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ALLATOSTATIN DECREASES STOMATOGASTRIC NEUROMUSCULAR TRANSMISSION IN THE CRAB *CANCER BOREALIS*

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Summary

The effects of insect allatostatins (ASTs) 1–4 were studied on the stomach musculature of the crab *Cancer borealis*. Of these, Diploptera-allatostatin 3 (D-AST-3) was the most effective. D-AST-3 (10^{-6} mol l^{-1}) reduced the amplitude of nerve-evoked contractions, excitatory junctional potentials and excitatory junctional currents at both cholinergic and glutamatergic neuromuscular junctions. Muscle fiber responses to ionophoretic applications of both acetylcholine and glutamate were reduced by the peptide, but D-AST-3 produced no apparent change in the input resistance of the muscle fiber. D-AST-3 reduced the amplitude of muscle contractures evoked by both acetylcholine and glutamate, but had no effect on contractures induced by a high [K^+]. These data suggest that D-AST-3 decreases the postsynaptic actions of both neurally released acetylcholine and glutamate. Because an AST-like peptide is found in peripheral sensory neurons that innervate stomatogastric muscles and in the pericardial organs, we suggest that an AST-like peptide may play a role in controlling the gain of the excitatory neuromuscular junctions in the stomach.

Key words: crustacean, acetylcholine receptors, glutamate receptors, neuropeptides, neuromodulation, crab, *Cancer borealis*.

Introduction

In motor systems, the nervous system generates motor neuron discharge patterns that result in movement. At many invertebrate neuromuscular junctions, the amplitude of the muscle contractions produced by a given temporal pattern of motor neuron discharge can be influenced by neuromodulatory substances, which are either released as cotransmitters from the terminals of the motor neurons themselves (Adams and O'Shea, 1983; Cropper *et al.* 1990) or act as circulating hormones (Keller, 1992; Weimann *et al.* 1997). In either case, the actual movements evoked by a motor pattern may be considerably altered by peripheral neuromodulatory control. In the present paper, we show that a peptide of the allatostatin family also probably functions as a modulator of arthropod neuromuscular junctions.

The muscles of the crustacean stomach receive excitatory innervation from motor neurons located in the stomatogastric ganglion (STG) (Maynard, 1972; Mulloney and Selverston, 1974*a*,*b*). As is common in arthropods, many of the motor neurons in the STG are glutamatergic (Hooper *et al.* 1986; Lingle, 1980; Marder, 1976). However, an unusual feature of the crustacean stomatogastric system is that some of the STG neurons make excitatory cholinergic synapses onto the muscles they innervate (Lingle, 1980; Marder, 1974, 1976). Also, unlike many other arthropod neuromuscular systems, stomatogastric muscles do not receive any direct inhibitory innervation (Govind *et al.* 1975).

The STG produces a large repertoire of motor pattern outputs under modulatory control (Harris-Warrick *et al.* 1992; Marder and Calabrese, 1996; Marder and Weimann, 1992). In the crab *Cancer borealis*, neuroactive substances are present in modulatory neurons located in a pair of commissural ganglia (CGs) and in the oesophageal ganglion (OG). These neurons provide direct modulatory inputs to the crab STG (Coleman *et al.* 1992, 1995; Coleman and Nusbaum, 1994; Norris *et al.* 1996). The same neuroactive substances are often found in the eye stalks and pericardial organs (Christie *et al.* 1995), the important neurohemal structures of these animals. Given that STG motor neurons are not known to contain modulatory substances, modulation of most stomatogastric muscles must occur *via* neurohemal control (Jorge-Rivera, 1997; Jorge-Rivera and Marder, 1996; Lingle, 1981; Weimann *et al.* 1997). However, the four gastropyloric receptor (GPR) sensory neurons contain acetylcholine (ACh), serotonin and an allatostatin (AST)-like peptide (Beltz *et al.* 1984; Katz *et al.* 1989; Skiebe and Schneider, 1994), and innervate several of the gastric mill muscles of the stomach. Thus, it is possible that some of the muscles of the stomach could be modulated by serotonin or

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AST-like peptides if these are liberated by the sensory neuron terminals on the surface of the muscles.

A number of modulatory substances increase the amplitude of stomatogastric muscle contractions in response to motor neuron discharge (Jorge-Rivera and Marder, 1996; Lingle, 1981; Meyrand and Marder, 1991; Meyrand and Moulins, 1986; Weimann *et al.* 1997), but to date none has been reported to decrease the gain of the movements evoked by stomatogastric ganglion motor patterns.

The allatostatins (ASTs) have been isolated from a number of insects: D-ASTs from the cockroach *Diploptera punctata* (Pratt *et al.* 1989, 1991; Woodhead *et al.* 1989), P-ASTs from the cockroach *Periplaneta americana* (Ding *et al.* 1995), M-ASTs from the moth *Manduca sexta* (Kramer *et al.* 1991) and C-ASTs from the blowfly *Calliphora vomitoria* (Duve *et al.* 1993). The cDNA and genomic DNA sequences of D-ASTs and P-ASTs show that there are 13 peptide members of the AST family in *D. punctata* and 14 members in *P. americana* (Ding *et al.* 1995; Donly *et al.* 1993). The ASTs inhibit juvenile hormone (JH) biosynthesis by the corpora allata in insects (Tobe and Stay, 1985). AST-like immunoreactivity has also been demonstrated in a number of insect species (Stay *et al.* 1992; Veelaert *et al.* 1995; Yoon and Stay, 1995).

The allatostatins are the only neuropeptides known to inhibit STG motor patterns (Marder *et al.* 1994; Skiebe and Schneider, 1994). In the crab, AST-like peptides are found in 12–19 cell bodies in each CG, two cell bodies in the OG, and in the stomatogastric nerve that carries the axons of many modulatory inputs to the STG (Skiebe and Schneider, 1994). In addition to the four GPR sensory neurons, other potential sources of AST-like peptides include the pericardial organs and sinus glands (Christie *et al.* 1995; Marder *et al.* 1994; Skiebe and Schneider, 1994). Given the presence of AST-like peptides both in sensory neurons that innervate stomatogastric muscles and in neurohemal organs, we wanted to determine whether the ASTs have direct actions on the gain of motor-neuron-evoked movements in the stomatogastric musculature. In the present paper, we demonstrate that one member of the cockroach AST family, D-AST-3, is effective at decreasing the efficacy of the neuromuscular junctions in the crab stomatogastric nervous system.

Materials and methods

Animals and solutions

Experiments were performed on 175 male *Cancer borealis* Stimpson purchased from local fishermen in Boston, MA, USA, and held in aerated saltwater aquaria at 12 °C until used. Physiological saline had the following composition (in mmol l^{-1}): NaCl, 440; KCl, 11.3; $CaCl_2$, 13.3; $MgCl_2$, 26.3; Trizma base, 11.0; maleic acid, 5.2, pH 7.4–7.6.

D-Allatostatins 1–4 were purchased from Bachem. Each peptide was dissolved in distilled water at 10^{-3} or 10^{-2} mol l^{-1} and stored at –20 °C. Samples were diluted in saline to the desired concentration minutes before experiments. Salines containing high K^+ concentrations, glutamate or ACh were freshly prepared prior to experiments. Glutamate, ACh and edrophonium chloride were purchased from Sigma. Chlorisondamine was a gift from the Ciba-Geigy Corporation.

Physiology

Muscle names follow the standard stomatogastric system nomenclature (Maynard and Dando, 1974). Neuromuscular preparations were isolated from the foregut of the crab and pinned into 5 ml chambers. Preparations were superfused continuously with a gravity-fed system at 10–15 ml min^{-1} unless indicated otherwise. Solutions were bath-applied by means of a switching port on the inflow of the superfusion system. Bath volume was approximately 3 ml. The saline temperature was held between 10–12 °C by means of a Peltier cooling system and was continuously monitored with a thermoelectric probe in the bath.

Innervating nerves were stimulated with trains of pulses through extracellular pin electrodes or suction electrodes. Recordings of excitatory junctional potentials (EJPs) were made using conventional intracellular microelectrodes with resistances of 10–15 MΩ and filled with 2.5 mol l^{-1} KCl. Excitatory junctional currents (EJCs) were measured using the two-electrode voltage-clamp technique (TEVC, Axoclamp 2A). The resistance of the electrodes was typically 8 MΩ. The distance between the electrodes was less than 50 μm. The diameter of clamped fibers was typically 150 μm. A grounded shield was placed between the electrodes to reduce the capacitative coupling between the two electrodes. The Axoclamp was used with a gain of 90–100.

Glutamate and ACh responses in muscle were obtained using ionophoretic electrodes filled with 1 mol l^{-1} glutamate or 1 mol l^{-1} ACh. Pulse durations ranged from 200 ms to 1 s, and ionophoretic current ranged between 200 and 800 nA (positive current pulses were used for ACh, and negative pulses were used for glutamate). Reliable responses were typically found near small branches of the motor nerve. Ionophoretic responses were considered stable if the peak amplitude and time course of the depolarization varied by less than 10 % for several pulses. The effects of D-AST-3 were studied after the duration of the pulse and the amount of current injection had been adjusted to evoke 75 % of the maximal response to ensure that saturation did not occur. ACh- and glutamate-evoked currents were recorded using TEVC with the ionophoretic electrode positioned within 100 μm of the recording and current-passing electrodes.

Muscle contraction recordings were obtained by attaching the muscle to a modified movement transducer (FT03, Grass Instruments). One of the muscle insertions was pinned down in the dish and the other end was attached to a 15.2 cm thread connected to the transducer at an angle of 45 °. In this configuration, stimulation of the motor nerve produced muscle shortening. To obtain ACh- and glutamate-mediated contractures, the muscle was superfused at a rate of 15 ml min^{-1} at a given agonist concentration. After maximal contracture for a given concentration had been obtained, the preparation was returned to control saline. There was a 15 min interval between each agonist application.

Results

The stomach of the crab is moved by a large number of striated muscles (Fig. 1). The intrinsic muscles insert on two ossicles of the stomach and contract to move these ossicles together. The extrinsic muscles connect one insertion on the surface of the stomach to another on the hypodermis and suspend the stomach in the body cavity. All of the extrinsic muscles are innervated by cholinergic motor neurons, whereas most of the intrinsic muscles are innervated by glutamatergic motor neurons (Hooper *et al.* 1986; Lingle, 1980; Marder, 1974, 1976; Weimann *et al.* 1991). The muscles and their transmitters used in the present paper are listed in Table 1. The original nomenclature (Maynard and Dando, 1974) used the designation 'gm' for the muscles that move the teeth of the gastric mill, the designation 'p' for the muscles of the pyloric chamber, and the designation 'cpv' for the muscles of the cardiopyloric valve.

Table 1. *Types of transmitter present in motor neurons innervating specific muscles stomach*

Muscle	Motor neuron	Transmitter
gm1	Gastric mill, GM	Acetylcholine
gm2	Gastric mill, GM	Acetylcholine
gm4	Dorsal gastric, DG	Glutamate (also acetylcholine extrajunctional receptors)
gm6	Lateral gastric, LG	Glutamate
gm8	Lateral gastric/medial gastric, LG/MG	Glutamate
cpv4,6	Lateral pyloric, LP	Glutamate
p8	Pyloric, PY	Glutamate

From Hooper *et al.* (1986); Lingle (1980).
gm, gastric muscle; cpv, cardiopyloric muscle; p, pyloric muscle.

D-AST-3 decreases the amplitude of nerve-evoked contractions

We wanted to determine whether any of the allatostatin peptides alter the amplitude of nerve-evoked muscle contractions in the stomatogastric nervous system. Fig. 2A shows the effect of four cockroach allatostatin peptides (D-ASTs 1–4) on nerve-evoked gm1 and gm4 contractions. The gm1 muscle is innervated by the cholinergic gastric mill (GM) neurons, whereas the gm4 muscle is innervated by the glutamatergic dorsal gastric (DG) motor neuron. D-AST-1, -2 and -4 (10^{-6} mol l^{-1}) produced a relatively small reduction in peak nerve-evoked contraction, but D-AST-3 had a much greater effect. All four peptides were applied to the same preparations in numerical order. Fig. 2B shows that 10^{-6} mol l^{-1} D-AST-3 reduced the amplitude of the gm1 contractions by more than 50 % and of the gm4 contractions

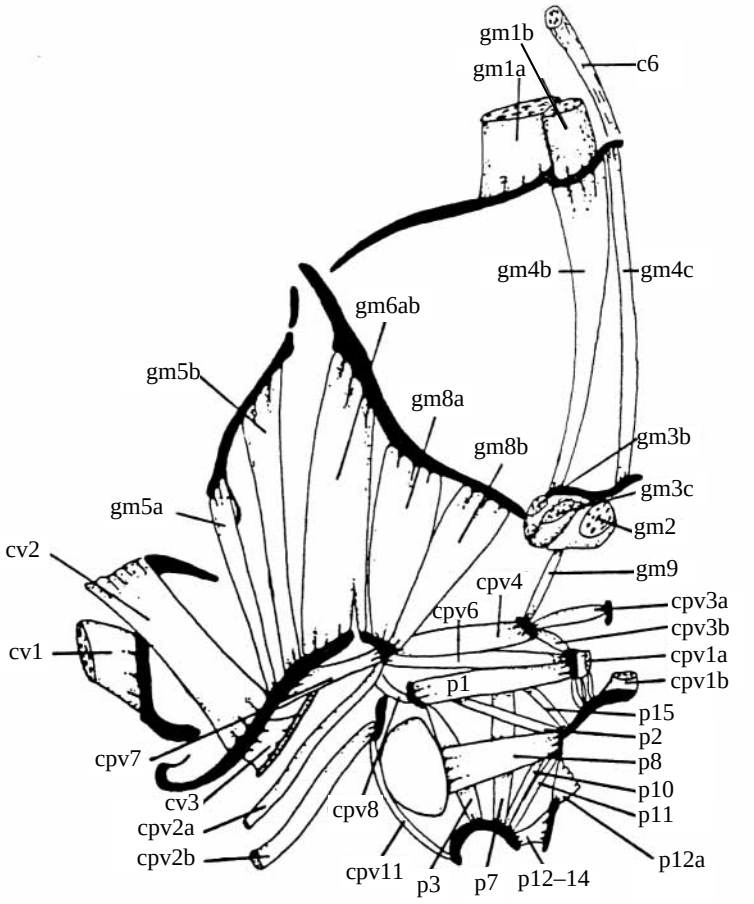

Fig. 1. Stomatogastric musculature. The stomatogastric muscles lie in the external surface of the crustacean stomach. Individual neuromuscular preparations are isolated for physiological experiments after the stomach has been split along the ventral midline and laid flat. Here, only the left side is shown. Posterior is down, and medial is to the right. Gastric muscles (labeled gm) lie in the anterior portion of the stomach. These muscles control the movements of a pair of lateral teeth and of a medial tooth located in the interior of the stomach. Pyloric muscles (labeled p) lie in the posterior portion of the stomach and control the movements of the pyloric chamber, which filters food and absorbs nutrients. Cardiopyloric muscles (labeled cpv) lie between the gastric and pyloric muscles and are involved in the movement of the cardiopyloric valve. Illustration from Weimann *et al.* (1991) with permission.

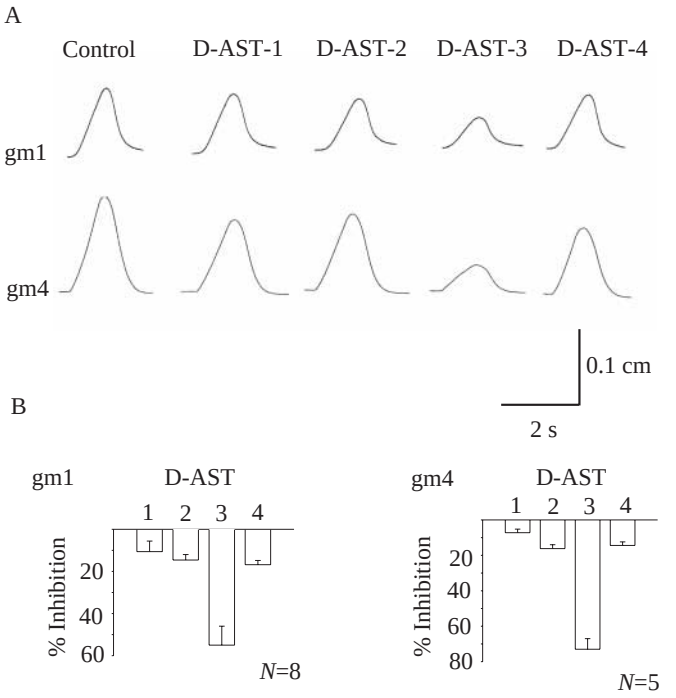

Fig. 2. Effect of 10^{-6} mol l^{-1} D-AST-1, -2, -3 and -4 on nerve-evoked contractions in gm1 and gm4. (A) The motor nerves were stimulated at 20 Hz for 1 s and muscle contraction was monitored with a movement transducer. Each peptide was bath-applied separately with a 15–20 min interval between applications. The contractions returned to control peak amplitude between each peptide application. (B) Histograms showing pooled data for each muscle. D-AST-3 reduced the peak amplitude of nerve-evoked gm1 and gm4 contractions more effectively than did D-AST-1, D-AST-2 and D-AST-4. Error bars are standard errors of the mean.

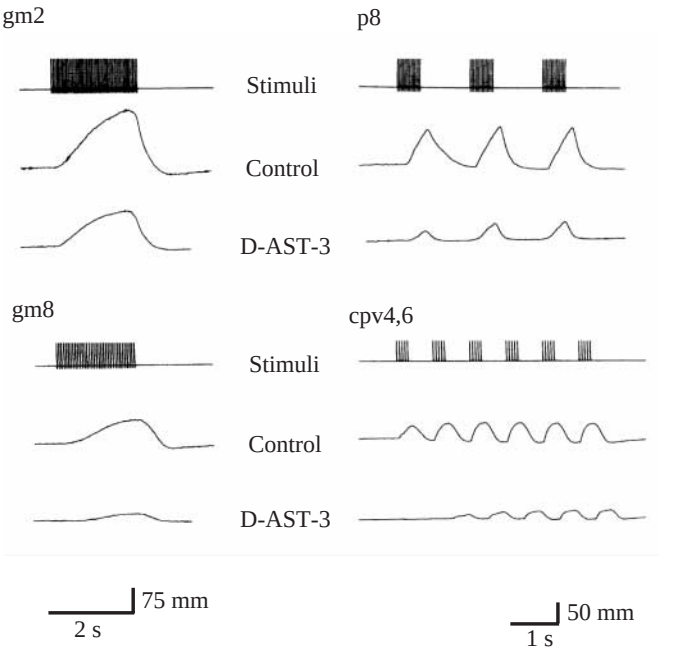

Fig. 3. Effect of 10^{-6} mol l^{-1} D-AST-3 on nerve-evoked contractions. Gastric muscle contractions were obtained by stimulating the motor nerve in 'gastric time' (gm2, 20 Hz for 2 s; gm8, 15 Hz for 2 s), whereas p8 and cpv4,6 muscle contractions were obtained by stimulating the motor nerve in 'pyloric time' (15 Hz for 0.5 s every 1 s for p8 and 10 Hz for 0.5 s every 0.75 s for cpv4,6).

by more than 70 %. All of these data were taken from responses to single trains of stimuli.

Because D-AST-3 was the most effective of the allatostatins tested, all further studies were carried out with this peptide. We used nerve-evoked contractions to screen the effect of D-AST-3 in other stomatogastric muscles to determine whether the effects of D-AST-3 were specific to the first muscles tested. Fig. 3 shows the effect of bath application of 10^{-6} mol l^{-1} D-AST-3 on the gm2, gm8, cpv4,6 and p8 muscles. These muscles include two additional gm muscles, the cholinergic gm2 muscle and the glutamatergic gm8 muscle as well as one glutamatergic p muscle, p8, and the glutamatergic cpv4,6 muscles. Each muscle was stimulated with a spike train that approximately mimics the pattern of action potential discharge of its innervating motor neuron. Fig. 3 shows that D-AST-3 decreased the peak nerve-evoked contractions in all four muscles. Note that the percentage decrease in the contraction amplitude of the muscles activated with repetitive pyloric-rhythm-timed stimuli (p8 and cpv4,6) was not constant, but that the difference between the control and D-AST-3 recordings decreased with successive stimuli. We did not study the effects of D-AST-3 on contractions evoked by more than four or five successive stimuli. In these cases, the amplitude of the contraction in response to the last stimulus was smaller than that of the control.

D-AST-3 decreases the amplitude of nerve-evoked EJPs

To determine whether the decrease in nerve-evoked contractions produced by D-AST-3 was associated with a decrease in the amplitude of nerve-evoked EJPs, we examined the effect of D-AST-3 on gm1, gm6 and gm8 EJPs. The EJP recordings seen in Fig. 4 show that these synapses display both considerable facilitation during the spike train and considerable summation of the postsynaptic potential. D-AST-3 decreased the final peak depolarization in all three muscles (paired *t*-test, $P \leqslant 0.01$). The histograms on the right of Fig. 4 show the pooled data for each muscle. Note that the amplitude of the first EJP decreases more in the presence of D-AST-3 than does the amplitude of the final membrane potential reached at the end of the train. However, the final peak depolarization is a combined result of the summation and of the unitary EJP amplitudes, and we therefore measured the amplitudes of the first and last EJPs in control saline and in the presence of D-AST-3. (The amplitude of the last EJP was measured by extrapolating the decay of the previous EJP to find the baseline membrane potential at the time of the last EJP, and then measuring the amplitude from that baseline.) If the facilitation index (F_i) is defined as: F_i =EJP amplitude (last)/EJP amplitude (first) minus 1, we find that D-AST-3 produces an increase in F_i from 2.7 to 6.9 ($P<0.05$) in the gm8 muscle, but only statistically insignificant changes in F_i in the other muscles.

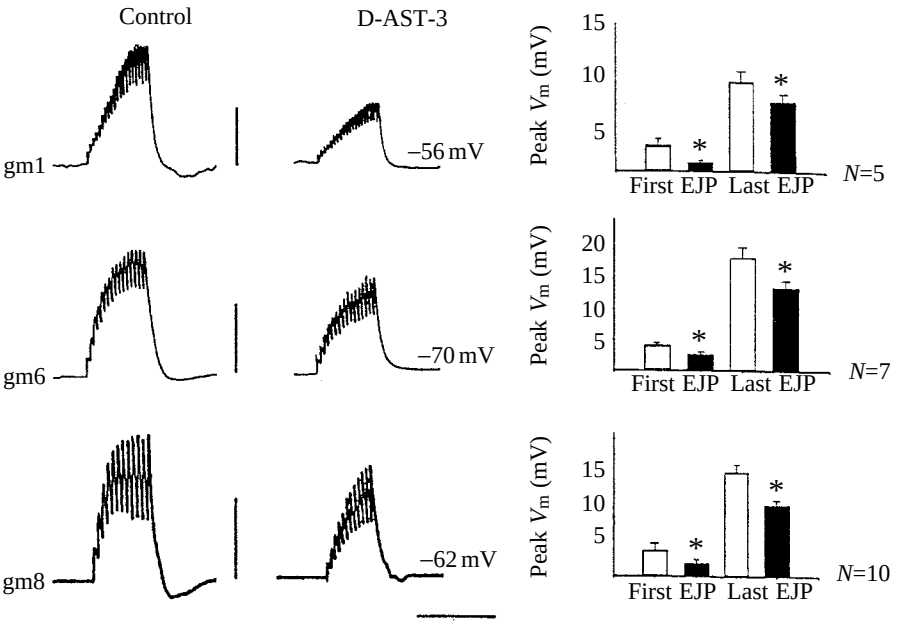

Fig. 4. Effect of 10^{-6} mol l^{-1} D-AST-3 on nerve-evoked EJPs and peak membrane potential (V_m). gm1 EJPs were obtained by stimulating the motor nerve at 10 Hz for 2 s. gm6 EJPs were obtained by stimulating the motor nerve at 10 Hz for 2 s. gm8 EJPs were obtained by stimulating the motor nerve at 12 Hz for 1 s. Scale bars: gm1, 5 mV, 2 s; gm6, 10 mV, 2 s; gm8, 6 mV, 1 s. The histograms show the peak membrane potential reached by the first EJP and at the end of the train of EJPs for pooled data. Open bars, control; filled bars, D-AST-3. *$P \leqslant 0.01$, in paired *t*-tests. Error bars are standard errors of the mean.

To measure the effect of D-AST-3 on the synaptic current, muscle fibers were voltage-clamped, and EJCs were evoked in control saline and in the presence of the peptide. Fig. 5 illustrates the results of one such experiment on the cholinergic EJCs in gm1. As was the case in the recordings of EJPs (Fig. 4), the reduction in the amplitude of the EJC was markedly larger for the first (smaller) EJC than for the last (larger) EJC in the train.

D-AST has no obvious effect on resting muscle fiber input impedance

Current–voltage plots were constructed to determine whether D-AST-3 produced a direct effect on muscle fiber input impedance. Fig. 6 shows examples of current *versus* voltage plots for the gm1, gm4 and gm6 muscles measured with two microelectrodes in control saline and in saline containing 10^{-6} mol l^{-1} D-AST-3. In all cases, the input impedance of muscle fiber did not change over the range of membrane potentials tested. Pooled data show that the change in input impedance was less than 5 % (N=4 for each muscle; not shown). Unfortunately, we were unable to measure reliably any changes in input impedance at potentials considerably above the resting potential because depolarizing pulses produced fiber contraction that expelled the microelectrodes from the recording site. In three preparations, we tested fiber impedance at depolarized potentials in the presence of 20 mmol l^{-1} Mn^{2+} to decrease muscle contraction, and we did not detect a change in impedance in the presence of D-AST-3 (results not shown).

D-AST-3 reduces the amplitude of exogenous agonist-evoked muscle contractures

The effects of D-AST-3 on nerve-evoked contractures and synaptic responses could be produced by effects on the presynaptic release of neurotransmitter, the postsynaptic response to the transmitter, or both of these. To investigate these possibilities further, we wished to determine whether D-AST-3 reduced the amplitude of postsynaptic responses to exogenously applied ACh or glutamate. In a first set of experiments, we performed dose–response curves with agonists in the presence and absence of D-AST-3. Fig. 7A shows gm4 contractures evoked by bath application of glutamate at concentrations from 3×10^{-5} to 10^{-3} mol l^{-1}. As can be seen, D-AST-3 inhibits glutamatergic contractures at 5×10^{-5} and 10^{-4} mol l^{-1} but not at 3×10^{-4} or 10^{-3} mol l^{-1}. We took advantage of the fact that the gm4 muscle expresses extrajunctional cholinergic receptors in addition to its glutamatergic junctional receptors (Lingle, 1980) to compare the effects of D-AST-3 on cholinergic contractures in the same muscle. In these experiments, we bath-applied ACh from 10^{-7} to 5×10^{-3} mol l^{-1} in the presence of 5×10^{-5} mol l^{-1} edrophonium chloride, an acetylcholinesterase inhibitor. As

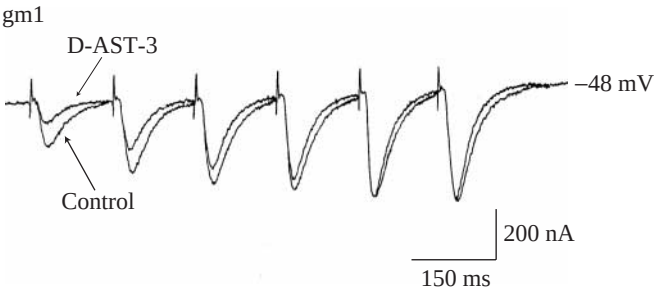

Fig. 5. Effect of 10^{-5} mol l^{-1} D-AST-3 on nerve-evoked EJCs. A gm1 muscle fiber was voltage-clamped with two microelectrodes at resting potential. The motor nerve was stimulated at 8 Hz for 750 ms, and EJC recordings were obtained under control conditions and upon bath-application of 10^{-5} mol l^{-1} D-AST-3 (shown superimposed).

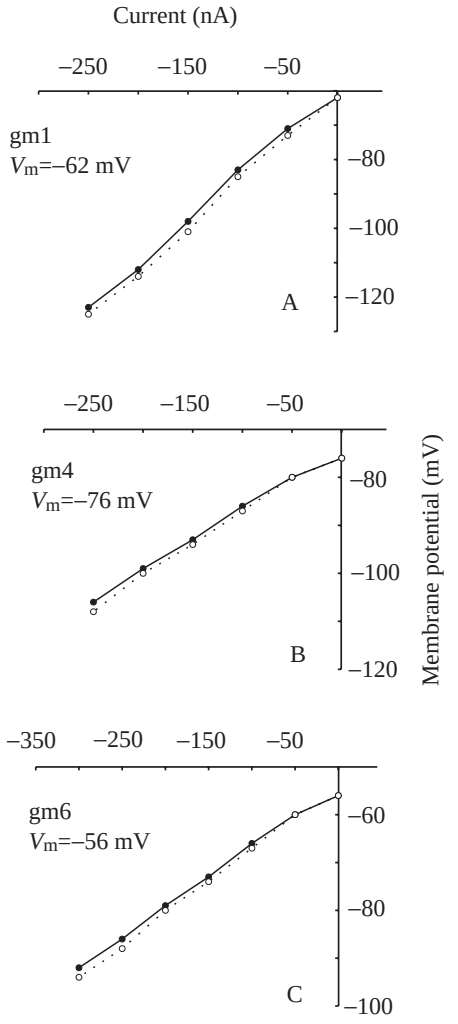

Fig. 6. Effect of 10^{-6} mol l^{-1} D-AST-3 on muscle fiber impedance. (A) gm1, (B) gm4 and (C) gm6 muscle fibers were impaled with two microelectrodes. Input impedance was measured in current-clamp mode by injecting a family of hyperpolarizing 1 sec pulses every 5 sec into the muscle fibers under control conditions (filled circles). 10^{-6} mol l^{-1} D-AST-3 was bath-applied and hyperpolarizing pulses of the same amplitude were given (open circles). The resting potential (V_m) of muscle fiber is indicated for each plot.

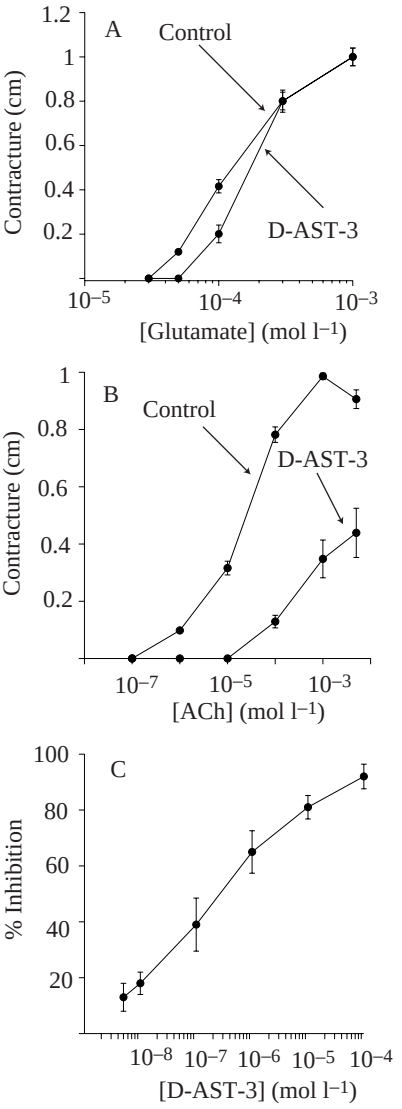

Fig. 7. Effect of 10^{-6} mol l^{-1} D-AST-3 on agonist-mediated contractures. (A) gm4 contractures were obtained by bath-application of glutamate from 3×10^{-5} to 10^{-3} mol l^{-1} with a 15 min interval between applications. After control contractures had been obtained, the same glutamate concentrations were co-applied with 10^{-6} mol l^{-1} D-AST-3 every 15 min (N=7). (B) Acetylcholine (ACh) was bath-applied from 10^{-7} to 5×10^{-3} mol l^{-1} in control saline and in the presence of 10^{-6} mol l^{-1} D-AST-3 (N=5). (C) The effect of D-AST-3 at concentrations ranging from 10^{-8} to 10^{-4} mol l^{-1} on contractures mediated by 5×10^{-5} mol l^{-1} ACh (N=5). There was a 15 min interval between each bath application. Values are means ± S.E.M.

with glutamatergic contractures, 10^{-6} mol l^{-1} D-AST-3 decreased the amplitude of the cholinergic contractures (Fig. 7B). Note that, under control conditions, 5×10^{-3} mol l^{-1} ACh produced slightly smaller contractures than 10^{-3} mol l^{-1} ACh (probably because of increased desensitization).

Dose-dependence of the effects of D-AST-3

The pronounced effect of repeated stimulation on the effectiveness of the D-AST-3-induced decrease in the amplitude of EJPs and EJCs precluded their use to determine the dose-dependence of the actions of D-AST-3. We therefore used agonist-evoked contractures to obtain a rough estimate of the dose-dependence of the effects of D-AST-3. Fig. 7C shows that the amplitude of contractures elicited by 5×10^{-5} mol l^{-1} ACh (in the presence of edrophonium chloride) was decreased in a dose-dependent manner by D-AST-3. The threshold for this effect was between 10^{-9} and 5×10^{-9} mol l^{-1} D-AST-3, with virtually complete inhibition of the contractures at 10^{-4} mol l^{-1} D-AST-3.

Does D-AST-3 act directly on the contractile mechanism?

The inhibition of agonist-mediated (ACh and glutamate) contractures does not distinguish between possible actions on the receptor–channel complex and on excitation–contraction

coupling. To look directly at the effects of AST on glutamate and ACh receptors, we applied ACh and glutamate ionophoretically to gm1 and gm8 muscles. Once a reliable ionophoretic response had been obtained, D-AST-3 was bath-applied. Fig. 8A shows that 10^{-6} mol l^{-1} D-AST-3 decreased the amplitude of the ACh response on gm1 by approximately 50 % (N=3), and Fig. 8B shows that 10^{-6} mol l^{-1} D-AST-3 decreased the amplitude of the gm8 glutamate response by approximately 40 % (N=3).

The percentage decrease in the ACh response elicited by D-AST-3 was voltage-dependent, as shown in experiments in which a gm1 muscle fiber was voltage-clamped with two microelectrodes and ACh was applied ionophoretically at different holding potentials. Fig. 9A shows ACh responses at holding potentials of −60, −70 and −80 mV before and during bath application of D-AST-3 (shown superimposed). The percentage decrease in the amplitude of the ACh response was more pronounced at more hyperpolarized membrane potentials. Fig. 9B shows pooled data from five experiments illustrating that the percentage decrease in amplitude produced by D-AST-3 was almost 50 % at −100 mV, but only approximately 30 % at −60 mV.

The experiments with ionophoretic applications of agonist demonstrate that D-AST-3 decreases agonist-evoked membrane currents, but do not eliminate the possibility that D-AST-3 also has a direct effect on the muscle contractile apparatus. To examine this possibility, we produced muscle contractures by depolarizing the muscles directly with saline containing an elevated [K^+]. To reduce the postsynaptic effects of any transmitter released from depolarized motor neuron terminals, we bathed the muscles in high concentrations of chlorisondamine, which blocks both glutamate- and ACh-activated conductances on stomatogastric muscles (Lingle, 1983; Lingle *et al.* 1981). Fig. 10 shows that 10^{-6} mol l^{-1} D-AST-3 did not decrease the amplitude of K^+-evoked contractures in gm4 (N=3), suggesting that the decrease in amplitude of agonist-evoked contractures (Figs 3, 7) can be accounted for by the decrease in amplitude of the postsynaptic responses to the agonists.

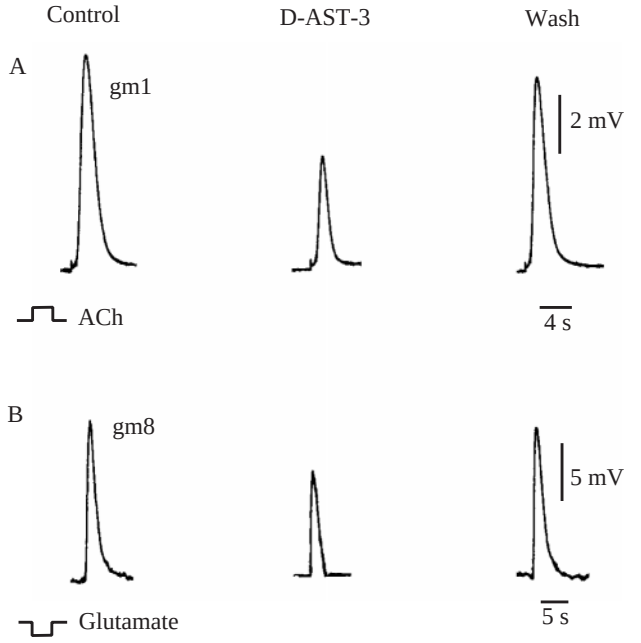

Fig. 8. Effect of 10^{-6} mol l^{-1} D-AST-3 on ionophoretic responses. (A) Acetylcholine (ACh)-mediated depolarization was obtained in a gm1 muscle fiber using a 1 mol l^{-1} ACh ionophoretic electrode in control saline and in saline containing peptide. The ionophoretic pulse was 300 nA for 200 ms. Resting potential, −53 mV. (B) Glutamate-mediated depolarization was obtained in a gm8 muscle fiber using a 1 mol l^{-1} glutamate ionophoretic electrode in control saline and in saline containing peptide. The ionophoretic current pulse was −500 nA for 400 ms. There was a 5 min interval between each ionophoretic response for each muscle. Resting potential, −61 mV.

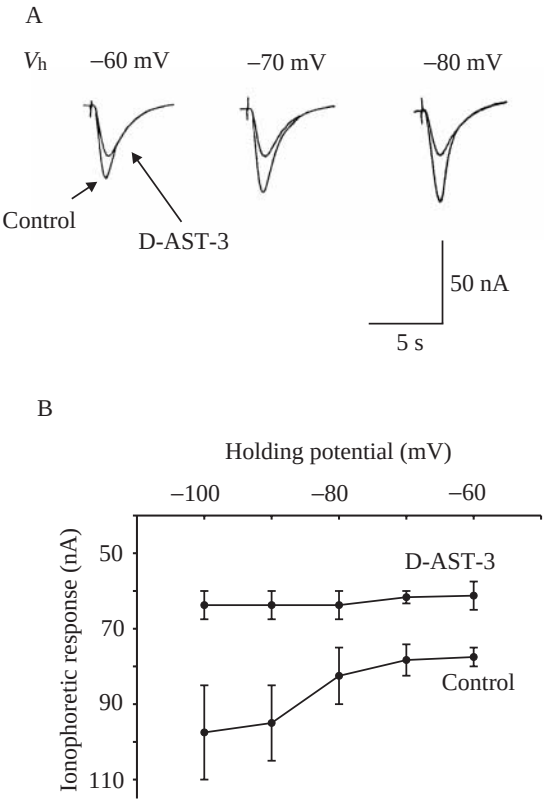

Fig. 9. The effect of D-AST-3 on acetylcholine (ACh) ionophoretic responses is voltage-dependent. (A) A gm1 muscle fiber was voltage-clamped with two microelectrodes placed on each side of an ionophoretic electrode filled with 1 mol l^{-1} ACh. The muscle fiber was clamped at a holding potential of −60 mV, −70 mV or −80 mV, and ionophoretic responses to ACh were obtained by injection of 400 nA of current for 250 ms. D-AST-3 was bath-applied at 10^{-6} mol l^{-1} and ACh responses were obtained as before. (B) Pooled data from five preparations. The error bars are standard errors of the mean.

Discussion

Modulation of the interaction between the motor neuron discharge pattern and muscle movement is known to occur in many invertebrate motor systems (Calabrese, 1989; Weiss *et al.* 1978; Whim and Lloyd, 1990). In many of the preparations

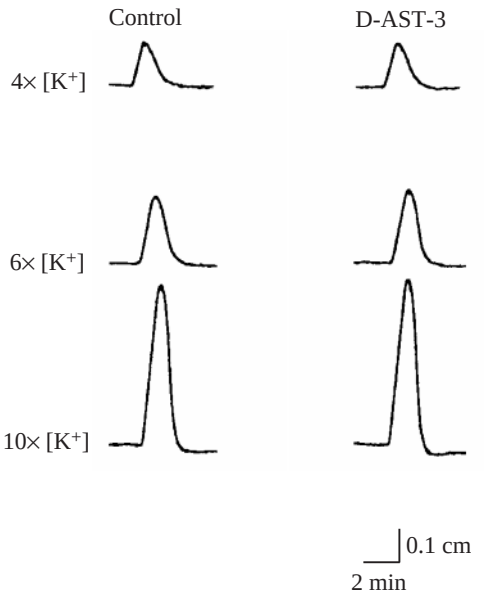

Fig. 10. The effect of 10^{-6} mol l^{-1} D-AST-3 on K^+-mediated contractures in gm4. K^+ was bath-applied at 4, 6 and 10 times its normal concentration in the presence of 10^{-3} mol l^{-1} chlorisondamine. After obtaining control traces, K^+ was co-applied at same concentrations in the presence of 10^{-3} mol l^{-1} chlorisondamine and 10^{-6} mol l^{-1} D-AST-3. The muscle was allowed to rest for 10 min between each bath application.

in which peripheral neuromodulation has been most extensively studied, considerably less is known about the mechanisms by which the circuits that generate movements are modulated than is known about peripheral modulation. In contrast, in the stomatogastric nervous system, studies of modulation of the central pattern-generating circuits have preceded the extensive study of the effects of neuromodulators on the periphery. It is now clear that many of the same neuromodulators that act on the central pattern circuits in the stomatogastric ganglion also act peripherally to modify the interaction between the motor pattern and movement of some or many of the stomach muscles (Jorge-Rivera, 1997). Many peripheral neuromodulatory actions will amplify the effect of central neuromodulation. For example, crustacean cardioactive peptide (CCAP) strongly enhances burst firing of the lateral pyloric (LP) neuron of the STG and also increases the amplitude of nerve-evoked contractions in muscles innervated by the LP neuron (Weimann *et al.* 1997).

We have shown that D-AST-3 has direct physiological actions on the peripheral stomatogastric nervous system. D-AST-3 decreases the amplitude of nerve-evoked contractions of several of the muscles of the stomach. Previous work has already demonstrated that D-AST-3 decreases the frequency of the pyloric motor pattern and the firing rates of many of the stomatogastric ganglion neurons (Marder *et al.* 1994; Skiebe and Schneider, 1994). Interestingly, the effects of AST on the STG are most pronounced when it is applied to preparations that are already relatively weakly active (Skiebe and Schneider, 1994), and our data suggest that AST will be most effective peripherally under conditions of lower firing frequency (Fig. 5). Thus, as with CCAP, to a first approximation, the effects of AST on the periphery will tend to act synergistically with its central effects.

AST-like immunoreactivity is found in the GPR neurons (Skiebe and Schneider, 1994) and therefore could be released from the peripheral terminals of these neurons as they ramify over some of the gastric mill muscles. However, a number of the muscles studied here are not known to be innervated by any AST-immunoreactive neurons and, therefore, if the actions of D-AST-3 reported here have physiological relevance, AST would have to reach those muscles by a hormonal delivery route. AST-like immunoreactivity is found in both the pericardial organs and the sinus glands of *C. borealis* (Christie *et al.* 1995), and it is therefore likely that ASTs are released into the general circulation and could act on the muscles of the stomatogastric ganglion. However, if an AST-like peptide were to act as a circulating hormone, it would need to act at relatively low concentrations, as other circulating hormones in crustaceans are found in the hemolymph at concentrations between 10^{-11} and 2×10^{-9} mol l^{-1} (Kobierski *et al.* 1987; Stangier *et al.* 1988).

Features of the physiological actions of D-AST-3 precluded accurate dose–response measurements for AST-3. Specifically, the effects of D-AST-3 were both voltage-dependent (Fig. 9) and dependent on the amplitude of the synaptic potential (Figs 4, 5). The threshold for physiological action of D-AST-3 on ACh-mediated contractures was 5×10^{-9} mol l^{-1}. However, it will be necessary to purify the native crab AST-like peptide(s) before it is possible to determine unequivocally the threshold concentration of AST for physiological action. If we assume that the native peptide(s) is likely to be more potent than the insect peptides used in the present study, then it is likely that the physiological actions reported here could be produced by hormonally delivered peptides.

Mechanism of action

The possible mechanism(s) by which D-AST-3 decreases the amplitude of both ACh and glutamate receptor-mediated increases in conductance is worth some speculation. It is hard to imagine that the same peptide could competitively inhibit the binding both of ACh to its receptor and of glutamate to its receptor, although many of the actions of D-AST-3 do appear to be competitive. Specifically, smaller EJPs and EJCs are reduced in amplitude considerably more than larger ones. If one assumes that the smaller EJPs and EJCs produced early in the train represent lower concentrations of released transmitter than the larger ones that result from the facilitation of the terminal, this would be consistent with the apparently competitive-looking curves seen with agonist-induced contractures. One possibility consistent with all of our data is that D-AST-3 activates a second-messenger system that results in modification of both the ACh receptor and the glutamate receptor, producing ‘competitive-looking’ blocks of both, although not because the peptide is blocking the action of either ACh or glutamate for its

receptor. However, a full understanding of these phenomena will require single-channel biophysical measurements and a variety of other mechanistic studies.

Functional consequences

In conclusion, it is likely that allatostatin-like peptides could function to decrease the gain of the functional transfer from motor pattern to movement in the stomatogastric system, which lacks direct inhibitory innervation. AST liberated from terminals of the GPR neurons could selectively regulate the tension and contraction in those muscles in a peripheral feedback loop. It is also possible that circulating AST could play a role in setting the tone of many of the stomach muscles, since AST sensitivity is widespread in these muscles.

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