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Characterization of Muscles Associated With the Articular Membrane in the Dorsal Surface of the Crayfish Abdomen

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ABSTRACT The anatomy, physiology, and biochemistry of the dorsal membrane muscle (DMA) and the superficial extensor muscle accessory head (SEAcc) in the abdomen of the crayfish, *Procambarus clarkii* and lobster, *Homarus americanus*, are reported. These muscles have not been previously characterized physiologically or biochemically. The anatomy was originally described by Pilgrim and Wiersma (1963. J Morph 113:453–587). The arrangement of these muscles varies depending on the abdominal segment. The function of the dorsal membrane muscle is to retract the thin articulating membrane joining the cuticular segments so that the dorsal membrane does not evert during extension of the abdomen. Consequently, the articular membrane does not protrude, and thus potential damage to the membrane is minimized. Examination of nerve terminal morphology revealed strings of varicosities, usually only associated with tonic terminals. The electrophysiological data indicate that there are at least four tonic excitatory and one inhibitory motor neuron innervating these muscles. Facilitation indices and fatigue-resistance indicate physiologically the tonic nature of innervation. Anti-GABA antibodies demonstrate the anatomical presence of an inhibitor motor neuron. The SDS electrophoretic analysis of myofibrillar proteins and Western blots of key protein isoforms for these muscles in crayfish and lobsters also indicate that the DMA and SEAcc muscles are tonic phenotype. *J. Exp. Zool. 287:353–377, 2000.*

The neuromuscular junctions of the crayfish have been repeatedly shown to be an excellent system for investigating the physiology of chemical synaptic transmission and have served as model systems in our general understanding of vesicular (i.e., quantal) transmitter release (Fatt and Katz, '53; Dudel and Kuffler, '60, '61a,b; Atwood, '62; Dudel, '65). Because of its simplicity, with one to a few motor neurons innervating entire muscles, the properties of individual, identifiable motor neurons can be ascertained and compared for their anatomical, biochemical and physiological nature. The suitability of the crayfish not only holds for motor neurons but also for examining muscle fiber properties as well as the intact whole motor unit (nerve and muscle). The majority of the abdominal musculature and a substantial amount of the musculature in the limbs have been examined and their phenotypes characterized (Huxley, 1880; Wiersma, '33; Pilgrim and Wiersma, '63; Atwood, '67, '72, '73, '76, '77, '82; Silverman and Charlton, '80; Govind and Atwood, '82). Thus, most of the musculature of crayfish has been described and characterized. The task remains to continue describing and understanding the muscles and motor neurons that have not been examined and to determine their functional role.

Crustacean skeletal muscle fibers, like those of vertebrates, can be biochemically defined based on a muscle's activity profile and contraction velocity (Ogonowski and Lang, '79; Mellon, '91; Günzel et al., '93; see Mykles, '97, for review). Phasic muscles have greater amounts of enzymes associated with glycolytic pathways, whereas tonic muscles contain greater amounts of enzymes associated with aerobic oxidative metabolism. Such differences are readily revealed by examining staining patterns after the precipitation of cobalt sulfide in cross-sections of muscles, patterns that reflect the enzymatic activity of their ATPases

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(Günzel et al., '93). However, more subtle and distinct differences among fiber types are provided by examining myofibrillar protein isoforms by SDS gel electrophoresis (Costello and Govind, '96; Mykles '85a,b, '88; Neil, et al., '93; Quigley and Mellon, '84; Sakurai et al., '96). Fast fibers contain a 75-kDa regulatory protein (P75) absent from slow fibers (Mykles 1985a,b, '88; Neil et al., '93). Slow-tonic (S_2) fibers contain a 55-kDa isoform of troponin-T (TnT_1) not present in slow-twitch (S_1) fibers (Mykles, '85a,b, '88; Ismail and Mykles, '92; Neil et al., '93; Galler and Neil, '94).

The information gathered on various neuromuscular junctions of the crayfish reveals that there are consistent morphological and physiological characteristics for particular motor neurons. For example, phasic motor neurons have terminals that are very thin and traverse much of the fiber they innervate. The tonic motor neurons are quite different in their terminal morphology, since they contain swellings (i.e., varicosities) intermittently along their terminals. In some leg musculature, like the leg extensor muscle, these swellings have been found to be as large as 12 μm in diameter for an adult animal (Atwood and Cooper, '96b). The terminology of "phasic" and "tonic" arose from their physiological measured and behavioral properties. As in vertebrates, phasic musculature is used for rapid, ballistic movements and if the muscle is repetitively stimulated in a short period of time it will undergo fatigue; the tonic muscle, however, is used for slow gradual movements or to maintain a body position. Tonic muscles are more fatigue resistant with repetitive stimulation.

The general comparison to the vertebrates holds for the most part when describing crustacean muscles; however, there are some differences. In crustaceans, some fibers within a muscle are innervated only by tonic motor neurons (i.e., leg opener) or only by phasic motor neurons (DEL_1, DEL_2, and DEM of the abdomen), but in other muscles (i.e., the leg extensor and closer) single fibers are innervated by both phasic and tonic motor neurons. In addition, these muscles receive direct inhibitory motor innervation. The excitatory motor neurons use glutamate as their transmitter and the inhibitory ones use GABA. The single muscle fibers that are dually innervated by tonic and phasic motor neurons do not allow one to call the motor unit fast twitch or slow-twitch (S_1) and/or slow-tonic (S_2). Interestingly, the motor neurons still possess the properties of being phasic or tonic. This is illustrated by the fact that the tonic motor neuron can continually release transmitter for long periods of time without synaptic depression during high frequency stimulation, whereas the phasic terminals show depression. Also the tonic nerve terminals show pronounced enhancement in releasing vesicles with high frequency stimulation resulting in a postsynaptic facilitated electrical membrane potential. The phasic terminals can also show some facilitation but not as extreme. The underlying cause of depression is not fully understood but may very likely correlate with ATP stores within the motor nerve terminals. The tonic and phasic terminals so far investigated among various types of neuromuscular preparations reveal that the tonic terminals contain many more mitochondria than the phasic ones (Bradacs et al., '97). This difference of mitochondrial content exists for adjacent terminals innervating the same target, as well as in purely tonic or phasic innervated muscles.

Both long thin terminals and varicose terminals can co-exist on the same target fiber, as in the leg extensor and closer muscles. Through chronic, electrical conditioning of phasic motor neurons, in vivo, they will transform physiologically and anatomically to tonic-like motor neurons which will persist for days upon cessation of the stimulation. This condition is termed long-term adaptation (LTA) (Lnenicka and Atwood, '85; Lnenicka, '91; Lnenicka et al., '91). Phasic–tonic differentiation of the type found in crustaceans also appears to occur in other organisms, including vertebrates (Robbins, '80; Sterz et al., '83) and insects (Hardie, '76; Rheuben, '85). LTA can be induced within 1 week for the motor nerve terminals, but it takes at least 3 weeks of continuous conditioning before the muscle starts to show biochemical signs of transformation (Cooper et al., '98). Plasticity, in the form of facilitation at the crustacean neuromuscular junction, also exists in shorter time scales and has been intensely investigated over the years (Sherman and Atwood, '71; Zucker, '73, '74a,b; Parnas et al., '82a–d; Dudel, '83, '89a–d; Zucker and Lara-Estrella, '83; see review by Atwood and Wojtowicz, '86; Atwood et al., '94; Winslow et al., '94). Two forms of facilitation can result from electrical activity. Short-term facilitation (STF), which lasts only a few milliseconds to a few minutes, can be induced and measured by three experimental procedures: (1) a test pulse directly following a single stimulus as with paired pulse facilitation (i.e., twin); or (2) with the test pulse appearing some time following a train of stimuli (i.e., delayed); or (3) in comparing the first

pulse within a train to the last pulse in the same train (i.e., train stimulation) (Crider and Cooper, '99, 2000). In comparison, long-term facilitation (LTF) lasts several minutes to hours and requires a tetanic stimulation of 20 Hz for 5–10 min (Dudel, '65; Wojtowicz and Atwood, '86; Dixon and Atwood, '89). The single test pulses, given at various time intervals after this type of induction, are enhanced substantially for the first 10 min after the high-frequency conditioning stimulation.

Combining synaptic structural information of the various nerve terminals (from serial sections and 3-D rendering of electron micrographs) along with the physiology provides a unique ability to correlate structure to function (Sherman and Atwood, '72; Cooper et al., '95b; Atwood and Cooper, '96a,b). With this foundation, the differential influences in synaptic transmission induced by neuromodulators on nerve terminals can be addressed (Ruffner and Cooper, '98; Crider and Cooper, '99; Shearer and Cooper, '99). This knowledge allows one to begin understanding the underlying mechanisms of synaptic plasticity and differentiation of tonic, phasic, and transformed nerve terminals and also lends hope that understanding the fundamental basics of synaptic transmission in this model system will be directly relevant to all neural systems (Bailey and Kandel, '93).

The rationale for this investigation was to understand the functional nature of these important articular associated muscles and whether the physiology of the muscles are correlative to the regular rapid and slow movements of the abdomen. Only the anatomical location has been described for the first two abdominal segments by Pilgrim and Wiersma ('63) without reference to differences in the remaining abdominal segments. Our purpose is to further describe the anatomy of dorsal membrane abdomen muscle (DMA) and the superficial extensor muscle accessory head (SEAcc) for each abdominal segment. This study also characterizes crayfish physiology and phenotypic biochemistry related to the function of controlling the articular membrane movement and position. The function of the dorsal membrane muscle appears to be to retract the articular membrane between the cuticular segments so that the membrane does not evert during abdominal movement. In this way the membrane is prevented from being exposed to the external surface and possible damage. The superficial extensor muscle accessory may also aid, in a minor way, extension of the abdomen. Preliminary findings of this study have been presented in abstract form (Sohn and Cooper, '99; Sohn et al., '99a,b).

MATERIALS AND METHODS

Animals

Mid-sized crayfish (*Procambarus clarkii*) measuring 6–10 cm in body length and weighing approximately 10.5 g were obtained from Atchafalaya Biological Supply Co. (Raceland, LA). Smaller animals (6–7 cm) were used for the nerve terminal morphology. Animals were housed in an aquatic facility within the laboratory in individual tanks set at a temperature of 23°C (16 hr/8 hr light/dark cycle) and fed fish food pellets (Aquadine) every 3 days. Only male crayfish in the intermolt stage was used. Lobsters were bought fresh from a local supermarket and dissected within 1 hr of being taken out of their seawater aquaria. The lobsters were 25–30 cm in body length and weighed approximately 0.5 kg.

Dissection

For these studies various tonic and phasic muscles were used. These included the lateral head of the superficial extensor muscle of the abdomen (SEL), which is known to be purely tonic and the two muscles located more medial are attached to the dorsal articular membrane between segments and the cuticle within a segment. These latter two muscles are termed the dorsal membrane abdomen muscle (DMA) and the superficial extensor muscle accessory head (SEAcc). The nomenclature of these muscles follows the work of Pilgrim and Wiersma ('63). In the past (Cooper et al., '98), SEL and SLE as well as SEM and SME were used interchangeably but from now on we prefer to remain with the original nomenclature. An additional change we would like draw to the reader's attention is the fact that the older nomenclature of the abdominal deep extensors L_1, L_2, and M do not fit with the superficial SEL and SEM, so we propose using deep extensor lateral group1 (DEL_1), deep extensor lateral group2 (DEL_2), and deep extensor medial (DEM) to replace the older, less descriptive terms.

To expose these muscles, a cross-section was made between the thorax and abdomen. After cutting along the longitudinal axis of the abdomen at the lateral midline on each side, the ventral side of the abdomen was removed to reveal the ventral surface of the deep abdominal extensor muscles. The dorsal half was spread apart and pinned down to expose the dorsal abdominal cav-

ity. The residual flexor muscle and connective tissue were removed for visual identification of the deep extensor muscles (DEL_1, DEL_2, and DEM) and the superficial lateral extensor muscle (SEL). The DMA and SEAcc are easily observed after removing the deep extensor muscles (DEL_1, DEL_2, and DEM).

The tissue was pinned out in a Sylgard dish for viewing with a Nikon Optiphot-2 upright fluorescent microscope using a 40× (0.55 NA) Nikon water-immersion objective. During experiments, crayfish preparations were maintained continuously in crayfish saline, a modified Van Harreveld's solution (205 mM NaCl; 5.3 mM KCl; 13.5 mM $CaCl_2 \cdot 2H_2O$; 2.45 mM $MgCl_2 \cdot 6H_2O$; 0.5 mM HEPES adjusted to pH 7.4) while lobsters were held in a lobster saline (520 mM NaCl; 12 mM KCl; 12 mM $CaCl_2 \cdot 2H_2O$; 10 mM $MgCl_2 \cdot 6H_2O$; 2.5 mM Tris-malate adjusted to pH 7.4).

Anatomy

The various extensor muscle groups were stained with methylene blue (1 mM) for 7 min and observed under a dissecting microscope (Hartman and Cooper, '94). Morphological investigation of terminal branches was performed to show the innervation pattern and to visualize the nerve terminals on the muscle fiber studied using the vital fluorescent dye, 4-Di-2-Asp (Molecular Probes, Eugene, OR; Magrassi et al., '87). The concentration of the dye was 2–5 μM in crayfish saline. The living preparation was stained for 2–5 min and then washed in crayfish saline.

Immunofluorescence

Whole mount preparations were pinned to a Sylgard dish with the muscle in a stretched position. They were fixed with 2.5% (v/v) glutaraldehyde, 0.5% (v/v) formaldehyde dissolved in a PBS buffer (0.1 M) with 4% sucrose for 1 hr with two changes of solution. The preparation was then placed into vials and washed in PBS buffer containing 0.2% (v/v) Triton X-100 and 1% (v/v) normal goat serum (Gibco/BRL, Grand Island, NY) for 1 hr with three changes at room temperature. The tissue was then incubated with primary antibody to GABA (Sigma, 1:1000 in PBS buffer) on a shaker at 4°C for 12 hr. The tissue was washed three times and incubated in secondary antibody (goat anti-rabbit IgG conjugated with Texas Red, Sigma) diluted 1:200 with PBS buffer at room temperature for 2 hr, followed by 2 washes in buffer. The synaptic locations were revealed by immunocytochemistry as previously shown for nerve terminals (Cooper et al., '96b; Cooper, '98). Fluorescent images of the nerve terminals were viewed with a Leica DM IRBE inverted fluorescent microscope using a 63× (1.2 NA) water immersion objective with appropriate illumination. The composite images of the various focal planes (Z-series) were collected with a Leica TCS NT confocal microscope for illustration.

Electron microscopy

The number of axons that innervate the DMA and SEAcc muscles was revealed by using transmission electron microscopy (TEM). In preparation for TEM, buffer for fixation and wash buffer were prepared as described previously (Cooper et al., '96a). The buffer (0.1 M sodium cacodylate, 4% sucrose, 0.022% $CaCl_2$; pH 7.4) was used to make a fixing solution containing 0.5% paraformaldehyde and 2.5% glutaraldehyde. The buffer for wash was the same used to make the fixing solution. Each preparation was fixed for 2 hr and rinsed in buffer. Next, the tissues were rinsed for 20 min in wash buffer. After washing, the nerve bundles of interest were trimmed out of the preparation and placed into individual vials. After being rinsed with buffer wash for 1.5 hr, each tissue was post-fixed for 1 hr in buffered 2% osmium tetroxide (wash buffer). Dehydration in a graded ethanol series (50%, 60%, 70%, 80%, 90%, 95%, three times 100%) was followed by embedding in an Araldite/Epon resin. Blocks were thin-sectioned (60–90 nm) and placed on Formvar-coated copper slot grids with evaporated carbon film. Specimens were viewed with a Hitachi H7000 transmission electron microscope.

Physiology: excitatory postsynaptic potentials (EPSPs)

Intracellular recordings were performed with microelectrodes filled with 3 M KCl. The resistance of the electrode in the bath was 30–60 MΩ. Responses were recorded with the Axoclamp 2A intracellular electrode amplifier (Axon Instrument). Electrical signals were recorded on VHS tape, and on-line to a Power Mac 9500 via a MacLab/4s interface. EPSPs were recorded at an acquisition rate of 10 kHz. All events were appropriately scaled to known test pulses applied through the electrode and directly measured on an oscilloscope. The corrected scale was then adjusted with MacLab Scope software (version 3.5.4). A Grass S-88 stimulator and stimulus isolation unit (Grass) with leads to a standard suction electrode set-up (Cooper et al., '98) was used to stimu-

late the excitatory nerves in each preparation. The stimulation paradigm was to produce trains of ten stimuli at frequencies of 20 or 40 Hz with 10-sec intervals. Twin pulses were provided at paired intervals of 20–350 msec. The fatigue resistance of synaptic transmission was assayed during continuous stimulation either at 5, 10, or 20 Hz as indicated.

The entire nerve root to the superficial and deep extensor muscles was stimulated with a suction electrode. While stimulating the nerve root, responses were measured in the muscles of interest. By varying the stimulation intensity or polarity, individual axons, or groups, were recruited and their responses measured.

The facilitation ratio (*Fe*) was calculated for trains by collecting the peak amplitudes of the first, third, and tenth pulses. The measured three peak amplitudes were then calculated for the facilitation of trains using the following equations:

$$Fe_{(10/1)} = ([EPSP_{10}]/[EPSP_1]) - 1;$$
$$Fe_{(10/3)} = ([EPSP_{10}]/[EPSP_3]) - 1,$$

where $[EPSP_1]$, $[EPSP_3]$, and $[EPSP_{10}]$ are the peak amplitudes of first, third, and tenth pulses, measured from the base in the trough prior to the event to the peak of the event (Crider and Cooper, '99, 2000).

Analysis of myofibrillar proteins with SDS–polyacrylamide gel electrophoresis and Western blotting

The muscles of interest were removed and placed in a tube on dry ice and stored at –70°C until processing. Muscle fibers were incubated in glycerination buffer (20 mM Tris-HCl, pH 7.4; 0.1 M KCl; 1 mM EDTA; 0.1% Triton X—00; and 50% glycerol) on ice for 3 hr to extract membrane and cytoplasmic proteins (Mykles, '85a). Single fibers were solubilized overnight in 25–100 µl SDS sample buffer (62.5 mM Tris-HCl, pH 6.8; 12.5% glycerol; and 1.25% SDS) at room temperature and heated at 90°C for 3 min. Myofibrillar proteins (4–6 µg) were separated in discontinuous 10% SDS–polyacrylamide gels (1 mm thickness) using a Bio-Rad Mini-Protean gel apparatus (Laemmli, '70). Proteins in gels were either fixed in 10% glutaraldehyde and stained with silver (Wray et al., '81; Mykles, '85a,b) or transferred to PVDF membrane for Western blotting (Towbin, et al., '79).

Polyclonal antibodies to lobster myofibrillar proteins were raised in rabbits as described (Mykles et al., '98). Briefly, proteins were purified from lobster deep abdominal muscle by chromatography (tropomyosin, troponin-T, and troponin-I_3; Miegel et al., '92) or preparative SDS–PAGE (P75; Mattson and Mykles, '93). Except for anti-troponin-I_3, IgG was purified from antisera by protein A-Sepharose chromatography (Beyette and Mykles, '92). Western blots were stained with colloidal gold (Chevallet, '97; Covi, '99) before blocking with 5% nonfat milk in TBS (20 mM Tris-HCl, pH 7.5; 0.5 M NaCl). Blots were incubated for 1 hr with antibodies to P75 (7.3 µg IgG/ml), tropomyosin (4 µg IgG/ml), troponin-I_3 (1:5,000 dilution of antiserum), or troponin-T (0.8 µg IgG/ml) in TTBS (0.05% Tween-20 in TBS). After washing in TTBS, blots were incubated with anti-rabbit IgG-HRP conjugate (1:10,000 dilution in TTBS) for 1 hr followed by chemiluminescent detection (Covi, '99). After detection with anti-P75, antibody was removed with stripping buffer (67.5 mM Tris-HCl, pH 6.7; 2% SDS; and 100 mM β-mercaptoethanol) at 60°C for 30 min and blots were reprobed with anti-tropomyosin IgG. Similarly, a second set of blots was probed with anti-troponin-T, stripped, and reprobed with anti-troponin-I_3.

RESULTS

Anatomy

The anatomical arrangement of the DMA and SEAcc musculature is different for each of the abdominal segments (Fig. 1). In the first abdominal segment (A1) the muscle's rostral attachment is on the articular membrane between the thorax and abdomen and the caudal attachment is on the articular membrane between segments A1 and A2. In the remaining segments, the caudal attachment of the muscles is on the articulating membrane but the rostral attachment is on the dorsal cuticle of the segment. This difference in attachment points in the first segment may likely arise due to the fact that this dorsal region between the abdomen and thorax is the area that splits open upon eclosion during each molt cycle. The split in the articular membrane occurs just rostral to the attachment of these muscles. In addition the muscles in this segment are longer than those of more caudal segments. These muscles in A1 are also more in parallel to each other, making it harder to distinguish between them. The muscles show a greater difference in the remaining segments. The SEAcc muscles are attached more toward the base of the articular membrane close to the ridge of the following segment (Fig. 2A) and contain longer fibers since the other end of the

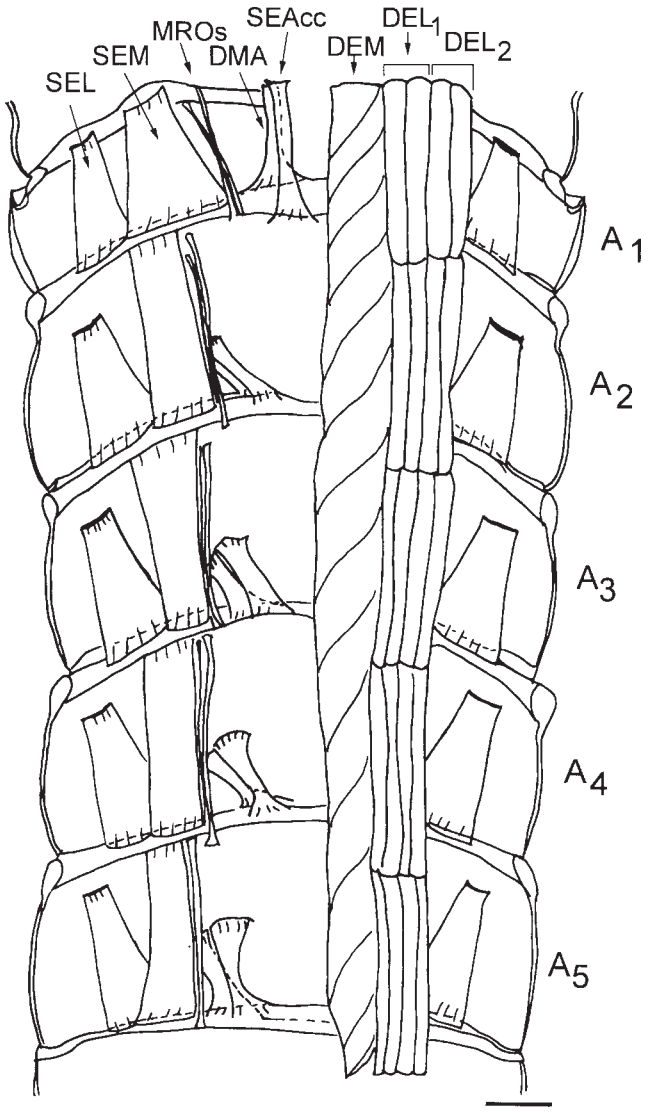


Fig. 1. Schematic drawing from a ventral view of the dorsal part of the crayfish abdomen showing the extensor musculature of each segment. The dorsal membrane abdomen muscle (DMA) and the superficial extensor accessory head muscle (SEAcc) occur in segments 1 through 5 of the abdomen with a different orientation for each segment. With the exception of segment 1, these muscles have their attachment sites at their anterior end to the calcified tergite and at the posterior end in the articular membrane. In segment 1, the homologous muscles have their anterior attachment sites to the articular membrane located between the thorax and abdomen. The illustration was based upon photographic montages of methylene blue stained preparations. On the left side of the figure all the deep extensor muscles have been removed to show the dorsal superficial extensor muscles. Scale = 2.35 mm.

muscle attaches more rostrally in the segment as compared to DMA (Fig. 2B). The SEAcc also makes a larger angle to the midline than the DMA muscle in the more caudal segments (Figs. 1 and 2A). Both muscles have more of a lateral attachment at the rostral ends in the more caudal abdominal segments (Fig. 1).

The motor neurons that innervate the DMA and SEAcc muscles have their cell bodies in the segmental ganglion. Their axons travel in the same nerve bundle (second root) as the axons to the other dorsal abdominal extensor muscles. As the nerve bundle transverses past the SEL and SEM muscles, the nerve bundle shows some branching for neurons associated with the MROs. One branch of the nerve continues toward the DMA and SEAcc muscles as shown from methylene blue staining of an exposed representative preparation which was used to illustrated schematically for easy identification (Fig. 3A). The nerve shows distinct branching as it approaches the DMA muscle with one branch remaining associated to the DMA and the other to the SEAcc. Morphology of nerve terminals reveals information as to the potential phenotype of the terminal. Following the application of the vital stain 4-Di-2-Asp, the terminals are observable within minutes (Fig. 3C and D). No obvious differences could be observed among the terminals on the DMA and SEAcc. Notice that there are multiple terminals that all appear to have strings of varicosities along their length. This morphology is indicative of tonic nerve terminals (Jahromi and Atwood, '74; Hill and Govind, '81; Atwood and Cooper, '96b).

To determine the extent of inhibitory innervation, staining for GABAnergic reaction was performed. There was a single axon within the nerve bundle that was immunoreactive. Since single strings of terminals were seen at terminal branch points, it is likely that the individual muscles are innervated by a single inhibitory axon. Representative terminals are shown in Fig. 3B for an inhibitory neuron on DMA. The terminals showed no differences among the DMA and SEAcc muscles.

To help in identifying the number of axons in the nerve bundle between the muscle receptor organs (MROs) and the DMA and SEAcc muscles, cross-sections were obtained at this point (Fig. 4A). In two different preparations 10 axons were observed and in another preparation 11 axons. Small axon profiles sometimes appear to be present in sections made just medial to the MROs. Possibly these small axons are branches of the motor neurons or the axons of sensory neurons associated with the dorsal carapace which may be contained within the nerve bundles. Cross-sections closer to the DMA and SEAcc indicate that the axons do bifurcate on the muscle since the

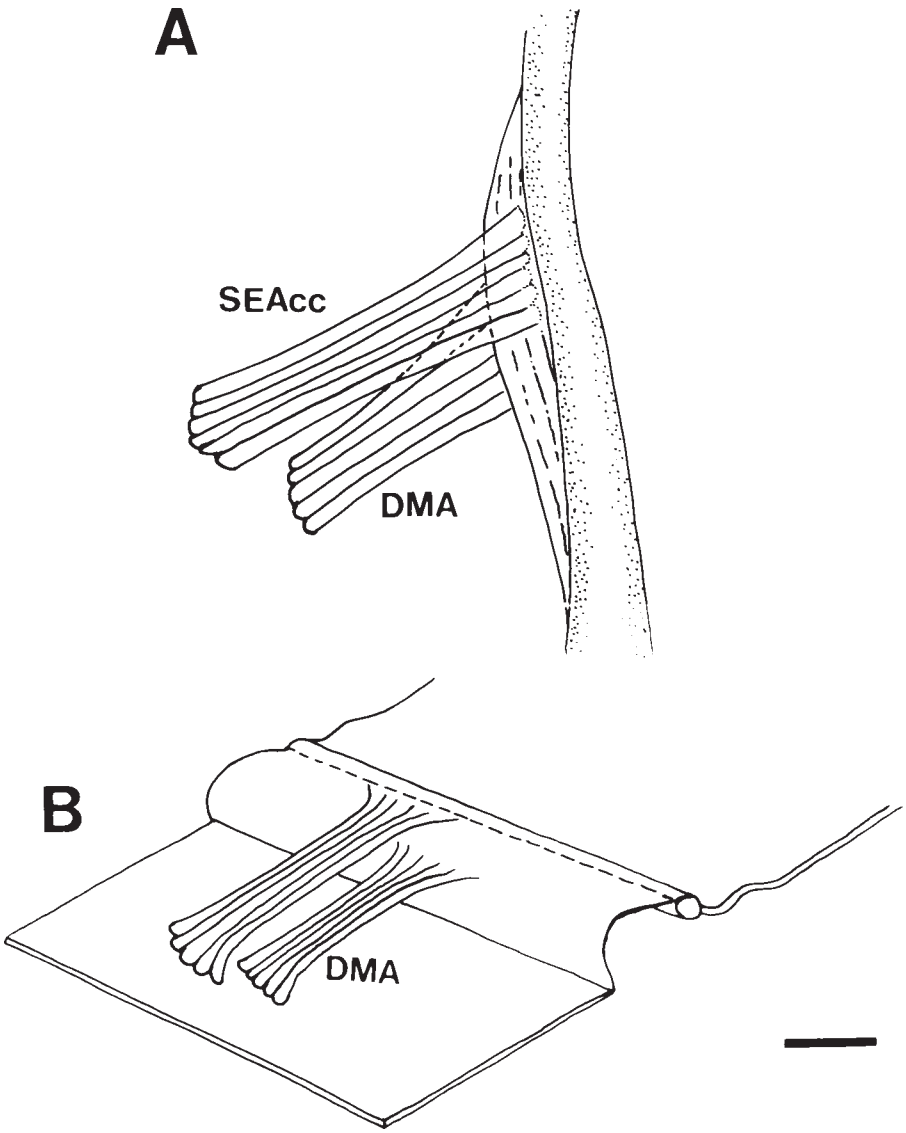


Fig. 2. Detailed schematic drawing of segment 2 showing the retraction of the articular membrane by the dorsal membrane abdomen muscle (DMA) and the superficial extensor accessory head muscle (SEAcc). **A:** Ventral view illustrates that the posterior end of SEAcc is attached to the most caudal area of the articular membrane. **B:** Side view shows the relative positions on the articulating membrane of these two muscles. Scale = 0.96 mm.

same axon profiles are seen in two separate different bundles, but of smaller diameters. To examine the structure of the muscles, cross-sections of the muscles were obtained as illustrated in Fig. 4B. The cross-sections revealed highly invaginated individual fibers throughout the muscle. The total number of single fibers within a particular bundle varied. More samples need to be obtained for each segment and at different developmental stages before comparisons can be made among segments and among differently sized crayfish. The terminal structures of motor neurons on DMA and SEAcc show multiple synapses within the varicose regions of the terminals. There were no obvious differences that we could observe among terminals of the DMA and the SEAcc muscles. Terminal morphology was not pursued since one could not identify which of the four excitatory motor neurons was being observed in the electron micrographs.

Physiology

The observations gathered from 10 crayfish and multiple measurements among segments of the DMA and SEAcc muscles, in some crayfish, illustrate that there are four different excitatory mo-

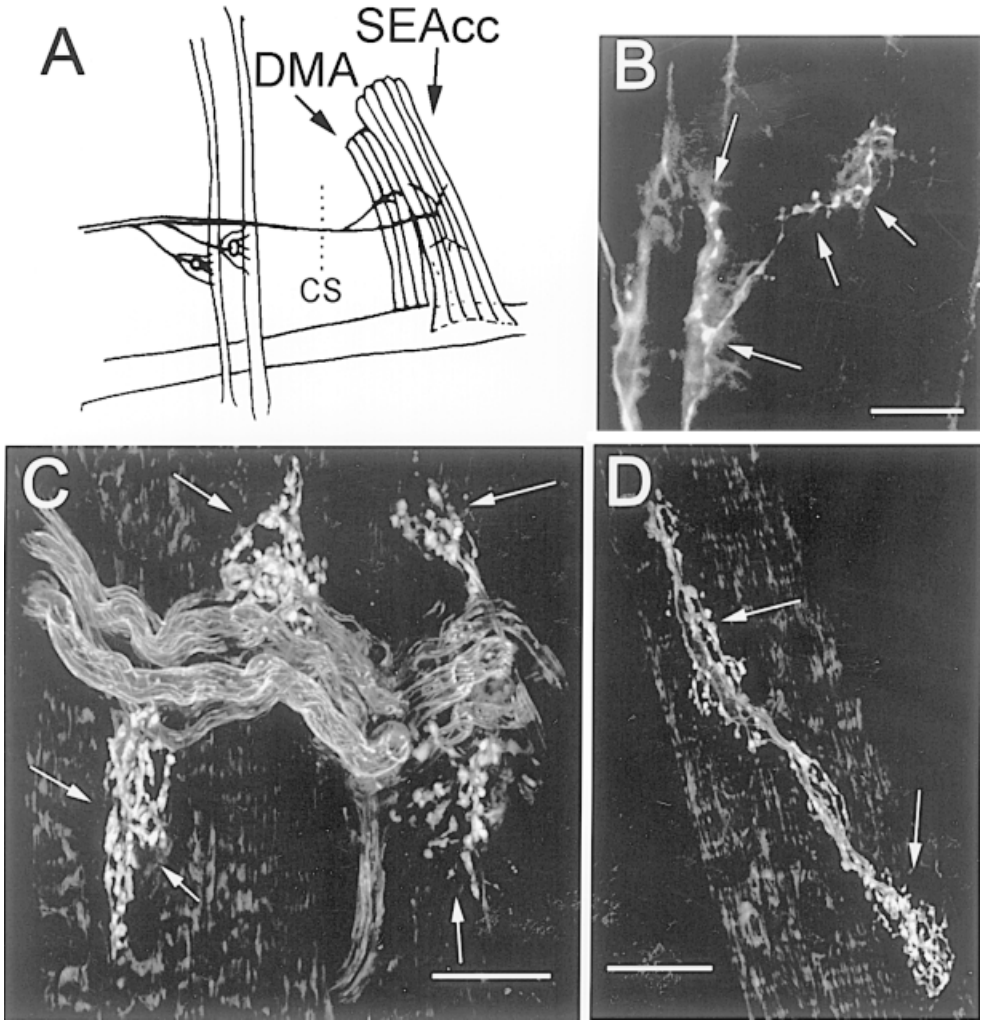
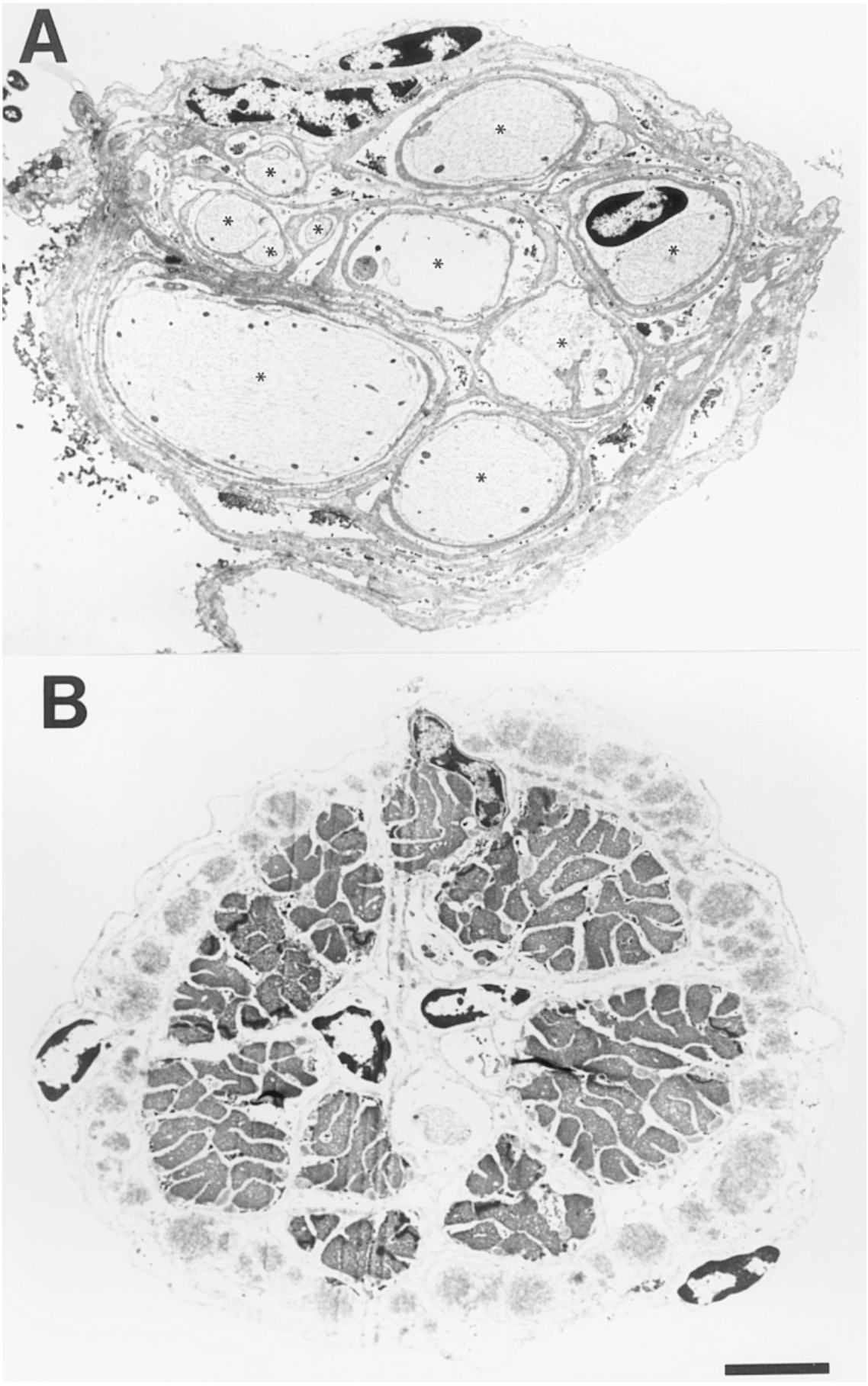


Fig. 3. Innervation pattern of the DMA and the SEAcc muscles of the crayfish. **A:** Schematic of the intact musculature of DMA and SEAcc. On the left side of DMA, MROs and SEM muscles are shown. The nerve bundle leaves the superficial extensor medial muscle (SEM), which then passes the two large sensory cell bodies of the neurons associated with the slowly adapting and rapidly adapting muscle receptor organs (MROs). The dotted line and region labeled as "cs" indicate the section and corresponding figure (Fig. 4). The nerve bundle approaches the muscles after traversing the MROs and it bifurcates on the DMA and SEAcc muscles. Staining the muscles with an antibody for GABA reveals a neuron with its terminals innervating both DMA and SEAcc (**B**). The selective staining of the single axon and terminals substantiates anatomically the presence of inhibitory innervation. The morphology of nerve terminals stained with 4-Di-2-Asp is shown on DMA (**C**) and SEAcc muscles (**D**). Note the multiple varicosities and terminals. These varicosities are found on muscles that are innervated by tonic excitatory and inhibitory motor neurons. Phasic terminals are thin and filiform which are not observed on the DMA and SEAcc muscles. Scale bars = 60 μm.

tor neurons that can be recruited and the presence of an inhibitory motor neuron. All four responses were difficult to obtain in some preparations because of the intermittent recruitment of the inhibitory axon. Without the use of the harsh compound, picrotoxin, to selectively block GABAnergic innervation, we instead varied the stimulus polarity, voltage amplitudes, and durations of the single stimulus pulses (0.3–0.55 msec) to recruit individual axons or composites of multiple axons. Representative responses for both DMA and SEAcc muscles are shown in Fig. 5. Since some neurons are very low in synaptic efficacy, single pulses re-

Fig. 4. Electron micrographs of the motor units associated with DMA and SEAcc muscles of the crayfish. **A:** Cross section of the nerve bundle reveals the number of axons (asterisks) and their sizes. **B:** Section of the DMA muscle bundles allowed individual fibers to be counted. Scale bar = (**A**) 4 μm, (**B**) 16 μm.


A
B

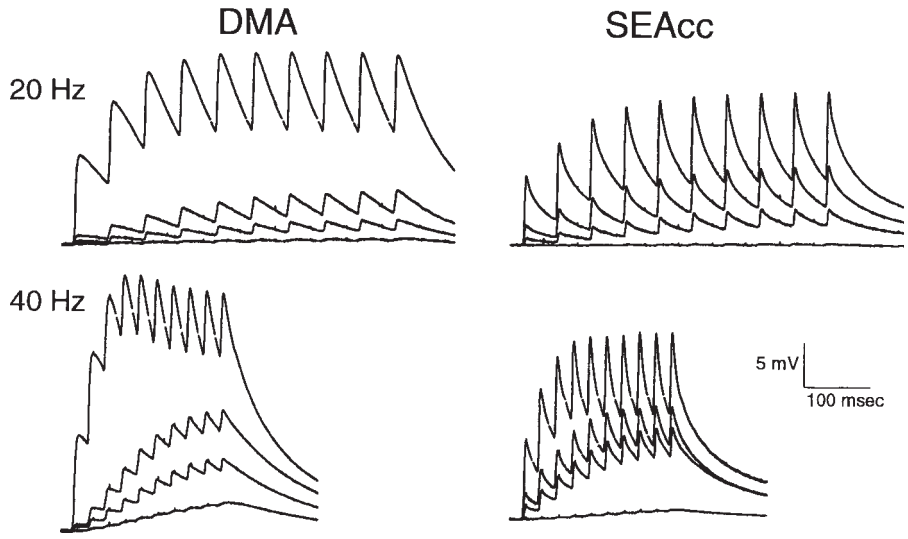


Fig. 5. Illustrations of the EPSPs recorded from DMA and SEAcc muscles of the crayfish. By providing trains of stimuli at 20 Hz (top panels) and 40 Hz (bottom panels) for at least ten pulses within a train, it is apparent that at least four distinctly different excitatory motor units could be recruited in both the DMA (left panel) and the SEAcc (right panel) muscles. The 40-Hz stimulation resulted in a more pronounced facilitation than 20-Hz stimulation. The responses among preparations are tabulated in Table 1. The entire nerve bundle was stimulated with a suction electrode at various voltages to recruit different units.

sulted in undetectable responses but became very obvious upon the induction of short-term facilitation and temporal summation provided by trains of stimuli at either 20 or 40 Hz. Trains of stimuli were used to characterize the various types of innervation on the muscles because of the ease in observation and measurement. In preparations in which the inhibitory axon was not recruited, the individual excitatory axons could routinely be recruited in the order of lowest synaptic efficacy to the greatest synaptic strength as revealed by the amplitudes of the postsynaptic potentials (EPSPs). This approach produced responses that were spatially summated when an additional axon was recruited. Therefore, we refer to the EPSPs as "responses 1, 2, 3, or 4" for the number of axons when composites were observed (Fig. 5). The response amplitudes were measured for a few representative preparations for comparisons among the DMA and SEAcc muscle preparations and within preparations for the various segments (Table 1). In addition, facilitation indices were calculated for each of the 4 responses of the same preparations (Table 1; see Materials and Methods for calculation).

In cases when contraction did not produce artifacts in the EPSP trains, the various responses could be subtracted from each other to obtain the synaptic response from the individual axons. These isolated responses are referred to as being from "axons 1, 2, 3, or 4" for the same "responses 1, 2, 3, and 4" as shown in Fig. 5 for both DMA and SEAcc muscles during 20 and 40 Hz stimulations (Fig. 6). The amplitudes and facilitation indices were recalculated to determine how varied the values would be between the composite responses 1, 2, 3, and 4 and for axons 1, 2, 3, and 4 (Table 2). Of course, when the smallest responses are recruited first then there is no difference among the two approaches. The facilitation values are larger for $Fe_{10/1}$ and $Fe_{10/3}$ when the individual axons are analyzed, but the values decline for the 3rd and 4th axons (Tables 1 and 2). The negative values arise from the 1st or 3rd EPSP response being larger in amplitude than the 10th EPSP within a train. It is apparent that the composite traces with underlying responses add to the amplitudes and offset the *Fe* values.

The inhibitory neuron could be recruited by varying the polarity and voltage of the stimulus. In some cases, the inhibitory postsynaptic potentials (IPSPs) responses can be recorded in isolation from excitatory responses (Fig. 7A) and in other cases the EPSPs and IPSPs can be mixed within a stimulus train (Fig. 7B). IPSPs are known to facilitate as demonstrated in other crayfish muscles (Vyshedskiy and Lin, '97a–c). These types of responses are observed from both DMA

TABLE 1. EPSP amplitudes and facilitation measures for each of the four responses during train stimulation at 20 Hz and 40 Hz for both DMA and SEAcc muscles

Prep.		Resp	1st	3rd	10th	Fe(10/1)	(10/3)
DMA	1	1	0.17	0.21	0.32	0.93	0.52
(20 Hz)		2	0.49	0.98	1.35	1.79	0.38
		3	1.37	2.31	3.01	1.20	0.31
		4	12.25	10.39	10.51	–0.14	0.01
	2	1	0.04	0.07	0.06	0.37	–0.13
		2	0.70	1.06	1.22	0.73	0.16
		3	1.27	1.67	1.79	0.42	0.08
		4	5.82	5.57	6.02	0.04	0.08
	3	1	0.22	0.36	0.69	2.13	0.93
		2	0.81	1.09	1.89	1.33	0.74
		3	1.17	2.02	3.27	1.79	0.62
		4	4.30	5.95	8.13	0.89	0.37
	4	1	0.06	0.05	0.22	2.35	3.03
		2	0.34	0.53	0.79	1.31	0.49
		3	0.54	0.93	1.77	2.30	0.90
		4	0.61	1.25	2.62	3.32	1.09
	5	1	0.14	0.19	0.29	1.06	0.53
		2	0.21	0.30	0.88	3.22	1.90
		3	0.60	1.32	2.16	2.63	0.64
		4	3.75	5.33	6.78	0.81	0.27
	6	1	0.06	0.10	0.12	1.04	0.17
		2	0.55	1.20	1.64	1.95	0.36
		3	3.23	4.67	6.00	0.86	0.28
		4	4.90	5.48	6.08	0.24	0.11
Mean		1	0.12 (0.03)	0.16 (0.05)	0.28 (0.09)	1.31 (0.31)	0.84 (0.46)
(±SEM)		2	0.52 (0.09)	0.86 (0.15)	1.30 (0.17)	1.72 (0.35)	0.67 (0.26)
		3	1.36 (0.40)	2.15 (0.54)	3.00 (0.65)	1.53 (0.35)	0.47 (0.12)
		4	5.27 (1.57)	5.66 (1.18)	6.69 (1.06)	0.86 (0.52)	0.32 (0.16)
DMA	1	1	0.25	0.38	0.42	0.71	0.12
(40 Hz)		2	0.60	1.43	1.65	1.75	0.16
		3	0.99	2.94	2.79	1.81	–0.05
		4	13.00	10.23	8.66	–0.33	–0.15
	2	1	0.08	0.19	0.19	1.22	–0.03
		2	0.19	0.41	0.54	1.81	0.32
		3	1.28	2.31	2.11	0.65	–0.09
		4	7.29	5.76	4.35	–0.40	–0.24
SEAcc	1	1	0.09	0.13	0.21	1.46	0.64
(20 Hz)		2	0.91	1.71	2.07	1.29	0.21
		3	2.80	4.24	5.34	0.91	0.26
		4	9.45	10.93	11.85	0.25	0.08
	2	1	0.36	0.54	0.67	0.87	0.25
		2	1.68	2.79	2.82	0.68	0.01
		3	3.79	5.60	4.47	0.18	–0.20
		4	14.01	10.73	10.83	–0.23	0.01
	3	1	0.16	0.20	0.22	0.40	0.09
		2	0.24	0.27	0.21	–0.13	–0.22
		3	0.60	1.10	1.18	0.98	0.08
		4	1.05	1.46	1.15	0.10	–0.21
	4	1	0.32	0.38	0.58	0.79	0.52
		2	1.34	1.23	1.74	0.31	0.42
		3	1.15	1.79	2.51	1.19	0.40
		4	3.42	5.02	6.24	0.82	0.24
	5	1	0.18	0.23	0.22	0.23	–0.06
		2	0.28	0.35	0.45	0.60	0.29
		3	0.56	1.84	3.24	4.81	0.77
		4	1.39	4.20	6.81	3.90	0.62
	6	1	0.06	0.07	0.05	–0.07	–0.24
		2	0.88	0.87	1.29	0.47	0.48
		3	1.36	2.24	3.30	1.43	0.47
		4	5.44	6.47	8.60	0.58	0.33

(*continued*)

TABLE 1. (continued).

Prep.		Resp	1st	3rd	10th	Fe(10/1)	(10/3)
Mean		1	0.19 (0.05)	0.26 (0.07)	0.33 (0.10)	0.61 (0.22)	0.20 (0.14)
(±SEM)		2	0.89 (0.23)	1.20 (0.39)	1.43 (0.41)	0.54 (0.19)	0.20 (0.12)
		3	1.71 (0.53)	2.80 (0.71)	3.34 (0.60)	1.58 (0.67)	0.30 (0.14)
		4	5.79 (2.07)	6.47 (1.53)	7.58 (1.57)	0.90 (0.62)	0.18 (0.12)
SEAcc	1	1	0.10	0.17	0.23	1.22	0.38
(40 Hz)		2	1.79	3.16	3.75	1.10	0.19
		3	3.68	5.09	5.06	0.37	–0.01
		4	10.93	10.74	10.93	0.01	0.02
	2	1	0.32	0.53	0.73	1.33	0.39
		2	1.20	2.24	2.41	1.01	0.08
		3	4.28	6.37	3.58	–0.16	–0.44
		4	13.69	15.05	9.53	–0.30	–0.37

and SEAcc muscles. In order to reliably recruit the inhibitory axon, the stimulus voltage sometimes had to be higher than the voltage to recruit the largest producing EPSP axon. This may also indicate that the size of the inhibitory axon is small or that a different density of voltage-sensitive ion channels is present which alters its threshold level.

It is common among single muscles of crustaceans to have regional variation in the sizes of EPSPs. In some cases there is a consistent relationship of amplitudes to muscle region among some types of muscles even if the muscle is innervated by a single excitatory motor neuron. For example, the leg opener has larger responses in the proximal fibers as compared to the central fibers. Likewise the DAFM muscle in lobsters has regional differences (Meiss and Govind, '79). We did not conduct an exhaustive study in regional differences across fibers of the DMA and SEAcc within a segment but some results indicate that fibers do show differences in the EPSPs within a muscle. In cases when the resting membrane potentials were equal, differences were observed for fibers in both muscles (Fig. 8). To illustrate this point, a single preparation was sampled in the various regions indicated (Fig. 8A) and responses recorded for the DMA and SEAcc muscles (Fig. 8B).

Another form of short-term facilitation (STF), besides those induced by trains of stimuli, is that produced by twin pulses of stimuli with a short delay between the pairs of pulses (Linder, '74; Dudel, '89a–d). Since the responses from axon 1 are very small and most of the time are undetectable for the first and second EPSPs within a train, we did not investigate twin pulses for this axon.

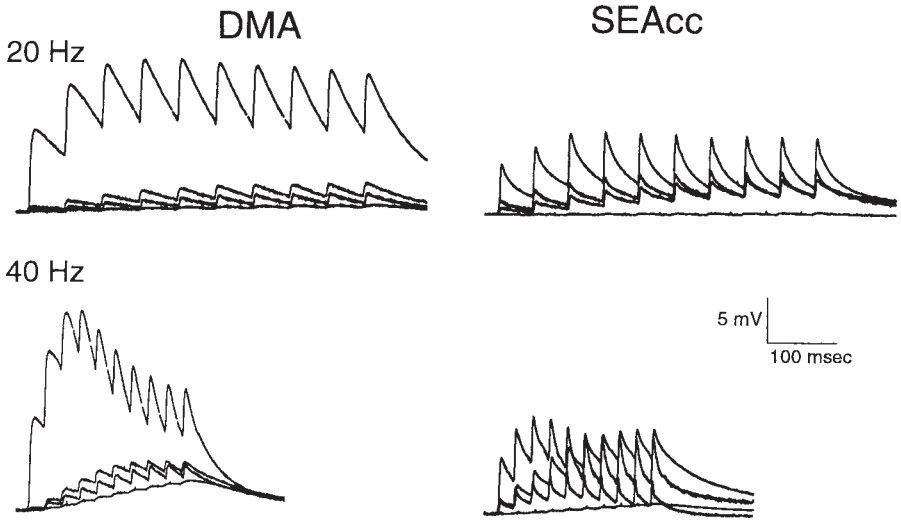


Fig. 6. To determine each neuron's own contribution to the summated postsynaptic response, the previous response was subtracted. This is shown, as in Fig. 5, for both the DMA and SEAcc at 20 and 40 Hz. The responses among preparations are tabulated in Table 2.

TABLE 2. EPSP amplitudes and facilitation measures for each of the four individual neurons during train stimulation at 20 Hz and 40 Hz for both DMA and SEAcc muscles

Prep.		Resp	1st	3rd	10th	Fe(10/1)	(10/3)
DMA	1	1	0.17	0.21	0.32	0.93	0.52
(20 Hz)		2	0.40	0.82	1.28	2.17	0.56
		3	0.67	1.33	1.56	1.31	0.17
		4	9.27	7.13	6.65	–0.28	–0.07
	2	1	0.04	0.07	0.06	0.37	–0.13
		2	0.75	1.11	1.36	0.81	0.22
		3	0.62	0.72	0.73	0.18	0.02
		4	4.87	4.19	4.57	–0.06	0.09
	3	1	0.22	0.36	0.69	2.13	0.93
		2	0.61	0.84	1.26	1.06	0.50
		3	0.39	0.90	1.45	2.74	0.61
		4	3.03	3.85	4.77	0.57	0.24
	4	1	0.06	0.05	0.22	2.35	3.03
		2	0.35	0.56	0.66	0.89	0.18
		3	0.21	0.39	0.98	3.75	1.53
		4	0.19	0.50	1.15	5.18	1.31
	5	1	0.14	0.19	0.29	1.06	0.53
		2	0.17	0.26	0.74	3.40	1.84
		3	0.49	1.19	1.38	1.82	0.16
		4	3.06	3.92	4.55	0.49	0.16
	6	1	0.06	0.10	0.12	1.04	0.17
		2	0.69	1.54	2.09	2.01	0.36
		3	3.45	4.56	5.62	0.63	0.23
		4	2.15	1.40	1.10	–0.49	–0.22
Mean		1	0.12 (0.03)	0.16 (0.05)	0.28 (0.09)	1.31 (0.31)	0.84 (0.46)
		2	0.50 (0.09)	0.86 (0.18)	1.23 (0.21)	1.72 (0.41)	0.61 (0.25)
		3	0.97 (0.50)	1.51 (0.62)	1.95 (0.75)	1.74 (0.55)	0.46 (0.23)
		4	3.76 (1.26)	3.50 (0.96)	3.80 (0.91)	0.90 (0.87)	0.25 (0.22)
DMA	1	1	0.25	0.38	0.42	0.71	0.12
(40 Hz)		2	0.46	1.10	1.43	2.11	0.30
		3	0.57	1.42	1.25	1.20	–0.12
		4	10.40	6.48	5.27	–0.49	–0.19
	2	1	0.08	0.19	0.19	1.22	–0.03
		2	0.26	0.36	0.49	0.88	0.36
		3	1.08	1.75	1.82	0.68	0.04
		4	5.07	3.43	2.47	–0.51	–0.28
SEAcc	1	1	0.09	0.13	0.21	1.46	0.64
(20 Hz)		2	0.67	1.55	1.89	1.82	0.22
		3	1.54	2.48	3.11	1.02	0.25
		4	5.66	5.91	5.65	–0.01	–0.04
	2	1	0.36	0.54	0.67	0.87	0.25
		2	1.25	2.08	2.14	0.71	0.03
		3	1.89	2.57	1.75	–0.07	–0.32
		4	8.75	4.92	5.66	–0.35	0.15
	3	1	0.16	0.20	0.22	0.40	0.09
		2	—	—	—	—	—
		3	0.37	0.76	0.93	0.51	0.21
		4	—	—	—	—	—
	4	1	0.32	0.38	0.58	0.79	0.52
		2	1.16	1.17	1.81	0.56	0.54
		3	—	0.70	0.97	—	0.39
		4	2.48	3.47	4.15	0.68	0.20
	5	1	0.18	0.23	0.22	0.23	–0.06
		2	0.16	0.15	0.26	0.62	0.73
		3	0.35	1.43	2.81	6.95	0.96
		4	0.87	2.40	3.88	3.47	0.61
	6	1	0.06	0.07	0.05	–0.07	–0.24
		2	0.91	1.09	1.65	0.82	0.51
		3	1.03	2.00	3.19	2.09	0.60
		4	5.35	5.65	7.02	0.31	0.24

(*continued*)

TABLE 2. (continued)

Prep.		Resp	1st	3rd	10th	Fe(10/1)	(10/3)
Mean		1	0.19 (0.05)	0.26 (0.07)	0.33 (0.10)	0.61 (0.22)	0.20 (0.14)
(±SEM)		2	0.83 (0.20)	1.21 (0.32)	1.55 (0.33)	0.91 (0.23)	0.41 (0.12)
		3	1.04 (0.31)	1.66 (0.34)	2.13 (0.43)	2.30 (1.21)	0.35 (0.17)
		4	4.62 (1.37)	4.47 (0.67)	5.27 (0.57)	0.82 (0.68)	0.23 (0.12)
SEAcc	1	1	0.10	0.17	0.23	1.22	0.38
(40 Hz)		2	1.58	2.66	3.21	1.03	0.21
		3	1.72	2.45	2.62	0.52	0.07
		4	6.23	5.10	5.26	–0.16	0.03
	2	1	0.32	0.53	0.73	1.33	0.39
		2	0.95	1.71	1.87	0.96	0.09
		3	2.79	3.55	1.81	–0.35	–0.49
		4	8.26	7.79	5.21	–0.37	–0.33

Likewise, to simplify the analysis we do not show the responses for axon 2, but only for the more reliable composite responses of 3 and 4 (Fig. 9A). For quantifying responses, the amplitudes of the 1st and 2nd EPSPs in the pair were measured. To index twin pulse facilitation, the same procedure (2nd/1st – 1 = *Fe*) as for the trains was used to calculated the *Fe* and the results at various delays graphed (Fig. 9B). The composite 4th responses showed much longer times before full decay occurred.

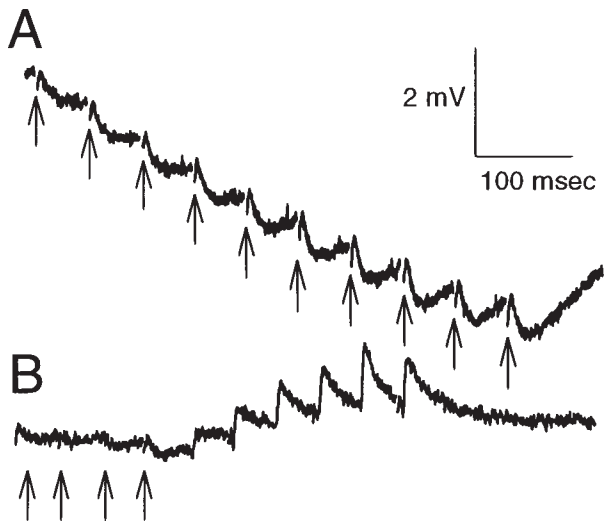


Fig. 7. Recordings from the crayfish DMA indicating the physiological responses when the inhibitory neuron is recruited alone (**A**) or during a mixed response (**B**). In the top trace the stimulation frequency was 40 Hz with an arrow denoting each stimulus pulse. In the bottom panel a 60-Hz stimulus was given and the IPSPs can only be observed for the first four pulses (arrows) followed by EPSPs. Trains of ten stimuli were provided with varying polarity and voltage of the stimulus until the inhibitory axon was recruited for illustrative purposes.

Besides anatomical data of terminal morphology indicating the terminals to be tonic in phenotype on both the DMA and SEAcc muscles, confirmation was obtained by physiological criteria. Fatigue resistance of synaptic transmission is used as a standard way to characterize a motor neuron as tonic or phasic. At 5-Hz stimulation, phasic neurons in the crayfish will show fatigue after several minutes and at 20-Hz stimulation in just a few minutes (Lnenicka and Atwood, '85; Cooper et al., '98). We are not aware of any reports that demonstrate EPSP of phasic motor terminals lasting an hour or more with continuous stimulation at 10 Hz. Because four excitatory axons innervate the muscles, a stimulation paradigm was used to recruit the smallest-response EPSP amplitudes first followed in sequence by the next larger sized EPSP. With this approach, each response could be assayed in order and for a set amount of time to be examined for synaptic depression. The results of a representative preparation are shown in Fig. 10 for a DMA in the first segment. The smallest EPSP response was continuously evoked at 10 Hz for 30 min without any sign of synaptic depression before recruiting the next largest EPSP response unit. These two neurons were stimulated for about 80 min without any signs of depression. Next, the third-largest EPSP response was recruited for about 1 hr until the largest EPSP response was recruited. The largest response did show depression after about 10 min but the decrease was small (~1.5 mV) over the next 2 hr. There may actually be a small depression in the EPSP amplitude, indicating that possibly a smaller response became fatigued, but in any case that would have been after continuous stimulation of over 250 min (4.2 hr). The data shown and those obtained from other preparations in which the

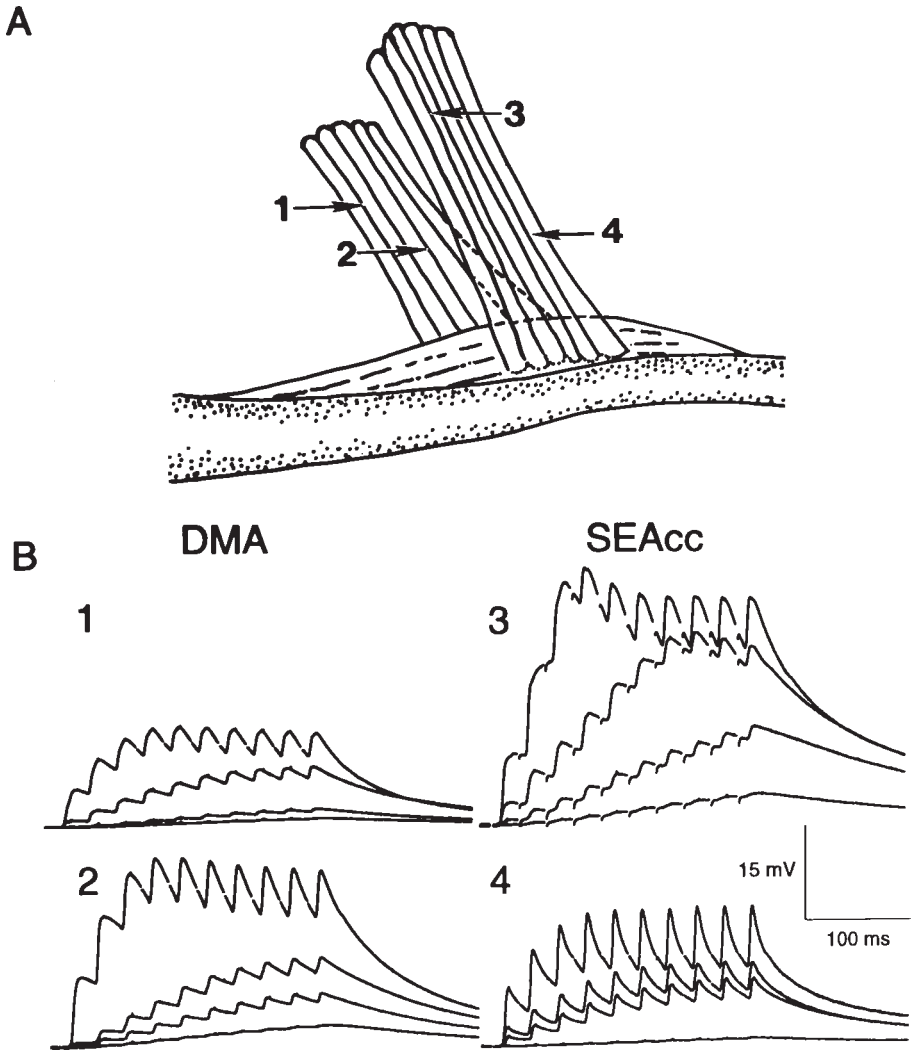


Fig. 8. To illustrate variation in the synaptic potentials for the four excitatory motor neurons among different regions of the DMA and SEAcc muscles, multiple fibers were examined within a given preparation. The various locations are shown in the schematic shown in (**A**), and the responses are indicated in (**B**) for each of the four responses. The resting membrane potentials were the same for each penetration although there were substantial differences recorded across the preparations.

smaller EPSP responses were continuously stimulated indicate that all four terminals are tonic in their physiological profiles. In addition, the responses from DMA and SEAcc from the multiple segments, A1 through A5, did not reveal any substantial differences in the fatigue resistance.

Analysis of myofibrillar protein isoforms

Analysis of terminal morphology and physiological responses indicate that since there are only tonic motor neurons innervating the DMA and SEAcc muscles, it is likely that the muscle fibers would show a biochemical composition of a slow-tonic phenotype. SDS–PAGE analysis of myofibrillar protein was used to distinguish between slow-twitch (S_1) and slow-tonic (S_2) fiber types (for review, see Silverman et al., '87; Mellon, '91; Mykles, '97). The results for both crayfish and lobster are shown in Fig. 11. In addition to the DMA and SEAcc, other muscles of previously identified muscle phenotype are included for comparison. The abdominal deep extensor muscles (DEL_1, DEL_2, and DEM) and the cutter claw closer muscle (Cu) contain fast-twitch fibers, based on morphological, histochemical, and physiological criteria (Mellon, '91; Govind, '92). Fast-twitch fibers are easily distinguished from slow fibers by the presence of the P75 regulatory protein (Costello and

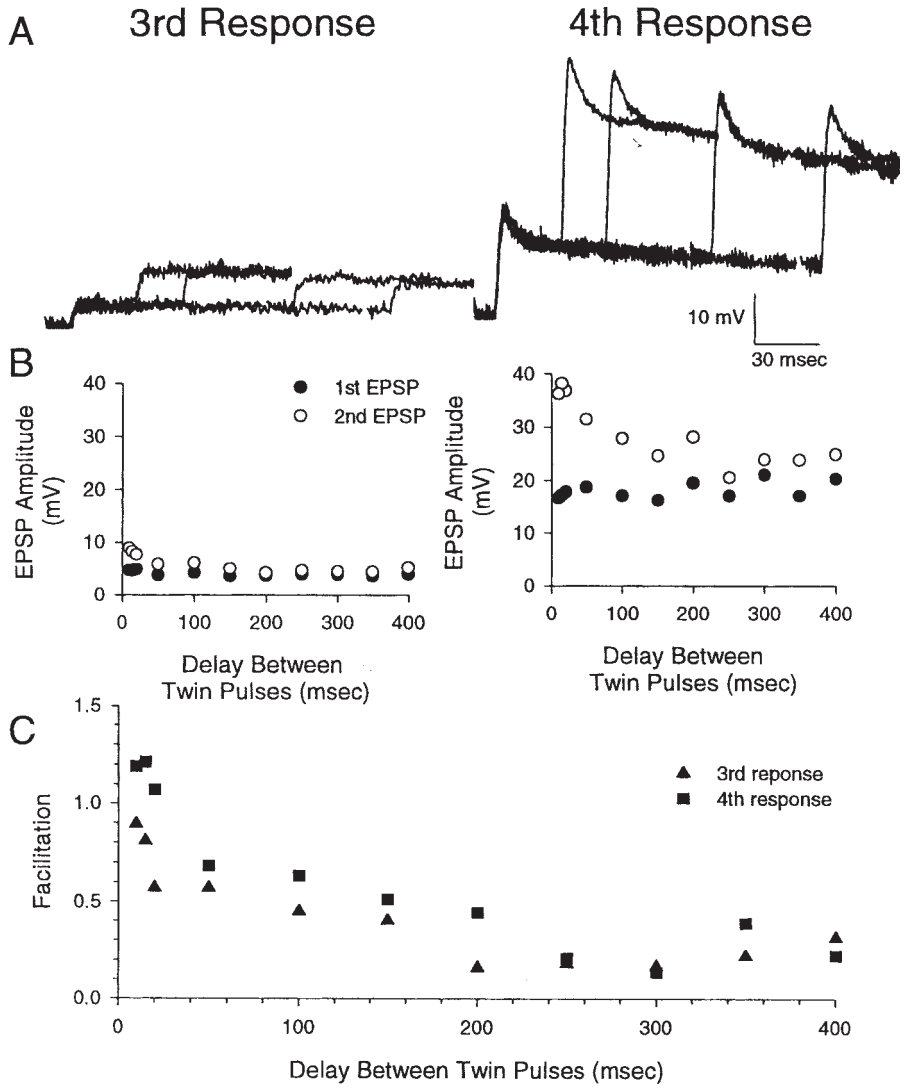


Fig. 9. Twin pulse facilitation of the 3rd and 4th composite responses showed slow decay times for the facilitation. The third and fourth responses were the most reliable for this type of measure since the first pulse in the pair is readily identifiable (**A**). The amplitude of the second responses is graphed at the various delays to quantify the time course of the decay (**B**). The facilitation index (Fe) for both third and fourth responses was determined by the ratio in the amplitudes of the 2nd and 1st responses, minus the numerical one (**C**).

Govind, '84; Mykles, '85a,b, '88; Silverman et al., '87). The two slow fiber types differ in troponin-T isoforms; slow-tonic (S_2) fibers contain TnT_1 (55 kDa), which is not present in slow-twitch (S_1) fibers (Mykles, '85a,b, '88; Silverman et al., '87; Ismail and Mykles, '92; Galler and Neil, '94). The crusher claw closer muscle (Cr) contains S_1 fibers; the abdominal superficial extensor medial (SEM) and superficial flexor medial muscles contain mostly S_2 fibers; and the abdominal superficial extensor lateral (SEL) and superficial flexor lateral muscles contain a mixture of S_1 and S_2 fibers (Mykles, '85a,b, '88; Neil et al., '93; Galler and Neil, '94). The DMA and SEAcc possess TnT_1, indicating that both muscles consist of S_2 fibers (Fig. 11A, lanes f and g; Fig. 11B, lane j).

Western blot analysis was used to show more clearly the differences in myofibrillar proteins in crayfish and lobster muscles (Figs. 12 and 13). The same samples in Fig. 11 were separated by SDS–PAGE and transferred to PVDF membrane; blots were probed with antibodies to P75, tropomyosin, troponin-T, and troponin-I. The antibodies to P75 and tropomyosin cross-reacted, suggesting some sequence similarity between the two proteins (panels B and C in Figs. 12 and 13).

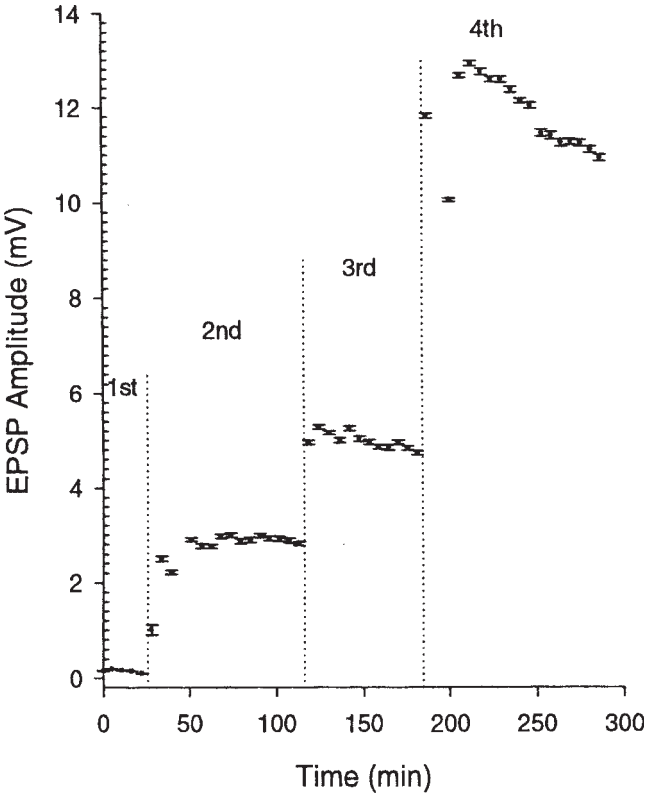


Fig. 10. Fatigue resistance of synaptic transmission was assayed by continuous stimulation at 10 Hz for each of the four different motor units in DMA. In sequential order, the neuron that gives rise to the smallest response was continuously stimulated at 10 Hz for 30 min without any sign of synaptic depression. In this particular experiment, the next largest response was then recruited for about 80 min also without depression. Finally, the third largest response was recruited for about 1 hr and lastly the largest response was recruited. The largest response did show depression after about 10 min but the decrease was small (~1.5 mV) over the next 2 hr. It is not known if the small decrease may be due to the depression of one of the previous three axons recruited, since they were continually stimulated throughout the entire period of time. Each EPSP was measured during the 10-Hz stimulation but only the average value for each 5 min is shown along with the standard error of the mean.

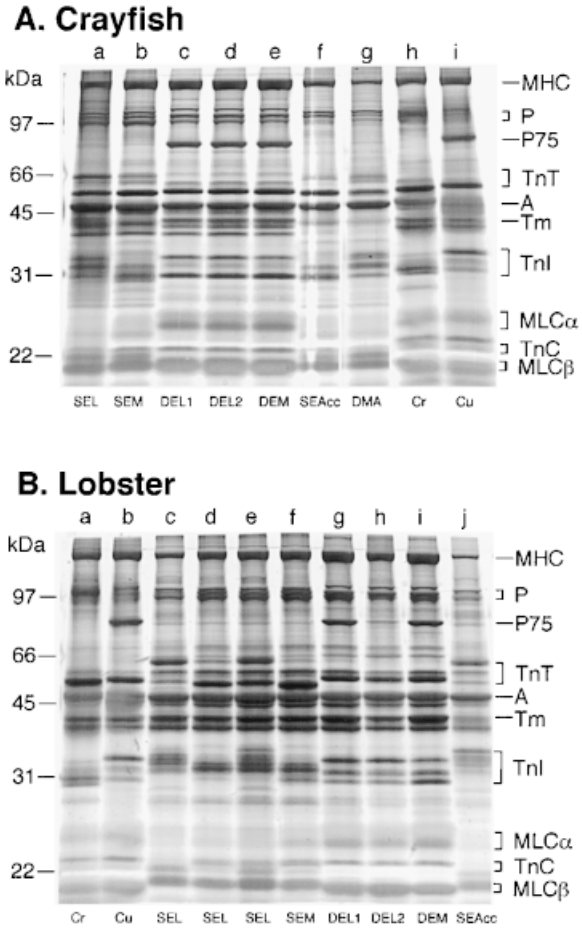


Fig. 11. Myofibrillar proteins of crayfish (**A**) and lobster (**B**) muscle fibers. Proteins from single glycerinated fibers were separated by SDS–PAGE and stained with silver (see Materials and Methods). Fibers analyzed were from superficial extensor lateral (SEL) and medial (SEM) muscles, deep extensor lateral 1 and 2 (DEL_1 and DEL_2) and medial (DEM) muscles, and superficial extensor accessory head (SEAcc) muscle from both species; dorsal membrane abdomen (DMA) muscle from crayfish; and crusher (Cr) and cutter (Cu) claw closer muscles from lobster. Fast fibers (DEL_1, DEL_2, DEM, and Cu) contain P75 regulatory protein absent from slow fibers (SEL, SEM, SEAcc, DMA, and Cr). Fibers also show variation in troponin-T (TnT), troponin-I (TnI), troponin-C (TnC), and myosin α and β light chain (MLC) isoforms. Three fibers from lobster SEL (lanes c–e) show isoform diversity within a single muscle. Other abbreviations: A, actin; P, paramyosin; MHC, myosin heavy chain; Tm, tropomyosin. Positions of molecular mass markers indicated at left.

P75 and TnT_2 were expressed exclusively in fast-twitch fibers (Fig. 12B and D, lanes b and g–i; Fig. 13B and D, lanes c–e and h). TnT_1 was only expressed in slow-tonic (S_2) fibers, although the amount varied reciprocally with a lower-mass isoform, TnT_3 (Fig. 12D, lanes c–e and j; Fig. 13D, lanes a and b). This reciprocity was shown previously in S_2 fibers from land crab claw closer muscle (Mykles, '88). Slow-twitch (S_1) fibers contained only TnT_3 (Fig. 12D, lanes a and f; Fig. 13D, lane f). The anti-troponin-I antibody recognized 6–7 isoforms in lobster fibers and 4–5 isoforms in crayfish (panel E in Figs. 12 and 13). There was significant variation in troponin-I isoform compositions between the different fiber types, and even within a particular fiber type (discussed below).

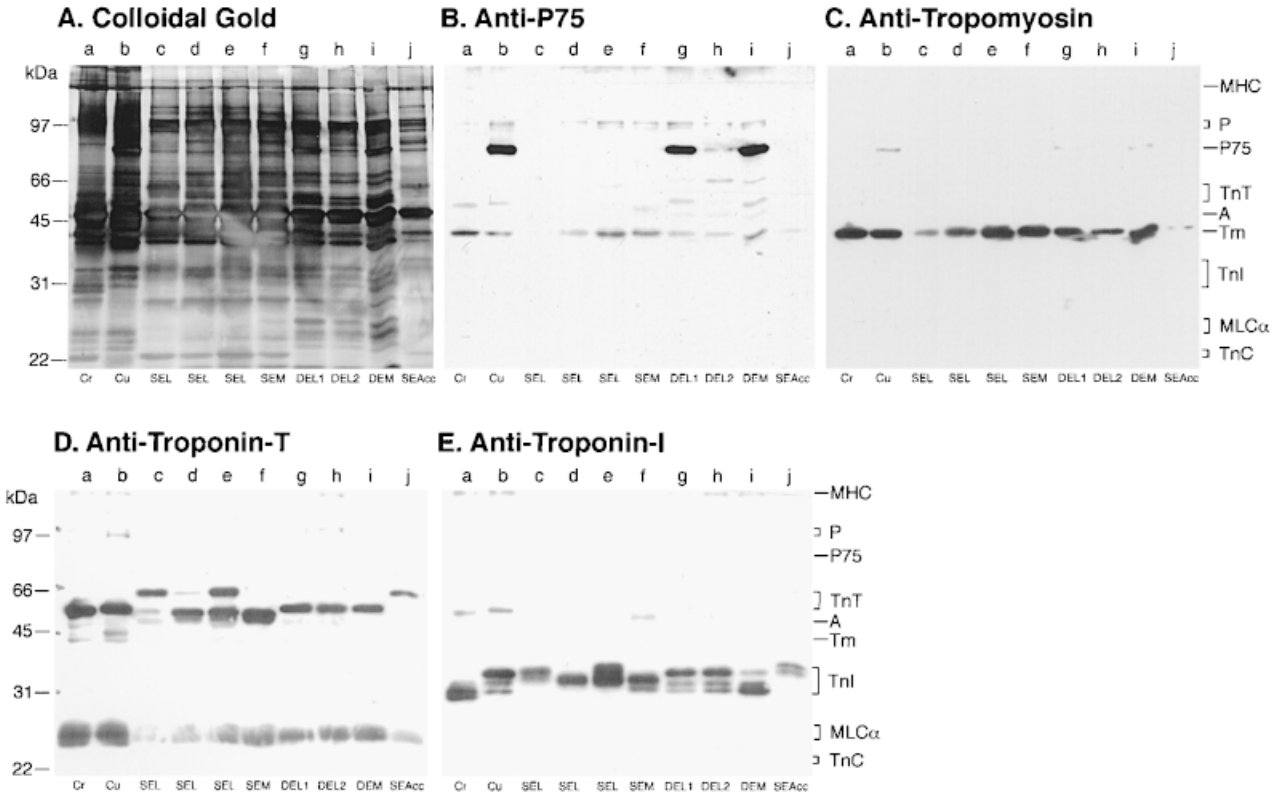


Fig. 12. Western blot analysis of lobster myofibrillar proteins. Proteins were separated by SDS–PAGE and transferred to PVDF membrane; blots were stained with colloidal gold (**A**) and probed with antibodies to P75 (**B**), tropomyosin (**C**), troponin-T (**D**), or troponin-I (**E**) (see Materials and Methods). P75 and tropomyosin (Tm) antibodies cross-reacted, indicating some sequence similarity between the two proteins. Fast fibers (lanes b and g–i) contained P75 and a single variant of troponin-T (TnT_2). Slow-tonic, or S_2, fibers (lanes c–e and j) contained a 55-kDa isoform of troponin-T (TnT_2), which varied reciprocally in abundance with TnT_2; these fibers also had higher-mass isoforms of troponin-I. Slow-twitch, or S_2, fibers (lanes a and f) contained only TnT_2. The diffuse staining in panel (D) between the 22- and 31-kDa markers was an artifact associated with the TnT antibody. See legend to Fig. 11 for abbreviations. Positions of molecular mass markers indicated at left.

DISCUSSION

The purpose of this study was to characterize the anatomical, physiological, and biochemical nature of the motor units associated with the dorsal articular membrane in the abdomen of the crayfish. The two muscles that were investigated, DMA and SEAcc, were first named by Pilgrim and Wiersma ('63). Amazingly, their known existence has escaped scientific inquiry since that time. Since it appears their functional role is to retract the articular membrane between the segments during abdominal movements, one would assume that they need to be regulated for fine gradual movements, during slow extension and flexion of the abdomen, as well as during the rapid tail flip movements used during escape behaviors.

Anatomy

The anatomical layout of the DMA and SEAcc muscles among the abdominal segments is quite intriguing. The angle of attachment from the articular membrane to the dorsal cuticle, in respect to the midline, varies for each segment. In the first abdominal segment (A1), the two muscles are about the same length and they are parallel to each other and to the midline, but in each subsequent caudal segment, the cuticular attachment is more lateral and there is a greater difference in the relative lengths between the muscles. The more medial SEAcc muscle becomes longer than the DMA from A1 to A5. In A5 the muscles cross each other at the ends of the cuticle attachment, strikingly different from the parallel arrangement

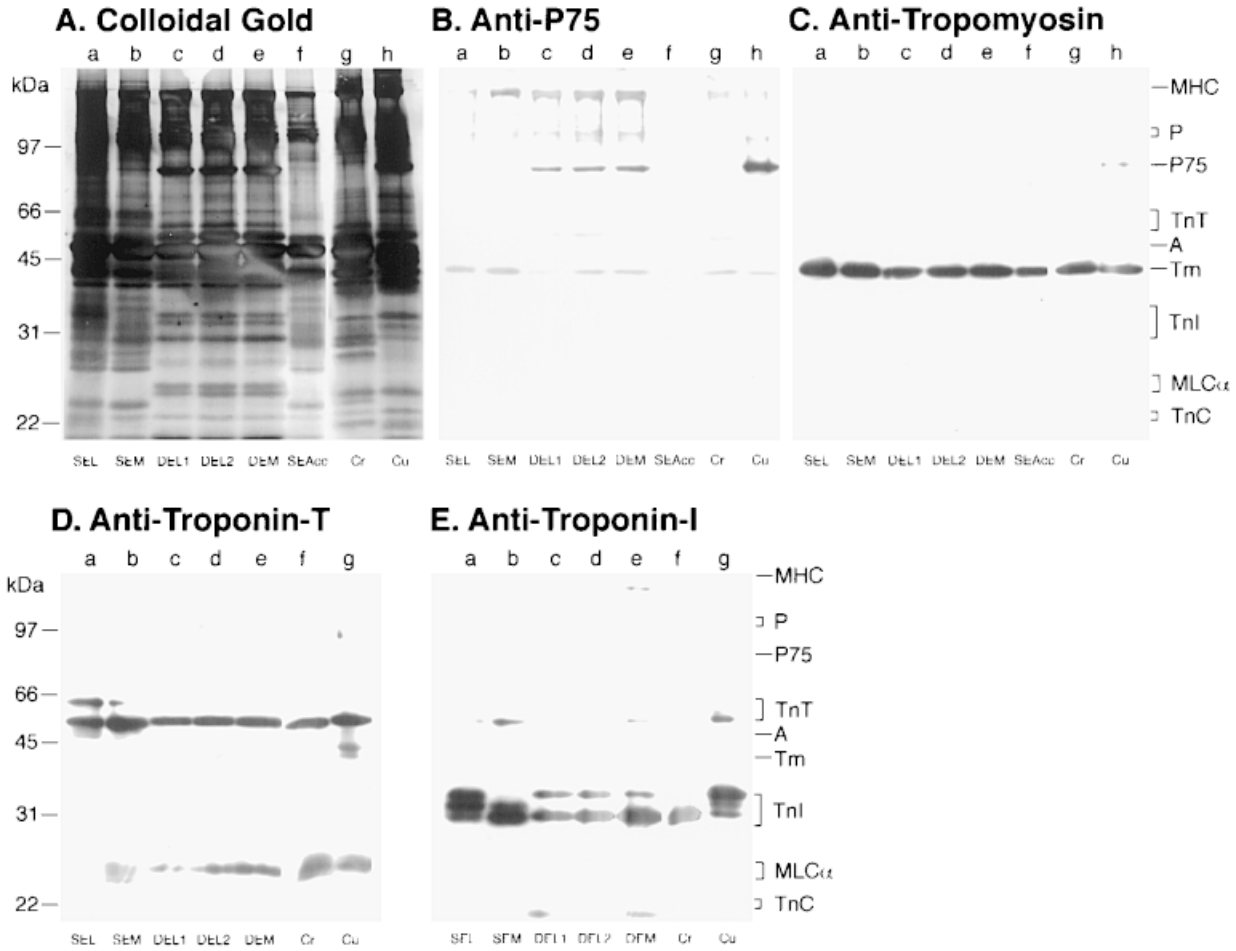


Fig. 13. Western blot analysis of crayfish myofibrillar proteins. Proteins were separated by SDS–PAGE and transferred to PVDF membrane; blots were stained with colloidal gold (**A**) and probed with antibodies to P75 (**B**), tropomyosin (**C**), troponin-T (**D**), or troponin-I (**E**) (see Materials and Methods). P75 and tropomyosin (Tm) antibodies cross-reacted, indicating some sequence similarity between the two proteins. Fast fibers (lanes c–e and h) contained P75 and a single variant of troponin-T (TnT_2). Slow-tonic, or S_2, fibers (lanes a and b) contained TnT_1 and TnT_3. Slow-twitch, or S_1, fibers (lane f) contained only TnT_2. The diffuse staining in panel (D) between the 22- and 31-kDa markers was an artifact associated with the TnT antibody. See legend to Fig. 11 for abbreviations. Positions of molecular mass markers indicated at left.

in A1. The functional significance for the shifts in angular attachments may likely be due to the range of movements in each segment. The rostral joints between the segments use a greater arc of movement as compared to the more caudal joints. Also, the segments are wider and have a larger surface area of articular membrane to control than the more caudal articular membranes. The muscular arrangements and area of membrane within an arc of movement for the abdominal joints could prove to be an interesting mechanical question for further investigation. In addition, we did not observe DMA or SEAcc muscles associated with the A6 articular membrane. Electron micrographs of the muscle attachments with the articular membrane show an extensive branching network of the cells into the articular membrane, likely to spread out the force of pulling on the membrane to a wider area.

As mentioned previously (see review by Atwood and Cooper, ’96b), the varicose nature of the terminals revealed by the 4-Di-2Asp staining, indicate that the motor neurons are tonic in nature, based on comparative data from other muscles in which physiology and terminal morphology have been compared in the crayfish: leg opener (Cooper et al., ’96a,b); leg extensor (King et al., ’96; Bradacs et al., ’97; Msghina et al., ’98); leg closer (Lnenicka and

Atwood, '88); and abdominal deep extensors (Lnenicka and Atwood, '85). The physiology and anatomical nature of these newly described motor neurons have now also been confirmed in the DMA and SEAcc abdominal muscles. Since the 4-Di-2Asp staining does not distinguish between excitatory and inhibitory terminals, immunocytochemistry was used to identify GABAnergic terminals (Dudel et al., '97). The results show that at least one axon in the nerve bundle and strings of varicose terminals spreads out on both DMA and SEAcc muscles. The axon profiles in the electron micrographs of the nerve bundle preceding the branching on to the DMA and SEAcc muscles show differences in diameters. This anatomical difference would supports our finding that individual units can be recruited separately by altering the intensity of the stimulus, since most likely it will take varying levels of stimulation to reach threshold among the different sized axons. The axon counts match reasonably well the number of physiologically and immunocytochemically identified units.

Future studies in identifying the density and dimensions of synapses and active zones in these low- and high-output tonic terminals observed among the four different axons on the DMA and SEAcc would be interesting and informative for comparative purposes, to determine if structural differences accounted for the synaptic differentiation in synaptic efficacies. Such undertakings have been accomplished for single tonic motor neurons that give rise to low- and high-output terminals (Jahromi and Atwood, '74; Govind et al., '94; Cooper et al., '96b,c) as well as for phasic and tonic terminals innervating the same muscle fiber (Walrond et al., '93; Bradacs et al., '95; King et al., '96). The higher output terminals have more complex synapses containing more active zones per synapse. Such correlations have now also been described for motor nerve terminals in *Drosophila* (Atwood et al., '93; Atwood and Cooper, '95, '96a; Stewart et al., '96).

Physiology

The physiological observations mirrored the anatomical findings in suggesting that the motor neurons innervating the DMA and SEAcc muscles are of the tonic phenotype. Also, the single sized amplitudes of IPSPs induced by trains of stimuli match the presence of a GABAnergic containing nerve on the muscles. Since the gross terminal anatomy cannot resolve differences among tonic excitatory terminals in their level of synaptic efficacy, physiological measures are required. Possibly at some later date when numbers of synapses and synaptic complexity can be examined with good spatial resolution, immunocytochemical approaches can be utilized to distinguish types of tonic terminals at the light microscopic level.

The induction of short-term facilitation, in the form of stimulus trains, allowed easy assessment of the various types of nerve terminals present on these muscles. The four different EPSP train profiles were common for both the DMA and SEAcc muscles for each of the abdominal segments from A1 to A5. However, in each segment tested there were differences among the fibers for a given muscle. More detailed studies will be needed to determine whether trends exist for a lateral to medial gradation in responses among the fibers for each of the four different excitatory responses in both the DMA and SEAcc muscles. For now, we can state with confidence that the responses do show some variation among fibers within the muscles. The axon that gives rise to the smallest EPSP responses would have been missed by physiological detection if facilitation studies using high enough frequencies were not employed. When 10-Hz trains were tested for 10 pulses in a train, the responses were not obvious above the noise level, but as the trains were tested at 10 Hz with at least 20 pulses in the train or at 20 Hz and higher frequencies for 10 pulses, then responses became detectable. Likewise, the use of only twin pulse facilitation would not have allowed detection of this low output axon. This stresses the point of using various stimulation and facilitation paradigms when assaying synaptic transmission (Crider and Cooper '99, 2000). Since the axon that gives rise to the smallest responses is recruited at low stimulus voltages and the axon which gives rise to the largest EPSPs requires larger voltages, it may indicate the axonal dimension of the corresponding axons in the nerve bundle. The subtracted responses allowed a better assessment of the individual properties of the four motor neurons without contamination of the measurements by the underlying responses. The ability to subtract out smaller responses from larger ones in the trains of EPSPs was not always possible since the larger EPSPs induced muscle contraction that resulted in movement artifacts in the recorded traces. Many preparations could not be used for analysis for this reason. In addition, the recruitment of the inhibitory axon during a train of EPSPs would result in responses not useable for quantification. The same phenomenon would result for the multiple excitatory

axons, such that during a train another axon would be recruited. This would result in large jumps in the EPSP amplitudes during the train. Such experimental problems were increased during trains of 20 pulses in duration and at the higher frequencies of stimulation, but were for the most part overcome by using 10 pulses within 20- and 40-Hz trains.

The clearest indication of the tonic nature in the four excitatory axons was the ability of each neuron to show fatigue resistance for hours during continuous 10-Hz stimulation. This stimulation paradigm would cause phasic neurons to depress much sooner (Atwood, '76; Bryan and Atwood, '81; Lnenicka et al., '86; Mercier and Atwood, '89; Atwood and Cooper, '96b; Cooper et al., '98).

Biochemistry

The skeletal muscles of decapod crustaceans express multiple isoforms of myofibrillar proteins. These include myosin HC and LC, paramyosin, tropomyosin, and troponin-T, -I, and -C (Costello and Govind, '84; Quigley and Mellon, '84; Mykles, '85a,b, '88; Kobayashi et al., '89; Li and Mykles, '90; Miyazaki et al., '90; Nishita, and Ojima, '90; Garone, '91; Ismail and Mykles, '92; Miegel et al., '92; Cotton and Mykles, '93; Günzel et al., '93; Miyazaki et al., '93; Neil et al., '93; Galler and Neil, '94; Sakurai et al., '96; Mykles et al., '98; LaFramboise et al., '99). Three major types, fast-twitch (F), slow-twitch (S_1), and slow-tonic (S_2), have been identified using myofibrillar protein isoform composition as well as physiological, morphological, and histochemical criteria. Qualitative and quantitative differences in isoforms that differ in molecular mass, such as myosin αLC, paramyosin, and troponin-I, -T, and -C, are useful for analysis of fibers with SDS–PAGE (Costello and Govind, '84; Mykles, '85a,b, '88; Nishita and Ojima, '90; Ismail and Mykles, '92; Miegel et al., '92; Neil et al., '93; Galler and Neil, '94; Sakurai et al., '96). Isoforms of myosin HC and tropomyosin have similar masses and thus cannot be distinguished on conventional single-dimension gels (Mykles, '85a,b; Mykles et al., '88; Li and Mykles, '90; Ishimoda-Takagi et al., '97). The most useful markers are proteins, such as P75 and troponin-T_1, that are expressed in one fiber type and not in the others. P75, which is exclusively expressed in fast-twitch fibers, is a regulatory protein related to tropomyosin, since antibodies to the two proteins cross-react (Figs. 12 and 13). Crustacean P75 may be homologous to troponin-H, an 80-kDa tropomyosin with a hydrophobic proline-rich extension, which is expressed in asynchronous muscles of insects (Karlik et al., '84; Bullard et al., '88). Troponin-T_1 occurs only in S_1 fibers; TnT_2 occurs only in fast fibers; and TnT_3 occurs in both S_1 and S_2 fibers (Figs. 11, 12, and 13). Thus, the presence and/or absence of P75 and troponin-T isoforms allow for unambiguous identification of the three fiber types.

Quantitative variation in troponin-I and -T isoforms show some degree of heterogeneity within fiber types. The fast-twitch fibers from lobster cutter claw and abdominal DEL and DEM muscles express TnI_1, TnI_3, and TnI_4 (Fig. 12E). However, TnI_4 is the dominant isoform in DEM, whereas TnI_1 is dominant in cutter, DEL_1, and DEL_2. In contrast, the fibers from crayfish abdominal DEL and DEM muscles have identical TnI isoform compositions (Fig. 13E). As many as three distinct fast-fiber subtypes that differ in contractile properties and pH lability of myosin ATPase have been identified in walking leg closer muscle of crab and crayfish (Maier et al., '84; Galler and Rathmayer, '92; Günzel et al., '93), but the isoform content was not analyzed in these studies. S_1 fibers from lobster crusher claw and abdominal SEM muscles differ in the relative amounts of TnI_2 and TnT_4; TnI_2 is greater than TnI_4 in SEM, while TnI_4 is greater than TnI_2 in crusher claw (Fig. 12E). S_2 fibers show the greatest diversity in troponin-I and -T isoform patterns. Three fibers from lobster abdominal SEL muscle represent the range of compositions observed in that muscle (Fig. 12D, lanes c–e). As shown previously in the land crab (Mykles, '88), TnT_1 varies reciprocally with a lower-mass variant (TnT_3) in the SEL fibers. These fibers also differ substantially in troponin-I isoforms (Fig. 12E, lanes c–e). The S_2 fibers from crayfish abdominal SEL and SEM muscles have different troponin-I isoform assemblages (Fig. 13E, lanes a and b). In addition, S_2 fibers from lobster DMA and SEAcc muscles have a similar TnI composition, which differ from those of S_2 fibers in SEL muscle (Fig. 11B, lane j; Fig. 12E, lane j; data not shown for DMA). In contrast, the S_2 fibers from crayfish DMA and SEAcc differ in TnI isoforms (Fig. 11A, compare lanes f and g). These data suggest that there is considerable heterogeneity in TnI isoform expression in S_2 fibers within a single muscle. Further work is needed to determine whether this heterogeneity has any physiological function. As a step toward that goal, we plan to analyze myofibrillar protein isoforms with respect to position within the muscle and innervation pattern.

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