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FIBER COMPOSITION AND INNERVATION PATTERNS OF THE LIMB CLOSER MUSCLE IN THE LOBSTER *HOMARUS AMERICANUS*

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Abstract

The closer muscle in the first walking leg of the lobster, *Homarus americanus*, is composed of mostly (78%) tonic fibers with low levels of myofibrillar adenosine triphosphatase (ATPase) activity. The remainder are phasic fibers, which have high ATPase activity and are restricted to a dorsal bundle. Based on histochemical demonstration of reduced nicotinamide dinucleotide (NADH) diaphorase, phasic fibers have a lower oxidative capacity than tonic fibers. The tonic fibers have mitochondria criss-crossing the entire cross-sectional profile or restricted to the periphery.

Fiber types based on myosin ATPase activity correlated closely with those based on resting sarcomere and A-band lengths on the dorsal surface of the closer muscle. Thus phasic fibers with high ATPase activity had short (<6 μm) sarcomeres and tonic fibers with low ATPase activity had long (>6 μm) sarcomeres. Innervation patterns of the dorsal fibers revealed all possible combinations between the two types of muscle fibers (phasic and tonic) and the two types of axons (fast and slow) except phasic fibers innervated only by slow axons. The distribution of fast and slow axons therefore serves to broaden the contractile performance of the closer muscle.

Introduction

The organization of the motor system in the thoracic appendages of decapod crustaceans is relatively simple (Wiersma, 1961; Atwood, 1973, 1976). Each of the three distal segments has two major antagonistic muscles innervated by relatively few (2–5) motoneurons. These few motoneurons bring about the full range of movements of the limbs. It is therefore not surprising to find them differentiated into fast and slow types. The neuromuscular synapses arising from each type of motoneuron vary in their transmitter release and facilitation effects on individual muscle fibers. Furthermore, muscle fibers are specialized into three broad types: phasic, tonic, and intermediate. Thus differentiation of motoneurons, neuromuscular synapses, and muscle fibers provides the basis for a wide range of contractile behaviour.

At one extreme of this range is the rapid twitch contraction produced by fast axons innervating phasic fibers, as in deep abdominal extensor and flexor muscles in crayfish (Kennedy and Takeda, 1965a, b). At the other extreme is the slow tonic contraction brought about by slow axons innervating tonic fibers, as in the crayfish superficial abdominal muscles. The closer muscle in the first two pairs of walking

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Abbreviations: FCE, fast closer excitor motoneuron; SCE, slow closer excitor motoneuron; ATPase, adenosine triphosphatase; NADH, nicotinamide adenine dinucleotide; ejp, excitatory junctional potential.

legs of the lobster (*Homarus americanus*) produces each type of contraction upon stimulation of its fast closer excitor (FCE) and slow closer excitor (SCE) motoneurons respectively (Wiersma, 1955). However, we do not know whether the different contractions can be accounted for by phasic and tonic fibers. Nor do we know the extent to which the distribution of innervation by each of the axons regulates contractile behaviour. The present study addresses these questions.

Materials and Methods

Lobsters (*Homarus americanus*) weighing 300–500 g were used throughout this study. The larger animals were purchased locally in Toronto and kept in artificial sea water at 10–11°C. The smaller animals were captured in the waters around Woods Hole and kept in running sea water at ambient temperatures. The closer muscle in the first and second walking legs were examined with the techniques described below.

Histochemistry

We followed the methods described by Ogonowski and Lang (1979) for detection of myofibrillar adenosine triphosphatase (ATPase) and reduced nicotinamide adenine dinucleotide (NADH) diaphorase in lobster muscle. Briefly, an intact propus of a walking leg was frozen and serially cross-sectioned (15 μm thick). The sections were placed on glass cover slips and stained by modifications of the methods of Padykula and Herman (1955) for myofibrillar ATPase and of Nachlas *et al.* (1958) for NADH diaphorase. The stained sections were mounted on glass slides for subsequent examination and photography. The muscles in propuses of the first walking legs from three separate lobsters were assayed histochemically.

The average number of fibers in the closer muscle was estimated by counting the number of fast and slow fibers (based on intensity of staining for ATPase) in three cross-sections from each of the proximal, central, and distal regions of the muscle. The three regions were far enough apart so that fibers did not overlap.

Light microscopy

Muscle fiber types were also determined by measuring the average resting length of the sarcomeres and A-bands in the myofibrils by the following technique. The dorsal surface of a closer muscle was exposed by removing the overlying opener muscle and the muscle was held at rest length, by appropriate manipulation of its dactyl, while being fixed in alcoholic Bouins fluid for 24 h. Muscle fibers were teased into fine strands in 70% alcohol on glass slides and viewed at 600 magnification under a light microscope. Five consecutive sarcomeres and three A-bands were measured with an ocular micrometer to obtain an average value for each fiber (Govind *et al.*, 1977). A total of 75 fibers were sampled in two separate closer muscles.

Electrophysiology

The innervation of muscle fibers by FCE and SCE axons was determined using conventional electrophysiological techniques. Recording from fibers of the closer muscle was done in lobster physiological saline of the following composition: NaCl, 472 m*M*; KCl, 10 m*M*; $MgCl \cdot 6H_2O$, 5 m*M*; CaCl, 16 m*M*; glucose, 11 m*M*; Tris maleate, 10 m*M*; pH 7.4. The saline was continuously perfused over the

preparation at a rate of about 200 ml/h and kept at 12–15°C (Meiss and Govind, 1979).

The dorsal aspect of the closer muscle was exposed (Fig. 1) by removing the antagonistic opener muscle and the connective tissue separating the two muscles. This also exposes the major branches of the closer nerve on the muscle surface. The main closer nerve was stimulated with a pair of fine platinum wire electrodes, near the point where it divides in the carpus. The resulting action potentials were monitored with an extracellular suction electrode on a primary branch of the closer nerve. At the same time, excitatory junctional potentials (ejp's) were recorded from individual muscle fibers by intracellular glass microelectrodes filled with 2 *M* KAc solution and having resistances of 5–15 MΩ. Brief square wave pulses of 0.1–0.15 msec were applied to the stimulating electrodes. Under these conditions the preparations were viable for at least 4–8 h. A total of 15 preparations were made from 12 separate lobsters.

Results

Fiber composition based on enzyme histochemistry

Two distinct types of fibers were defined on the basis of different staining intensities for myofibrillar ATPase in cross-sections of the closer muscle (Fig. 2A–D). The intensely staining fibers demonstrated high activity for this enzyme, which is characteristic of phasic muscle (Hajek *et al.*, 1973; Lehman and Szent-Györgi, 1975). Conversely, fibers staining less intensely denoted low activity for myofibrillar ATPase characteristic of tonic muscle. Thus the closer muscle in the first walking leg of lobster is composed of phasic and tonic fibers. The proportions of phasic and tonic fibers were estimated to be 22% and 78% respectively.

Phasic and tonic fibers also have a regional distribution in the closer muscle. The phasic fibers originate as a dorsal bundle on either side of the tendon in the distal part of the pinnate muscle (Fig. 2C, D) and insert on the exoskeleton in the proximal part (Fig. 2A, B).

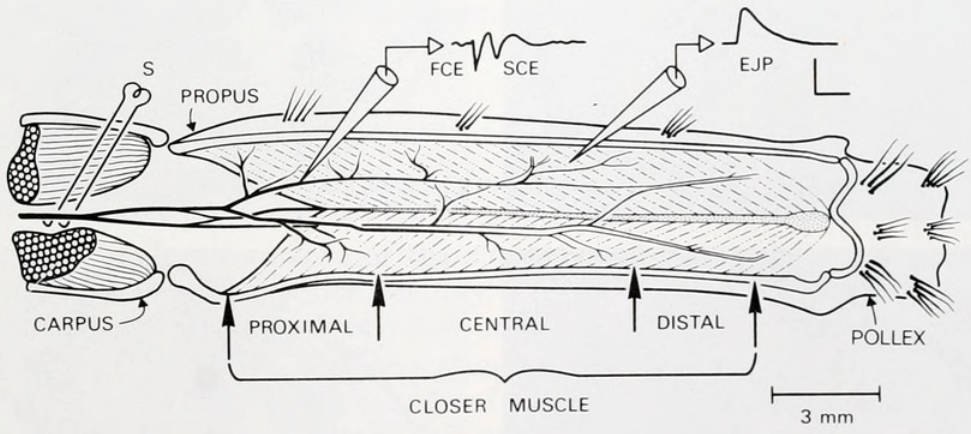
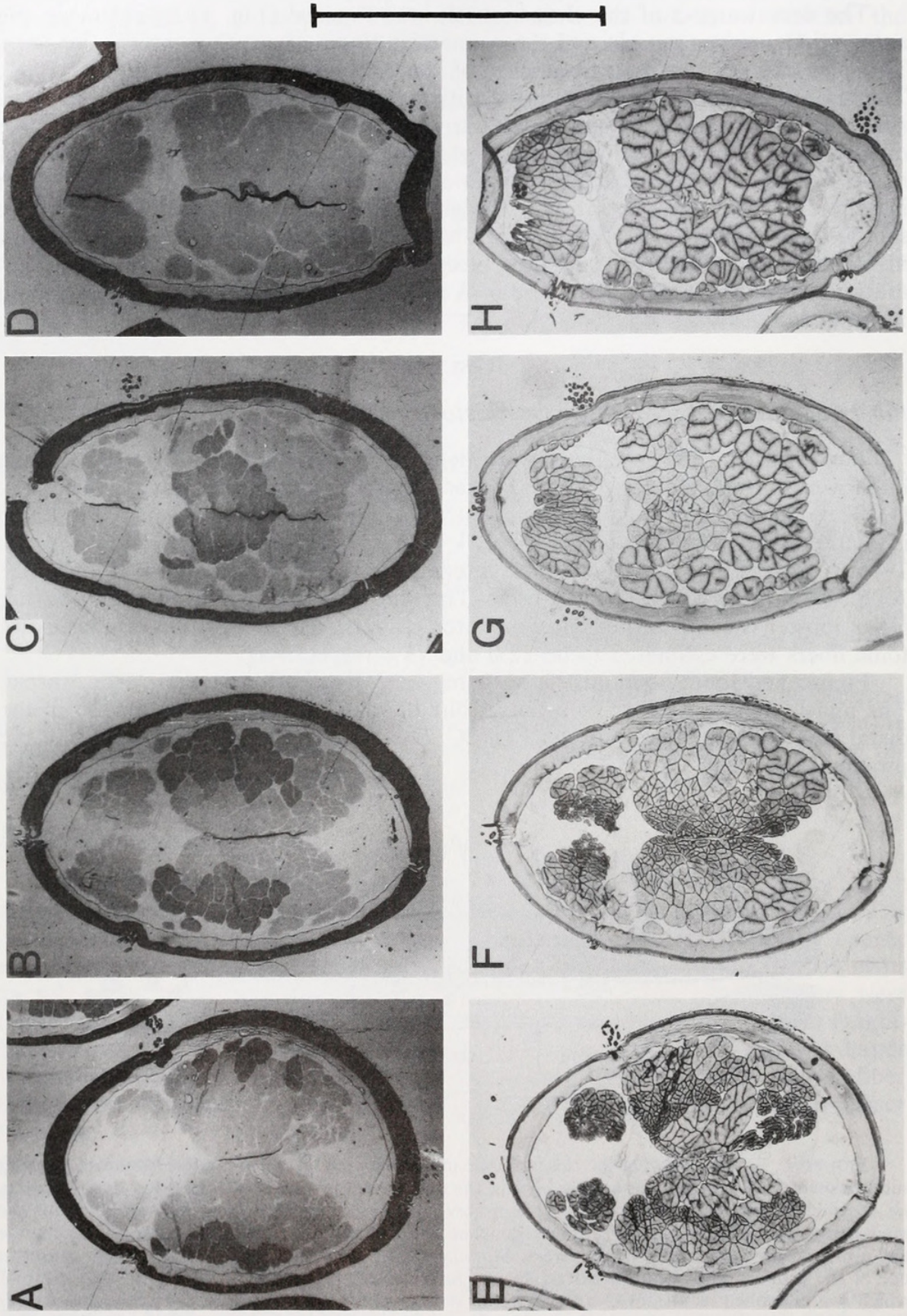


FIGURE 1. Dorsal aspect of the closer muscle in the first walking leg of lobster, *Homarus*, showing subdivision of the fibers into those at the ends of the muscle, *i.e.* proximal and distal, and those between, *i.e.* central. The branching pattern of the closer nerve as revealed by methylene blue staining shows the main nerve in the carpus dividing into two branches which ramify over the dorsal surface of the muscle and also send branches to the ventral areas. Stimulation (S) of the main nerve in the carpus evokes in one of the primary branches two extracellularly recorded action potentials, the faster conducting of which was identified as the FCE axon and the slower conducting as the SCE axon. Simultaneous intracellular recording from one of the central fibers gave an EJP in response to FCE stimulation but not to SCE stimulation. Calibration bar; vertical 5 mV; horizontal 30 msec.


D
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Alternate cross-sections of the propodite were stained for NADH diaphorase to measure oxidative capacity (Fig. 2E–H). The diformazan granules formed denoted the density and location of mitochondria in the muscle fibers. In general, phasic fibers had a lower oxidative capacity than tonic fibers. Thus in the proximal and central parts of the closer muscle (Fig. 2E–G) fibers with the weakest staining reaction for NADH diaphorase have the same location as those staining intensely for ATPase (Fig. 2A–C). These are phasic fibers. Fibers staining more heavily for NADH diaphorase in the distal part (Fig. 2H) are the same ones that stain weakly for ATPase. These are tonic fibers. The tonic fibers also have more pronounced staining of their boundaries than the phasic fibers (Fig. 2G), denoting greater concentration of subsarcolemmal mitochondria. Thus in the closer muscle there appears to be good correlation between fiber type based on ATPase activity and the oxidative capacity of the fiber, *i.e.*, phasic fibers have a low oxidative capacity and tonic fibers a high oxidative capacity.

Among the tonic fibers the staining reaction for NADH diaphorase shows one of two patterns (Fig. 3). In some fibers the staining is restricted principally to the periphery of the fiber while in others it also criss-crosses the fiber. The proximal region of the closer muscle (Fig. 2E, F) has both types of tonic fibers whereas the distal area (Fig. 2G, H) has fibers with the mitochondria distributed along their boundaries. Phasic fibers also have their mitochondria restricted largely to the periphery (Fig. 3), where they appear in lower concentration than those in tonic fibers.

Since cross-sections of the entire propodite were taken, the antagonistic opener muscle was also treated histochemically, along with the closer muscle (Fig. 2). Fibers of this muscle stained lightly for myofibrillar ATPase, indicating that low activity is characteristic of tonic muscle (Fig. 2A–D). Alternate sections stained for NADH diaphorase (Fig. 2E–H) indicated relatively high oxidative capacity with some regional variation: Fibers in the proximal part stained more intensely (Fig. 2E, F) than those in the distal part (Fig. 2G, H).

In summary, based on myofibrillar ATPase activity, the closer muscle in the lobster walking leg appears to be composed of a minority of phasic fibers, restricted to a dorsal bundle, and a majority of tonic fibers. The phasic fibers have a lower oxidative capacity than the tonic fibers, which themselves have variable oxidative capacities.

Fiber composition based on sarcomere length

Fibers on the dorsal surface of the closer muscle were characterized by their resting sarcomere lengths, which reliably indicate contractile speed: Phasic fibers have relatively short sarcomeres (2–4 μm) and tonic fibers longer sarcomeres (>6 μm) (Atwood, 1973, 1976). The resting sarcomere length can vary considerably depending on the stretch or contraction of the fiber when fixed. Consequently, the length of the A-band was measured as a precaution. Though changes in the length

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FIGURE 2. Representative cross sections of the propus from the first walking leg of lobster, *Homarus*, stained for myofibrillar ATPase (A–D) and NADH diaphorase (E–H). The opener muscle is located dorsally and is small compared to the closer muscle occupying most of the propus. Adjacent sections were stained for ATPase and NADH diaphorase and these are shown from the proximal (A, E), central (B, C, F, G), and distal (D, H,) regions. Scale mark, 5 mm.

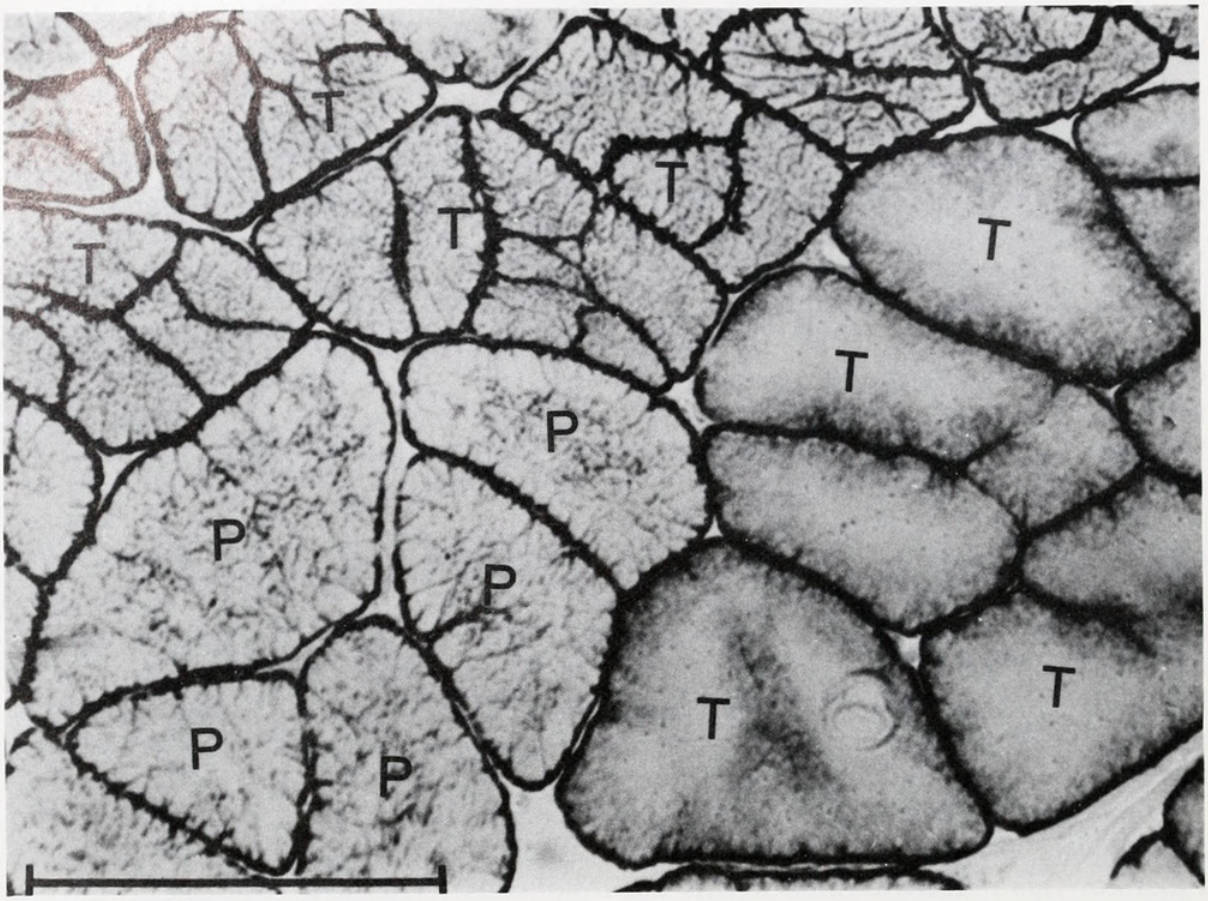

FIGURE 3. Enlarged view of part of Figure 2F showing cross-section of closer muscle fibers stained for NADH diaphorase. Staining is restricted to the circumference in all phasic (P) and some tonic (T) fibers; in other tonic (T) fibers the staining subdivides the fiber extensively, denoting a more widespread distribution of mitochondria. Scale mark, 0.5 mm.

of the A-band have been reported during contraction (Dewey *et al.*, 1977) they are so minute as to escape detection by conventional measuring techniques.

Whereas the determination of fiber types based on enzyme histochemistry dealt with the entire closer muscle, that based on sarcomere length was restricted to the dorsal surface (Fig. 1). For determining sarcomere length, fibers were taken from the two ends of the muscle, *i.e.*, proximal and distal areas, and from the center, thus avoiding overlap in sampling among the three regions. A total of 25 fibers were examined from each area. Two distinct types of fibers were found, and these occurred regionally (Fig. 4). Fibers with short sarcomeres and A-bands characteristic of phasic type occurred in the central region, while those with long sarcomeres and A-bands characteristic of tonic type were at the two ends of the muscle (Fig. 4).

Innervation by FCE and SCE axons

Innervation received by muscle fibers from the two excitor axons, FCE and SCE, on the dorsal surface of the closer muscle was determined by electrophysiological techniques. Since the FCE axon has a higher conduction velocity than the SCE axon (Wiersma, 1955; Hoyle and Wiersma, 1958; Govind and Lang, 1974), its action potential preceded that of the SCE axon in extracellular recordings from the closer nerve (Fig. 1). Recordings of muscle excitatory junctional potentials

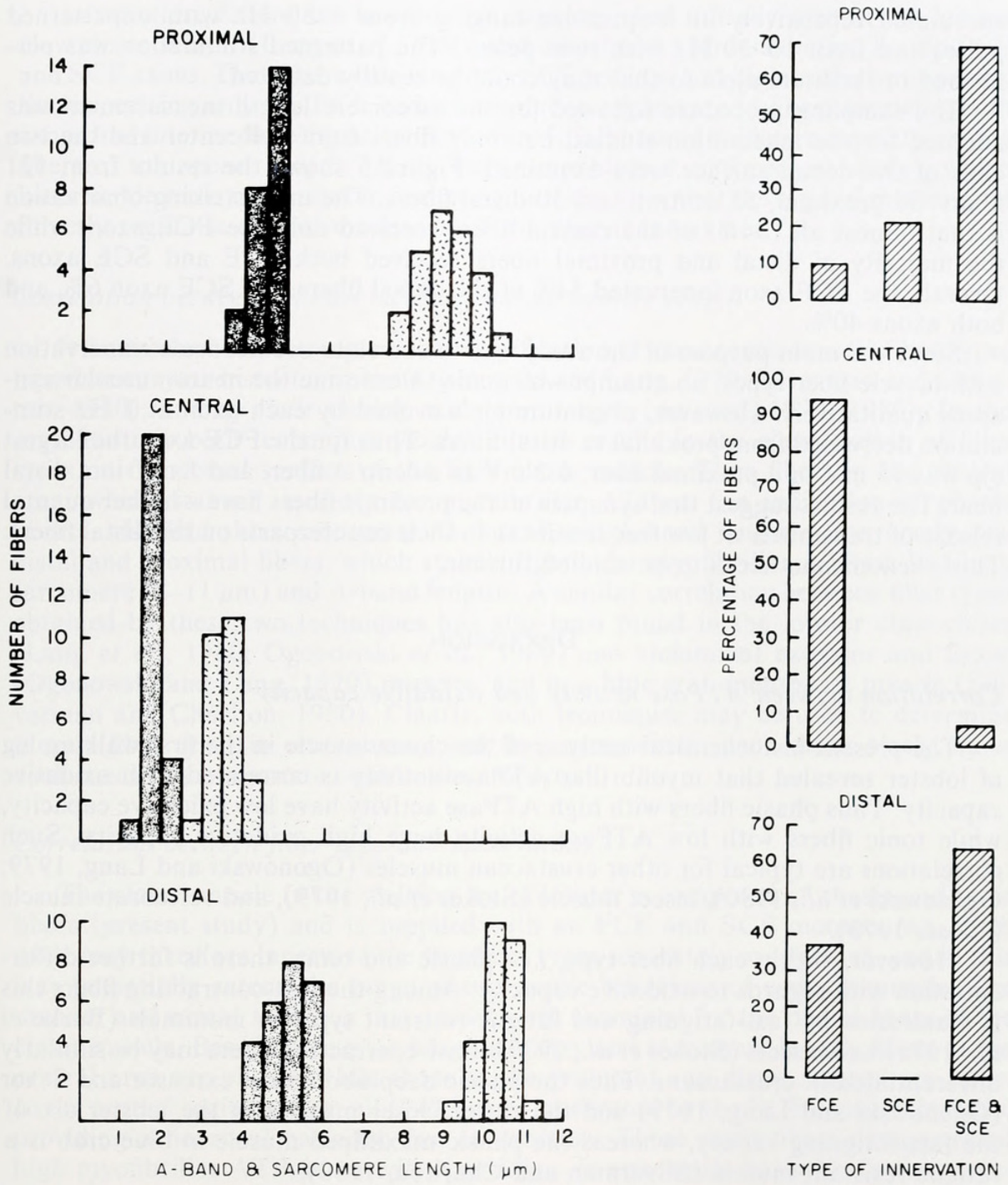

FIGURE 4. (Left) Distribution of fiber types based on A-band (darker stipple) and sarcomere length (light stipple) of proximal, central, and distal fibers from the dorsal surface of the closer muscle in first walking leg of lobster, *Homarus*.

FIGURE 5. (Right) Histogram of percent innervation by FCE, SCE, and both FCE and SCE axons in proximal, central, and distal fibers from the dorsal surface of the closer muscle in the first two pairs of walking legs of lobster, *Homarus*.

(ejp's) corresponding to each action potential confirmed that they were excitor axons. Selective stimulation of FCE and SCE axons was done in one of two ways. Either the two axons were physically separated in the carpus or each was recruited at a lower voltage by appropriate placement of the stimulating electrode. In order to determine whether a muscle fiber received endings from an axon, the axon was

stimulated repetitively at frequencies ranging from 1–50 Hz with unpatterned pulses and from 10–30 Hz with twin pulses. The patterned stimulation was performed to facilitate ejp's so that they could be readily detected.

The sampling procedure followed for the sarcomere length measurement was adopted for the innervation studies, *i.e.*, only fibers from the center and the two ends of the dorsal surface were examined. Figure 5 shows the results from 121 fibers: 38 proximal, 53 central, and 30 distal fibers. The most striking observation is that almost all (94%) of the central fibers received only the FCE axon, while the majority of distal and proximal fibers received both FCE and SCE axons. Overall, the FCE axon innervated 54% of the dorsal fibers, the SCE axon 6%, and both axons 40%.

Since the main purpose of the study was to correlate occurrence of innervation with muscle fiber types, no attempt was made to examine the neuromuscular synapses qualitatively. However, maximum ejp's evoked by each axon at 1 Hz stimulation decreased from proximal to distal fibers. Thus for the FCE axon the largest ejp was 15 mV in a proximal fiber, 6.2 mV in a central fiber, and 1 mV in a distal fiber. The results suggest that synapses on the proximal fibers have a higher quantal release of transmitter at low frequencies than their counterparts on the distal fibers. This phenomenon needs to be studied further.

Discussion

Correlation between ATPase activity and oxidative capacity

The present histochemical analysis of the closer muscle in the first walking leg of lobster revealed that myofibrillar ATPase activity is correlated with oxidative capacity. Thus phasic fibers with high ATPase activity have low oxidative capacity, while tonic fibers with low ATPase activity have high oxidative capacity. Such correlations are typical for other crustacean muscles (Ogonowski and Lang, 1979; Ogonowski *et al.*, 1980), insect muscle (Stokes *et al.*, 1979), and vertebrate muscle (Close, 1973).

However, within each fiber type, *i.e.*, phasic and tonic, there is further differentiation with regards to oxidative capacity. Among the fast-contracting fibers this is manifested by fast-fatiguing and fatigue-resistant types in mammals (Burke *et al.*, 1971) and insects (Stokes *et al.*, 1979). Fast-contracting fibers may be similarly differentiated in crustaceans. Thus the phasic deep-abdominal extensor and flexor (Ogonowski and Lang, 1979) and the cutter closer muscles in the lobster are of the fast-fatiguing variety, whereas the phasic maxilliped muscle in blue crab is a fatigue-resistant muscle (Silverman and Charlton, 1980).

Among slow-contracting fibers, which are characteristically fatigue-resistant in vertebrates, a fast-fatiguing variety has been found in the mesocoxal muscles of cockroaches (Stokes *et al.*, 1979). Our studies on lobster muscle have revealed at least two types of tonic fibers based on enzyme histochemistry. The most convincing demonstration of such differentiation is found among muscles composed entirely of tonic fibers, such as the claw opener muscle and the closer muscle of the crusher claw (K. S. Kent, Boston Univ., unpublished). In these muscles a few fibers have much lower myofibrillar ATPase activity and much higher oxidative capacity than the other tonic fibers. The present study on the closer muscle in the first walking leg of lobster revealed at least two types of tonic fibers, based on their distribution of mitochondria.

In addition, differences in oxidative capacity were initially seen in the lobster claw closer muscle (Lang *et al.*, 1980) and correlated with innervation by the FCE and SCE axons. Thus fibers receiving only SCE endings had high oxidative capacity, those receiving only the FCE had low oxidative capacity, and fibers receiving both axons had intermediate capacity. However, differences in oxidative capacity among fibers of the opener muscle (K. S. Kent, unpublished; and present study), which has a single tonic motoneuron, argues against the view that the type of motoneuron directly regulates oxidative capacity of the fiber it innervates.

Correlation between ATPase activity and sarcomere length

It is worthwhile to compare the classification of crustacean muscle fiber types based on enzyme histochemistry (Ogonowski and Lang, 1979; Silverman and Charlton, 1980), and by their resting sarcomere length (Atwood, 1973, 1976). In the present study both techniques yielded similar results in the classification of fiber types on the dorsal surface of the limb closer muscle. Thus fibers in the central area, which stained intensely for myofibrillar ATPase (an indication of phasic fibers), also had short sarcomere (2–4 μm) and A-band lengths. Conversely, the distal and proximal fibers, which stained lightly for myofibrillar ATPase had long sarcomere (8–11 μm) and A-band lengths. A similar correlation between fiber types obtained by these two techniques has also been found in the lobster claw closer (Lang *et al.*, 1980; Ogonowski *et al.*, 1980) and abdominal extensor and flexor (Ogonowski and Lang, 1979) muscles, and in a blue crab mouthpart muscle (Silverman and Charlton, 1980). Clearly, both techniques may be used to determine muscle fiber types in crustaceans; and they complement each other nicely, since each measures a separate contractile property.

Correlation between fiber type and innervation

The closer muscle in the walking leg of lobster is composed of phasic and tonic fibers (present study) and is supplied with an FCE and SCE motoneuron. Each axon may therefore innervate the two fiber types separately or together, and in this way potentially extend the range of contractile behaviour of a single muscle. The manner and extent to which this occurs were examined for the dorsal fibers of the closer muscle. Based on enzyme histochemistry and sarcomere length, fibers of the central area are phasic, while those of the proximal and distal regions are tonic. In the central region almost all (94%) were innervated by the FCE axon exclusively, providing a neuronal basis for fast contraction. These same fibers demonstrated high myofibrillar ATPase activity, providing an enzymatic basis for fast contractions (Lehman and Szent-Gyorgi, 1975). Furthermore, the short sarcomeres characteristic of these central fibers provide a structural basis for fast contractions, as the speed of shortening is directly related to the number of sarcomeres in series per unit length of the fiber (Huxley and Niedergerke, 1955; Jahromi and Atwood, 1969). Thus, in terms of their innervation and fiber characteristics, the central fibers would constitute a phasic unit within the closer muscle. Whether the location of this unit within the closer muscle is mechanically especially favorable for producing fast contractions remains to be determined.

In other areas of the closer muscle the two types of motoneurons (FCE and SCE) innervate the two types of muscle fibers (phasic and tonic) in all possible combinations except the one where phasic fibers are innervated by SCE only. In

functional terms, therefore, the closer muscle has five different contractile units, all serving to enhance its behavioural repertoire.

In the present study phasic fibers did not receive the SCE axon exclusively. Nor was this combination found in the homologous closer muscle in the cutter claw of lobster (Lang *et al.*, 1980). Since this is the only combination of neuron and muscle fiber type not found in the closer muscle, it may be important in revealing how motoneurons select their target muscle fibers or how muscle fibers regulate their innervation.

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