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THE LOCUST OVIPOSITOR OPENER MUSCLE: PROCTOLINERGIC CENTRAL AND PERIPHERAL NEUROMODULATION IN A CENTRALLY DRIVEN MOTOR SYSTEM

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Summary

The pentapeptide proctolin has multiple effects on the locust oviposition digging system. At the neuromuscular junction of the ventral opener muscle, it has a concentration-dependent range of modulatory effects. At low concentrations (10^{-10} mol l^{-1}), proctolin causes an increase in the frequency of miniature excitatory junctional potentials, but has no apparent effects on the muscle membrane or contractile properties. In the middle range of concentrations (approximately 10^{-9} mol l^{-1}) proctolin increases neurally evoked twitch tension three- to fourfold with little change in the basal tension. At high concentrations (>10^{-8} mol l^{-1}), proctolin causes a large increase in basal tension, upon which is occasionally superimposed a slow (approximately 0.3–0.5Hz) myogenic rhythm. Stimulation of the ventral opener nerve at 30Hz for 5min releases approximately 8% of the proctolin store of the muscle. *In vitro* ganglion–muscle preparations which are expressing the oviposition digging rhythm produced in the terminal abdominal ganglion release about 25% of the store of endogenous proctolin during 5min of superfusion. This declines to below the level of detectability over about 20min of superfusion. Muscle contractions decline and then cease over the same period, although the patterned neural input and muscle electromyogram responses are still present. Superfusion of 10^{-9} mol l^{-1} proctolin restores the muscle contractions to their original magnitude. Superfusion of 10^{-8} mol l^{-1} proctolin over preparations in which the oviposition digging pattern has slowed results in the frequency of the rhythm being restored to its original levels. We suggest that, rather than having a facultative modulatory role in this neuromuscular system, proctolin is required for it to function normally. Furthermore, proctolin may maintain the functional integrity of the central systems driving oviposition digging.

Introduction

Recent years have seen an explosion in the number of identified myotropic neuropeptides in insects. It is still far from clear, however, why insect systems possess so

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many different signal molecules, or even whether all of these signals are used in a physiological manner. The first of these myotropic peptides to be discovered was proctolin, isolated and sequenced over fifteen years ago by Brown and Starratt (1975). In the interim, proctolin has been shown to have a wide range of effects in several different phyla (reviewed by O'Shea and Adams, 1986; Orchard *et al.* 1988). Virtually all of these effects are modulatory, typically characterized by a slow onset and long duration. This has made it difficult to elucidate what, if any, specific roles in motor control are being played by the peptide. A major stumbling block in this effort has been a lack of preparations in which it is possible to correlate peptide effects with the natural performance of the system.

In the preceding paper (Belanger and Orchard, 1993), we described a new insect neuromuscular preparation based on the ventral opener muscle of the locust ovipositor. It is possible in this preparation simultaneously to record efferent activity, intracellular muscle potentials, electromyographs and behaviourally relevant muscle force during the production of a centrally driven behaviour. The ventral opener muscle is a typical insect skeletal intermediate-type muscle and its major function is in digging the oviposition hole, although it is also involved in egg-laying and in producing the frothy cap of the egg pod (Uvarov, 1966; Thompson, 1982, 1986). The motor pattern driving this behaviour is produced by a central pattern generator (CPG) in the terminal abdominal ganglion (Thompson, 1986) and this ganglion also contains the five opener muscle motoneurones, at least one of which appears to be proctolinergic (Belanger and Orchard, 1993). In the present paper, we show that proctolin has a dose-dependent range of effects at the ventral opener neuromuscular junction: increased frequency of miniature excitatory junctional potentials (mEJPs) at low concentrations, enhanced neurally evoked twitch tension at midrange concentrations and dose-dependent increases in basal tension at high concentrations. Proctolin is released both by electrical stimulation of the motor nerve and by activity of the digging CPG. We further show that proctolin appears to be required for the normal functioning of the muscle. Muscle contractions produced by the oviposition digging pattern decrease over time *in vitro* and this decrease is paralleled by a decrease in proctolin released into the superfusate. Superfusing proctolin restores the contractions to their original magnitude. In addition, proctolin affects the oviposition digging CPG, restoring the initial frequency of the rhythm in preparations in which it has slowed. Preliminary accounts of some of these results have appeared previously (Belanger and Orchard, 1988, 1992*a*; Orchard *et al.* 1988).

Materials and methods

Details of the animals and most of the experimental methods used were presented in the preceding paper (Belanger and Orchard, 1993). To ensure that all the animals started the experiments with similar amounts of presynaptic stores, all the experiments were conducted on animals that had been interrupted during oviposition and then kept overnight in a container which held no suitable oviposition substratum. Any animals that released their eggs during this period were not used.

Measurement of proctolin release

Release of proctolin by the opener muscle system was measured using a modification of the methods of Orchard and Lange (1986). In preparations undergoing patterned digging activity, the superfusate was collected over sequential 5min periods. For experiments on stimulated release, the abdominal ganglia were removed and the opener nerve was stimulated continuously for 5min at 10 or 30Hz while the superfusate was collected. Superfusate samples from either protocol were treated identically. They were conditioned through Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) to desalt and concentrate the samples. After the superfusate samples had been run through three times, the cartridges were washed with 8ml of water and then extracted with 3ml of 30 % propanol. The propanolic extracts were then dried down in a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY) and the samples reconstituted with 40 μl of normal saline containing 10^{-6} mol l^{-1} phentolamine. This was to prevent possible effects of released octopamine on the bioassay (see Orchard and Lange, 1985). The extracts were then tested, in 20 μl volumes, for proctolin-like bioactivity on the locust oviduct. The quantity of material present was assessed by comparing these responses to those produced by proctolin standards. Locust oviducts have a log–linear dose-dependent response to proctolin in the range of approximately 2–200fmol (Lange *et al*. 1986). In the previous paper (Belanger and Orchard, 1993), we showed that at least 75% of the proctolin-like bioactivity associated with the opener muscle is chromatographically indistinguishable from proctolin.

Presentation and analysis of data

There was considerable variation present in the time-dependence of some of the effects presented here. For that reason, we frequently present results from one preparation, but these are all typical of results obtained from at least six preparations. 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

Spontaneously produced motor spikes differentially affect muscle tension

Analysis of simultaneous recordings of muscle tension and patterned activity from the digging CPG revealed that, in every preparation examined, not all units in the opener nerve appear to contribute equally to tension production (Fig. 1). In the example shown, recorded shortly after the start of superfusion and before definite patterned activity had begun, there are five clearly identifiable units and they have easily distinguishable effects on muscle tension. Two of the units appear to be 'fast'-type axons, in that they produce easily discernible twitches. The other units produce increases in tension that are comparable in size, but are much slower and smoother and require a higher frequency of firing. This is typical of insect 'slow' axons. In the ten preparations examined in this way, in every case it was possible to discern similar populations of spikes. A detailed analysis of these revealed that the majority of the activity in the opener nerve was accounted for by

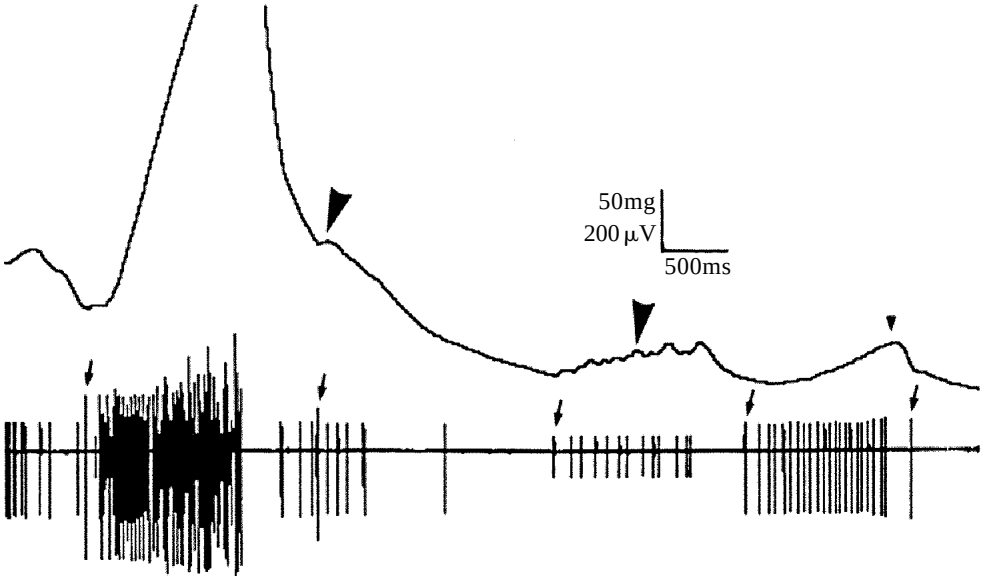

Fig. 1. Variation in the contributions of opener motoneurone action potentials to the production of muscle tension. The extracellular trace (lower) shows spontaneous activity in the opener nerve resulting from the digging CPG in the terminal ganglion, shortly after the start of superfusion with normal saline. Five clearly different spike sizes can be seen (arrows). Two of these produce discrete twitches of the muscle (top trace, single twitch at the first large arrowhead, a series at the second). One produces a slow, smooth increase in muscle tension (small arrowhead). Note that the peak of the large muscle contraction is clipped.

one population of spikes (Fig. 2). This spike population was never one that produced discernible twitches of the muscle in response to single spikes, but always appeared to be of the slow type. Associated with this, the general structure of opener nerve bursts was usually the same, displaying an increasing frequency of slow spikes throughout the burst, with fast spikes appearing in the latter portion of the burst (Fig. 3). In the examples shown, the fast spikes are larger than the slow ones, but we have deliberately chosen to show examples where this was the case, because it makes it easier to see the relative firing times and rates. There were also preparations where fast spikes were smaller than the slow ones (e.g. Fig. 1).

The fact that the spike frequencies were changing throughout the burst means that it is not particularly useful to describe the burst by its average frequency. This is especially true since the bursts are of different lengths, so that different bursts that have the same average frequency may be delivering different numbers of action potentials to the muscle. This combination of effects prevented us from simply relating spontaneous neural input to muscle output. We were, however, able to relate electrically evoked neural input to muscle tension (see below).

Modulatory effects of proctolin on the opener muscle

The apparent differences in the efficacy of the various motoneurone spikes led to

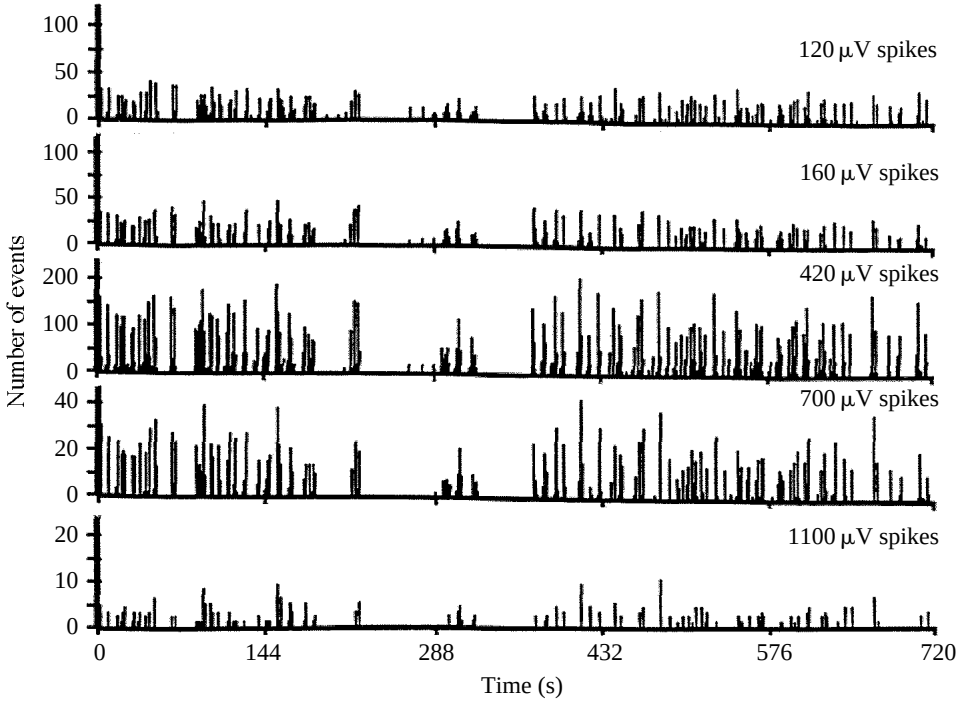

Fig. 2. Most of the patterned activity in the opener nerve was produced by one population of spikes. These are rate histograms for each of the spike populations that could be identified in one preparation (smallest unit is at top, largest at bottom). Five different spike populations could be reliably identified (120, 160, 420, 700, 1100 μV), but most of the activity in each burst was produced by the 420 μV spikes. (Note the differences in scale of the histograms.) The ordinate represents the number of occurrences of each unit within the 1.6s histogram bins and the graph spans 12min.

consideration of the possibility that one or more populations of neurones were releasing a cotransmitter(s), as has been shown for other insect motoneurones (O'Shea, 1985). Since our previous work suggested (Belanger and Orchard, 1993) that at least one of the opener motoneurones is proctolinergic, we looked for effects of proctolin on the muscle. Superfusion of ventral opener muscles with proctolin in normal saline at a concentration of 10^{-10} mol l^{-1} significantly (N=6) increased the frequency of miniature excitatory junctional potentials (mEJPs) from an average of 4.6Hz (range 0.9–13.7Hz) in control saline to 7.2Hz (range 1.1–16.7Hz) in proctolin saline. The average in each replicate was based on 500 successive mEJPs recorded in normal saline or after 5min of superfusion with proctolin. Fig. 4A presents an interevent interval histogram using data from a typical experiment, in which the average frequency increased from 1.6 to 2.4Hz. There is a notable increase in the number of events in the shorter interval ranges. In this same preparation, there was also a change in the amplitude distribution of the mEJPs (Fig. 4B), with more large mEJPs appearing in the presence of proctolin. This resulted in a significant increase in the average, but not the range, of amplitude of mEJPs from 1.13mV (range 0.3–4.1mV) to 1.36mV (range 0.3–4.1mV). We did not see any changes

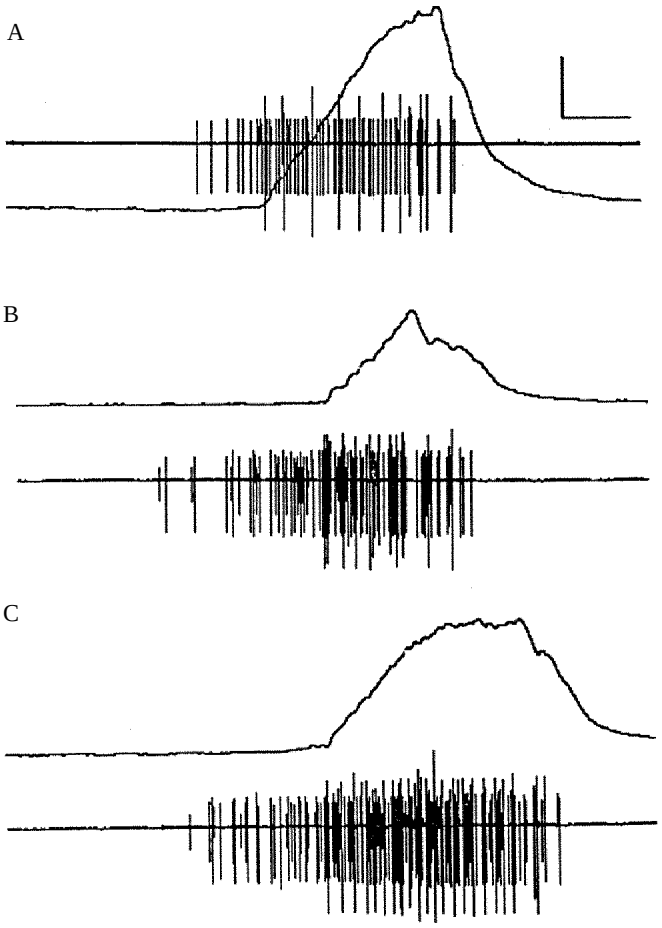

Fig. 3. Examples of opener nerve activity and the resulting muscle tension from three different preparations to show the typical structure of opener activity. In each case, slow axons fire at the beginning of the burst, but there is little change in tension until the fast units fire. Also, the firing frequency of the slow units continues to increase throughout the burst. Each of the examples was taken within 10min of the start of superfusion, during patterned activity of the digging CPG. Scale bars, A 350 μV, 50mg, 500ms; B 100mg, 500 μV, 500ms; C 50mg, 500 μV, 500ms.

in input resistance, as measured using constant-current hyperpolarizing pulses. Similar increases in mEJP amplitude, with corresponding changes in the amplitude distribution, occurred in the five other preparations examined. Both the frequency and the amplitude effects could be reversed by washing with normal saline, but the reversal generally took tens of minutes.

When higher concentrations (10^{-9} mol l^{-1}) of proctolin were superfused over opener muscles, several effects were seen. Occasionally (2 of 6 preparations), a slight depolarization (<5mV) was seen, but there was never any change in input resistance, as measured using constant-current hyperpolarizing pulses and there was very little, if any, change in basal tension. We experienced considerable difficulty in assessing possible

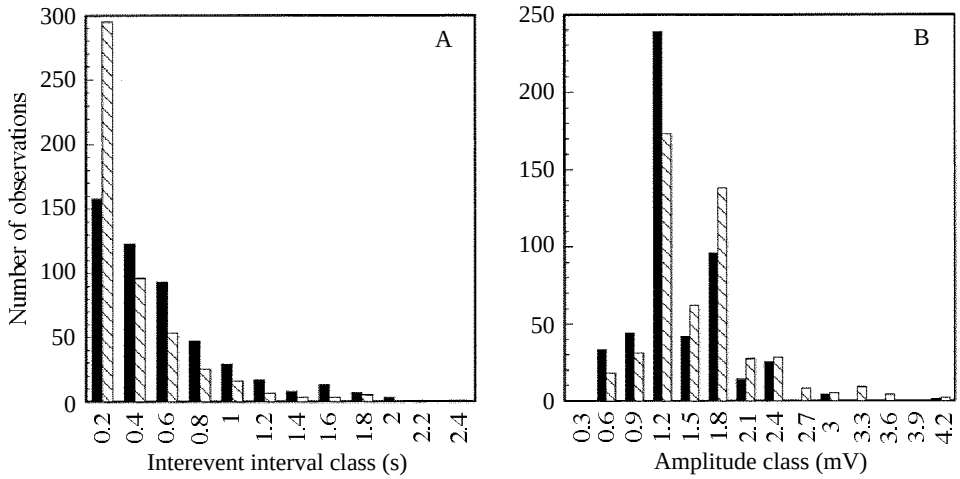

Fig. 4. Properties of mEJPs in a typical preparation measured in control saline (filled bars) or after 5min of superfusion with 10^{-10} mol l^{-1} proctolin (hatched bars). (A) Interevent interval histogram of mEJPs in a preparation in which proctolin significantly increased the frequency from 1.6 to 2.4Hz. The altered frequency is reflected in the increase in events in the smaller bins. (B) Proctolin perfusion did not change the observed size range of mEJPs, but did significantly increase the mean size (1.13 to 1.36mV) and altered the amplitude distribution of mEJPs. Each figure is based on data from 500 successive mEJPs in normal saline and after 5 min of proctolin superfusion in the same preparation.

effects of proctolin on EJPs in response to trains of pulses, because the resulting muscle twitches invariably displaced the intracellular electrode. In ten preparations where single stimulus pulses were used, recruiting all units in the nerve, proctolin had no significant effect on EJP size. However, in all ten preparations, tension produced by the muscle in response to short trains of pulses applied to the opener nerve increased three- to fourfold (Fig. 5).

At very high concentrations (>10^{-8} mol l^{-1}) of proctolin, there was a large, dose-dependent increase in the basal tension of the muscle (Fig. 6). Occasionally, a slow, 0.3–0.5Hz myogenic rhythm also appeared, superimposed upon this contracture. Most frequently (6 of 8 preparations), the myogenic rhythm was confined to only one bundle of muscle fibres, generally amongst the most ventral fibres of the muscle. In these cases, the resulting twitches were not detectable by the force transducer, although the twitches could be seen by looking at the muscle. The size of the induced contracture prevented examination of alterations in input resistance, as the muscle movements themselves produced resistance changes by shifting the intracellular electrodes. There were no significant changes in resting membrane potential.

Proctolin alters tension/frequency curves

We looked next at whether the effect of proctolin could account for any of the observed differences in motoneurone efficacy (Fig. 7). 500ms trains of pulses of varying frequency were delivered to the opener nerve and the peak tension produced by the

muscle was measured. Varying the stimulating voltage recruited differentially at least either the largest two units in the nerve or all of the units in the nerve. In every case, we made certain that every stimulus-evoked action potential in a train was the same size and contained the same number of inflection points, to avoid introducing artefacts from

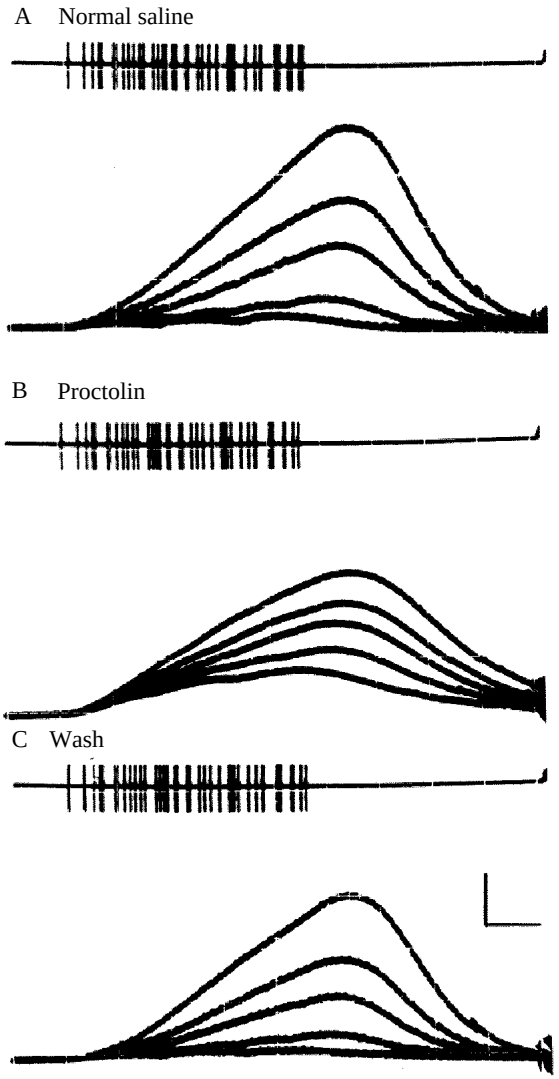

Fig. 5. Proctolin increases neurally evoked muscle contractions. The figures show muscle tension increases (bottom traces) in response to stimulation of all units in the opener nerve (top trace) at 5, 10, 15, 20 and 25Hz in normal saline (A), 10^{-9} mol l^{-1} proctolin (B) and after a 10min wash in normal saline (C). The resulting contractions are enhanced considerably in the presence of proctolin (note the larger scale for B), but return to control levels after washing. Since the nerve records contain overlapping traces of each of the stimulus trains applied, they should only be used as markers of stimulus onset. Scale bars: A,C 40mg, 100ms; B 100mg, 100ms.

recruiting different populations of spikes within a given train. The most typical result was that, predictably, recruiting all the units in the nerve in normal saline produced larger contractions than recruiting just the two largest units. The difference became more pronounced as the stimulation frequency was increased. However, when the experiment was repeated after 10min of superfusion with 10^{-9} mol l^{-1} proctolin, there was no difference between recruiting all the opener nerve units or just the two largest. This suggested that the effect of the three smaller units in the nerve in this preparation could be imitated by proctolin superfusion. In five other preparations we found similar effects. In three preparations, however, where this effect could not be repeated, we suspect it was because we could not recruit the two largest axons without recruiting the presumed proctolinergic fibre(s).

Proctolin is released during neural activity

To test the hypothesis that we were recruiting proctolinergic populations of neurones in some stimulation experiments, but not in others, we directly measured proctolin release induced by electrical stimulation. Based on their spontaneous activity during the first 5 min of superfusion, preparations were selected in which the largest units appeared to be fast axons (three of the six used are shown in Fig. 3). The abdominal ganglia were removed and the opener nerve was stimulated *via* a suction electrode. Again, we monitored the evoked spikes to ensure that the same population was recruited throughout the stimulation period. In the three preparations in which we were able to stimulate

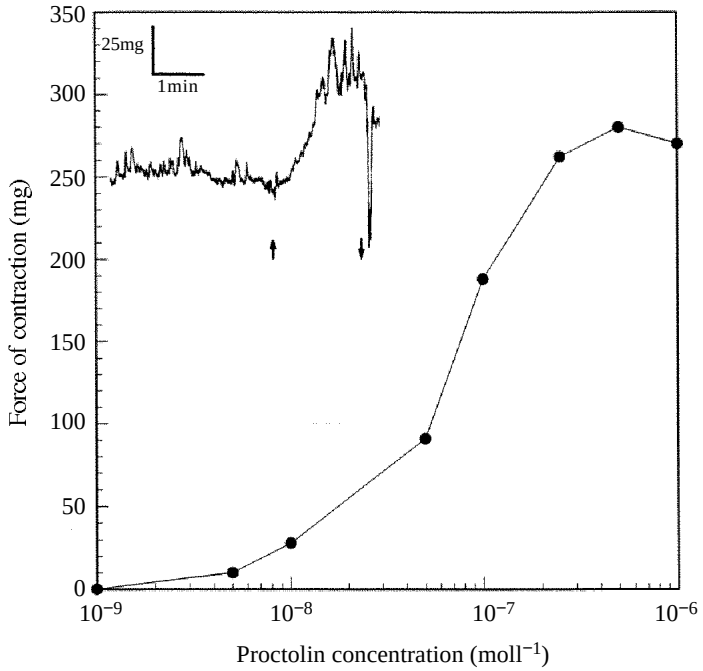

Fig. 6. A typical dose–response curve for the peak amount of tension produced by the ventral opener muscle in response to superfused proctolin. The inset shows a typical contraction produced by 5×10^{-8} mol l^{-1} proctolin, added at first arrow.

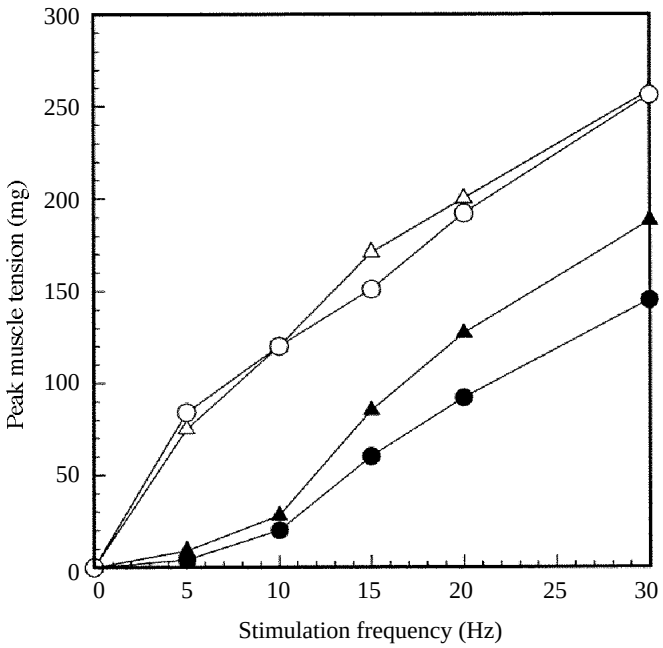

Fig. 7. The effect on muscle tension of varying the frequency of opener nerve spikes in normal saline (filled symbols) and after 10min in 10^{-9}mol l^{-1} proctolin (open symbols). In normal saline, stimulating all units (triangles) of the opener nerve with 500ms trains of pulses at varying frequencies consistently produced more tension than stimulating just the two largest units (circles). With proctolin superfusion, there was no difference in tension produced by recruiting all units or just the largest two.

reliably either just the two largest units in the opener nerve or all of the units, no detectable proctolin release occurred during stimulation of the large units at either 10 or 30Hz. The sensitivity of the bioassay is about 4fmol, so any proctolin release into the superfusate was below this level. However, when the stimulus intensity was increased to recruit all units, measurable amounts of proctolin were released during 30Hz, but not 10Hz, stimulation. An average of 23fmol (range 15–35fmol) of proctolin was released during the 5min stimulation. This represents about 8% of the total store of proctolin associated with the muscle (approximately 300fmol per ventral opener muscle, Belanger and Orchard, 1993). In three other preparations, we were unable to release proctolin differentially, probably for the reasons noted above. In these preparations, there was again no detectable release of proctolin at 10Hz, but 30Hz stimulation of the largest units released an average of 33fmol (21–53fmol) of proctolin, while stimulation of all units released an average of 28fmol (19–41fmol).

If proctolin plays a role in the normal functioning of the opener muscle, we surmised that it should be possible to measure its release during activity of the oviposition digging CPG. Therefore, we collected samples of superfusate from such preparations and tested them for proctolin-like bioactivity. Typical results are shown in Fig. 8, where 81fmol of proctolin was released during 5min of activity of the digging CPG. [The times used are those during which the preparation was being superfused, although these are generally

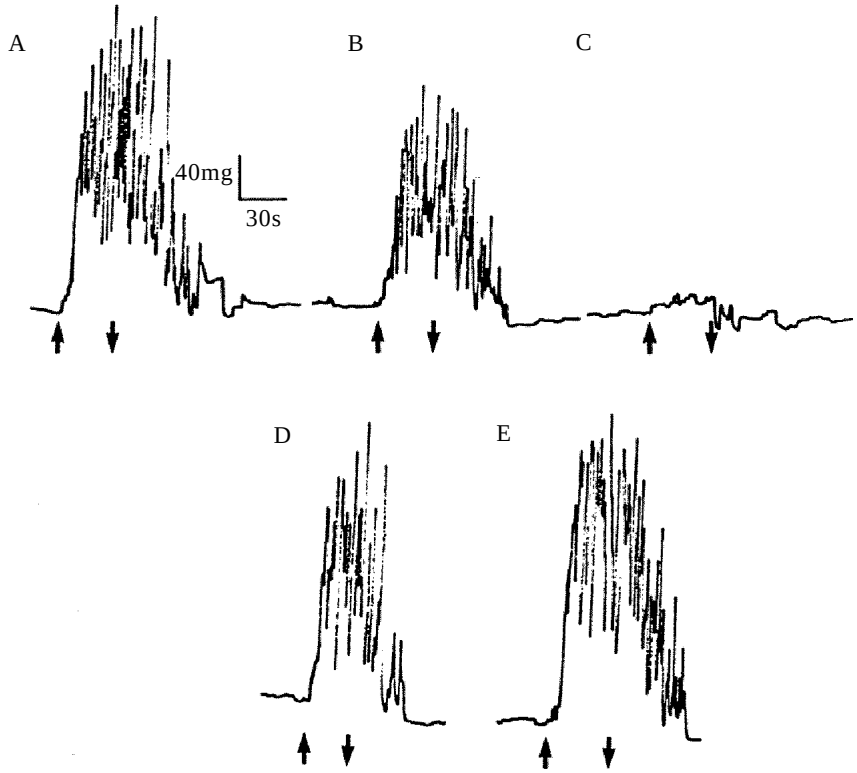

Fig. 8. Release of proctolin, as measured using the locust oviduct bioassay, during spontaneous activity of the oviposition digging CPG. The top traces show responses of the locust oviduct to extracts of superfusate collected after (A) 10, (B) 15 and (C) 20min of superfusion. The earliest superfusate samples produce responses characteristic of proctolin-like bioactivity, but the size of the response declines over the course of the superfusion. The lower traces show the responses of the same oviduct to (D) 20 and (E) 50fmol of proctolin. Samples were applied at the upward arrow and washed off at the downward arrow.

several minutes shorter than the time during which the CPG was running. Since this initial running of the CPG was in a relatively small (approximately 0.5ml) volume of low-calcium saline, the amount of proctolin released prior to superfusion should have been minimized.] This represents about 27% of the average proctolin store of an opener muscle (Belanger and Orchard, 1993). In subsequent 5min samples in the example shown, the amount of proctolin recovered fell to 12fmol and then below the level of detectability of the assay (about 4fmol). Similar amounts released initially (range of 25–130fmol), and a similar subsequent decline to below detectability levels, were seen in each of the six preparations examined, although the time course varied from preparation to preparation.

Parallel decreases in muscle tension and endogenous proctolin release

Since oviposition digging in these animals typically takes about 30–40min, the above-noted decline in proctolin release could be of physiological significance during the normal activity of the opener muscle. When we superfused opener muscles for extended periods, the muscle contractions ceased even though the patterned activity in the opener

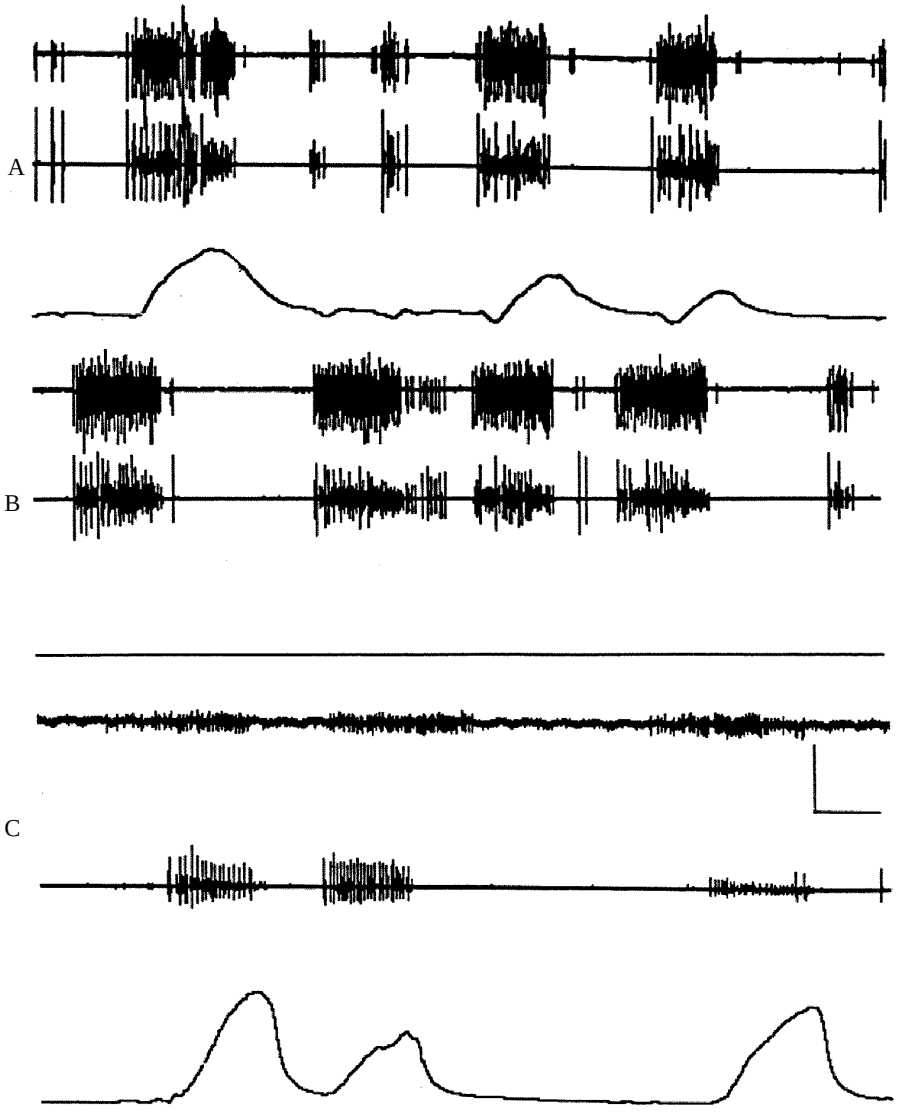

Fig. 9. Muscle contractions decrease with time in spontaneously active preparations, but exogenous proctolin restores their initial levels. Each set of traces shows opener nerve activity (top), opener EMG (middle) and opener muscle tension (bottom) recorded after (A) 10min and (B) 20min of superfusion with normal saline. While nerve activity and substantial EMG responses are present in B, there are no resulting muscle twitches. C is recorded 5min after starting superfusion with 10^{-9} mol l^{-1} proctolin. While there is less EMG activity than in B, the resulting muscle contractions are similar in size to those in A. (The nerve spikes recorded in C are reduced because the proctolin-enhanced contractions generally pulled the opener nerve out of the suction electrode, attenuating the recording.) Scale bars, 200 μV, 50 μV, 100mg, 1s.

nerve was still present and large electromyogram (EMG) responses could still be recorded (Fig. 9A,B). This shows that conventional neurotransmission was still functioning. Concomitant with this decrease in tension production, there was a decrease in the quantity of proctolin released into the superfusate (Fig. 10). When 10^{-9} mol l^{-1} proctolin was added to the superfusate, the contractions were restored to their original levels. Although the time course for the effect varied from preparation to preparation, in each of six cases there was a parallel decrease in muscle tension and proctolin release, which could be reversed by the application of exogenous proctolin.

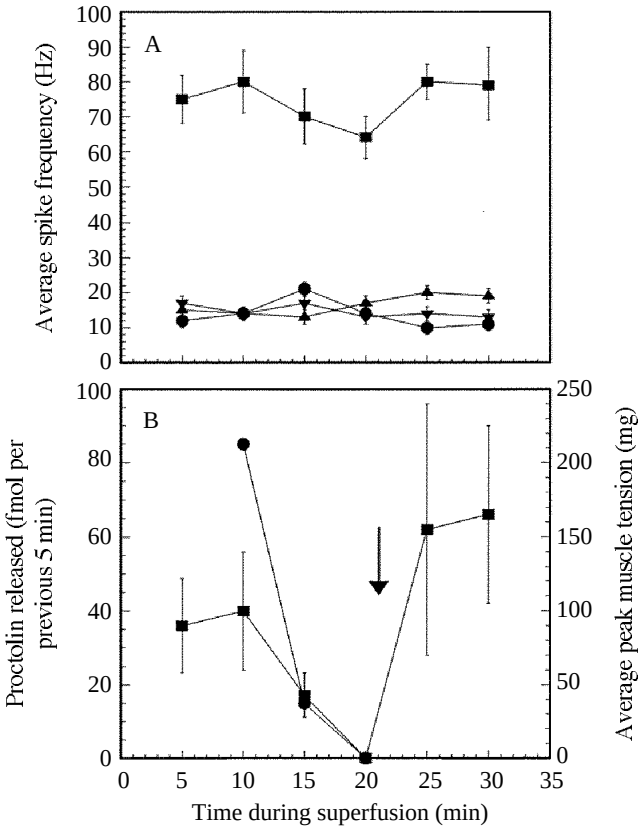

Fig. 10. Time course of the effects of naturally occurring proctolin depletion on muscle contraction in one preparation. (A) The average firing frequency of four different spike classes over the course of the experiment. Each point is the average ± S.D. for that particular spike class, measured in 3s bins over the previous 1min. (B) The amount of proctolin (measured as proctolin-like bioactivity on the locust oviduct) released into the superfusate during the previous 5min (filled circles) and the average ± S.D. peak muscle tension during contractions over the previous 1min. Although there is little change in neural input to the muscle, the amount of released proctolin declines and muscle contractions fall to zero. At the arrow, superfusion with 10^{-9} mol l^{-1} proctolin is begun. The muscle contractions return to their original levels, but there is little effect on the neural activity.

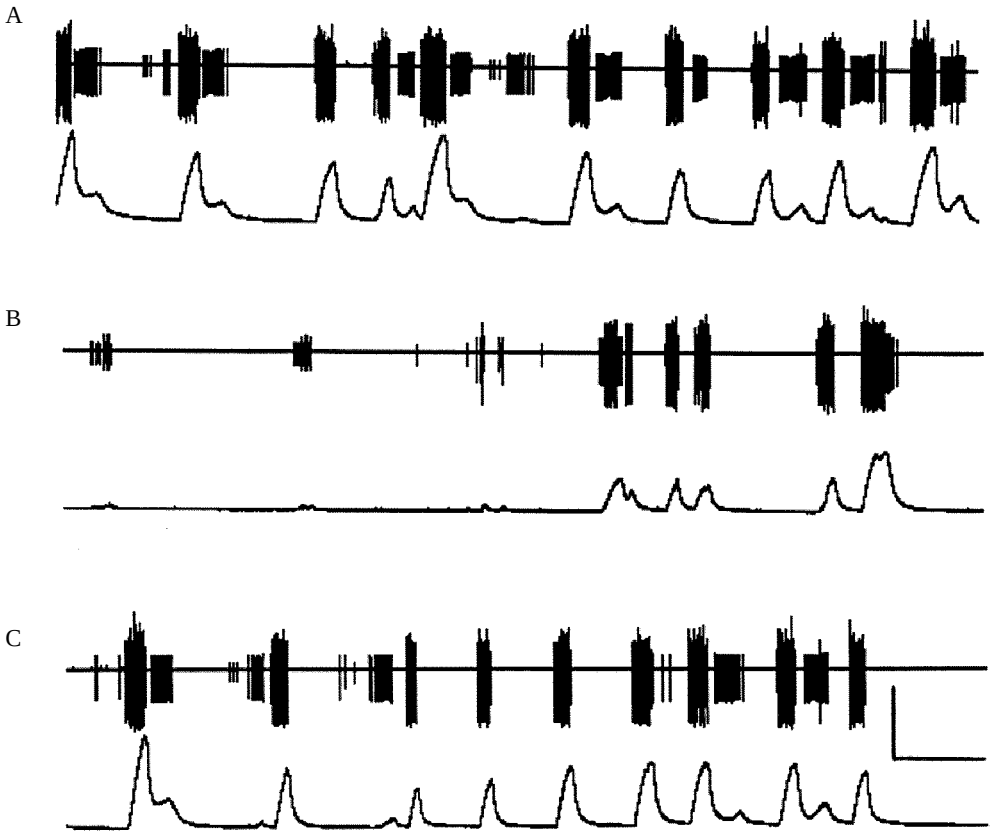

Fig. 11. Effects of proctolin superfusion on activity of the oviposition digging CPG. Each pair of traces shows extracellularly recorded opener nerve activity (top) and opener muscle tension (bottom). (A) Characteristic activity of the digging CPG after 10min *in vitro* being superfused with normal saline. Rhythmic bursting and muscle twitches are evident. (B) After 20min, much of the rhythmic activity has subsided, except for irregular bursts. The resulting muscle twitches are reduced. (C) After 5min of superfusion with 10^{-8} mol l^{-1} proctolin, the rhythmic activity returns and the muscle twitches are restored to their original levels. Scale bars, 200 μV, 100mg, 5s.

Central effects of proctolin on the motor pattern

Since proctolin has been shown to affect CPGs in other systems (see Discussion), it was possible that its ability to restore faded muscle contractions represented a central effect, in addition to the peripheral effects described above. Therefore, we examined the centrally generated activity in the opener nerve in the presence and absence of proctolin (Fig. 11). When we superfused ganglion–muscle preparations in which the oviposition digging pattern had slowed considerably (this occurs after some time in the absence of input to the ganglion, see Belanger and Orchard, 1992*b*) with 10^{-8} moll^{-1} proctolin, we found that the pattern could be restored to its original levels of activity. This effect was seen in six of the ten preparations examined.

Discussion

In this paper we have presented evidence for a physiological role for proctolin in the locust oviposition digging system. We believe it is the first time that an identified insect myotropic neuropeptide has been shown to be released during activity typical of a defined, natural behaviour. The peptide has a number of modulatory effects on the ventral opener muscle and, indeed, appears to play a necessary role in the normal functioning of this system.

The simplest explanation for the observed effects of proctolin on mEJPs is that proctolin is presynaptically increasing the rate at which quanta of transmitter are released, either by affecting existing release sites or by recruiting new ones. This would explain the increase in observed frequency and could also explain the altered amplitude distributions, if release sites electrotonically closer to the recording electrode are being recruited. An increase in the length constant of the membrane would produce a similar effect, but this should have produced a change in the input resistance, which was not seen. It would also shift the peaks of the mEJP amplitude histogram, which was also not seen. Recruitment of previously inactive release sites has been proposed as a mechanism for enhancing synaptic efficacy at crustacean neuromuscular junctions (Wojtowicz and Atwood, 1986). Unfortunately, this hypothesis is probably not testable in this preparation. It would involve performing quantal analysis, which would be difficult because the opener muscle fibres are multiterminally innervated and too large to be regarded as isopotential, precluding straightforward intracellular analysis of EJPs and the synapses are located deep within the muscle (Belanger and Orchard, 1993), preventing focal recording of postsynaptic currents. There are few other reports of presynaptic effects of peptides in insects. Schiebe and Walther (1988) found that some RFamides (FMRFamide and YGGFMRFamide) increase p, the probability of release of transmitter, and may also increase n, the number of release sites. Either of these possibilities would be consistent with our data.

The small depolarization of the muscle fibres that was occasionally seen in proctolin could be a result of the increased mEJP frequency producing an increased tonic level of glutamate in the synaptic cleft, but it seems inconsistent that proctolin should alter the frequency of mEJPs and apparently not affect evoked EJPs. This apparent discrepancy between the effects of substances on mEJPs and evoked responses has been seen before at insect neuromuscular junctions (see Walther, 1979), but the reasons behind it are unclear.

The other effects of proctolin on the ventral opener muscle are fairly typical of its actions in a number of arthropod neuromuscular systems (reviewed in Orchard *et al.* 1989). In all of these systems in which proctolin has been shown to have an effect, it acts to increase the efficacy of neuromuscular transmission either by enhancing neurally evoked tension responses or by inducing contracture. In most cases, its mechanism of action is not clear, although second messengers have been implicated in several systems (e.g. inositol phosphate in locust oviduct, Lange, 1989; cyclic AMP in crayfish skeletal muscles, Bishop *et al.* 1991) and, in all cases, part of the effector pathway seems to involve the mobilization of calcium. The only system in which there is a clear picture of the effects of proctolin is the tonic flexor muscle of the crayfish (Bishop *et al.* 1987, 1991). In this system, proctolin contributes significantly to the tension produced by the

muscle, at least in part by increasing the sensitivity to depolarization of large-conductance calcium channels in the plasma membrane.

The range of concentrations over which proctolin's effects were seen is also comparable to that of other systems (see Tables 3 and 5 of Orchard *et al.* 1989). The induction of myogenic activity in the muscle, at what are rather high concentrations (10^{-8} mol l^{-1}), is somewhat unusual. Certainly there are many other tissues where proctolin has a similar effect (e.g. locust extensor tibialis, Piek and Mantel, 1977; locust oviduct, Lange and Orchard, 1984), but generally at concentrations two to three orders of magnitude lower. In over a hundred opener muscle preparations, we have never seen myogenic activity in the absence of exogenously applied proctolin. We feel, therefore, that this effect is artefactual and that the opener muscle is probably never exposed to these concentrations of proctolin *in vivo*. It is quite possible that this concentration is high enough to drive the calcium-regulating systems of the muscle fibres out of their normal operating ranges and into a range where they are susceptible to the type of oscillatory behaviour seen in other systems (for a discussion of this, see Meyer and Stryer, 1988).

The amounts of proctolin released by neural stimulation were likewise similar to those observed in other systems. Using a comparable paradigm, Orchard and Lange (1986) obtained release of about 6% of the total proctolin store from the oviduct muscles of *Locusta migratoria*. Whim and Lloyd (1989) obtained similar results for the release of small cardioactive peptides (SCPs) from buccal muscles in *Aplysia californica.* However, when they stimulated at high frequencies (50Hz, 10% duty cycle) they were able to produce depletions of about 25% of the total stores during a 1h stimulation period. Bishop *et al.* (1987) also found that prolonged stimulation of proctolinergic neurones could significantly deplete peptide stores as well as reduce muscle tension in response to neuronal input. The much higher rates of release seen in locust opener muscle preparations expressing the digging rhythm are probably a result of the much higher frequency of spiking produced during the behaviour. Fig. 2 shows that mean firing frequencies in a burst are commonly above 60Hz for some axons. As noted in the results, the mean frequency is a substantial underestimate of the maximum frequency reached during a burst, particularly for the slow axons which increase their firing rate throughout much of the burst.

Our data imply that the release of proctolin by a slow motor axon is an integral part of the normal functioning of the ventral opener muscle. This conclusion is based on several pieces of evidence. First, motor axons which physiologically appear to be of the slow type can release proctolin in response to neural stimulation. Second, the pattern of opener nerve bursts, in which the slow axons fire before the fast ones, is consistent with the idea that a cotransmitter is being released locally to amplify the effects of the fast EJPs on muscle tension. The fast axons are not necessarily required to produce tension, as the slow axons alone can do this (see Fig. 1). This is probably a reflection of the fact that the putative proctolinergic axons also appear to contain a conventional transmitter, probably glutamate (Belanger and Orchard, 1993). Therefore, slow axon spikes probably release glutamate and proctolin simultaneously, but the postsynaptic potentials due to this are too small to produce much tension without amplification. This was often seen in records of

proctolin-depleted preparations, where fast axon spikes could produce small twitches, but slow spikes had no effect (data not shown).

Based on the observations of the decline with time of tension production in superfused preparations, the concomitant decline in proctolin release into the superfusate and the subsequent reversal of the tension decrease by superfused proctolin (Fig. 10), we suggest that proctolin is necessary for normal oviposition digging. The ventral ovipositor muscle has associated with it approximately 300fmol of proctolin (Belanger and Orchard, 1993), which is sufficient to produce a concentration of 10^{-9} mol l^{-1}, the order of magnitude seen for enhanced neurally evoked tension (Fig. 5), in a volume of 300 μl. This is probably several thousandfold larger than the volume of the synaptic clefts into which proctolin is being released and is about half the total haemolymph volume of an adult female *L. migratoria* (Loughton and Tobe, 1969). However, since our preparations are being constantly superfused, at least some of the peptide is being washed out. To our knowledge, there are no reported reuptake systems for proctolin and, being a peptide, it almost certainly must be synthesized on ribosomes in the cell body and transported down the axon to the presynaptic terminals. This is not the case for glutamate, which can be both taken up and synthesized at the presynaptic terminals (Hoyle, 1983). Thus, proctolin, but not glutamate, may be susceptible to a physiologically significant depletion of presynaptic stores as the peptide is continuously washed out of the synaptic cleft by the superfusion. This would explain why the levels of tension production declined before the levels of EJPs.

An alternative explanation for these results, of course, is that the condition of the muscle is deteriorating with time *in vitro* and that adding proctolin simply enhances neuromuscular transmission enough to produce measurable contractions of the muscle. We tend not to favour this explanation for several reasons. First, the most likely source of deterioration of the muscle is probably oxygen deprivation: Chesler and Fourtner (1981) have reported that a cockroach slow muscle loses its ability to produce tension unless oxygen is continuously bubbled through the saline. This decline takes about 15–30min and is reversible. While we did not routinely oxygenate the saline, we ran several control experiments in which oxygen was continuously bubbled through the saline and this did not affect the results. Furthermore, it is probable that the ovipositor muscles are resistant to anoxia, as this is a condition that they are likely to encounter during oviposition. The normal flow of air through the orthopteran tracheal system is in through the thoracic spiracles and out through the abdominal ones (Wigglesworth, 1972). It is difficult to see how this flow could not be compromised with the abdomen buried in moist soil, the favoured oviposition site. Second, rhythmic movements of the ovipositor valves can continue for at least 10h (data not shown) in semi-intact preparations which are not being superfused. Finally and most compellingly, there is the fact that the decline in muscle tension is paralleled by a decrease in the measurable release of proctolin into the superfusate.

The possibility that the opener muscle requires proctolin for its normal functioning has interesting parallels with the crayfish tonic flexor muscle (Bishop *et al.* 1987). In this system, too, the use-associated declines in neurally induced muscle tension can be reversed by adding exogenous proctolin. These authors suggested that proctolin is acting

to increase the efficiency of the system by decreasing the number of action potentials required to produce and maintain a given level of tension. However, while maintaining a given level of tension of the locust opener muscle may be important during the tonic gaping observed during egg-laying, it does not seem likely to be important during the phasic movements associated with digging. It may be that proctolin is used in the opener system to produce the large amounts of tension required within the time windows imposed by the CPG. In the absence of proctolin, the opener muscle can still produce large amounts of tension, provided that a high enough input frequency or a long enough train of input is used (Fig. 7). During normal behaviour, however, these two factors are constrained by physiological limits on the one hand and by relative phase requirements on the other. In the intact animal, sensory information is also available which may be used to enhance tension production during digging as required, but this information is not available to the CPG in the isolated preparation we used.

Proctolin's effect on the oviposition digging CPG is novel. While the concentration (10^{-8} mol l^{-1}) at which this effect was seen is high for a peptide, this may be because the peptide has difficulty penetrating the sheath surrounding the central nervous system. Indeed, Stevenson (1989) has suggested that an octopamine concentration of 10^{-1} mol l^{-1} in the superfusate would cause effects equivalent to 10^{-6} mol l^{-1} octopamine in the ganglionic neuropile. Assuming that peptides have similar difficulty penetrating the neural sheath, the effective concentration of proctolin in the neuropile in our experiments is likely to be well within the physiological range. In fact, it is rather surprising that proctolin was able to penetrate the ganglionic sheath at all. It probably occurred because of the number of major nerve trunks that were cut during the dissection, providing routes into the neuropile for the peptide.

We are only aware of two other instances of central effects of proctolin in insects. The first is a sensitization of the cercal escape reflex in *Periplaneta americana* (Fitch and Djamgoz, 1988). The second, more pertinent one, is a transient increase in the spontaneous firing rate of dorsal unpaired median (DUM) neurones in the cockroach (Washio and Sato, 1991). This is interesting because octopamine, which is released by many DUM neurones, has been suggested to have (inhibitory) effects on the digging CPG (Sombati and Hoyle, 1984). In crustaceans, proctolin has a number of actions on CPGs. For instance, it can initiate rhythms in the stomatogastric ganglion (STG) in all of the decapods which have been examined (reviewed in Marder, 1987). In many cases, proctolin produces its effects by enabling conditional bursters, turning on membrane conductances that endow the cells with endogenous burst capabilities (Heinzel and Selverston, 1988). It is not yet clear just how important endogenous burst capabilities are in neurones constituting insect CPGs, but it has recently been shown that members of respiratory and flight CPGs in the locust can have such properties induced by octopamine (Ramirez and Pearson, 1991). Surprisingly, given the volume of work on crustaceans, there are very few reports of modulation of insect CPGs and they all involve biogenic amines. Bellah *et al.* (1984) reported alterations in the frequency of the ventilatory rhythm in *Corydalus cornutus*, and Sombati and Hoyle (1984) and Classen and Kammer (1986) reported effects on the flight motor programmes in *Schistocerca gregaria* and *Manduca sexta*, respectively. Given Washio and Sato's (1991) finding of excitatory

proctolin effects on DUM neurones, it is difficult to assess the possible directness of proctolin's effect on the digging CPG. Proctolin could be exciting DUM neurones, which are then releasing octopamine, which could be the active agent on cells in the CPG. The only way to answer this question is to find the neurones that are members of the digging CPG and to look for direct physiological effects of proctolin on those cells.

In conclusion, we wish to make two points of general interest to students of arthropod motor control. The first is that not all action potentials are created equal. It is not possible simply to equate overall motoneurone activity with muscle performance. This is shown clearly by the observation that different excitatory units in the motor nerve can produce different effects (Fig. 1). This general point has long been known in the context of fast *versus* slow motor axons (e.g. Hoyle, 1975), but it has been slow to be widely acknowledged with respect to the release of transmitter substances with vastly differing effects. Therefore, it is vital to identify the relative activities of different units in any given system and to attempt to correlate the contributions of the specific units to muscle output under a variety of conditions. The second point is that the recent activity of any axons under study is of profound importance in attempting to make functional interpretations of how neuronal input influences motor output. Since physiologically significant depletion of transmitters can occur over a physiologically relevant short time period, extreme caution must be exercised in interpreting the results of neural stimulation experiments.

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