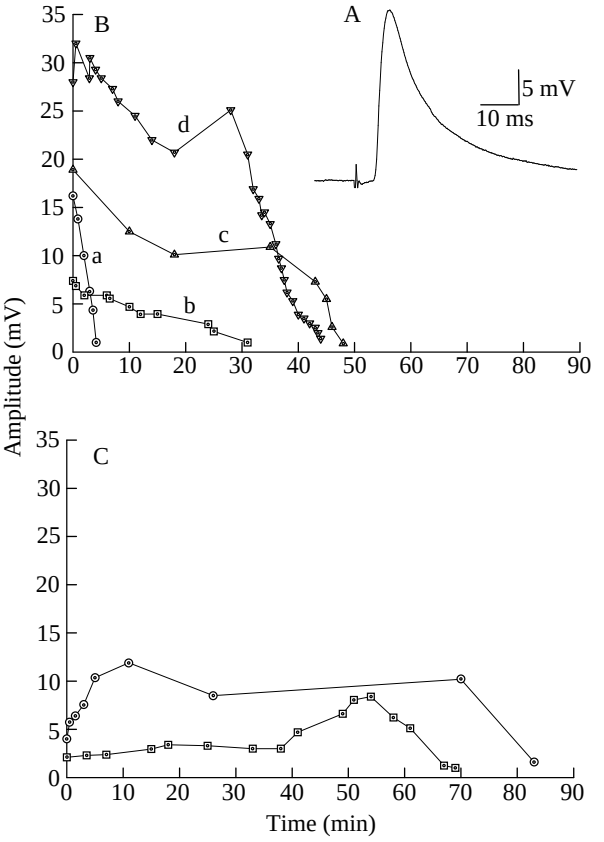

Fig. 8. Synaptic depression of the phasic motoneurone showing variable rates for different preparations. (A) A single excitatory postsynaptic potential (EPSP) evoked at 1 Hz before depression. Taken from the preparation that produced trace c in B. (B) Rates of synaptic depression measured in single fibres of four recently obtained animals of 5–6 cm in length (a, b, c and d). Depression was induced by selectively stimulating the phasic axon continuously at 5 Hz. Note the varied rates of depression in these preparations. (C) Rates of synaptic depression measured in two older animals that had been held for 3 months. The initial EPSP amplitudes and depression curves for these animals were markedly different from those of the recently obtained animals.

while the second axon fires rarely. Thus, one axon fires with a much more tonic pattern than the other, and the difference in total impulse production is extremely large, as shown in other cases (Pahapill *et al.* 1985; Atwood *et al.* 1991).

Excitatory postsynaptic potentials

Selective focal stimulation (or intracellular stimulation) of the two excitatory axons evoked characteristic EPSPs. The EPSPs of the phasic axon were relatively large and easily detected over the entire inner surface of the muscle in all fibres (Fig. 7). Typically, they were 5–30 mV in amplitude and showed moderate facilitation with successive closely spaced impulses. When the frequency was increased from 1 to 5 Hz, the potentials facilitated slightly, increasing to between 1.1 and 1.4 times their initial amplitude. The EPSP amplitudes sometimes showed a gradient within the muscle, with larger potentials in the more proximal (fast) fibres (Fig. 7), but this was not seen consistently in all preparations examined. With maintained stimulation at 5–10 Hz, EPSPs exhibited depression, declining slowly in amplitude over many minutes.

We observed that the rate of depression of phasic synaptic transmission was quite variable in different preparations (Fig. 8). The form of the depression curve appeared to depend in part upon the previous history of the animal. Freshly acquired specimens generally exhibited rapid depression and had large initial EPSPs (Fig. 8B). In contrast, specimens held for a long time (2 months or more) frequently had much smaller EPSPs and showed less rapid initial depression (Fig. 8C; Table 1). Although these differences were not investigated further in the present study, they are entirely consistent with adaptational effects which have been well studied in the crayfish closer muscle. The past history of the

animal is known to have a major effect on phasic EPSP properties (Lnenicka, 1991).

When the tonic axon was stimulated, its EPSPs were invariably very small, but facilitated dramatically with a train of stimuli delivered at a frequency of 10 Hz or greater (Fig. 7B). Very little could be seen at lower frequencies. Similar results were reported by Wiens (1985, 1993), who previously sampled this muscle. At frequencies of 10–40 Hz for 30 min, the EPSPs of the tonic axon showed no depression. Although all muscle fibres sampled showed very pronounced short-term facilitation during a train of impulses, there was considerable fibre-to-fibre variation within a muscle (Figs 7, 9), with deeper (outer) fibres usually producing larger EPSPs. These fibres are probably the small-diameter fibres evident in cross sections of the muscle (Fig. 1). We encountered no poorly facilitating EPSPs generated by 'high-output' terminals of the type invariably found for the tonic excitatory axons of the crayfish opener muscle (Iravani, 1965; Bittner, 1968; Atwood *et al.* 1994), crab opener and stretcher muscles (Atwood, 1965; Atwood and Bittner, 1971; Sherman, 1977) and the lobster accessory flexor muscle (Walrond *et al.* 1993). Since the leg extensor muscle contains many fibres, it remains possible that, in spite of extensive probing, we may have failed to find such poorly facilitating 'high-output' EPSPs.

For the exposed inner surface of the muscle, the extreme variability in EPSP amplitude observed for the phasic axon in different specimens was not encountered for EPSPs of the tonic axon (Table 1). Thus, the EPSPs of the tonic axon are less affected by the previous history of the animal.

The very small EPSPs observed for the tonic axon at low frequencies, or at the beginning of a train of impulses, pose a problem for muscle activity: how does the muscle develop tension when total depolarization produced by tonic EPSPs at 20–40 Hz is rather small in most fibres? As in other muscles, some fibres are likely to be recruited at lower frequencies than most (Fig. 9). This helps to explain frequency-dependent gradation of muscle contraction. Another factor, not well studied previously, concerns the potentiating effect of haemolymph. The tonic EPSPs of the extensor muscle summate more effectively when a solution containing haemolymph is added (Fig. 10). The effect is mainly attributable to a postsynaptic action, since the EPSP peak-to-peak amplitude does not increase much, but the rate of decay of the evoked depolarization becomes slower. The decay of the last EPSP in a train displayed rapid (t_1) and slow (t_2) components of temporal decay, both of which were prolonged in haemolymph-containing solution (Fig. 10). A similar effect was observed in crab muscles when a more haemolymph-like physiological solution was employed (Lang *et al.* 1979). These observations suggest that the muscle may perform differently in the animal than when isolated in an artificial solution. In particular, the observations suggest that the muscle is likely to be more effectively depolarized and to develop

Table 1. *EPSP amplitudes of phasic and tonic motoneurones sampled across the leg extensor muscle with a comparison of results for recently obtained animals and for animals held for longer than 3 months*

Preparation	Phasic amplitude (mV)	Tonic amplitude (mV)
Fresh animals		
1	14.1±3.2 (9)	0.90±0.95 (8)
2	18.5±4.8 (8)	0.77±0.87 (9)
3	20.9±8.5 (13)	0.38±0.35 (10)
Animals held for longer than 3 months		
1	7.9±2.02 (13)	0.56±0.22 (8)
2	6.2±1.3 (22)	0.74±0.39 (8)
3	4.02±1.3 (13)	0.53±0.59 (8)
4	1.7±0.4 (6)	0.42±0.27 (8)
5	1.3±0.3 (10)	0.34±0.20 (8)

Values are means ± S.D. (*N*).

Tonic amplitude (sixteenth pulse) is a measure of the peak amplitude of the last EPSP riding on the decay of the preceding EPSP after averaging three consecutive pulse trains of 300 ms duration and 50 Hz stimulation within the train.

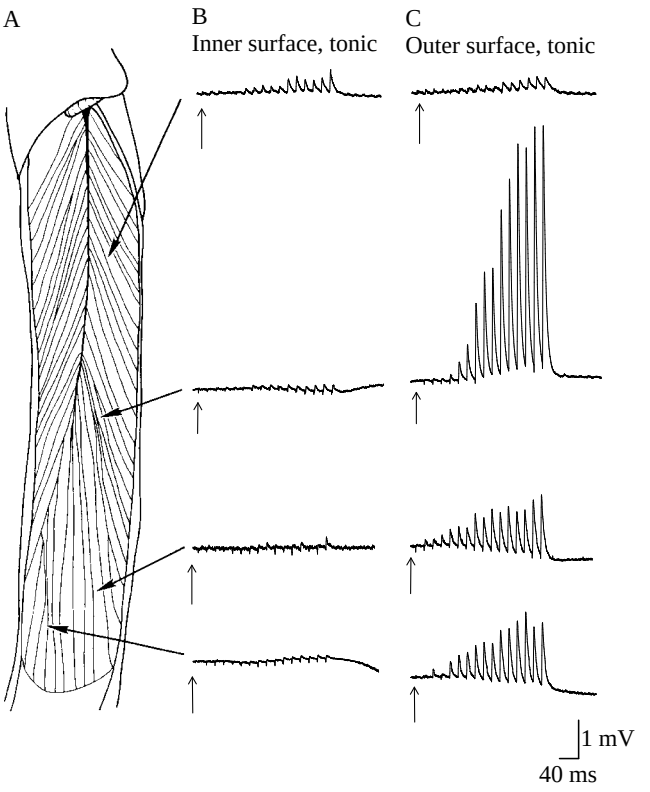

Fig. 9. Tonic excitatory postsynaptic potentials (EPSPs) recorded in individual fibres of the inner and outer surfaces of the muscle, showing the range of responses encountered and the generally larger amplitude of the EPSPs in outer fibres. All recordings are displayed at the same gain. (A) Drawing of the inner surface of the extensor muscle to show the locations at which the recordings were taken. The distal end is at the top. (B) Facilitation of tonic EPSPs recorded from the inner surface with train durations of 300 ms and 50 Hz stimulation frequency. (C) Recordings made under the same conditions as in B, but from the outer muscle fibres.

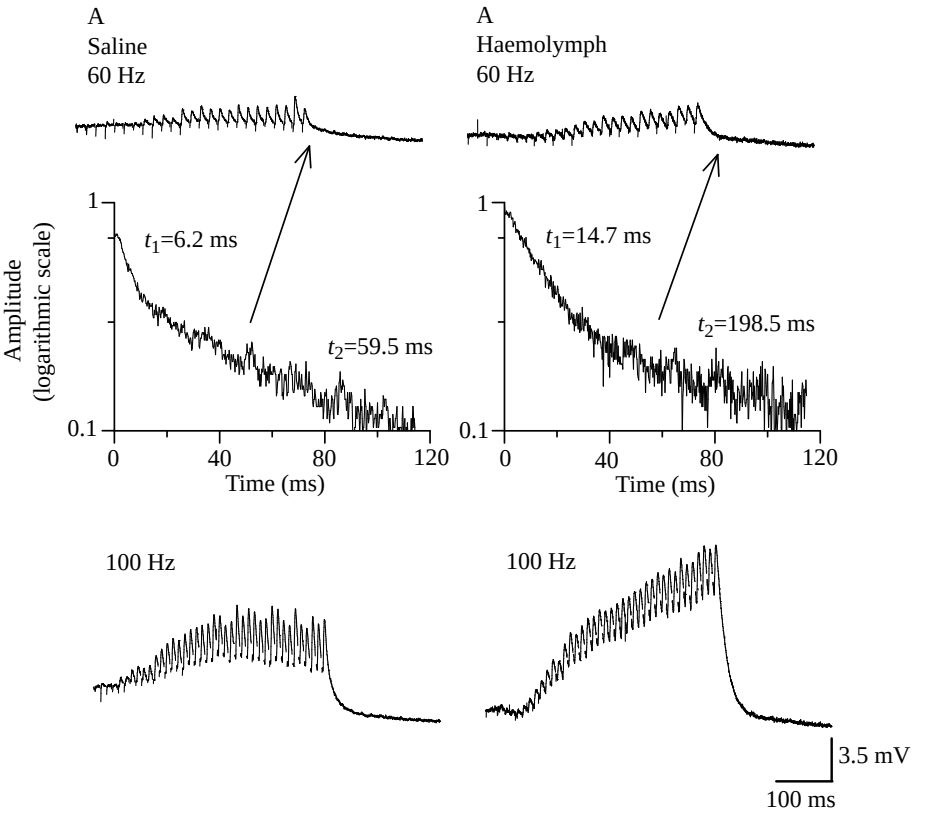

Fig. 10. Enhancement of the depolarization produced by tonic EPSPs of a fibre on the inner surface of the muscle by focal axonal stimulation in the presence of haemolymph-containing solution. Recordings in crayfish solution (A) and 50 % haemolymph-containing solution (B) are compared. The second panel is a magnified semi-logarithmic plot of the last EPSP in the 60 Hz train to illustrate that the decay of the rapid phase is longer in the presence of haemolymph. Curves fit best to double exponentials; the time constants (t_1, t_2) of the two exponential curves are indicated beside the traces.

tension more effectively at lower frequencies of activation in haemolymph. To ensure that physiological results obtained from isolated preparations can be related to the operation of the muscle in the intact animal, attention must be given to the factors in haemolymph that affect muscle performance, both pre- and postsynaptically (see Stewart *et al.* 1994).

Focal recording from nerve terminals

In preparations stained with 4-Di-2-Asp, recordings of transmission were obtained from individually visualized terminal structures with appropriately sized macro-patch electrodes. A very large difference in transmission was observed at low frequencies of stimulation at phasic and tonic terminals (Fig. 11). At tonic terminals (even at large proximal varicosities), many impulses evoked no release, even though the nerve terminal potential (ntp) was recorded, while others evoked currents indicative of one (or rarely two) quantal events of variable latency. Quantal contents were always less than 1 at low frequencies of stimulation (10 Hz or less). Averaged recordings typically showed a synaptic current smaller than the nerve terminal potential (Fig. 11), because of many included failures of transmission. At phasic terminals, in contrast, recorded events were always multi-quantal and included no failures. Quantal content could be assessed by measuring a number of individual quantal events (often seen as 'late releases', as in Fig. 11A) and taking the averaged result as the mean quantal unit size. The averaged evoked response was then divided by the quantal unit size to obtain an estimate of the mean quantal content (Cooper *et al.* 1995*b*). Such estimations (Fig. 11C,D) showed that individual patches of the 'phasic' terminal had quantal contents of 5–15 at low frequencies of stimulation. These values are many times greater than those recorded with the same electrode for the varicosities of the tonic axon. The ratio of quantal contents for the two types of terminal was generally 50–200 at 1 Hz. This is a much greater difference than that found between high- and low-output terminals of the crayfish opener motor axon (Wojtowicz *et al.* 1994; Cooper *et al.* 1995*a*). The result suggests a radical difference in regulation of synaptic transmission for the phasic and tonic terminals.

Discussion

The present study provides an overview of the physiology and motor terminal structure for the extensor muscle of the crayfish leg. This preparation is particularly favourable for investigating differences in synaptic transmission of identified phasic and tonic motoneurones. In each specimen, the two neurones can be compared side by side. Both axons, and their respective endings, are very accessible for stimulation, intra-axonal injections and focal recording. The terminals of the two axons, which are markedly different in morphology, can be investigated using fluorescence microscopy, focal recording electrodes and electron microscopy. The relatively large size of the muscle and its flat inner surface aid experimentation.

The overall phasic–tonic differences in normal activity, synaptic physiology, metabolic properties and terminal morphology are in general agreement with previous studies on

other crustacean muscles. The tonic axon of the extensor muscle is much more active than its phasic counterpart and contains more mitochondria, which probably have a higher average membrane potential. The implication from this and other studies is that a relatively high oxidative metabolism supports the vigorous ongoing activity of the tonic axon (Nguyen and Atwood, 1994).

Unlike other muscles with dual excitatory innervation, such as the crayfish claw closer muscle, the extensor muscle is supplied by a tonic axon that is larger in diameter than its phasic counterpart. The terminals of the tonic axon of the leg extensor, like those of other tonic axons, are prominent and varicose, while those of the phasic axon are relatively thin and filiform (Atwood and Wojtowicz, 1986). The axonal difference in mitochondrial content is also found in the terminals.

Despite its much more prominent terminals, the tonic axon has a strikingly lower transmitter output at low frequencies of nerve impulse activity. This was shown conclusively in the present experiments by focal recordings of quantal emission at visually identified varicosities of the terminals. In the leg extensor muscle, tonic EPSPs are vanishingly small at low frequencies, but facilitate strongly at high frequencies in all fibres sampled. Phasic EPSPs are large at low frequencies and facilitate slightly at higher frequencies, but much less strongly than the tonic EPSPs. With maintained stimulation, in this preparation as in others, phasic EPSPs undergo depression, though at variable rates in specimens from different animals, while tonic EPSPs do not show any depression when stimulated at 20–40 Hz for 30 min or longer (see Atwood, 1976; Atwood and Wojtowicz, 1986).

The large phasic–tonic difference in EPSP properties is due in large measure to distinctive properties of quantal emission at the nerve terminals. The probability of transmitter release at individual synapses must be much higher at low frequencies for the phasic axon since, for a small region of terminal sampled by a macro-patch electrode, the quantal content is many times greater, and yet the frequency of occurrence of synapses in

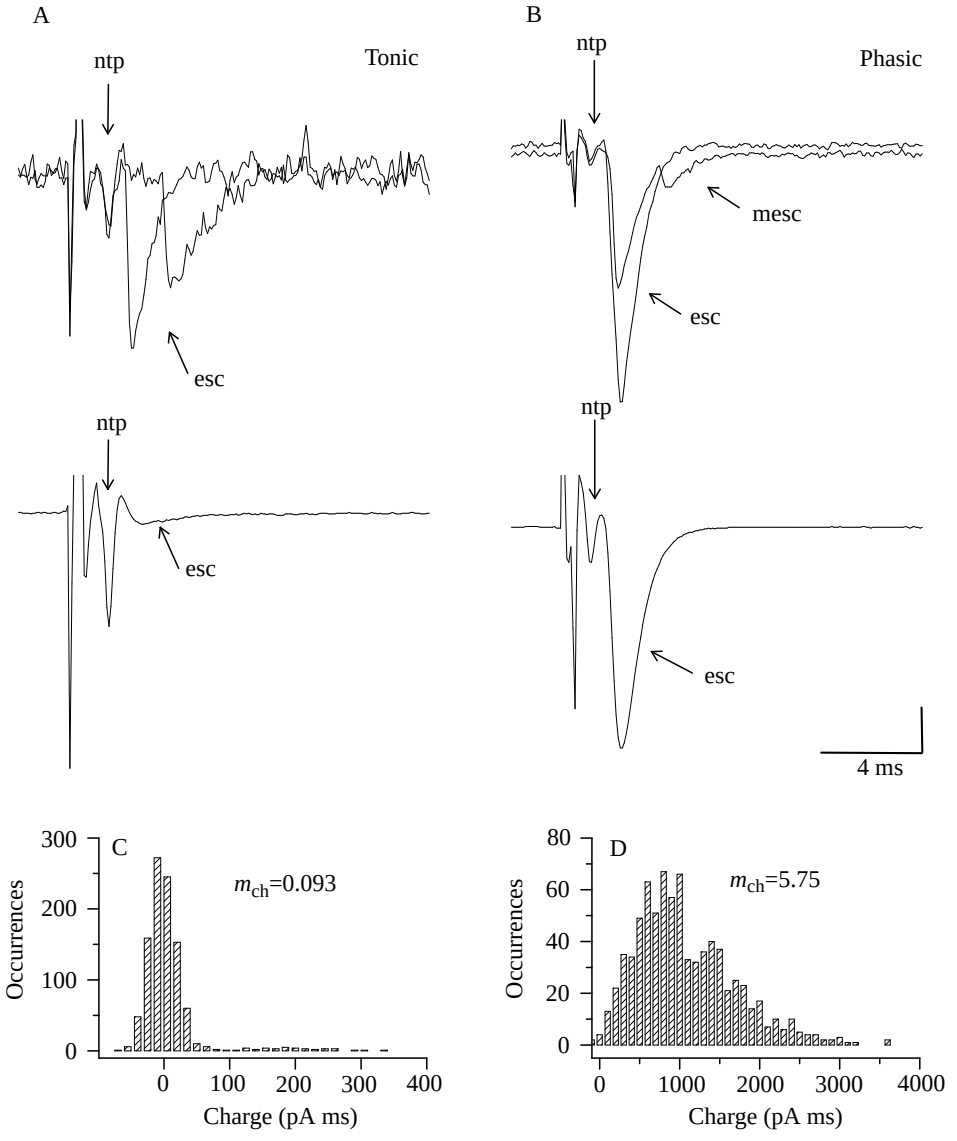

Fig. 11. (A,B) Focal recording of extracellular synaptic currents (esc) at visualized terminals of tonic (A, left) and phasic (B, right) endings. Top traces are superimposed single sweeps containing evoked responses; a spontaneous miniature extracellular synaptic current or quantal event (mesc) appears in the phasic record. Bottom traces are the averages of 1000 evoked events. Vertical arrows mark the nerve terminal potential (ntp); diagonal arrows mark evoked release. Scale bars: top panel, A, 54 pA; B, 200 pA; bottom panel, A and B, 80 pA. (C,D) Quantal events (integrated charge measurements) of paired tonic (C) and phasic (D) endings, recorded with the same macro-patch electrode, showing the typical difference in quantal content (m_{ch}, quantal content determined from charge measurements). In this example, the phasic:tonic ratio of quantal contents was 62. All recordings (A–D) were obtained while stimulating at 1 Hz.

electron micrographs is lower for the smaller phasic terminals. In a recently completed serial-section electron microscopy study, 2.6 synapses per μm terminal length were found on phasic terminals, and 3.6 synapses per μm on tonic terminals (King *et al.* 1996). The larger EPSP of the phasic axon is thus attributable to a higher probability of quantal release per synapse, together with a more extensive spreading of the phasic terminals on the surface of the muscle fibres. Since these different types of terminal morphology and transmission occur together on the same postsynaptic targets (muscle fibres), the differences cannot be due primarily to influences of the target on the nerve terminal (see Frank, 1973; Davis and Murphey, 1994), but must be due instead to differences arising in the motoneurones themselves. How these differences arise is a topic for further investigation, since several mechanisms are possible.

All muscle fibres sampled in the extensor muscle received both excitatory axons, despite evidence for differences in fibre type. A bundle of fast fibres in the proximal region stained more darkly with the myosin ATPase reaction and had shorter sarcomeres than in other regions of the muscle (confirming previous observations for this muscle in other crayfish species; Girardier *et al.* 1963; Law and Atwood, 1971). These fibres, like those elsewhere in the muscle, exhibited both phasic and tonic EPSPs. Throughout the muscle, tonic EPSPs were highly facilitating, though variable in amplitude. We did not locate any poorly facilitating tonic EPSPs, as found in many other crustacean limb muscles (Atwood and Bittner, 1971; Rathmayer and Erxleben, 1983). Thus, the extensor muscle differs from several other well-studied limb muscles in having very few, or possibly none, of the poorly facilitating 'high-output' terminals for the tonic axon and in having all fibre types within the muscle fully innervated by both excitatory axons.

A possible factor in the occurrence of predominantly small, highly facilitating EPSPs for the tonic axon is the degree and pattern of activity in this neurone. Myographic observations of its activity in walking animals have indicated a more continuous and higher average level of activity than for the motor axon of the opener muscle, another example of the 'tonic' type of neurone. The more frequent occurrence of muscle fibres exhibiting larger, less highly facilitating EPSPs in the opener muscle (Bittner, 1968) suggests that these may be functionally related to a more intermittent pattern of activity or to the production of significant muscle tension at low frequencies. Further quantitative comparisons of activity patterns in different muscles would be required to test this proposal. At the very least, the present observations indicate that not all 'tonic' axons to leg muscles follow the same pattern of intramuscular nerve terminal differentiation.

The present observations on the extensor muscle illustrate its similarities to, and differences from, other crustacean neuromuscular preparations and suggest that the problem of phasic–tonic synaptic differentiation can be further investigated advantageously in this preparation.

Electron micrographs were obtained through the assistance of Dr Leo Marin. Assistance in preparation of figures and manuscript was provided by Marianne Hegström-Wojtowicz, and assistance with muscle fibre sarcomere measurements by Carolins Hurtado. We thank Dr T. J. Wiens for reading a draft of this paper. This research was supported by grants from NSERC, Canada, and MRC, Canada.

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