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THE LOCUST OVIPOSITOR OPENER MUSCLE: PROPERTIES OF THE NEUROMUSCULAR SYSTEM

BY JIM H. BELANGER* AND IAN ORCHARD

Department of Zoology, University of Toronto, Toronto, Ontario, Canada, M5S 1A1

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Summary

A new preparation for studying properties of neuromuscular transmission and neuromodulation in insects, the ventral opener muscle of the locust ovipositor, is described. It consists of the complete musculoskeletal apparatus of the ventral opener muscle and the associated abdominal ganglia containing the central pattern generator that drives oviposition digging. In this preparation it is possible to record simultaneously motoneurone activity, muscle electromyograms, intracellular muscle fibre activity and behaviourally relevant tension production. The muscle displays ultrastructural and physiological characteristics typical of insect intermediate-type fibres: abundant sarcoplasmic reticulum, active Ca^{2+}-dependent membrane responses, phasic contractions when stimulated by high-potassium saline and a low twitch:tetanus ratio. Superfused glutamate (10^{-4} mol l^{-1}) and proctolin (10^{-8} mol l^{-1}) induce contractions of the muscle, while GABA (10^{-4} mol l^{-1}) reduces potassium-induced depolarizations. HPLC, bioassay and immunological methods show that proctolin is associated with all of the ventral ovipositor muscles, and combined back-filling/immunohistochemistry suggests that at least one of the ventral opener motoneurones is proctolinergic.

Introduction

Two of the major principles of invertebrate neuroethology are the concepts of central pattern generation and neuromodulation. It is well established that central pattern generators (CPGs) exist (Delcomyn, 1980; Roberts and Roberts, 1983; Grillner, 1985), although in many behaviourally relevant contexts sensory input forms an intrinsic part of the pattern-generating mechanism (e.g. in locust flight, Pearson and Wolf, 1987). It is equally well established that modulation, particularly by neuropeptides, plays a major role in the organization and generation of much invertebrate behaviour (Kravitz, 1988; Bicker and Menzel, 1989; Calabrese, 1989; Dickinson, 1989). What is very unclear at the moment is the precise role(s) played by this modulation. Considerable evidence shows that neuropeptides can alter the output of many pattern-generating circuits (see Marder *et*

*Present address: Arizona Research Laboratories, Division of Neurobiology, 611 Gould-Simpson Building, University of Arizona, Tucson, AZ 85721, USA.

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al. 1987; Harris-Warrick, 1988; Harris-Warrick and Marder, 1991). For example, proctolin can modulate both the gastric mill (Heinzel, 1988) and the pyloric CPGs (Hooper and Marder, 1987) in the lobster, and FMRFamide modulates the heartbeat pattern generator in the leech (Kuhlman *et al.* 1985). However, there are very few data on how modulatory neurones on the output side of CPGs are integrated into normal behaviour. Two of the few examples are Whim and Evans' (1988) study of the modulation of locust flight muscle, where octopamine enhances muscle twitch tension while increasing relaxation rate, and Whim and Lloyd's (1990) demonstration that the small cardioactive peptides (SCP_A and SCP_B) increase contractions of *Aplysia californica* buccal musculature. Given the importance of neuromodulators in invertebrate motor control (see, for example, Evans and Myers, 1986; Calabrese, 1989), it is essential to obtain some hard data on the relative contributions of peptidergic and 'conventional' motoneurones during the production of behaviourally meaningful muscle contractions. The goal in this and the following paper (Belanger and Orchard, 1993) is to introduce a preparation from which such data may be obtained.

Oviposition in many acridid insects consists of the female burying egg pods at depths of several centimetres in loose, moist soil (for a comprehensive review, see Uvarov, 1966). The excavation is accomplished by two pairs of heavily sclerotized, shovel-shaped ovipositor valves, located at the tip of the abdomen. The ovipositor valves are pressed into the substratum, and cyclical opening and closing of the valves 'ratchets' the abdomen into the ground (Vincent, 1975). The digging mechanism of the ovipositor consists of the valves, a pair of internal apodemes and ten pairs of muscles (Snodgrass, 1935). In an elegant series of studies, Thompson (1986*a*,*b*) described the functional anatomy and the motor programme underlying oviposition digging in *Schistocerca gregaria*. The behaviour is driven by a CPG located in the terminal abdominal ganglion (Thompson, 1982, 1986*a*), although sensory input appears to be important in the maintenance of the rhythm (Belanger and Orchard, 1992).

The largest ovipositor valve muscles are the dorsal and ventral openers, so-called because their contraction results in the valves swinging open. These muscles insert onto the bases of their respective valves posteriorly and along the ovipositor apodemes anteriorly. This allows the entire functional unit of the openers to be removed from the animal with its innervation intact. Since severing the ventral nerve cord is generally sufficient to initiate the digging motor programme (Thompson, 1986*b*), this preparation is ideal for examining the motor output from a CPG and its implementation at the muscular level. In this study, we describe the properties of the ventral opener muscle, its innervation and the characteristics of the opener neuromuscular junction. We show that the muscle responds to glutamate, gamma-aminobutyric acid (GABA) and the pentapeptide proctolin. We further show, using several independent methods, that proctolin is associated with all of the ventral ovipositor muscles, and that at least one of the five opener motoneurones may be proctolinergic. In a subsequent paper (Belanger and Orchard, 1993), we show that proctolin may not be simply a modulator *per se* in this system, but that it appears to be required for normal functioning of the muscle. Preliminary accounts of some of these results have appeared previously (Belanger and Orchard, 1987, 1988; Orchard *et al.* 1989).

Materials and methods

Animals

Mature (>15 days post final moult) adult female *Locusta migratoria* from a crowded colony at the University of Toronto were used. (During preliminary experiments we noticed a large amount of variability in the responses, to proctolin in particular. Much of this variability disappeared when experiments were conducted solely on animals interrupted during the process of oviposition. Therefore, only such animals were used for the physiological and pharmacological experiments.) The locusts were maintained at 30˚C under a 12h:12h L:D light regime and fed on wheat seedlings and bran. All experiments were performed at 26–28˚C.

Anatomical methods

The gross anatomy of the opener muscles was examined using either Methylene Blue (0.003%) or Janus Green (0.02%) for comparison with Thompson's (1982, 1986*a*) findings for *Schistocerca gregaria*. Backfills of the opener nerve were made using 10 % cobalt lysine in distilled water. The preparations were kept for 24h at 4˚C, after which the tissues were processed and subsequently silver-intensified following Davis (1982). They were then photographed, and drawn using a *camera lucida.*

For electron microscopy, ovipositor muscles, still attached to their apodemes, were dissected out in low-calcium saline (see below). The tissues were fixed for 0.5h in 1:1 low-calcium saline:4% glutaraldehyde in Millonig's buffer. They were then transferred for 0.5h to 4% glutaraldehyde in Millonig's buffer, after which small (<1mm radius) strips of muscle were cut and fixed for a further 22h at 22˚C in Millonig's buffer (pH7.4) containing 4% glutaraldehyde. The tissues were then washed, postfixed for 1h in 1 % OsO_4, washed, stained *en bloc* with 0.5% uranyl acetate, washed, dehydrated and embedded in Spurr's resin. Thin sections (80nm) were cut, stained with uranyl acetate and lead citrate, and examined on a Phillips 201 or Hitachi 7000 transmission electron microscope.

Physiology

Animals were anaesthetized by a brief (30s) exposure to CO_2. (If this was not performed, the animals tended to initiate the digging rhythm unpredictably during the dissection. With anaesthesia, the digging rhythm generally did not begin until shortly after the abdominal nerve cord was severed; see Thompson, 1986*b*.) They were then waxed down in a dish, covered with low-calcium saline (see below), and the ovipositor valve-opener muscle assembly was removed. Fig. 1 illustrates the preparation used for most of the physiological experiments reported here. It consisted of one pair of the ovipositor valves, their associated apodemes and opener musculature and, for some experiments, the final two ganglia of the abdominal nerve cord. It maintained the ventral opener muscle innervation from the terminal abdominal ganglion and the normal attachment sites of the muscles on the bases of the ovipositor valves and along the ovipositor apodeme. For pharmacological and high-potassium (see below) experiments, the dorsal opener muscle was disabled by cutting the tendon by which it attaches to the

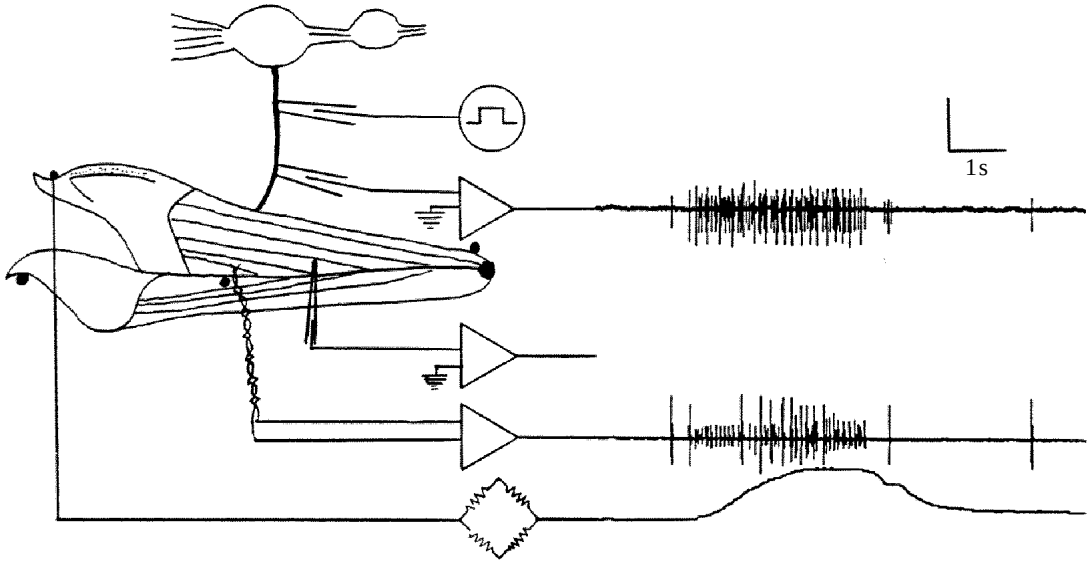


Fig. 1. Schematic diagram showing the isolated ganglion–muscle preparation used for most of these experiments. Suction electrodes were used to stimulate and record from (top trace) the opener nerve. Intracellular recordings and electromyograms (middle trace) were made from the ventral opener muscle. (No intracellular trace is shown because movement artefacts were too large in normal saline.) Ventral opener muscle tension was recorded using a force transducer attached to the ventral opener valve (bottom trace). Locations of the pins used to stabilize the preparation are shown by large black dots. The traces show one burst of opener activity driven by the digging CPG in the terminal abdominal ganglion. Scale bars: top trace 1 mV, middle trace 100 μV, bottom trace 100mg.

dorsal valve. This was done because contraction of the dorsal opener muscle also opens the ovipositor valves. It was not cut for experiments involving stimulation of the opener nerve, so that the ventral opener muscle could be examined in as near to its normal state as possible. For pharmacological experiments, as well as those involving neural stimulation, the abdominal ganglia were removed by cutting the eighth sternal nerve close to its emergence from the terminal ganglion. This was generally performed after the preparation had been expressing the oviposition digging rhythm in normal saline in the superfusion chamber for 30min. The preparation was placed in a recording chamber with a volume of approximately 0.5ml, where it was superfused with saline (see compositions below) at a rate of approximately $1 ml min^{-1}$. It was fastened by a minuten pin passing between the dorsal closer muscles and their apodeme (Fig. 1). A second pin was used as a ‘stop’ for the dorsal valve, while a third pin served as a stop for the anterior end of the apodeme. Tension in the muscle was recorded isotonically by attaching the ventral ovipositor valve to an AE875 miniature force transducer (Aksjeselskapet Mikroelektronikk, Norway) using a small hook made from piano wire. The transducer was always attached to the hollow of the small ‘hook’ at the posterior end of the valve. In this position, any behaviourally meaningful contraction of the ventral opener muscle resulted in an opening of the ovipositor valves, which is the natural action of the muscle

in vivo. Small contractions of single muscle fibres could occur without moving the ovipositor valve, so would not be detected by the force transducer. Therefore, before any stimulus was taken as producing no contraction, the muscle was visually monitored using a dissecting microscope.

Suction electrodes were used for extracellular nerve recording and stimulation. The recording electrode was positioned on the final branch of the nerve projecting to the opener muscle, while the stimulating electrode was placed on the nerve proximal to the recording site. Electromyographic recordings were made using a bipolar electrode fashioned from copper armature wire. This was inserted between the ventral opener muscle and its apodeme, approximately at the middle of the muscle. Intracellular recordings from opener muscle fibres were made using glass microelectrodes filled with $3\,\mathrm{mol\,l^{-1}}$ potassium acetate and having resistances of 30–60MΩ. All signals were amplified conventionally, and were generally stored on magnetic tape for later analysis. Some data were analyzed using an RC Electronics (Goleta, CA) EGAA digital data acquisition and analysis system. The minimum sampling rates used for digitization were 50kHz for extracellular nerve recordings, 10kHz for electromyograms and intracellular membrane potential and 1kHz for muscle tension recordings. The physiological experiments reported here are based on recordings from over one hundred animals.

Proctolin detection and immunohistochemistry

The presence of proctolin in various tissues of the ovipositor was determined using high performance liquid chromatography (HPLC) followed by bioassay on the locust oviduct. Details are given in Lange *et al.* (1986). To determine whether the bioactivity could be recognized by an anti-proctolin antiserum, tissues were extracted as for HPLC, and then samples equivalent to approximately 60fmol of proctolin-like bioactivity were made up to 50 μl using saline containing approximately $0.5\mathrm{units\,ml^{-1}}$ of the peptidase inhibitor bacitracin. 2 μl of either a rabbit anti-proctolin antiserum (kindly provided by Dr Tim Kingan) or normal rabbit serum were then added to the samples. These were incubated overnight at 4°C, and then tested for bioactivity on the locust oviduct.

The immunohistochemical methods were a modification of those performed by Lange *et al.* (1986). To block axonal transport and so increase the quantities of peptide present in the cell bodies, animals were injected with 10 μl of 0.3% colchicine, and the next day abdominal ganglia were dissected out in low-calcium saline and fixed. For double-labelling experiments, no colchicine was used, and opener nerves were backfilled overnight with cobalt lysine. The cobalt was then precipitated with ammonium sulphide, and the tissues fixed. The samples were processed identically subsequent to this with anti-proctolin antisera kindly provided by Drs Norman Davis and Paul Taghert. Details may be found in Lange *et al.* (1986). Controls were performed in which the histochemical antisera were preincubated overnight in a solution of $0.5\mathrm{mg\,ml^{-1}}$ proctolin (Peninsula Laboratories, Belmont, CA). All antibody staining was abolished by this treatment.

Solutions

Most physiological experiments were performed using a saline (‘normal saline’)

consisting of (in mmol l^{-1}): NaCl 150; KCl 10; $CaCl_2$ 4; $MgCl_2$ 2; $NaHCO_3$ 4; Hepes 5; trehalose 5; sucrose 90; pH7.2. For some experiments involving intracellular recordings, muscle contractions were reduced by using a similar saline in which the calcium concentration was reduced to 2mmol l^{-1} ('reduced-calcium saline'), or a saline in which the calcium concentration was further reduced to 1mmol l^{-1} and the magnesium concentration was increased to 4mmol l^{-1} ('low-calcium saline'). 'Calcium-free saline' was produced from normal saline by omitting calcium and adding 1mmol l^{-1} of the calcium chelator EGTA. Osmotic balance was maintained in each of these salines by adjusting the sucrose concentration. Potassium-induced contractions of the muscles were produced using normal saline in which the KCl concentration was increased to 100mmol l^{-1} ('high-potassium saline'). Osmotic balance was maintained by reducing the NaCl concentration to 105mmol l^{-1} and omitting the sucrose. Pharmacological agents were dissolved in saline and added *via* the superfusion system. All chemicals, except where noted, were purchased from Sigma (St Louis, MO).

Unless otherwise noted, statistical significance of the results was determined using Wilcoxon's test, with the Holm procedure used to correct for multiple simultaneous tests (Krauth, 1988). The significance level was set at $P<0.05$.

Results

Anatomical organization and mechanics of the muscle–valve system

At the level of gross anatomy, no major differences were found between the neuromuscular organization of the ovipositor of *Locusta migratoria* and that described for *Schistocerca gregaria* by Thompson (1986*a*). Semi-diagrammatic illustrations of the opener muscle in its resting and maximally contracted states are shown in Fig. 2. These show how the ventral opener fibres insert posteriorly along the entire base of the ventral valve, and anteriorly along the ventral face of the ovipositor apodeme. In principle, contraction of the ventral opener simultaneously depresses the ventral valve and deflects the apodeme ventrally. In practice, movement of the apodeme is prevented, partly by its rigidity, but mostly by the simultaneous action of the dorsal opener flexing it in the opposite direction.

Consideration of Fig. 2 reveals that the force produced at the ovipositor valve face is not related linearly to the tension produced by the opener muscle. Rather, only the component of the muscle tension that is normal to the valve base will produce a torque about the valve hinge. Therefore, if the ovipositor valve is assumed to be rigid, then the force normal to the valve face is equal to the opener muscle tension multiplied by the sine of the angle formed by the muscle and the valve base (see Fig. 2C). Over the range of natural valve movements, this angle varies from about 30° (at rest) to about 90° (at maximal opener contraction). This translates into a range of forces, at the valve face, from about half to full ventral opener muscle tension.

Because the ventral muscle attaches at sites along the valve base from the hinge to the ventral edge of the base, not all muscle fibres contribute equally to tension production. The fibres that attach furthest from the hinge have a mechanical advantage over fibres

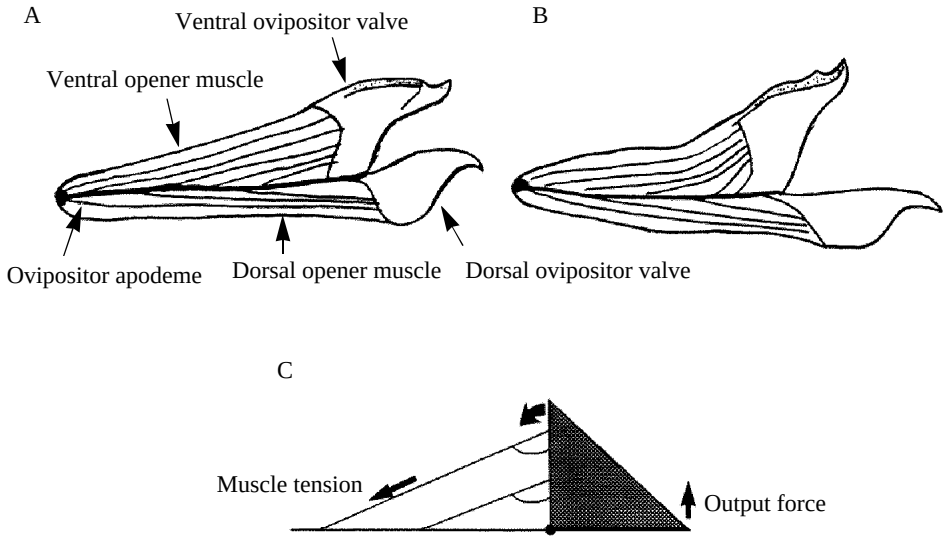
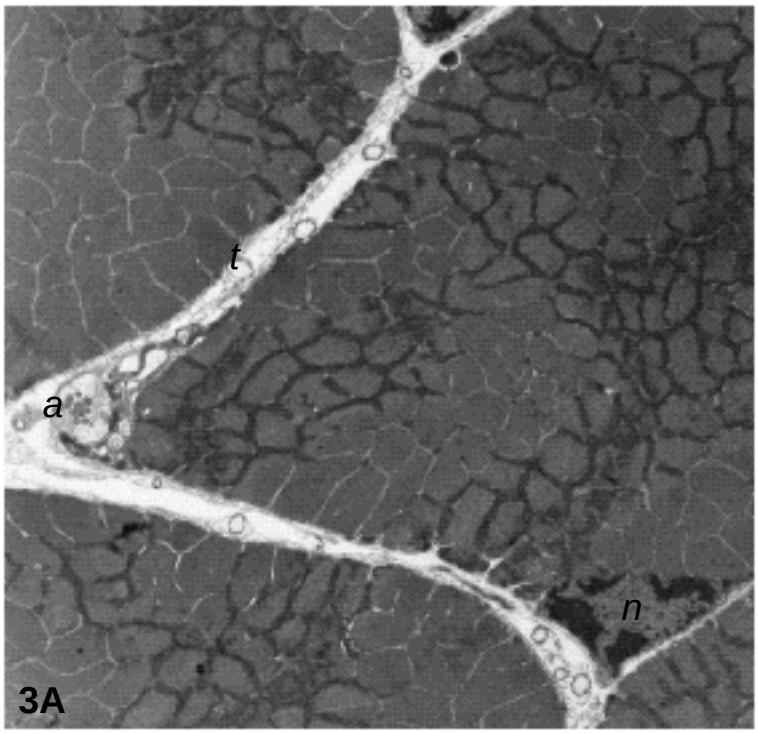


Fig. 2. Semi-diagrammatic illustrations (lateral views) of the opener muscle system with the opener muscle (A) relaxed and (B) contracted. The valves are shown in white, while the approximate orientation of the muscle fibres (although not their exact numbers) are shown by the lines. The ovipositor apodemes are dorsoventrally flattened cuticular structures that project anteriorly from the ovipositor valves and whose dorsal and ventral surfaces provide the anterior attachment sites for, respectively, the dorsal and ventral opener muscles. The apodemes are normally surrounded by the muscles and so are not visible, but their position is indicated by the heavy black line. The valves articulate where they meet the ovipositor apodemes. (C) Mechanics of the opener ovipositor valve system. The triangle represents the ventral valve, the horizontal line represents the ovipositor apodeme, and the diagonal lines indicate the orientation of the muscle fibres. The hinge site is indicated by the dot. Tension in the ventral opener produces a torque about the hinge between the ventral valve and the apodeme. Therefore, the force produced at the valve face, which was the muscle output measured in these studies, represents the actual tension in the muscle multiplied by the sine of the angle (indicated by the arcs) between the muscle and the valve base.

attaching close to the hinge. Since the fibres are all approximately parallel, if they were to contract simultaneously and at the same rate, the contribution each fibre would make to the total force would be based purely on its initial position. By contrast, the fibres of the dorsal opener muscle attach to a tendon which inserts at the dorsal edge of the base of the dorsal valve. Thus, all the fibres of the dorsal muscle have the maximum mechanical advantage in producing torque about the valve hinge. This may explain why the dorsal muscle can be so much smaller than the ventral muscle and still provide the opposition to the tension developed in the ventral muscle.

Histological sections (not shown) of the openers–apodeme assembly showed that the ventral muscle is composed of several hundred fibres, most of which run the entire length of the muscle. There was little heterogeneity amongst the fibres at the light microscope level. The sarcomere lengths ranged from approximately 6 to 9 μm, but the maximum range could frequently be seen in a single fibre, and there was no consistent variation correlated with fibre position. There was some variation in staining of the fibres but, as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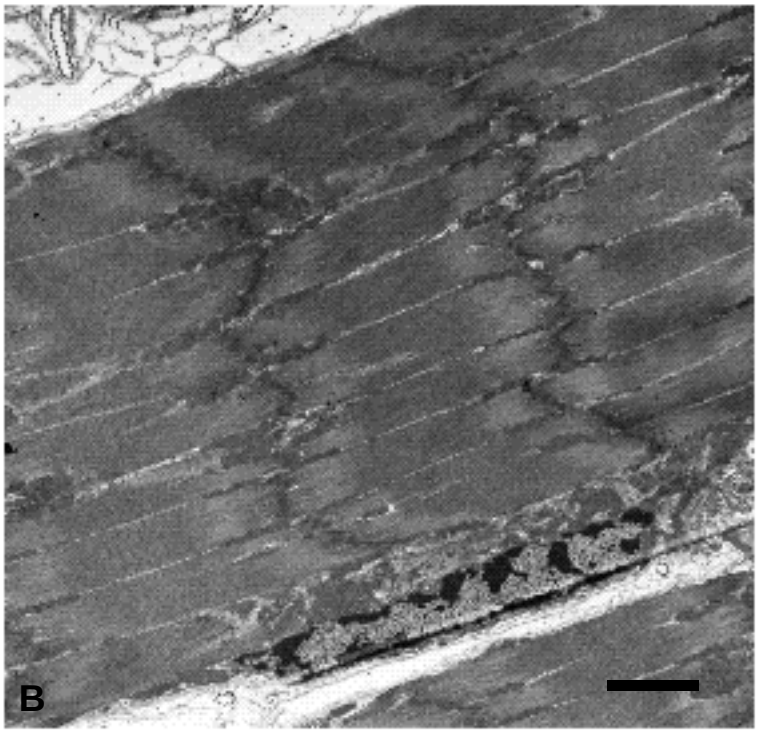
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Fig. 3. Ultrastructure of the ventral opener muscle. (A) Survey electron micrograph of a cross section through the ventral opener muscle, near the dorsal surface. Most of one muscle fibre is shown, along with the fibre nucleus (*n*), an associated axon (*a*) and numerous tracheae (*t*). The plane of this section is close to a Z-line, as indicated by the fact that some sarcomeres are separated by only sarcoplasmic reticulum (light areas), while others have mitochondria (dark areas) between them. (B) Survey electron micrograph of a longitudinal section through the ventral opener muscle, near the dorsal surface. The width of an entire fibre is shown to illustrate the relative proportions of sarcomere constituents. The fibre nucleus is also present. Scale bar, A 2.2 μm; B 2.0 μm.

this was seen even from section to section in the same tissue block, it was attributed to capriciousness of the stain.

Ultrastructure of the ventral opener muscle

At the ultrastructural level, the ventral opener muscle had an organization characteristic of close-packed insect skeletal muscle (Fig. 3). Seen in cross section, the sarcomeres were irregular polygons with variable dimensions (Fig. 3A). Their shape means that diameter is not a very useful descriptive term, but none of the myofibrils was more than about 0.4 μm from the sarcoplasmic reticulum. The thick filaments were arrayed in a typical hexagonal lattice, with a thin to thick ratio of about 6:1. In muscles fixed at approximately rest length, the sarcomere length was rather variable, ranging from approximately 6.5 to 9 μm (Fig. 3B). The A-bands were 3.5–5.5 μm long, and the I-bands 3–5 μm, with the A-bands consistently being about 50% of the sarcomere length. The mitochondria were clustered around the Z-lines, packed in single file for a distance of 10–20% of the sarcomere length towards the next Z-line. The Z-lines of the sarcomeres were generally in register, although there was some variation through any given fibre. There were no obvious M-lines. The sarcoplasmic reticulum was relatively abundant, and dyads were common. These were seen most frequently at the margins of the A-bands. Based on measurements from both longitudinal and cross sections, an estimate was made of the approximate volumes of the fibre occupied by myofilaments, mitochondria and the internal tubule systems (T-tubules and sarcoplasmic reticulum). Neglecting any other fibre constituents, these three occupied approximately 80% (range of about 70–90%), 10% (5–15%) and 10% (5–15%), respectively, of the fibre volume. We did not notice any systematic variation in ultrastructure, although it must be noted that only a small number (several sections from two different blocks from each of three preparations) of samples was examined.

Neuromuscular junctions were distributed regularly along the fibres (Fig. 4). These were always located deep within the muscle and never found on the surface (see Fig. 3A). The majority of the axonal profiles contained clear-core spherical vesicles approximately 45nm in diameter (Fig. 4). However, occasional profiles also contained electron-dense spherical granules of approximately 150nm diameter (Fig. 4 inset). These were only seen in profiles that also contained the smaller clear-core vesicles.

Innervation of the ventral opener muscle

Backfilling of the ventral opener nerve consistently revealed the presence of three large

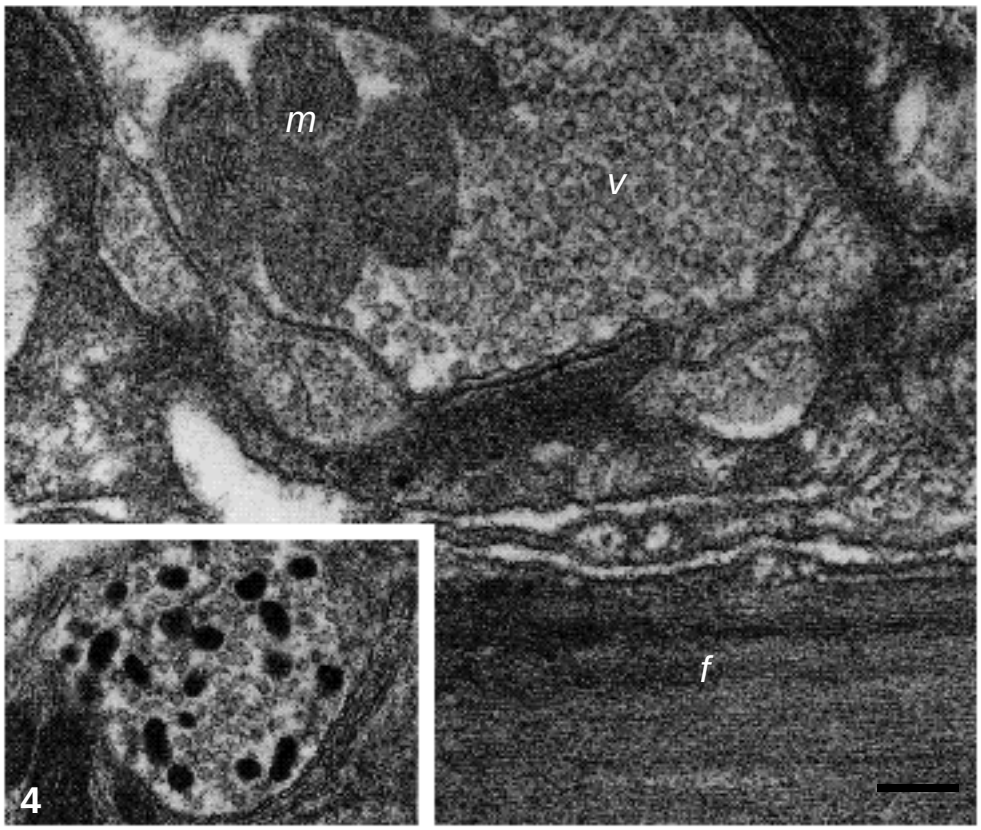

Fig. 4. Neuromuscular contacts of the ventral opener muscle. The figure shows an apposition between an axonal profile containing clear-core vesicles (*v*) and mitochondria (*m*) and an opener muscle fibre (*f*). The inset shows an axonal profile which contains large electron-dense vesicles in addition to the clear-core ones. Scale bar, 225nm; inset 450nm.

and two smaller lateral somata on the dorsal surface of the terminal abdominal ganglion (Fig. 5A) as well as, occasionally, one or two dorsal unpaired median (DUM) neurones. These findings were supported by extracellular recordings of activity in the opener nerve (Fig. 5B). In preparations expressing the digging rhythm, six spikes could usually be distinguished on the basis of size, with occasionally one much smaller spike. It was not, however, always possible to recruit each of these spikes in turn with increasing stimulation strength. Rather, by stimulating an opener nerve that was isolated from the ganglion, only four different spikes could usually be clearly recruited. Presumably, some of the axons were either too similar in size, or located inopportunely with respect to the stimulating electrode, to be differentially excited. In any given preparation, it was always possible to stimulate either just the two lowest-threshold axons or to stimulate all of the (presumed) motor axons together.

Intracellular muscle physiology

Intracellular recordings from the fibres of the opener muscle showed them to possess

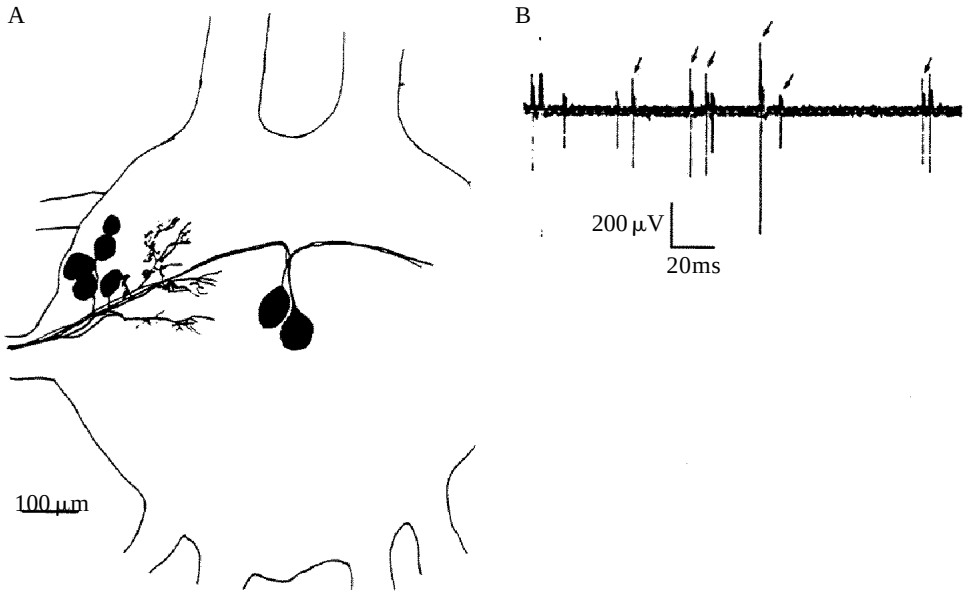

Fig. 5. Innervation of the ventral opener muscle. (A) *Camera lucida* drawing of a cobalt backfill preparation showing cells in the terminal abdominal ganglion which project to the ventral opener muscle. The somata of the five presumptive motoneurones of the muscle are grouped on the ipsilateral margin of the ganglion, while the two central somata appear to be DUM neurones. (B) Extracellular recording of spontaneous spikes in the opener nerve. This record is from a preparation expressing the oviposition digging rhythm. Six different spikes are distinguishable on the basis of size (arrows).

resting potentials, in normal saline, ranging from −45 to −75mV. Fig. 6 shows representative recordings from two fibres, using one electrode to pass current while monitoring membrane potential with a second. While the input resistances of these two fibres were the same (as were the approximate fibre diameters based on estimates with an ocular micrometer), one produced anode-break responses, while the other did not. This variation, plus the potential differences in muscle fibre tension contributions based on their position of attachment to the ovipositor valve, led us to look for variation in fibre properties that was related to location in the muscle. In six preparations that were systematically examined, there was no consistent correlation between resting membrane potential or input resistance and fibre position, but there was a lesser likelihood of obtaining anode-break responses from more ventral fibres. Approximately 50% of fibres on the dorsal half of the muscle produced anode-break responses, compared with less than 20% of the fibres on the ventral half.

Miniature excitatory junctional potentials (mEJPs) were invariably recorded (Fig. 7A). These were rather large, frequently having amplitudes of several millivolts, and were seen in the absence of activity in the opener nerve. To ensure that these were mEJPs, and not activity due to small axons whose spikes we were perhaps unable to detect, muscles were bathed in 10^{-5} mol l^{-1} tetrodotoxin. In the six preparations examined, this abolished all evoked responses from the opener muscle, but had no effect on the measured frequency of mEJPs (data not shown).

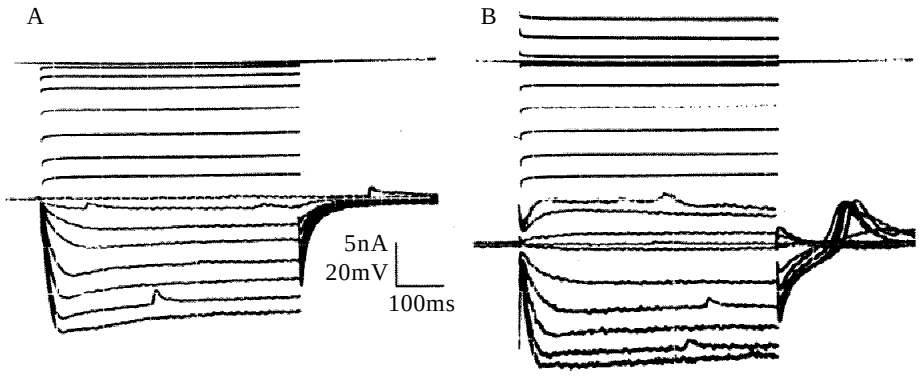

Fig. 6. Two-electrode investigations of intracellular responses to injected current of two different opener muscle fibres. The more ventral fibre (A) had a resting membrane potential of −70mV, while the more dorsal fibre (B) had a resting potential of −61mV and produced anode-break responses. The two fibres had similar input resistances and approximately similar diameters. The top traces monitor current injected *via* one electrode, while the bottom traces monitor membrane potential *via* the second electrode. The experiments were performed in normal saline.

Activity of the digging rhythm CPG in the terminal abdominal ganglion produced excitatory junctional potentials (EJPs) in opener muscle fibres (Fig. 7B). These could usually only be recorded in low-calcium saline (1mmol l^{-1} Ca^{2+} and 4mmol l^{-1} Mg^{2+}), as otherwise the resulting muscle contractions displaced the intracellular electrode. However, maintained perfusion with low-calcium saline induced considerable variation in the size of EJPs, which was seen clearly in data from evoked EJPs. In one experiment, performed after 30min of low-calcium saline perfusion, fifty successive stimuli applied to the opener nerve at 1Hz recruited the same population of axons (as judged from the extracellular record of opener nerve activity) each time, but 80% of the stimuli failed to produce EJPs in an opener muscle fibre (Fig. 7C), and none produced a detectable rise in muscle tension. The wide variation in the rate of rise of the evoked EJPs seen at the recording site makes it likely that many were produced at synapses of varying electrotonic distance from the recording electrode. Similar results were seen in muscle fibres from six other preparations tested in low-calcium saline. In no case was the likelihood of producing an EJP dependent on the number of preceding stimuli (i.e. the failures did not seem to be due to depletion of transmitter). In reduced-calcium saline (2mmol l^{-1} Ca^{2+} and 2mmol l^{-1} Mg^{2+}), failures were extremely rare. Therefore, to avoid the synaptic depression associated with low-calcium saline, most experiments involving evoked EJPs were performed in reduced-calcium saline. Under these conditions, most opener muscle fibres produced graded electrogenic potentials in response to EJPs (Fig. 7D). The active component often seen in these was only rarely seen in low-calcium saline, even though many of the EJPs were larger than those eliciting active responses in reduced-calcium saline. This suggests that the active response is due to a calcium influx.

At least some opener muscle fibres receive multiple excitatory innervation. Recruiting multiple units by increasing the electrical stimuli applied to the opener nerve increased the resulting EJPs in a stepwise manner in most of the fibres tested (Fig. 7D). Frequently,

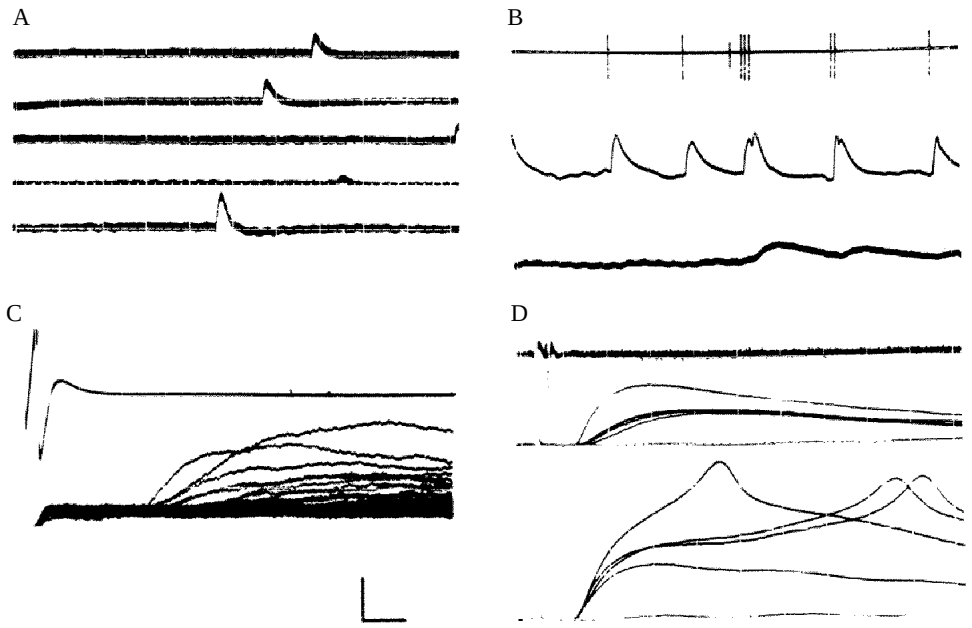

Fig. 7. EJPs in opener muscle fibres. (A) Miniature EJPs. This is a continuous a.c.-coupled recording from a fibre with a resting potential of −60mV. Scale bars, 5mV, 100ms. (B) EJPs in an opener fibre resulting from digging CPG activity in the opener nerve. The upper trace shows extracellular opener nerve activity, the middle trace shows intracellular muscle fibre potential, and the lower trace shows opener muscle tension in low-calcium saline. The EJPs match the large opener spikes one for one, but are not all associated with muscle twitches. Scale bars, 1mV, 10mV, 200mg, 50ms. (C) Fifty stimuli applied to the opener nerve at 1Hz after prolonged superfusion with low-calcium saline. The stimuli all recruited the same population of opener spikes (top trace), but only approximately 20% produced EJPs. Note the wide variation in EJP size and latency, probably resulting from the recruitment of synapses at different distances from the recording electrode. Scale bars, 5mV, 2mV, 2ms. (D) Simultaneous recordings from two muscle fibres showing recruitment in the opener muscle. Five superimposed sweeps of increasing stimulus intensity recruit sequentially more units in the opener nerve (top trace), producing increasing EJPs in a ventral (middle trace) and a dorsal (bottom trace) muscle fibre, resulting in active responses and twitches (not shown) in the dorsal, but not the ventral, fibre. Scale bars, 200 μV, 10mV, 10mV, 5ms.

the increase was sufficient to produce active responses and twitches in the fibres (Fig. 7D, lower trace).

Muscle contractile properties

There is considerable variation in the size of the ventral opener muscle. (Based on the quantity of protein present, there is at least a threefold variation in muscle mass.) Because of this, it is not very meaningful to average muscle contraction data across different preparations, and this has not been done. Rather, typical experimental results are presented, and only those findings that could be replicated at least six times. Further, we suspected early in this investigation that presynaptic depletion of modulatory transmitter substances might be of physiological significance in this system (see Belanger and

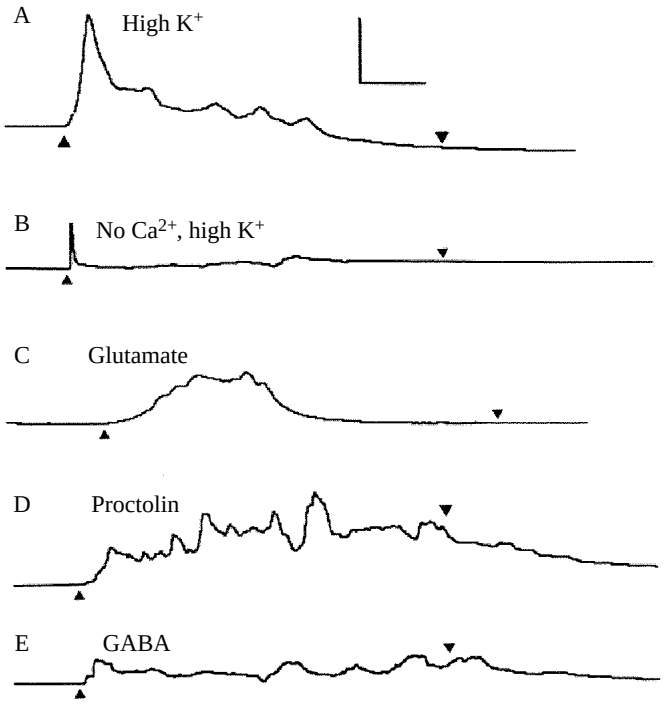

Fig. 8. Contractile properties of the ventral opener muscle. (A) Superfusion with high-potassium saline (between arrowheads) produces a phasic contraction which subsides, despite the continued presence of the stimulus. Scale bars, 200mg, 100s. (B) High-potassium saline in the absence of external Ca^{2+} produces a smaller, extremely brief response. Scale bars, 100mg, 100 s. (C) Superfusion with 10^{-4} mol l^{-1} glutamate also produces a phasic response. Scale bars, 100mg, 50s. (D) Superfusion with 10^{-8} mol l^{-1} proctolin produces a prolonged contraction, which persists for the duration of the stimulus. Scale bars, 200mg, 100s. (E) After continuous superfusion with saline containing 10^{-4} mol l^{-1} GABA, switching to high-potassium saline also containing 10^{-4} mol l^{-1} GABA produces very little response, suggesting that GABA is attenuating the potassium-induced depolarization. Scale bars, 50mg, 100s.

Orchard, 1993). Therefore, to ensure that we were examining purely muscle properties, the high-potassium and neural stimulation experiments reported here were performed on muscles that had been expressing the oviposition digging rhythm in normal saline superfusion for at least 30min. This should have been sufficient to exhaust presynaptic stores of cotransmitters (Belanger and Orchard, 1993).

Mechanical responses of the muscle to potassium depolarization were phasic, declining back to resting levels despite continued superfusion with high-potassium saline (Fig. 8A). The onset of the contraction was somewhat slow, but this is at least partly due to the turnover time of the superfusion chamber and partly to the mechanical properties of the muscle–valve system, discussed above. When muscles were depolarized after prolonged superfusion (15min) with calcium-free saline, there was still a phasic contraction (Fig. 8B), but the amplitude of these contractions was significantly smaller than those seen in normal saline (rank-sum test, N=6). The observation that a contraction

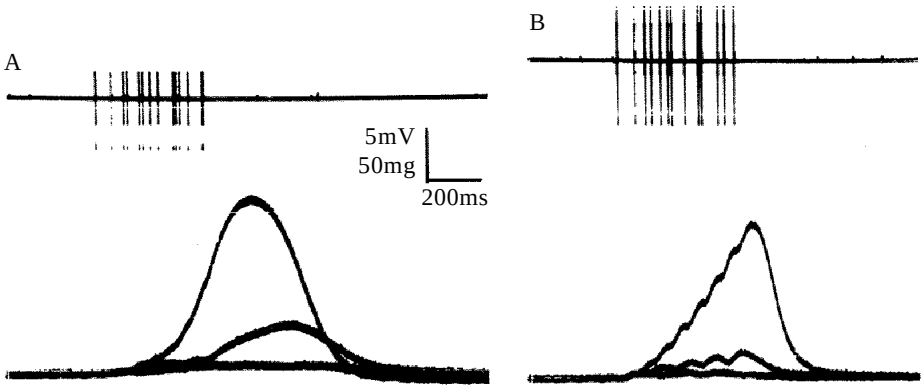

Fig. 9. Muscle contractile responses to neural stimulation. (A) Stimulation of just the two lowest-threshold units in the opener nerve with 500ms trains of pulses at 5, 10 and 15Hz produces slow, graded contractions. (B) Stimulation of all units in the same preparation at the same frequencies produces a contraction that both peaks and decays more quickly. Scale bars, top traces opener nerve 5mV; bottom traces muscle tension 50mg; 200ms.

occurred at all suggests that an influx of extracellular Ca^{2+} is not an absolute requirement for producing a contraction.

The muscle had a low twitch:tetanus ratio, with stimulation at less than 1Hz producing either no response or very slight twitches, typically less than 5mg of force (Fig. 9). As the frequency of stimulation increased, the resulting tension also increased significantly, up to hundreds of milligrams, depending on the size of the individual muscle. Stimulating different populations of opener axons resulted in differing tension responses. In the preparation illustrated, stimulation of just the two lowest-threshold units in the opener nerve produced smooth, graded contractions. When all units in the nerve were recruited, individual twitches could be seen in the response, and both the peak tension and the subsequent relaxation occurred more quickly.

Muscle pharmacology

At most insect neuromuscular synapses, the classical excitatory transmitter appears to be L-glutamate (Aidley, 1985). When opener muscles were superfused with 10^{-4} mol l^{-1} glutamate, phasic contractions, similar to those seen in high-potassium saline, were produced (Fig. 8C). Contractions were also produced when the muscle was superfused with the peptide proctolin (10^{-8} mol l^{-1}, Fig. 8D). Unlike the glutamate or potassium contractions, however, these contractions were tonic, persisting for as long as the peptide was present. Proctolin also produced a variety of other, dose-dependent, effects, which are dealt with in the following paper (Belanger and Orchard, 1993).

We never saw inhibitory junctional potentials (IJPs) during intracellular recordings of existing patterned activity. However, because many insect skeletal muscles receive inhibitory, GABAergic innervation (Aidley, 1985), and because IJPs can be difficult to detect, the opener muscle was tested for sensitivity to GABA. Muscles were superfused with 10^{-4} mol l^{-1} GABA, and then depolarized with high-potassium saline which also contained GABA (Fig. 8E). This resulted in contractions the amplitudes of which were

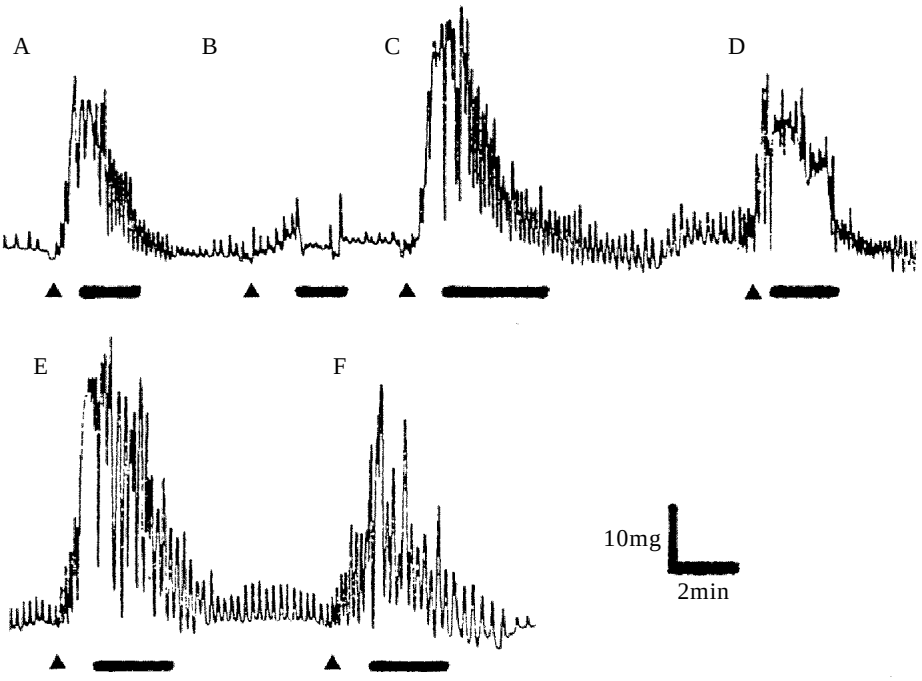

Fig. 10. Responses of the locust oviduct to extracts of the ventral ovipositor opener muscle. (A) Application of opener muscle extract (arrowhead) causes a proctolin-like response consisting of an increase in basal tension of the oviduct and increased myogenic contractions. These effects are reversed by washing (heavy line). (B) Incubating extracts overnight with anti-proctolin antiserum plus the peptidase inhibitor bacitracin abolishes the response. Incubating the extract with normal serum plus bacitracin (C), or with bacitracin alone (D), does not diminish the bioactivity. Bottom panel shows the oviduct responses to 20 (E) and 10 (F) fmol of proctolin.

significantly reduced (rank-sum test, N=6) compared to those in high-potassium saline in the absence of GABA.

Presence of a proctolin-like peptide

The observation that the opener muscle produced changes in basal tension when exposed to proctolin suggested that a proctolin-like peptide could be associated with the muscle tissues. Accordingly, the ventral ovipositor muscles were surveyed for proctolin-like bioactivity. When tested using the locust oviduct as a bioassay (Lange *et al.* 1986), proctolin-like bioactivity was found in extracts of the ventral opener muscle (Fig. 10A), the other ventral ovipositor muscles and the terminal two abdominal ganglia (data not shown).

We also tested for the possibility that the proctolin-like bioactivity could be recognized by an anti-proctolin antibody. In initial experiments, incubating muscle extracts with normal rabbit serum overnight at 4˚C resulted in the loss of all bioactivity. However, when the peptidase inhibitor bacitracin was added to the extracts, there was no loss of bioactivity (Fig. 10C). When the extracts were incubated with an anti-proctolin antiserum

Table 1. *Proctolin content* of various tissues of the ovipositor of* Locusta migratoria

Tissue	*N*†	fmol per tissue‡	pmol mg^{-1} protein‡
Ventral closer muscle	4	70±26	0.90±0.21
Ventral opener muscle	4	290±91	0.68±0.08
Ventral retractor muscle	5	57±33	0.85±0.32
Ventral protractor muscle	5	47±26	1.97±0.90
Abdominal ganglion VII	4	72±21	3.79±0.71
Abdominal ganglion VIII	4	241±66	9.39±2.63

*As determined from proctolin-like bioactivity on the locust oviduct.
†For each determination, 4–6 tissues were pooled.
‡Values are mean±S.E.M.

plus bacitracin, virtually all of the proctolin-like bioactivity was abolished (Fig. 10B). Muscle extracts containing bacitracin produced oviduct contractions not significantly different (rank-sum test, N=6) from the controls (Fig. 10D). Again, similar results were found for all of the ventral ovipositor muscles and the terminal two abdominal ganglia (data not shown).

To quantify the amount of material present, extracts were analyzed using HPLC, and then those fractions that co-eluted with proctolin were bioassayed on the locust oviduct. In this manner, we found that at least 87% of the proctolin-like bioactivity present in the tissues co-eluted with proctolin (data not shown). When this proctolin-like material was run through a second, sequential HPLC system, again at least 87% of the bioactivity co-eluted with proctolin (data not shown). Thus, approximately 75% of the bioactivity present was chromatographically indistinguishable from proctolin. The quantities of proctolin-like bioactivity, corrected according to these HPLC data, associated with each of the ventral ovipositor muscles and the final two abdominal ganglia are shown in Table 1.

The association of proctolin-like material with the opener muscles implied that at least one of the neurones projecting to the ventral opener should be proctolinergic, so we looked for cells corresponding to the opener muscle neurones that were proctolin-immunoreactive. The findings reported here are based on 30 preparations, 16 of which were stained with an antiserum provided by Dr Paul Taghert and 14 with antiserum provided by Dr Norman Davis. Cells on the dorsal surface of the terminal abdominal ganglion that stained repeatedly with both of the antisera are shown in Fig. 11A. Most notable was a cluster of six large cells grouped in a rosette in the centre of the ganglion, and a number of bilaterally paired clusters around the margin. We obtained reliable staining by both antisera of only three cell bodies on the ventral surface of the ganglion (Fig. 11B). Two of these were large midline cells, and one was an unpaired cell on the midline at the anterior margin of the ganglion. Both antisera stained a variable number of other cells on both surfaces of the ganglion, particularly in the posterior midline portion. However, since this staining varied from preparation to preparation and did not involve any cells potentially projecting to the opener muscle, we have not included these data. One of the dorsal groups of cells is in approximately the same position as the ventral opener motoneurone cell bodies, but it was quite difficult to obtain staining of this group

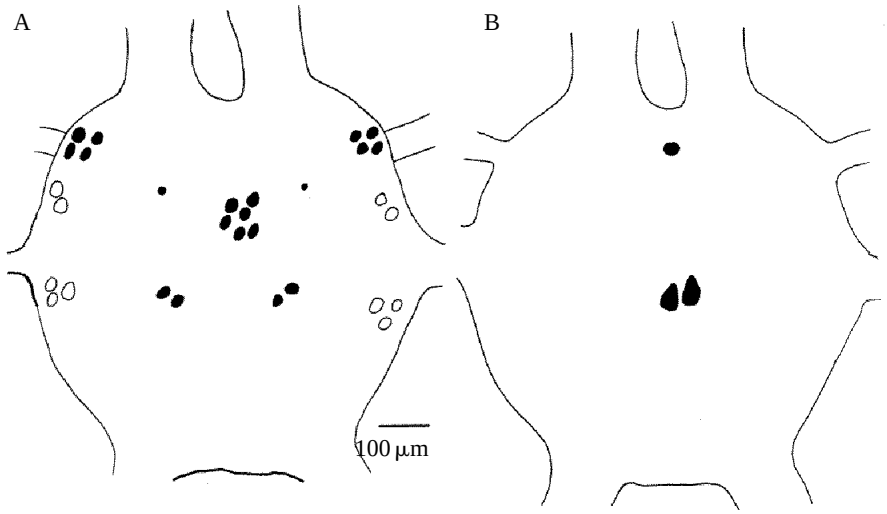

Fig. 11. Locations of neurones on the dorsal (A) and ventral (B) surfaces of the terminal abdominal ganglion which displayed proctolin-like immunoreactivity. Only cells that were reliably stained by two different anti-proctolin antisera are shown. Cells that were intensely stained are dark, while lightly staining cells are shown as outlines. Anterior is at the top.

(and several other of the lateral cells) in the absence of colchicine. Unfortunately, colchicine injection interfered with the backfilling experiments. Nonetheless, in five preparations, we obtained weak staining of these cells and simultaneous cobalt backfills of the opener motoneurones. (The combination of weak staining and cobalt precipitate made it impossible to obtain satisfactory photographs of these preparations.) Whenever this occurred, one of the bilaterally paired groups was weakly stained, while its contralateral homologue was absent, presumably because the cobalt precipitate was masking the fluorescent label. In the absence of cobalt, we never saw cases where only one member of a bilaterally paired group was stained, while the other was not. This suggests that at least one, and possibly two, proctolinergic motoneurones project to the ventral opener muscle.

Discussion

This study has presented a broad survey of basic morphological, physiological and pharmacological properties of the locust ovipositor ventral opener muscle. It is intended to form the basis for work using the opener muscle as a model system for the investigation of the role(s) of modulation in non-tonic insect muscle systems. The opener muscle displays most of the physiological and ultrastructural characteristics typical of an insect intermediate-type muscle. The most important ultrastructural characteristic in determining muscle contraction speed and duration appears to be the quantity of internal membrane systems, particularly of sarcoplasmic reticulum (Aidley, 1985; Hoyle, 1983; Josephson and Young, 1987) and the value of approximately 10% of total fibre volume found in this study agrees well with the range of 7–19% reported by Cochrane *et al.*

(1972) for phasic skeletal fibres in *Schistocerca gregaria*. It is also similar to the quantities found by Huddart and Oates (1970) in *Locusta migratoria* extensor tibialis (up to 16%, estimated from their data). The ratio of myofibrillar volume to tubule system volume, approximately 8:1, is considerably higher than the values of 2:1 to 4:1 found in cicada tymbal muscles (Josephson and Young, 1987), but the twitches of the opener are also considerably longer. The phasic contractions of several seconds duration are similar to those reported by Cochrane *et al.* (1972). The low twitch:tension ratio, however, is more typical of tonic muscles (Aidley, 1985).

The major functions of the ventral opener appear to be to pull the abdomen down into the oviposition hole (Vincent, 1975) and to oppose the displacement of the ovipositor apodeme during the digging thrust of the dorsal opener. Thus, it needs to shorten considerably, as well as to provide significant tension. The relatively long A- and I-bands of the sarcomeres are consistent with these tasks. The long I-bands mean that the muscle can shorten by up to 50% of its rest length, while the long A-bands provide more cross-bridges, and hence more tension. In connection with this, Aidley (1985) has suggested that high I:A ratios (like the 6:1 ratio found in the ventral opener) are associated with long A-filaments, and presumably act to reduce the strain on the individual I-filaments.

The variation in excitability of different fibres of the opener, seen in their differing likelihoods of producing anode-break responses and variation in the duration of the active component, is also seen in other insect skeletal muscles (see, for example, Deitmer and Rathmayer, 1976). It is not clear whether these differences are specifically adaptive for the opener muscle or merely reflect the general heterogeneity of insect muscle fibres. It is possible, although speculative, that the more dorsal muscle fibres show an increased likelihood of spiking as compensation for their low mechanical advantage in rotating the ovipositor valve.

Mechanical activation of the opener muscle in the apparent absence of extracellular calcium is rather unusual for an arthropod muscle (reviewed in Caillé *et al.* 1985). It is possible, however, that our methods did not completely remove extracellular calcium. While nominally Ca^{2+}-free saline plus a chelating agent such as EGTA would be expected to remove extracellular calcium, a concentration of $1\,mmol\,l^{-1}$ EGTA may be insufficient to remove all calcium ions from the T-tubule system (Barret and Barret, 1975). This could be a particular difficulty in the opener muscle, where there are extensive T-tubules (see Fig. 3). Thus, regardless of whether the extracellular calcium is acting as a trigger for release from an internal store, or is participating directly in myofibrillar activation, there could be a sufficient quantity remaining in the T-tubules to permit a phasic contraction. Based on the quantity of calcium likely to be present in the T-tubules (of the order of $mmol\,l^{-1}$, assuming equilibrium with the haemolymph), the relative volumes of the T-tubule system and the myofibrillar space and the fact that the calcium-free contracture is of the same order of magnitude as that seen in normal saline, it seems unlikely that extracellular calcium is playing a major role in the actual contractile process.

The combination of HPLC, bioassay and antibody data makes it fairly certain that the proctolin-like bioactivity associated with the ventral opener muscle is indeed proctolin.

The levels found (0.68pmolmg^{-1}protein) are quite similar to the values reported for other arthropod muscles (see Tables 2 and 4 of Orchard *et al.* 1989). Likewise, the amounts found in the abdominal ganglia and the other ventral muscles of the ovipositor (Table 1) are similar to values in the literature (Orchard *et al.* 1989). The large electron-dense vesicles seen in axon profiles (Fig. 4) may represent peptidergic material (Peters *et al.* 1991) and could contain proctolin. The smaller, clear-core vesicles are typical of neurotransmitter vesicles (Peters *et al.* 1991) and are therefore likely to contain either glutamate or GABA. Immunohistochemistry at the ultrastructural level could confirm these hypotheses.

Aidley (1985) has suggested that some correlation can be drawn between the complexity of innervation of a particular insect muscle and the diversity of mechanical output required from that muscle. In addition to oviposition digging, the ovipositor valves, and hence the opener muscles, are involved in egg-laying (gaping and some directing of the eggs as they issue from the oviduct) and in producing the frothy plug which caps the egg pod (Uvarov, 1966; Thompson, 1982, 1986*a*; J. H. Belanger, unpublished observations). It is not clear how much control over mechanical output is required for these activities. Certainly there are clear differences in the demands on the muscle during digging, a relatively phasic action, and the sustained gaping often produced during egg-laying. At least some of this variety is probably superimposed on the basic properties of the opener muscle *via* the action of neuromodulators such as proctolin (see Belanger and Orchard, 1993).

The apparent complexity of the control of insect muscle, as seen in the bewildering heterogeneity at the various levels of ultrastructure, innervation, transmitter and modulator types, etc., makes it essential to have data at all of these levels in order to pursue any understanding of function. Especially important, because of the possible multiple messengers involved, is the need specifically to correlate motoneurone input with muscle output. Thus, it has long been known that ‘fast’ and ‘slow’ motoneurones have different effects (Aidley, 1985), but it is now important to try to establish how much of this variety is due to differences in intrinsic physiology of the neurones, how much to the fact that they release different transmitters and cotransmitters, and how much to the interplay between these. Kupfermann (1991) has recently emphasized the need for systems in which it is possible to unravel these functional aspects of neuromodulation, which are probably widespread in the animal kingdom. With the aid of model systems such as the one presented here, some order can be imposed on this complexity.

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References

AIDLEY, D. J.(1985). Muscular contraction. In *Comprehensive Insect Biochemistry and Physiology*, vol. 5 (ed. G. A. Kerkut and L. I. Gilbert), pp. 407–437. Oxford: Pergamon Press.

BARRET, J. N. AND BARRET, E. F. (1975). Excitation–contraction coupling in skeletal muscle: blockade by high extracellular concentrations of calcium buffers. *Science* **200**, 1270–1272.

BELANGER, J. H. AND ORCHARD, I. (1987). Association of a proctolin-like peptide with the ovipositor muscles of *Locusta migratoria. Soc. Neurosci. Abstr.* **13**, 235.

BELANGER, J. H. AND ORCHARD, I. (1988). Proctolinergic modulation of neuromuscular transmission at an ovipositor muscle of *Locusta migratoria. Soc. Neurosci. Abstr.* **14**, 534.

BELANGER, J. H. AND ORCHARD, I. (1992). The role of sensory input in maintaining output from the locust oviposition digging central pattern generator. *J. comp. Physiol* A (in press).

BELANGER, J. H. AND ORCHARD, I. (1993). The locust ovipositor opener muscle: proctolinergic central and peripheral neuromodulation in a centrally driven motor system. *J. exp. Biol.* **174**, 343–362.

BICKER, G. AND MENZEL, R. (1989). Chemical codes for the control of behaviour in arthropods. *Nature* **337**, 33–39.

CAILLÉ, J., ILDEFONSE, M. AND ROUGIER, O. (1985) Excitation–contraction coupling in skeletal muscle. *Prog. Biophys. molec. Biol.* **46**, 185–229.

CALABRESE, R. L.(1989). Modulation of muscle and neuromuscular junctions in invertebrates. *Seminars Neurosci.* **1**, 25–34.

COCHRANE, D. G., ELDER, H. Y. AND USHERWOOD, P. N. R. (1972). Physiology and ultrastructure of phasic and tonic skeletal muscle fibres in the locust, *Schistocerca gregaria. J. Cell Sci.* **10**, 419–441.

DAVIS, N. T. (1982). Improved method for cobalt filling and silver intensification of insect motor neurons. *Stain Technol.* **57**, 239–244.

DEITMER, J. W. AND RATHMAYER, W. (1976). Calcium action potentials in larval muscle fibres of the moth *Ephestia kühniella* Z. (Lepidoptera). *J. comp. Physiol.* **112**, 123–132.

DELCOMYN, F.(1980). Neural basis of rhythmic behaviour in animals. *Science* **210**, 492–498.

DICKINSON, P.(1989). Modulation of simple motor patterns. *Seminars Neurosci.* **1**, 15–24.

EVANS, P. D. AND MYERS, C. M.(1986). Peptidergic and aminergic modulation of insect skeletal muscle. *J. exp. Biol.* **124**, 143–176.

GRILLNER, S. (1985). Neurobiological bases of rhythmic motor acts in vertebrates. *Science* **228**, 143–148.

HARRIS-WARRICK, R. M. (1988). Chemical modulation of central pattern generators. In *Neural Control of Rhythmic Movements in Vertebrates* (ed. A. H. Cohen, S. Rossignol and S. Grillner), pp. 285–331. New York: John Wiley and Sons.

HARRIS-WARRICK, R. M. AND MARDER, E. (1991). Modulation of neural networks for behaviour. *A. Rev. Neurosci.* **14**, 39–57.

HEINZEL, H. G. (1988). Gastric mill activity in the lobster. II. Proctolin and octopamine initiate and modulate chewing. *J. Neurophysiol.* **59**, 551–565.

HOOPER, S. L. AND MARDER, E. (1987). Modulation of the lobster pyloric rhythm by the peptide proctolin. *J. Neurosci.* **7**, 2097–2112.

HOYLE, G. (1978). Distributions of nerve and muscle fibre types in locust jumping muscle. *J. exp. Biol.* **73**, 205–233.

HOYLE, G.(1983). *Muscles and their Neural Control.* New York: John Wiley and Sons.

HUDDART, H. AND OATES, K. (1970). Ultrastructure of stick insect and locust skeletal muscle in relation to excitation–contraction coupling. *J. Insect Physiol.* **16**, 1467–1483.

JOSEPHSON, R. K. AND YOUNG, D. (1987). Fiber ultrastructure and contraction kinetics in insect fast muscles. *Am. Zool.* **27**, 991–1000.

KRAUTH, J.(1988). *Distribution-Free Statistics.* Amsterdam: Elsevier Science Publishers.

KRAVITZ, E. A.(1988). Hormonal control of behaviour: Amines and the biasing of behavioural output in lobsters. *Science* **241**, 1775–1781.

KUHLMAN, J. R., LI, C. AND CALABRESE, R. L. (1985). FMRFamide-like substances in the leech. II. Bioactivity on the heartbeat system. *J. Neurosci.* **5**, 2310–2317.

KUPFERMANN, I. (1991). Functional studies of cotransmission. *Physiol. Rev.* **71**, 683–731.

LANGE, A. B., ORCHARD, I. AND ADAMS, M. E.(1986). Peptidergic innervation of insect visceral muscle – The association of proctolin with oviduct visceral musculature. *J. comp. Neurol.* **254**, 279–286.

MARDER, E. E., HOOPER, S. L. AND EISEN, J. S. (1987). Multiple neurotransmitters provide a mechanism for the production of multiple outputs from a single neuronal circuit. In *Synaptic Function* (ed. G. M. Edelman, W. E. Gall and W. M. Cowan), pp. 305–327. New York: John Wiley and Sons.

ORCHARD, I., BELANGER, J. H. AND LANGE, A. B. (1989). Proctolin – a review with emphasis on insects. *J. Neurobiol.* **20**, 470–496.

PEARSON, K. G. AND WOLF, H.(1987). Comparison of motor patterns in the intact and deafferented flight system of the locust. *J. comp. Physiol.* A **160**, 259–268.
PETERS, A., PALAY, S. L. AND WEBSTER, H. DEF. (1991). *The Fine Structure of the Nervous System*, Third Edition. Oxford: Oxford University Press.
ROBERTS, A. AND ROBERTS, B. L. (eds) (1983). *Neural Origin of Rhythmic Movements.* Cambridge: Cambridge University Press.
SNODGRASS, R. E. (1935). The abdominal mechanisms of a grasshopper. *Smithson. misc. Collns* **94**, 1–87.
THOMPSON, K. J. (1982). The neural basis of the motor program for grasshopper oviposition digging. PhD dissertation, University of Oregon.
THOMPSON, K. J. (1986*a*). Oviposition digging in the grasshopper. I. Functional anatomy and the motor program. *J. exp. Biol.* **122**, 387–411.
THOMPSON, K. J. (1986*b*). Oviposition digging in the grasshopper. II. Descending neural control. *J. exp. Biol.* **122**, 413–425.
UVAROV, B.(1966). *Grasshoppers and Locusts.* Cambridge: Cambridge University Press.
VINCENT, J. F. V.(1975). How does the female locust dig her oviposition hole? *J. Ent.* **50**, 175–181.
WHIM, M. D. AND EVANS, P. D.(1988). Octopaminergic modulation of flight muscle in the locust. *J. exp. Biol.* **134**, 247–266.
WHIM, M. D. AND LLOYD, P. E. (1990). Neuropeptide cotransmitters released from an identified cholinergic motor neuron modulate neuromuscular efficacy in *Aplysia*. *J. Neurosci.* **10**, 3313–3322.