MODULATION OF THE BUCCAL MUSCLE CONTRACTION BY IDENTIFIED SEROTONERGIC AND PEPTIDERGIC NEURONS IN THE SNAIL *ACHATINA FULICA*

MASAYUKI YOSHIDA[1] AND MAKOTO KOBAYASHI[2],*

[1]*Laboratory of Fish Physiology, Faculty of Applied Biological Science, Hiroshima University, Higashi-Hiroshima, Japan* and [2]*Physiological Laboratory, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima, Japan*

Summary

Serotonergic and peptidergic modulation of buccal muscle contraction at an identified neuromuscular synapse in the African giant snail *Achatina fulica* was examined. A pair of excitatory motoneurons of the radula protractor was identified in the buccal ganglia and these were named B10 neurons. Pharmacological experiments revealed the B10s to be cholinergic. The serotonergic cerebral neuron v-CDN enhanced B10-evoked contraction of the radula protractor, and it is suggested that this effect is mediated postsynaptically by serotonin released from the v-CDN terminals.

Immunohistochemical analyses revealed that the B10 motoneurons contained an *Achatina* cardioexcitatory peptide-1 (ACEP-1)-like substance in their cell body, axon and terminals in the radula protractor. From electron microscopic observation, the ACEP-1-like immunoreactive substance was found to be localized in dense-cored vesicles but not in clear vesicles in the nerve terminals. ACEP-1 applied to the radula protractor markedly enhanced B10-evoked contraction of the muscle by increasing the amplitude of excitatory junction potentials (EJPs). The increase of EJP amplitude in the presence of the peptide was probably due to the increased release of acetylcholine from the terminals of B10. It is suggested that the cholinergic motoneuron B10 uses ACEP-1 as an excitatory cotransmitter.

Key words: African giant snail, *Achatina fulica*, identified synapse, serotonin, ACEP-1.

Introduction

The aim of this study was to reveal the features of peptidergic and serotonergic modulatory mechanisms in an identified neuromuscular system of *Achatina fulica*.

Recently, the modulation of muscle movement by peptide cotransmitters has been closely examined in some molluscan species (Buckett *et al.* 1990; Cropper *et al.* 1990; Weiss *et al.* 1992). A molecular genetic approach is also being used to investigate the physiological and evolutionary importance of the co-existence of multiple peptides in neuromuscular synapses (Linacre *et al.* 1990; Miller *et al.* 1993*a*,*b*). Neuronal activity-dependent release of a peptide modulator may enable a single neuron to exert a subtle regulation of muscle contraction in various circumstances. Such a mechanism may well be useful for animals, such as pulmonates and opisthobranchs, that have a relatively small number of central neurons involved in the control of their movements.

In the African giant snail *Achatina fulica*, many bioactive peptides have been isolated from various tissues, and the amino acid sequences of some of them have been determined (Fujimoto *et al.* 1990, 1991; Ikeda *et al.* 1992; Harada *et al.*

1993). Among them, *Achatina* cardioexcitatory peptide-1 (ACEP-1), an undecapeptide isolated from the atria, shows both cardioexcitatory activity and an enhancing effect on the contraction of several muscles, including the buccal muscles, in this snail (Fujimoto *et al.* 1990).

Serotonergic modulation of buccal muscle movement has been widely studied in gastropod molluscs (Weiss *et al.* 1978, 1979; Granzow and Rowell, 1981; Zoran *et al.* 1989; Yoshida and Kobayashi, 1991). In the buccal motor system of *A. fulica*, serotonergic modulation of radula retractor contraction elicited by the identified cholinergic motoneuron B4 has been demonstrated (Yoshida and Kobayashi, 1991). It has also been suggested that serotonin is involved in the control of the buccal motor rhythm in *A. fulica* (Yoshida and Kobayashi, 1992). In this species, it has been suggested that the widely distributed innervation by the serotonergic cerebral neuron v-CDN has a role in raising the excitability of the feeding system overall (Yoshida and Kobayashi, 1991, 1992).

The rhythmic motor pattern in the buccal mass of *A. fulica* can be divided into three phases; the radula protraction phase,

*Author for correspondence.

the radula retraction phase and an inactive phase. So far, several pairs of motoneurons that are active during the radula retraction phase have been identified and their mode of action has been analysed (Yoshida and Kobayashi, 1991). In the present study, a pair of motoneurons, designated B10s, that are active during the protraction phase has been identified. It is suggested that these contain ACEP-1 in addition to the main transmitter, acetylcholine (ACh). The way in which radula protractor contraction is modulated by ACEP-1, as well as by serotonin, has been investigated physiologically.

Materials and methods

Animals and physiology

The African giant snails *Achatina fulica* Férussac used in this study were captured in Okinawa and transported to Hiroshima by air. The animals were reared in moist containers at 25 °C and fed on sweet potatoes.

For physiological experiments, the radula protractor was dissected from the animal with the buccal ganglia attached *via* buccal nerve 2 (see Fig. 1 of Yoshida and Kobayashi, 1991). The rostral part of the thin connective tissue sheath covering the ganglia was then surgically removed. The experimental chamber was separated into two compartments by a partition containing a slit through which buccal nerve 2 was passed. These two compartments, one for the ganglia and the other for the muscle, were perfused independently. The electrical activities of neurons and muscle fibers were recorded by means of conventional electrophysiological technique. Muscle tension was recorded through a force transducer connected to an amplifier. The recordings obtained were stored on FM tape for later analyses.

The physiological solution used in the experiments contained (in $mmol\,l^{-1}$): NaCl, 61; KCl, 3.3; $CaCl_2$, 10.7; $MgCl_2$, 13; glucose, 5; Hepes, 10 (pH 7.5). To block the intraganglionic chemical synapses, the ganglia compartment was perfused with high-Mg^{2+} (3× normal), Ca^{2+}-free solution. To block the polysynaptic connections, the preparation was perfused with high-Mg^{2+} (3× normal), high-Ca^{2+} (3× normal) solution.

All physiological experiments were performed at room temperature (20–25 °C).

Immunocytochemistry

Light microscopy

The buccal ganglia and the radula protractor were dissected from the animal. The radula protractor was fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS) containing $0.1\,mol\,l^{-1}$ sucrose. The ganglia were incubated in saline containing Protease (Sigma type IV, $12\,mg\,ml^{-1}$ saline) for 1 h, then fixed in the above solution. Both the ganglia and the muscle were then processed in the same way, with all procedures being carried out at 4 °C. The fixed tissues were washed in PBS containing 1 % Triton X-100 for 20 h and then incubated in a blocking solution (1 % bovine serum albumin,

1 % normal goat serum, 1 % Triton X-100 and 0.05 % NaN_3 in PBS) for 2 h. The tissues were incubated in a 1:1000 dilution of primary antibody (rabbit polyclonal antiserum raised against ACEP-1) for 72 h. They were washed in PBS for 24 h and then incubated with a 1:600–1000 dilution of Rhodamine-labeled secondary antibody for 12–24 h. The preparations were washed in PBS for 48 h and cleared in a 6:1 mixture of glycerol and PBS overnight. The preparations were viewed and photographed using a fluorescence microscope.

When PBS was substituted for the primary antibody, no structure was labeled by Rhodamine. In addition, when one half of the bilaterally symmetrical tissue was incubated in the primary antibody while the other half was treated with antibody preincubated with $10^{-4}\,mol\,l^{-1}$ ACEP-1 for 24 h at 4 °C, the fluorescent staining of the preincubated side was greatly reduced.

Electron microscopy

The radula protractor was dissected from the animal in high-Mg^{2+}, Ca^{2+}-free solution. The dissected muscle was fixed in 3 % glutaraldehyde in PBS containing $0.1\,mol\,l^{-1}$ sucrose for 3 h at room temperature. The fixed muscle was washed in $0.1\,mol\,l^{-1}$ phosphate buffer (PB) for 2 h. The tissue was dehydrated and embedded in LR-White resin. The resin was then polymerized by ultraviolet illumination at −20 °C for 48–72 h. Ultra-thin sections were cut and plated on nickel mesh.

The sections were incubated with 1 % bovine serum albumin in PBS for 1 h and then incubated with a 1:600 dilution of primary antibody raised against ACEP-1 for 6 h at room temperature. They were washed in PBS containing Tween-20 for 15 min and incubated with a 1:50 dilution of protein-A–gold (10 nm particle diameter) for 3 h at room temperature. The sections were washed in PBS containing Tween-20 for 15 min and refixed in 1 % glutaraldehyde for 10 min. They were then electron-stained with uranyl acetate and lead citrate. The stained sections were viewed and photographed using a transmission electron microscope.

Results

Protractor motoneuron B10

A motoneuron innervating the radula protractor was identified from its position beneath a cluster of small cells located on the rostral surface of the buccal ganglion. This motoneuron was designated B10. The positions of the B10 motoneurons, as well as previously identified neurons in the buccal ganglia, are shown schematically in Fig. 1A. Fig. 1B shows a reconstruction of the morphology of B10 stained intracellularly with Lucifer Yellow. B10 has a single axon in n2, which innervates both the radula protractor and the radula retractor.

Fig. 2A shows an intracellular recording of spontaneous activity from B10 and an extracellular recording of activity in the radula protractor muscle. In addition to a 1:1 relationship
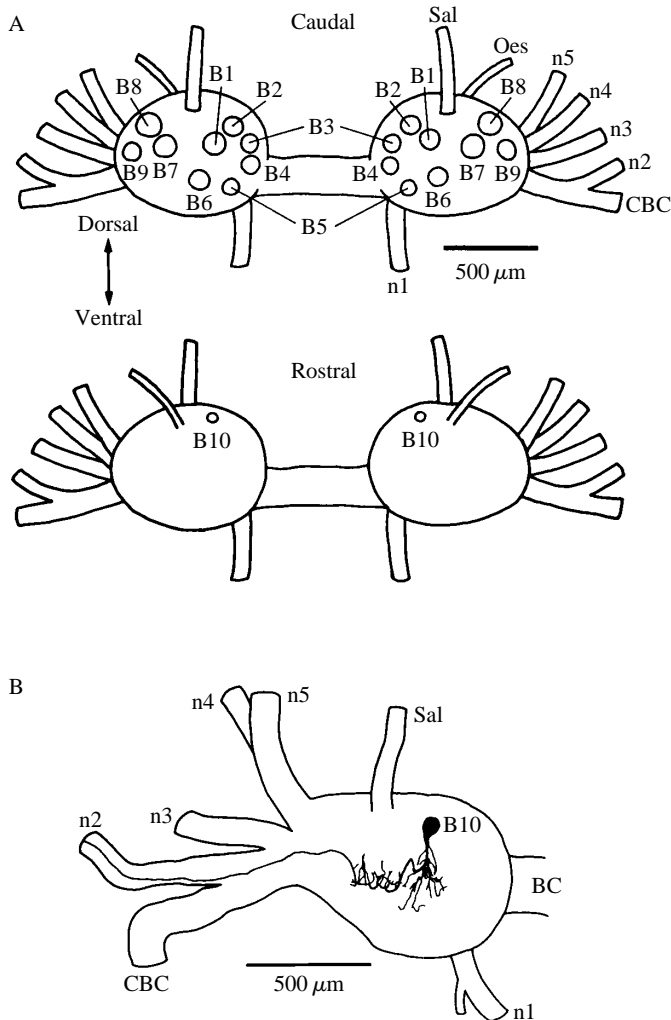
Fig. 1. (A) Schematic drawing of the buccal ganglia showing the positions of identified neurons (B1–B10). (B) Morphology of R-B10 stained by the injection of Lucifer Yellow. Rostral view of the right buccal ganglion is shown. BC, buccal commissure; CBC, cerebrobuccal connective; n1–n5, buccal nerves 1–5; Oes, oesophageal nerve; Sal, salivary nerve.

between the muscle EJPs and B10 firing, two different types of EJP activity were observed in the muscle (Fig. 2A, arrows). The radula protractor thus appears to be innervated by at least three excitatory motoneurons. Fig. 2B shows intracellularly recorded EJPs in the radula protractor evoked by the firing of B10. These EJPs showed summation as the frequency of B10 firing was increased (Fig. 2Bi,ii), finally reaching a plateau (Fig. 2Biii). No action potential was recorded from the muscle fibers of the radula protractor in the course of these experiments.

To test the monosynapticity of the connection between B10 and the radula protractor, intraganglionic chemical synapses were blocked by perfusing the ganglia compartment with high-Mg^{2+}, Ca^{2+}-free solution. Such conditions did not affect the contraction of the radula protractor evoked by B10 firing (Fig. 2C), suggesting that B10 makes a monosynaptic connection onto the muscle.

Excitatory transmitter of B10

In a previous study, an identified motoneuron, B4, innervating the radula retractor was shown to be cholinergic (Yoshida and Kobayashi, 1991). In the present series of experiments, we found that the protractor motoneuron B10 is also cholinergic. Perfusion of the muscle compartment with a cholinergic blocker, propantheline, at a concentration of $3\times10^{-5}\,mol\,l^{-1}$ completely blocked the B10-evoked protractor contraction (Fig. 3A). Partial recovery of the contraction was obtained after washing the muscle with saline. Fig. 3B shows the intracellularly recorded EJPs in the radula protractor evoked by B10 firing. The B10-evoked EJP in the radula protractor was also blocked by propantheline. In addition, contraction of the radula protractor induced by direct application of acetylcholine (ACh) to the muscle was blocked by perfusing the muscle with a solution containing $10^{-5}\,mol\,l^{-1}$ propantheline (Fig. 3C). Furthermore, another cholinergic blocker, hexamethonium at a concentration of $10^{-4}\,mol\,l^{-1}$, showed similar effects to those of propantheline in blocking the contraction and EJPs of the radula protractor elicited by B10 stimulation, although hexamethonium was less effective than propantheline (data not shown). These results suggest that the main excitatory transmitter of B10 is probably ACh.

Synaptic connection of B10

B10 was found to receive synaptic input from the identified buccal ganglionic neuron B1. B1 is a motoneuron of the radula retractor and it has been suggested that it is also involved in buccal rhythm generation (Yoshida and Kobayashi, 1992). Firing of B1 evoked by depolarizing current injection induced compound postsynaptic potentials (PSPs) in B10 (Fig. 4A). The PSPs appeared to contain two components; fast inhibitory PSPs (IPSPs) and gradually rising depolarizing PSPs that overcame the IPSPs at the end of B1 stimulation. The fast IPSPs persisted when the ganglia were perfused with high-Mg^{2+}, high-Ca^{2+} solution, which is known to block polysynaptic pathways by raising the spike threshold of interneurons in *A. fulica* (Furukawa and Kobayashi, 1987), whereas the depolarizing PSPs disappeared in this solution (Fig. 4B). In such conditions, unitary IPSPs can be observed in B10. The IPSPs in B10 followed the action potentials of B1 in a 1:1 manner with a constant latency of approximately 30 ms. The inhibitory connection between B1 and B10 thus appears to be monosynaptic. The depolarizing PSPs may be produced *via* activation of the buccal rhythm generator, which is excited by B1 firing (Yoshida and Kobayashi, 1992).

Serotonergic modulation of protractor contraction

An identified serotonergic modulatory neuron, v-CDN, has an enhancing effect on the contraction of the radula retractor evoked by a cholinergic motoneuron B4 in *A. fulica* (Yoshida and Kobayashi, 1991). In the present study, a similar enhancing effect of v-CDN was also found in the B10–protractor system. Firing of v-CDN enhanced the contraction of the radula protractor evoked by B10 firing

A

B10

Pro

40 mV

0.2 mV

10 s

Bi   Bii   Biii   C   Control   Ca²⁺-free

Pro

B10

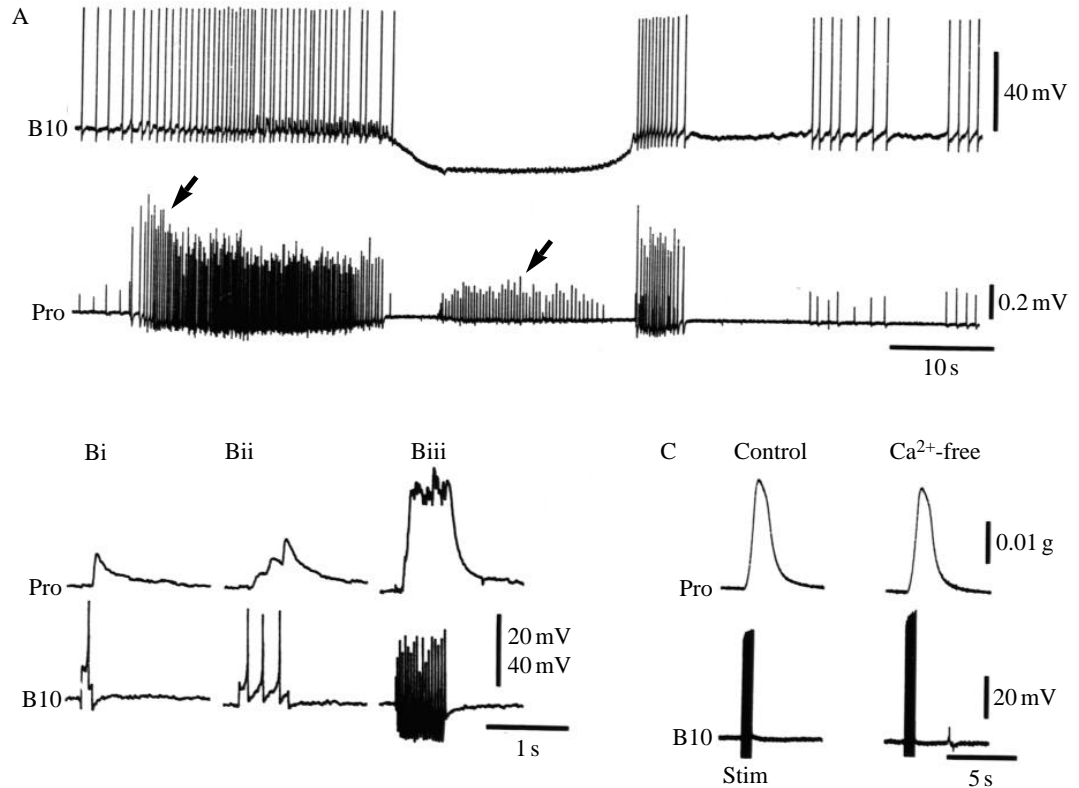20 mV
40 mV

1 s

Pro

B10

Stim

0.01 g

20 mV

5 s

Fig. 2. (A) Simultaneous recordings of spontaneous activity in B10 and in the radula protractor (Pro) made intracellularly from the neuron and extracellularly from the muscle. Firing of B10 causes muscle EJPs in 1:1 manner. Besides the EJPs elicited by B10 firing, two other EJP components (arrows) are observed. (B) Intracellularly recorded EJPs in the radula protractor elicited by artificially evoked B10 firing. (C) Contraction of the radula protractor evoked by B10 stimulation (Stim, 20 Hz, 1 s) was not reduced by perfusing the ganglia with high-Mg^{2+}, Ca^{2+}-free solution.

(Fig. 5A). In the central ganglia, B10 received slow and long-lasting inhibitory input from v-CDN (Fig. 5B).

To determine the site of action of v-CDN in enhancing the muscle contraction, we examined the effect of serotonin, which is a putative transmitter of the neuron, on the muscle contraction evoked by application of ACh. As shown in Fig. 5C, serotonin enhanced the ACh-induced contraction of the radula protractor. This suggests that v-CDN enhances the muscle contraction, at least in part, by affecting the muscle fibers directly.

Peptidergic modulation of protractor contraction
Localization of ACEP-1-like immunoreactive substance

Fig. 6 shows immunoreactivity of the buccal ganglia to the polyclonal antibody raised against ACEP-1. A pair of immunoreactive cells (arrows), each about 60 μm in cell body diameter, was found to be located in the same position as that of B10 neurons. To confirm that ACEP-1-like immunoreactive neurons were indeed B10s, immunostaining of the preparation was performed after physiological identification and intracellular staining of B10. Fig. 7Ai,ii shows the cell body and axon of a B10 neuron stained intracellularly with Lucifer Yellow after the cell had been physiologically identified by testing the direct excitatory innervation to the radula protractor. Fig. 7Bi,ii shows the ACEP-1-like immunoreactivity of the same regions as those in Fig. 7Ai,ii, thus confirming that a physiologically identified B10 contains an ACEP-1-like substance. In all preparations tested (N=4), B10 showed ACEP-1-like immunoreactivity. In buccal nerve 2, which

innervates the radula protractor and retractor, the axon of B10 showed ACEP-1-like immunoreactivity (Fig. 7Aii, Bii).

Fig. 8A shows ACEP-1-like immunoreactivity in the radula protractor. Many neural fibers and terminal-like structures contain ACEP-1-like immunoreactive substance. B10 is the only neuron, among all the cells so far examined, that shows both an innervation to the radula protractor and ACEP-1-like immunoreactivity. Thus, the neural fibers and the terminals showing ACEP-1-like immunoreactivity in the radula protractor are likely to be those of the B10 neuron.

From conventional electron microscopical observation, many neuronal elements in the radula protractor were found to contain two types of vesicles, clear and dense-cored vesicles (not shown). Intracellular localization of ACEP-1-like substance was examined by using the protein-A–gold method. In this process, to prevent the reduction of antigenicity of the ACEP-1-like substance, post-fixation with OsO_4 was not carried out, so the membrane structure of the immunostained section is not clearly visible. Nevertheless, by comparing the immunostained sections with sections processed by conventional electron microscopic procedure, the structures marked by arrows and arrowheads in Fig. 8B apparently correspond to the dense-cored vesicles (arrows) and clear vesicles (arrowheads) observed in conventional sections. Fig. 8B shows the neuronal elements in the radula protractor. Gold particles are specifically observed in dense-cored vesicles, indicating that ACEP-1-like material is localized in these vesicles, but not in the clear vesicles, which probably contain ACh.
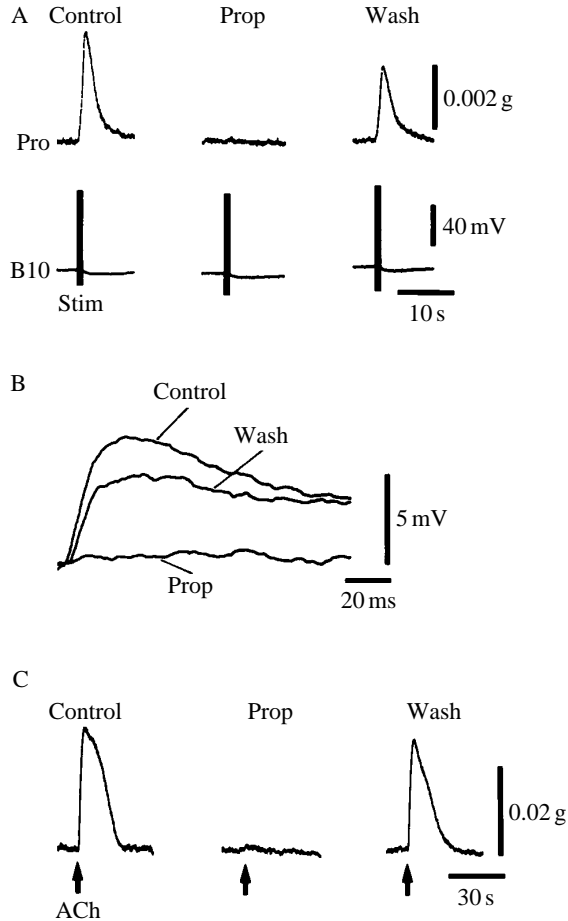
Fig. 3. (A) Effect of 3×10^{-5} mol l^{-1} propantheline (Prop) on contraction of the radula protractor in response to B10 stimulation (30 Hz, 1 s). Propantheline was applied to the muscle by perfusion for 5 min before the recording was made. (B) Effect of 3×10^{-5} mol l^{-1} propantheline on the B10-evoked EJP of the radula protractor. Each trace is an average of 15 EJPs evoked at 5 s intervals. Propantheline was applied to the muscle through a small pipette placed adjacent to the muscle. (C) Effect of 10^{-5} mol l^{-1} propantheline on contraction of the radula protractor in response to 10^{-5} mol l^{-1} acetylcholine (ACh). Propantheline was applied to the muscle by perfusion for 3 min before the recording was made.

Effects of ACEP-1 on the radula protractor

Fig. 9A shows enhanced contractions of the radula protractor in the presence of ACEP-1. Muscle contractions, induced by artificially evoked bursts of B10 with constant interburst intervals, were markedly enhanced when the muscle compartment was perfused with 10^{-8} mol l^{-1} ACEP-1. The contractions returned to normal levels after washing the muscle with saline. ACEP-1 itself did not cause contraction of the radula protractor when it was applied to the muscle (Fig. 9A). The threshold concentration of ACEP-1 was about 10^{-9} mol l^{-1}. The variation of the B10 spike size seen in Fig. 9A is due to difficulty in balancing the deflection caused by current injection from the recording electrode. Nevertheless, the frequency of evoked spikes can be precisely
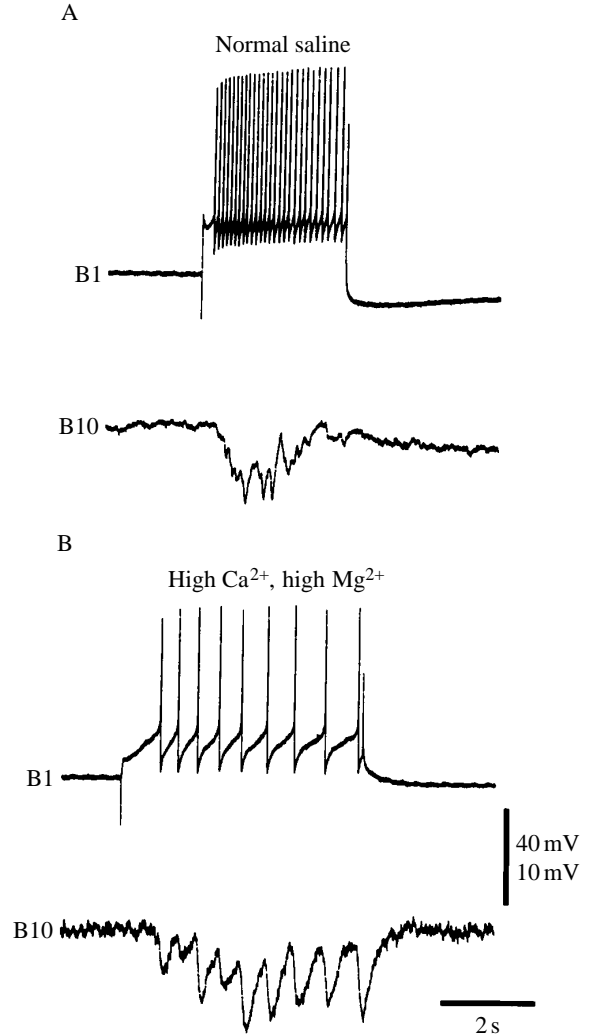

Fig. 4. The connection between B1 and B10 appears to be monosynaptic. B1 was made to fire by depolarizing current injection. (A) Ganglia were perfused with normal saline. (B) The same ganglia were perfused with high-Ca^{2+}, high-Mg^{2+} solution.

controlled. The interspike activity of B10 seen in Fig. 9A consists of synaptic inputs that did not cause action potentials in B10 during the recording period.

Fig. 9B shows the intracellularly recorded EJPs of the protractor evoked by B10 firing before, during and after the application of ACEP-1 to the muscle. While the EJPs were monitored, the peptide was applied to a small area of the muscle from a fine pipette placed immediately adjacent to the recording region to keep the enhancement of the muscle contraction as low as possible, thus enabling continuous recording. The concentration of the peptide at the recording region was probably lower than that in the pipette, since the rest of the muscle was continuously perfused with saline. B10-evoked EJPs in the protractor were apparently increased by ACEP-1 (Fig. 9B). Enhancement of the muscle contraction by ACEP-1, therefore, appears to be at least in part the result of the enlarged B10-evoked EJPs in the presence of the peptide.

To reveal the site of action of ACEP-1, we tested the effect

A

v-CDN

| 40 mV

B10

| 40 mV

Pro

| 0.01 g

1 min

B

v-CDN

B10

4 s | 100 mV
      | 5 mV

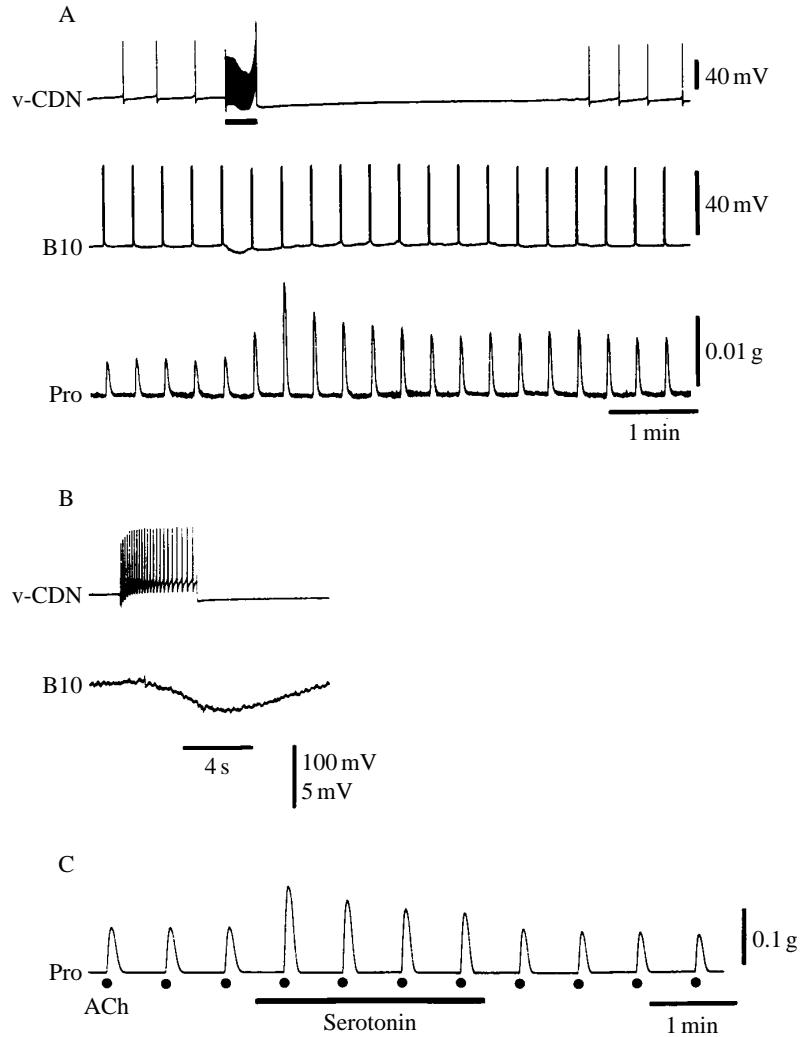C

Pro

| 0.1 g

ACh

Serotonin   1 min

Fig. 5. (A) The enhancing effect of v-CDN on contractions of the radula protractor evoked by B10 stimulation (20 Hz for 1 s at 20 s interburst intervals). v-CDN was made to fire by depolarizing current injection during the period indicated by the bar under the recorded trace. (B) Inhibitory connection from v-CDN to B10. v-CDN was made to fire by depolarizing current injection. (C) Enhancing effect of serotonin on contractions of the radula protractor evoked by brief application of 10^{-5} mol l^{-1} ACh (dots). 10^{-7} mol l^{-1} serotonin was applied to the muscle by perfusion during the period indicated by the bar under the recorded trace.

of the peptide on the contraction of the radula protractor induced by direct application of ACh to the muscle. As shown in Fig. 9C, ACEP-1 did not affect the tension of ACh-evoked contraction of the muscle. This result suggests that the enhancing effect of the peptide is mainly mediated presynaptically.

Discussion

The enhancing action of serotonergic v-CDN on the contraction of the radula retractor has been reported previously (Yoshida and Kobayashi, 1991). In the present study, we found that the contraction of the antagonistic muscle, the radula protractor, is similarly enhanced by the same neuron. Thus, the B10-evoked contraction of the radula protractor seems to be modulated by the postsynaptic action of serotonin released from v-CDN. Since the firing of v-CDN itself does not directly cause contraction of either muscle, enhancement of the contraction of one muscle by v-CDN during the buccal rhythm does not prevent the relaxation of the antagonist. When powerful and effective rasping movement is needed, the force and duration of radula retraction required would be larger than

those of protraction. The excitatory connection of v-CDN with retractor motoneuron B4 strengthens and extends the activity in the radula retraction phase (Yoshida and Kobayashi, 1991). The slow and long-lasting inhibitory effect of v-CDN on B10 (Fig. 5B) may shorten the burst duration of B10 during the radula protraction phase, resulting in a shortening of the duration of protractor contraction compared with retractor contraction; at the same time, v-CDN increases the tension of the protractor. Simultaneous *in vivo* monitoring of radular movement and v-CDN activity is required to reveal the precise role of v-CDN in an intact animal.

Many lines of evidence have emerged showing that neuropeptides modulate muscle contraction at the level of identified neuromuscular synapses in molluscs (Zoran *et al.* 1989; Kupfermann, 1991; Weiss *et al.* 1992). In *Aplysia californica*, several kinds of peptides released from identified cholinergic motoneurons modulate buccal muscle contraction (Cropper *et al.* 1987, 1988; Whim and Lloyd, 1990). One of these peptides, buccalin, is believed to act at the presynaptic terminal of the motoneuron, from which the peptide is released, decreasing the size of EJPs elicited by the motoneuron (Cropper *et al.* 1988). In contrast, ACEP-1 in *A. fulica* appears to act on
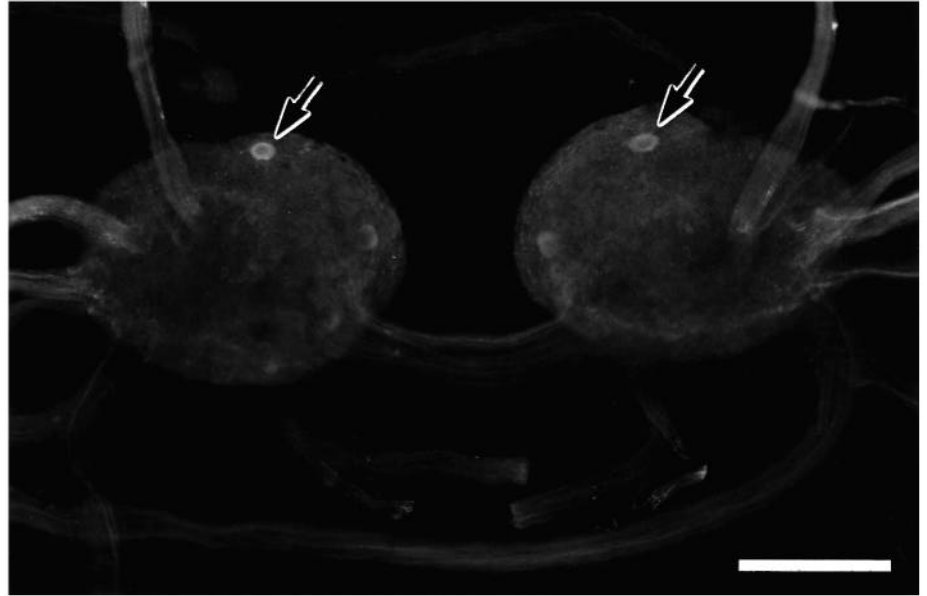
Fig. 6. ACEP-1-like immunoreactivity in the buccal ganglia (rostral view). A whole-mount preparation is shown. Arrows show a pair of ACEP-1-like immunoreactive neurons. Scale bar, 500 μm.
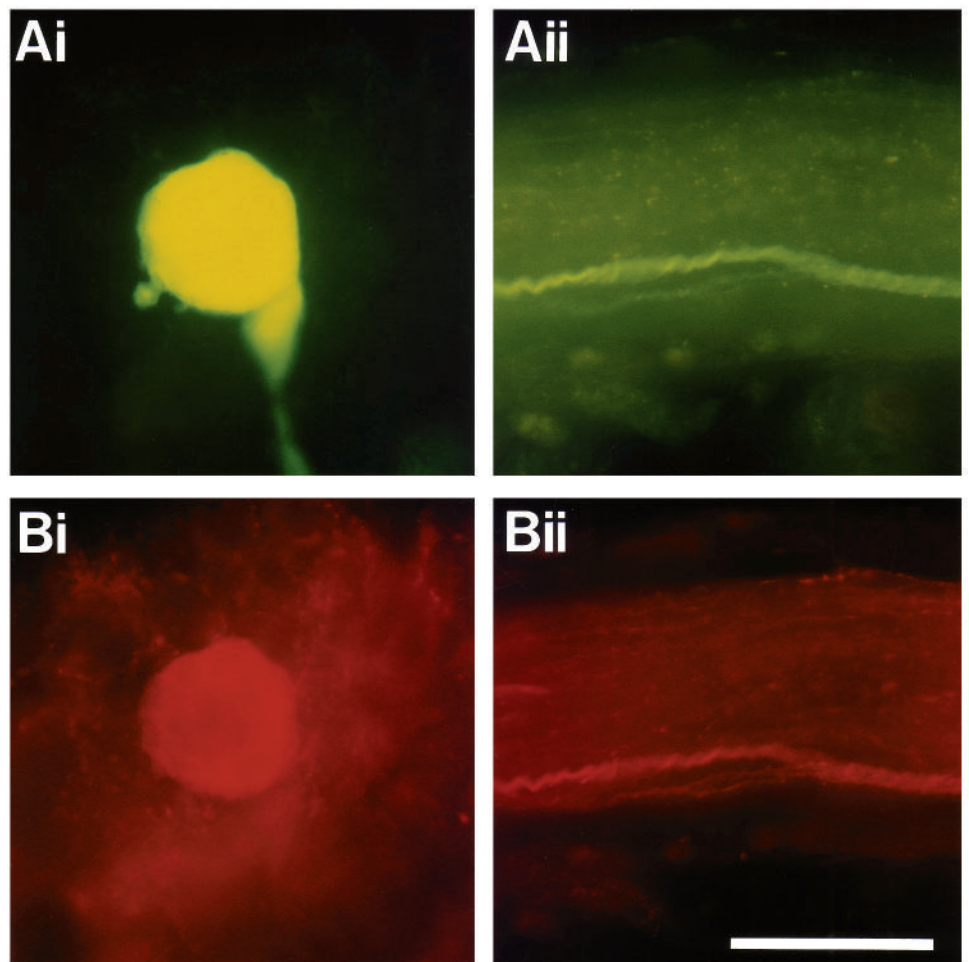

Fig. 7. (A) Neuron B10 stained intracellularly by the injection of Lucifer Yellow after physiological identification. (Ai) Cell body of B10. (Aii) Axonal projection of B10 in the buccal nerve 2. (Bi,ii) ACEP-1-like immunoreactivities in the same regions as shown in Ai and Aii, respectively. Scale bar, 100 μm.

the presynaptic terminal, increasing the size of EJPs elicited by the cholinergic motoneuron B10, from which the peptide may also be released. However, the possibility of a direct effect of the peptide on the muscle cells cannot yet be ruled out.

Since B10 is the only neuron that has, so far, been shown to innervate the radula protractor and to have ACEP-1-like immunoreactivity, it is possible that an ACEP-1-like substance, released from B10, affects neuromuscular
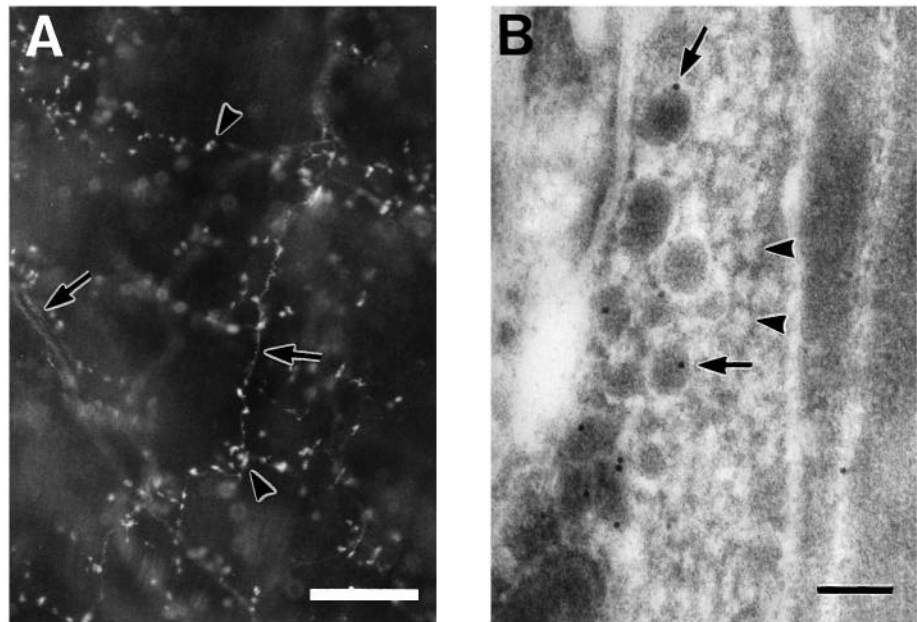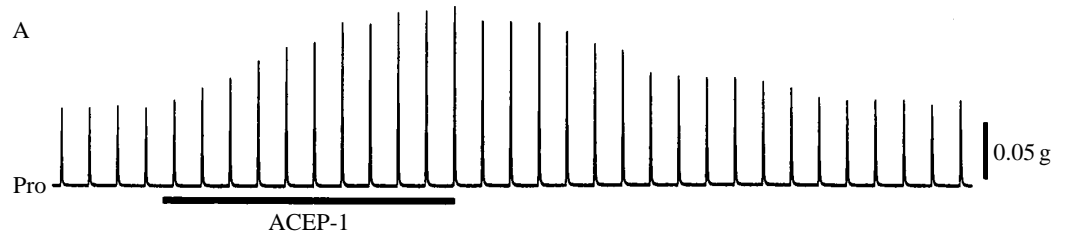
Fig. 8. ACEP-1-like immunoreactivity in the radula protractor. (A) A whole-mount preparation is shown. Many neural fibers (arrows) and terminals (arrowheads) show ACEP-1-like immunoreactivity. Scale bar, 50 μm. (B) Electron micrograph of a nerve terminal in the radula protractor. ACEP-1-like immunoreactive material is localized in large dense-cored vesicles (arrows) but not in clear vesicles (arrowheads). Scale bar, 0.1 μm.

Fig. 9. (A) Effect of ACEP-1 on contractions of the radula protractor evoked by stimulation of B10 (20 Hz for 1 s) at 30 s interburst intervals. 10^{-8} mol l^{-1} ACEP-1 was applied to the muscle by perfusion during the period indicated by the bar under the record. (B) Effect of ACEP-1 on the B10-evoked EJP of the radula protractor. Each trace is an average of 15 EJPs evoked at 5 s intervals. 10^{-6} mol l^{-1} ACEP-1 was applied to the muscle through a small pipette placed adjacent to the muscle. (C) Effect of ACEP-1 on the contraction of the radula protractor evoked by an application of 10^{-5} mol l^{-1} ACh to the muscle. 10^{-7} mol l^{-1} ACEP-1 was applied to the muscle by perfusion for 5 min before and during the recording.
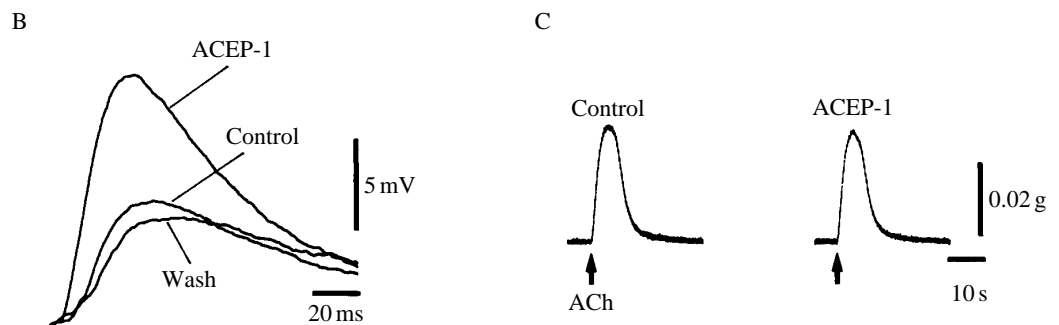
transmission. Electron microscopical evidence, indicating the localization of the ACEP-1-like immunoreactivity in dense-cored vesicles within the neural element of the radula protractor, supports this hypothesis. Clear vesicles co-existing with the dense-cored vesicles may contain the main excitatory transmitter ACh. If this were the case, the peptide modulator packed in its separate vesicles may well show a different pattern of release from that of ACh, depending on the state of activity of the motoneuron. In the buccal system of *Aplysia californica*, Whim and Lloyd (1989) have shown that the release of the peptide modulator co-existing with ACh in an identified motoneuron is dependent on the firing frequency of the neuron.

From the evidence presented in this study, it is not possible

to conclude that the ACEP-1-like substance detected immunocytochemically in B10 is ACEP-1 itself. Nevertheless, since exogenously applied ACEP-1 enhanced B10-evoked muscle contraction at a very low concentration (threshold concentration 10^{-9} mol l^{-1}), it is possible that ACEP-1 itself does have a modulatory role in the regulation of buccal muscle contraction in the intact animal.

Aminergic and peptidergic modulation of muscle contraction has also been extensively investigated in various systems in invertebrates other than molluscs (O'Shea and Evans, 1979; Kravitz *et al.* 1980; Fischer and Florey, 1983; Evans and Myers, 1986; Whim and Evans, 1988). Adams and O'Shea (1983) have described the release and action of a peptide cotransmitter at an identified neuromuscular junction in the cockroach, while the structure and function of crustacean neuropeptides have been reviewed from a comparative point of view by Keller (1992).

It appears that cotransmission mediated by multiple substances is a common feature of nervous systems in both vertebrates and invertebrates. However, investigation of cotransmission in vertebrates at the level of identified neurons is not easy because of the limited accessibility of the preparations used in much of the research. The identified neuromuscular synapse of *A. fulica* should provide a useful system for investigating the physiological functions of cotransmission.

References

ADAMS, M. E. AND O'SHEA, M. (1983). Peptide cotransmitter at a neuromuscular junction. *Science* **221**, 286–289.

BUCKETT, K. J., PETERS, M., DOCKRAY, G. J., VAN MINNEN, J. AND BENJAMIN, P. R. (1990). Regulation of heartbeat in *Lymnaea* by motoneurons containing FMRFamide-like peptides. *J. Neurophysiol.* **63**, 1426–1435.

CROPPER, E. C., MILLER, M. W., TENENBAUM, R., KOLKS, M. A. G., KUPFERMANN, I. AND WEISS, K. R. (1988). Structure and action of buccalin: A modulatory neuropeptide localized to an identified small cardioactive peptide-containing cholinergic motor neuron of *Aplysia californica. Proc. natn. Acad. Sci. U.S.A.* **85**, 6177–6181.

CROPPER, E. C., PRICE, D., TENENBAUM, R., KUPFERMANN, I. AND WEISS, K. R. (1990). Release of peptide cotransmitters from a cholinergic motor neuron under physiological conditions. *Proc. natn. Acad. Sci. U.S.A.* **87**, 933–937.

CROPPER, E. C., TENENBAUM, R., KOLKS, M. A. G., KUPFERMANN, I. AND WEISS, K. R. (1987). Myomodulin: A bioactive neuropeptide present in an identified cholinergic buccal motor neuron of *Aplysia. Proc. natn. Acad. Sci. U.S.A.* **84**, 5483–5486.

EVANS, P. D. AND MYERS, C. M. (1986). The modulatory actions of FMRFamide and related peptides on locust skeletal muscle. *J. exp. Biol.* **126**, 403–422.

FISCHER, L. AND FLOREY, E. (1983). Modulation of synaptic transmission and excitation–contraction coupling in the opener muscle of the crayfish, *Astacus leptodactylus*, by 5-hydroxytryptamine and octopamine. *J. exp. Biol.* **102**, 187–198.

FUJIMOTO, K., KUBOTA, I., YASUDA-KAMATANI, Y., MINAKATA, H., NOMOTO, K., YOSHIDA, M., HARADA, A., MUNEOKA, Y. AND KOBAYASHI, M. (1991). Purification of achatin-I from the atria of the African giant snail, *Achatina fulica* and its possible function. *Biochem. biophys. Res. Commun.* **177**, 847–853.

FUJIMOTO, K., OHTA, N., YOSHIDA, M., KUBOTA, I., MUNEOKA, Y. AND KOBAYASHI, M. (1990). A novel cardio-excitatory peptide isolated from the atria of the African giant snail, *Achatina fulica. Biochem. biophys. Res. Commun.* **167**, 777–783.

FURUKAWA, Y. AND KOBAYASHI, M. (1987). Neural control of heart beat in the African giant snail, *Achatina fulica* Férussac. II. Interconnections among the heart regulatory neurones. *J. exp. Biol.* **129**, 295–307.

GRANZOW, B. AND ROWELL, C. H. F. (1981). Further observations on the serotonergic cerebral neurones of *Helisoma* (Mollusca, Gastropoda): the case for homology with the metacerebral giant cells. *J. exp. Biol.* **90**, 283–305.

HARADA, A., YOSHIDA, M., MINAKATA, H., NOMOTO, K., MUNEOKA, Y. AND KOBAYASHI, M. (1993). Structure and function of the molluscan myoactive tetradecapeptides. *Zool. Sci.* **10**, 257–265.

IKEDA, T., YASUDA-KAMATANI, Y., MINAKATA, H., KENNY, P. T. M., NOMOTO, K. AND MUNEOKA, Y. (1992). *Mytilus*-inhibitory peptide analogues isolated from the ganglia of a pulmonate mollusc, *Achatina fulica. Comp. Biochem. Physiol.* **101**C, 245–249.

KELLER, R. (1992). Crustacean neuropeptides: Structures, functions and comparative aspects. *Experientia* **48**, 439–448.

KRAVITZ, E. A., GLUSMAN, S., HARRIS-WARRICK, R. M., LIVINGSTONE, M. S., SCHWARZ, T. AND GOY, M. F. (1980). Amines and a peptide as neurohormones in lobsters: actions on neuromuscular preparations and preliminary behavioural studies. *J. exp. Biol.* **89**, 159–175.

KUPFERMANN, I. (1991). Functional studies of cotransmission. *Physiol. Rev.* **71**, 683–732.

LINACRE, A., KELLETT, E., SAUNDERS, S., BRIGHT, K., BENJAMIN, P. R. AND BURKE, J. F. (1990). Cardioactive neuropeptide Phe-Met-Arg-Phe-NH$_2$ (FMRFamide) and novel related peptides are encoded in multiple copies by a single gene in the snail *Lymnaea stagnalis. J. Neurosci.* **10**, 412–419.

MILLER, M. W., BEUSHAUSEN, S., CROPPER, E. C., EISINGER, K., STAMM, S., VILIM, F. S., VITEK, A., ZAJC, A., KUPFERMANN, I., BROSIUS, J. AND WEISS, K. R. (1993a). The buccalin-related neuropeptides: isolation and characterization of an *Aplysia* cDNA clone encoding a family of peptide cotransmitters. *J. Neurosci.* **13**, 3346–3357.

MILLER, M. W., BEUSHAUSEN, S., VITEK, A., STAMM, S., KUPFERMANN, I., BROSIUS, J. AND WEISS, K. R. (1993b). The myomodulin-related neuropeptides: characterization of a gene encoding a family of peptide cotransmitters in *Aplysia. J. Neurosci.* **13**, 3358–3367.

O'SHEA, M. AND EVANS, P. D. (1979). Potentiation of neuromuscular transmission by an octopaminergic neurone in the locust. *J. exp. Biol.* **79**, 169–190.

WEISS, K. R., BREZINA, V., CROPPER, E. C., HOOPER, S. L., MILLER, M. W., PROBST, W. C., VILIM, F. S. AND KUPFERMANN, I. (1992). Peptidergic co-transmission in *Aplysia*: Functional implications for rhythmic behaviors. *Experientia* **48**, 456–463.

WEISS, K. R., COHEN, J. L. AND KUPFERMANN, I. (1978). Modulatory control of buccal musculature by a serotonergic neuron (metacerebral cell) in *Aplysia. J. Neurophysiol.* **41**, 181–203.

Weiss, K. R., Mandelbaum, D. E., Schonberg, M. and Kupfermann, I. (1979). Modulation of buccal muscle contractility by serotonergic metacerebral cells in *Aplysia*: evidence for a role of cyclic adenosine monophosphate. *J. Neurophysiol.* **42**, 791–803.

Whim, M. D. and Evans, P. D. (1988). Octopaminergic modulation of flight muscle in the locust. *J. exp. Biol.* **134**, 247–266.

Whim, M. D. and Lloyd, P. E. (1989). Frequency-dependent release of peptide cotransmitters from identified cholinergic motor neurons in *Aplysia*. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9034–9038.

Whim, M. D. and Lloyd, P. E. (1990). Neuropeptide cotransmitters released from an identified cholinergic motor neuron modulate neuromuscular efficacy in *Aplysia*. *J. Neurosci.* **10**, 3313–3322.

Yoshida, M. and Kobayashi, M. (1991). Neural control of the buccal muscle movement in the African giant snail *Achatina fulica*. *J. exp. Biol.* **155**, 415–433.

Yoshida, M. and Kobayashi, M. (1992). Identified neurones involved in the control of rhythmic buccal motor activity in the snail *Achatina fulica*. *J. exp. Biol.* **164**, 117–133.

Zoran, M. J., Haydon, P. G. and Matthews, P. J. (1989). Aminergic and peptidergic modulation of motor function at an identified neuromuscular junction in *Helisoma*. *J. exp. Biol.* **142**, 225–243.