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Fictive locomotion induced by octopamine in the earthworm

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Summary

We investigated the function of octopamine (OA) as a motor pattern inducer in the earthworm *Eisenia fetida*. We used semi-intact preparations, consisting of 20 sequential segmental ganglia of the ventral nerve cord (VNC) together with the body wall from the middle of the animal. Bath-application of 10^{-4} mol l^{-1} OA to the semi-intact preparation induced phasic muscle contractions, which are consistent with crawling. In the isolated VNC, OA induced bursts of motor neuron activity in the first lateral nerves. Burst frequency increased with OA concentration, with a large increase in activity observed in the range 10^{-6}–10^{-4} mol l^{-1}. At 10^{-4} mol l^{-1}, the coefficient of variation of burst periods (BPs) was smaller than that seen upon application of OA at other concentrations, which is indicative of rhythmic bursts. These rhythmic bursts propagated along the VNC from the anterior to posterior, with a propagation velocity ranging from 60 to 110 mm s^{-1}. This velocity is consistent with the propagation velocity of muscle contraction during crawling behavior in the intact earthworm. From these results, we conclude that fictive crawling motor patterns are observed at 10^{-4} mol l^{-1} OA, and that OA can induce rhythmic bursts in the isolated VNC of the earthworm.

Key words: central pattern generator, crawling, earthworm, *Eisenia fetida*, fictive locomotion, motor pattern, octopamine.

Introduction

Animal locomotion that consists of stereotypical repetition is controlled by a central pattern generator (CPG) (Delcomyn, 1980) located in the central nervous system (CNS). The CPG is a network of neurons capable of generating a rhythmic motor output in the absence of real movements provided by locomotor organs (for a review, see Grillner, 1999), which is usually termed fictive locomotion (for a review, see Buchanan, 1999). One way of inducing fictive locomotion is through the application of bioactive substances to the CPG. For example, in lamprey, the CPG activity responsible for generating swimming motor rhythm is located within its spinal cord (for a review, see Grillner et al., 1998) and was identified by the presence of rhythmic ventral root bursting in isolated spinal cord preparations perfused with glutamate (Cohen and Wallén, 1980). Moreover, serotonin-releasing neurons that regulate the spinal locomotor system in the river lamprey have also been reported (Zhang and Grillner, 2000).

Crawling locomotion in the earthworm may be also controlled by a CPG. When an earthworm crawls over both rough and dry surfaces, locomotion is produced by waves of body wall contractions and elongations with protraction and retraction of bristles (setae) on the lateral and ventral surfaces of the body (Friedländer, 1894; Gray and Lissmann, 1938). Peristaltic crawling in this animal was thought to be brought about by coordinated reciprocal contractions of longitudinal and circular muscle bands (for a review, see Gardner, 1976), and the motor neuron regulation of muscle activity *via* the lateral nerves has previously been investigated by several authors (Knapp and Mill, 1968; Günther, 1970; Drewes and Pax, 1974). Assmé and Chang (1988) identified in the earthworm a rhythmically discharging neuron located in the VNC, whose frequency of discharge remained constant at about 100 Hz. Although the anatomy and function of the earthworm nervous system have been investigated (for a review, see Mill, 1982), the CPG network for crawling locomotion in this animal is not fully elucidated because there have been no studies of fictive locomotion in this animal.

The monoamine octopamine (OA) is known to generate and enhance motor output pattern in the CNS of invertebrates (for a review, see David and Coulon, 1985). OA enhancement and/or inducement of the motor output pattern has been described in the leech (Hashemzadeh-Gargari and Friesen,

1989), moth (Kinnamon et al., 1984; Claassen and Kammer, 1986; Bowdan and Wyse, 2000), locust (Baudoux et al., 1998; Sombati and Hoyle, 1984; Stevenson and Kutsch, 1988) and lobster (Johnson and Harris-Warrick, 1990; Ayali and Harris-Warrick, 1999). In contrast, OA inhibits the swimming pattern in *Tritonia* (McClellan et al., 1994) and the crayfish (Mulloney et al., 1987). Moreover, OA modulation of motor patterns has been identified in the moth (Johnston et al., 1999), locust (Baudoux and Burrows, 1998; Burrows and Pflüger, 1995), lobster (Flamm and Harris-Warrick, 1986; Johnson et al., 1995; Skorupski, 1996; Johnson and Harris-Warrick, 1997) and crayfish (Gill and Skorupski, 1999).

In the central nervous system of the earthworm, *Eisenia fetida*, the distribution of OA-like immunoreactive neurons was investigated, and the concentration of endogenous OA assayed by high-performance liquid chromatography (Csoknya et al., 1996). Although previous evidence suggests that OA may function as a neurotransmitter in the CNS of the earthworm (Gardner, 1979), no definite physiological or biochemical role for OA has been identified in the earthworm nervous system. We therefore examined the effect of OA on motor output in the earthworm, and investigated the function of OA as an inducer and/or modulator of motor output pattern. Preliminary results of this work have been presented in abstract form (Mizutani et al., 2000).

Materials and methods

Animals

Earthworms, *Eisenia fetida*, were purchased from a local trader. Worms were maintained in a plastic case that contained a mixture of humid soil and sawdust at room temperature (22 °C). All experiments were performed at room temperature on sexually mature earthworms weighing more than 300 mg. Six animals were used for each experiment.

Recordings of circular muscle contraction

Before incision, earthworms were anesthetized in 10 % ethanol for 10 min. A tactile stimulus with tweezers was applied to ensure that no reflex activity was present, then the earthworm was pinned on a silicone plate and dissected from the dorsal side. The head and rear parts of the earthworm with gut were removed using a pair of fine forceps, and the 20 sequential segmental ganglia of the VNC with the body wall were isolated (semi-intact preparation) in an earthworm saline (125 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Tris-buffer, pH 7.4). The semi-intact preparation was then transferred to an experimental chamber with a silicone base, and the edges of the body wall were fixed using setting pins in readiness for the recording of circular muscle contraction. One side of the body wall in a segment located in the middle of the semi-intact preparation was pierced with a small hook, which was connected to a force transducer (Force transducer model 1030, AD Instruments com., USA), and the other side was fixed with a small setting pin. The force transducer was used to measure

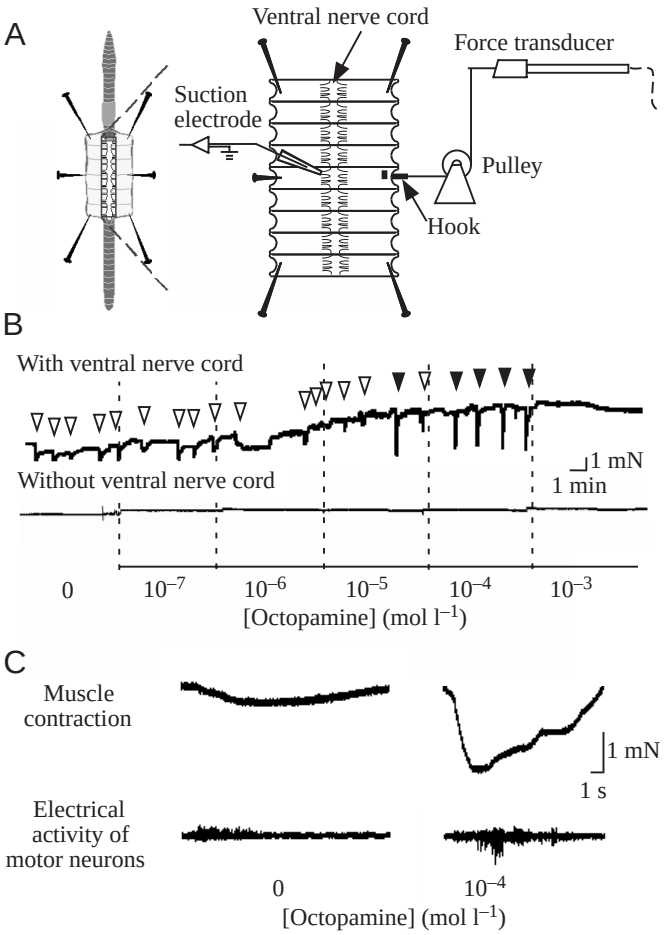

Fig. 1. Circular muscle contraction induced by OA. (A) Diagram of a semi-intact preparation. In the middle part of the dissected body wall, a small hook is connected to a force transducer *via* a pulley. Electrical activities of motor neurons were simultaneously recorded from the cut end of the first segmental nerve in the same segment using a glass suction electrode. (B) Strong phasic contractions could be observed on application of 10^{-4} mol l^{-1} OA (upper trace). In the absence of the ventral nerve cord, no contractions were observed under the same experimental conditions (lower trace). Downward deflections of the force response indicate a circular muscle contraction. The broken lines indicate when the concentration of OA was changed. Open triangles, resting level contraction; solid triangles, strong contraction associated with crawling behavior. (C) The results of simultaneous recording of muscle contraction and electrical activity from motor neurons. In control preparations, electrical activity was only seen at the onset of a contraction. At 10^{-4} mol l^{-1} OA, electrical activity occurred throughout much of the period of contraction.

contractile forces generated by circular muscle activity after the preparation had been pinned out flat (Fig. 1A). Simultaneously, body wall contraction could be observed with the aid of a light microscope. The analogue signal of the transducer output was AD-converted (sampling rate, 1 kHz; Powerlab/8SP, AD Instruments com., USA), and analyzed by PC (Power Macintosh G3, Apple computer Inc., USA) with data acquisition software (Chart version 3.6.1., AD

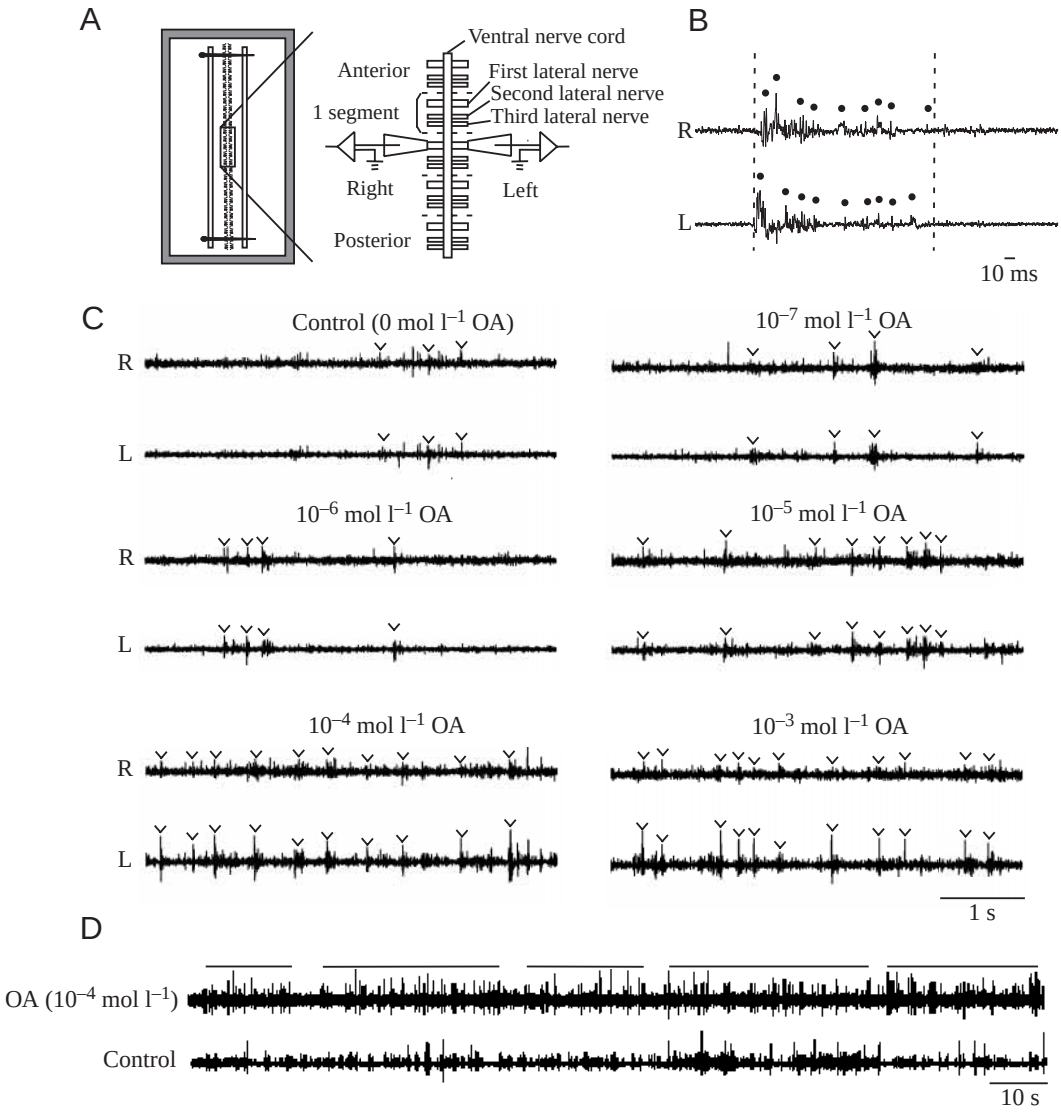

Fig. 2. Extracellular recordings of motor outputs from the first lateral nerves. (A) Schematic diagram showing configuration of suction electrodes. Right and left first lateral nerves in the same segment were suctioned to record motor activity patterns. (B) Neural activity from the right (R) and left (L) first lateral nerves was synchronized. The distance between broken lines signifies burst duration. The solid circles indicate spikes of large amplitude. (C) Extended recording time from the first lateral nerves. The arrowheads indicate burst activity. (D) At 10^{-4} mol l^{-1}, constant burst activity was observed in comparison with the control condition. The bars above each trace mark the occurrence of a crawling episode.

Instruments com., USA). The force transducer was calibrated with weights.

Electrophysiological recordings

Twenty sequential segmental VNC ganglia isolated from the body wall were transferred to an experimental chamber for electrophysiological recordings. The chamber consisted of a cover glass (40 mm×40 mm) whose edges were bordered by silicone rubber (Fig. 2A, left). Neural activities were recorded from the first lateral nerves with glass suction electrodes filled with earthworm saline. The distance between electrodes was measured using a vernier caliper. High- (>150 Hz) and low- (<3 kHz) pass filters were used for data acquisition with an amplifier (MEG-2100, Nihon Koden, Japan), and the electrophysiological data were analyzed with the PC and software described above. The sampling rate used was 10 kHz.

Octopamine application

Several different concentrations of DL-octopamine HCl (Sigma Aldrich Co., USA) were prepared in earthworm saline, and diluted by introduction into the experimental chamber with a pipette to give the final desired concentration. Before OA application, the preparation was washed for 30 min with fresh earthworm saline, and then allowed to stabilize for 15 min in the experimental chamber before beginning data acquisition. OA concentration was increased every 5 min to give intermediate OA levels ranging from 0 to 10^{-3} mol l^{-1}. All experiments were completed within 30 min.

Results

Circular muscle contraction induced by octopamine

The effect of different concentrations of OA (0–10^{-4} mol l^{-1}) on circular muscle contractile activity in the presence (Fig. 1B, upper trace) and absence (Fig. 1B, lower trace) of the VNC was measured. Under control conditions (0 mol l^{-1} OA), only sporadic contractions of circular muscle were seen. Application of a low concentration of OA (≤10^{-6} mol l^{-1}) in the presence of the VNC did not have any significant effect on the resting level of contractile activity, and the contractile tension remained low (open triangles, Fig. 1B, ≤1 mN in amplitude). The muscle gradually increased phasic contractions as the OA concentration was raised from 10^{-5} to 10^{-4} mol l^{-1}. At 10^{-5} mol l^{-1}, OA sometimes induced strong contractions, which probably mimicked muscular activity associated with crawling behavior (solid triangles, Fig. 1B, ≥2 mN in amplitude). At 10^{-4} mol l^{-1}, OA induced repetitive strong contractions (≥2 mN in amplitude). When earthworm saline with high concentrations of OA >10^{-3} mol l^{-1} was applied, tetanic-type contractions of the body wall could be observed. When the segmental ganglia of the VNC were removed from the body wall preparation, application of OA did not induce muscle contraction (Fig. 1B, lower trace). This result shows that the action of OA is directly on the segmental ganglion of the VNC directly rather than on the muscle it innervates.

Muscle contraction and motor neuron activity were recorded simultaneously in preparations with the VNC (Fig. 1C). Under control conditions (0 mol l^{-1} OA), burst activity was only seen at the onset of weak contractions, with approximately 30 spikes causing one contraction. At 10^{-4} mol l^{-1} OA, electrical activity was always seen during strong contractions, with approximately 100 spikes present for each contraction.

Rhythmic bursting induced by octopamine

We measured the electrical activities of motor neurons from the right and left first lateral nerves of the VNC isolated from the body wall (Fig. 2A). We chose to make measurements from the first lateral nerves because they form a pathway from motor neurons to muscles (Knapp and Mill, 1968; Günther, 1970; Drewes and Pax, 1978). Burst-like activity was observed, and bursts recorded from right and left lateral nerves were synchronized (Fig. 2B,C). During the bursting activities, several large spikes were observed (solid circles, Fig. 2B; the distance between broken lines indicates burst duration). Bursts were intermittent for OA concentrations from 0 to 10^{-6} mol l^{-1}, while from 10^{-5} to 10^{-3} mol l^{-1} burst frequency (number of bursts appearing per second) increased (Fig. 2C). At 10^{-4} mol l^{-1} OA in particular, bursts were observed with a constant period (Fig. 2D). Such bursts appeared from 2 to 5 min after OA application, and continued for more than 120 s. We defined these constant-period-bursts as motor patterns responsible for fictive locomotion. We measured the times at which the spikes with high amplitude occurred in a burst, and calculated burst periods (BPs, Fig. 3A). We also counted the number of bursts recorded from 2 to 5 min after application of each concentration of OA, and calculated burst frequency

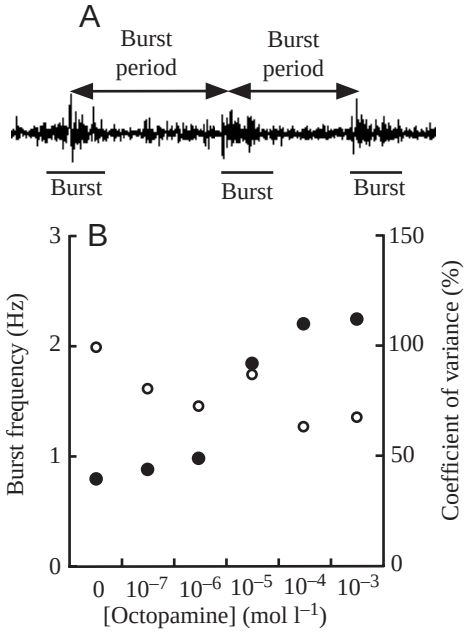

Fig. 3. Burst period (BP) and its coefficient of variance as a function of concentration (*N*=6). (A) We measured the BP and counted the number of bursts from 2 to 5 min after OA application. (B) A significant increase in the frequency of burst activity was observed for OA concentrations in the range from 10^{-6} mol l^{-1} to 10^{-4} mol l^{-1}. The coefficient of variation (the square root of BP variance divided by its mean) was small during fictive locomotion. Solid circles, burst frequency; open circles, coefficient of variance.

(burst number per second, Fig. 3B, solid circles, *N*=6). Burst frequency was found to increase with OA concentration. From 0 to 10^{-6} mol l^{-1}, burst frequency was constant and low. A significant increase in burst frequency was observed when OA concentration was increased from 10^{-6} to 10^{-4} mol l^{-1}, and saturated at 10^{-3} mol l^{-1}. Standard deviations of BPs decreased with increasing OA concentration, suggesting that OA stabilized bursting activities. The coefficients of variation of BP (the square root of BP variance divided by its mean) were small for bursting activity associated with fictive locomotion at 10^{-4} mol l^{-1} OA (Fig. 3B, open circles). In contrast, the coefficient of variation of BP at 10^{-5} mol l^{-1} OA was large. This finding is consistent with the results describing muscle contraction (Fig. 1B), given the interspersed weak and strong contractions at this concentration of OA. At 10^{-3} mol l^{-1} OA, the coefficient of variation of BP was small, and while the motor pattern was phasic, muscle contractile activity was tetanic in appearance (Fig. 1B). This result indicates that burst frequency is more regular in fictive locomotion at 10^{-4} mol l^{-1}.

Propagation of octopamine-induced burst activities

We simultaneously recorded electrical activities from the first lateral nerves of three segmental ganglia separated by intervening segments (Fig. 4A). OA-induced bursts propagated from the anterior to posterior segments (Fig. 4B). By timing the burst activity (arrowheads in Fig. 4B) we were able to determine burst latencies (solid triangles in Fig. 4B), from which the

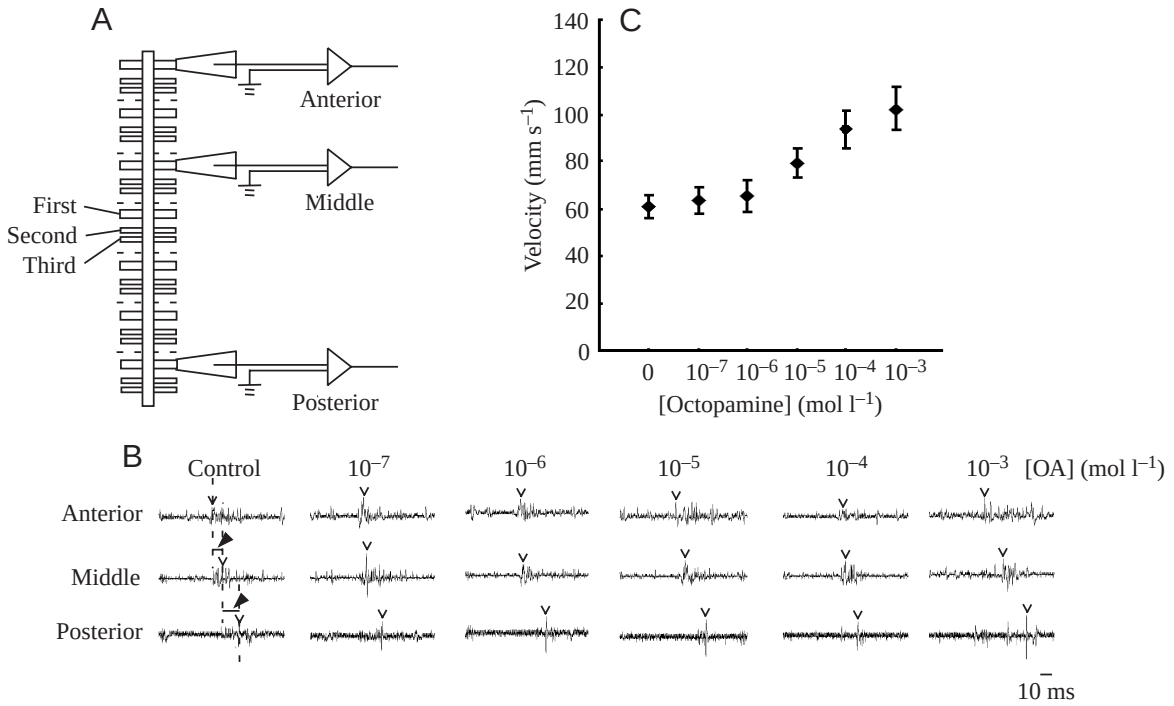

Fig. 4. Motor output recorded from first lateral nerves of three (anterior, middle and posterior) segmental ganglia separated by intervening segments. (A) Experimental setup. (B) Bursts propagated along VNC. Arrowheads signify the timing of highest amplitude spikes in bursts. Bars (indicated by solid triangles) are latencies. In this case, the distances between the anterior and middle electrodes, and middle and posterior electrodes, are 0.6 and 1.8 mm, respectively. (C) Propagation velocity of bursts along VNC (*N*=6). The propagation velocity was approximately 60–110 mm s^{-1}, and increased with increasing octopamine concentration. Values are means ± S.D. A noticeable increase in propagation velocity was observed for octopamine concentrations between 10^{-6} mol l^{-1} and 10^{-3} mol l^{-1}.

propagation velocities of OA-induced bursts could be calculated (Fig. 4C, *N*=6). We found that, for each OA concentration, latency increased linearly with distance from the preceding electrode and that bursts propagated at a constant velocity. The propagation velocity was in the range 60–110 mm s^{-1}, and increased with increasing OA concentration (10^{-6}–10^{-3} mol l^{-1}) (Fig. 4C). In the presence of 0 to 10^{-6} mol l^{-1} OA, burst velocity was constant and low, while at concentrations over 10^{-5} mol l^{-1}, OA increased the burst propagation velocity.

Discussion

Fictive locomotion

Rhythmic activity produced by the CPG, in the absence of real movements produced by locomotor organs, is usually termed fictive locomotion (for a review, see Buchanan, 1999). In this study, we have shown that OA induces fictive locomotion in the earthworm, *Eisenia fetida*. The fictive locomotion observed as periodic circular muscle contraction required the presence of the VNC in the experimental preparation (Fig. 1B). Periodic circular muscle contraction was observed at an OA concentration in excess of 10^{-4} mol l^{-1}. Burst activity frequency measured from the isolated lateral nerves was increased as OA concentration increased (Fig. 3B). At 10^{-4} mol l^{-1} OA, bursts were observed with a constant period. In addition, bursts propagated from the anterior to posterior segments at a constant velocity, which increased in magnitude as the OA concentration was raised from 10^{-5} mol l^{-1} to 10^{-3} mol l^{-1} (Fig. 4C). Given that swimming movements in the lamprey are generated by unitary oscillators located within the spinal cord, and propagate from anterior to posterior (Matsushima and Grillner, 1992), we suggest that crawling in the earthworm is also generated by a series of distributed oscillators located within the segmental ganglia of the VNC.

Burst propagation

Burst activities propagated along the VNC from anterior to posterior (Fig. 4B), with a propagation velocity in the range of 60–110 mm s^{-1} (Fig. 4C). In our previous observations of the earthworm's crawling behavior, periodic contractions of the middle part of the body were found to drag on the caudal part during crawling (Mizutani et al., 1999). The muscle contraction velocity of the middle part of the body wall during crawling in intact animals, estimated from the propagation velocity of the extracellular field potential of muscle origin, is approximately 50 mm s^{-1} (Drewes et al., 1978). This propagation velocity agrees with the burst propagation velocity along the VNC found in this study since it is known that, in the isolated preparation, the electrical wave propagation is slightly faster than the mechanical wave (Orlovsky et al., 1999). The propagation velocity of electrical activity along the VNC during fictive locomotion (10^{-4} mol l^{-1} OA) in this study

was approximately 90 mm s^{-1}, which is almost twice that of the propagation velocity of myoelectricity (approximately 50 mm s^{-1}) in intact animals. The propagation velocity appeared to become faster as the OA concentration was increased (Fig. 4C). This result suggests that OA controls the burst propagation velocity in the VNC, thereby regulating the propagation velocity of the contracting body wall during earthworm crawling. In another study, it was found that OA modulates facilitation of a feeding pattern in the pond snail *Lymnaea stagnalis* (Elliott and Vehovszky, 2000), and that this facilitation is specific to an OA-containing interneuron. Thus it is possible that the mechanism of control of burst propagation velocity by OA in this study may be an increase in the frequency of CPG activity in each segment and/or facilitation in OA interneurons between segments.

Cellular mechanisms

OA is a candidate neurotransmitter in the earthworm nervous system (Gardner, 1979). The distribution of OA-like immunoreactive neurons in the central nervous system of the earthworm, *Eisenia fetida*, was investigated by Csoknya et al. (1996), who also reported their OA content (12.6±0.17 pmol mg^{-1}, 0.88±0.17 pmol per ganglion). Neverthless, the physiological role of OA in the earthworm has not been identified. This work shows, for the first time, that OA induces fictive locomotion in the earthworm, thereby providing a physiological role for OA in fictive locomotion, and serving the same function as glutamate in the lamprey (Cohen and Wallén, 1980).

The presence of an OA-sensitive adenylate cyclase has been reported in the nervous tissue of the earthworm, *Lumbricus terrestris* (Robertson and Osborne, 1979). In isolated leech nerve cords, OA modulated swimming patterns in a manner that was mimicked by cyclic adenosine 3′,5′-monophosphate (cAMP) application (Hashemzadeh-Gargari and Friesen, 1989). An increase in phasic feeding-burst frequency at 10^{-4} mol l^{-1} OA was observed in an isolated *Manduca sexta* ganglion preparation (Bowdan and Wyse, 2000), in addition to the fact that OA has been shown to alter the level of excitation and/or effectiveness of synaptic transmission in central neurons (Kinnamon et al., 1984). We consider that OA may alter the level of excitation and/or effectiveness of synaptic transmission in the CPG by increasing neuronal cAMP, which is activated by an OA-sensitive adenylate cyclase.

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