Actions of a Pair of Identified Cerebral-Buccal Interneurons (CBI-8/9) in *Aplysia* That Contain the Peptide Myomodulin

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Xin, Yuanpei, Itay Hurwitz, Ray Perrins, Colin G. Evans, Vera Alexeeva, Klaudiusz R. Weiss, and Irving Kupfermann. Actions of a pair of identified cerebral-buccal interneurons (CBI-8/9) in *Aplysia* that contain the peptide myomodulin. *J. Neurophysiol.* 81: 507–520, 1999. A combination of biocytin back-fills of the cerebral-buccal connectives and immunocytochemistry of the cerebral ganglion demonstrated that of the 13 bilateral pairs of cerebral-buccal interneurons in the cerebral ganglion, a subpopulation of 3 are immunopositive for the peptide myomodulin. The present paper describes the properties of two of these cells, which we have termed CBI-8 and CBI-9. CBI-8 and CBI-9 were found to be dye coupled and electrically coupled. The cells have virtually identical properties, and consequently we consider them to be “twin” pairs and refer to them as CBI-8/9. CBI-8/9 were identified by electrophysiological criteria and then labeled with dye. Labeled cells were found to be immunopositive for myomodulin, and, using high pressure liquid chromatography, the cells were shown to contain authentic myomodulin. CBI-8/9 were found to receive synaptic input after mechanical stimulation of the tentacles. They also received excitatory input from C-PR, a neuron involved in neck lengthening, and received a slow inhibitory input from CC5, a cell involved in neck shortening, suggesting that CBI-8/9 may be active during forward movements of the head or buccal mass. Firing of CBI-8 or CBI-9 resulted in the activation of a relatively small number of buccal neurons as evidenced by extracellular recordings from buccal nerves. Firing also produced local movements of the buccal mass, in particular a strong contraction of the I7 muscle, which mediates radula opening. CBI-8/9 were found to produce a slow depolarization and rhythmic activity of B48, the motor neuron for the I7 muscle. The data provide continuing evidence that the small population of cerebral buccal interneurons is composed of neurons that are highly diverse in their functional roles. CBI-8/9 may function as a type of premotor neuron, or perhaps as a peptidergic modulatory neuron, the functions of which are dependent on the coactivity of other neurons.

**INTRODUCTION**

The nervous systems of mollusks and crustaceans contain ganglia with relatively few numbers of neurons and have provided model systems for the study of the mechanisms of the generation of rhythmic behaviors and of behavioral choice. In each group of animals, ganglia that control feeding and alimentary functions have received special attention (Arshavsky et al. 1989; Benjamin 1983; Delaney and Gelperin 1990b; Dickinson et al. 1988; Harris-Warrick and Marder 1991; Susswein and Byrne 1988). The stomatogastric ganglia of crustacea and the buccal ganglion of gastropod mollusks contain much of the neural circuitry that generates various interrelated rhythmic programs. These ganglia in turn are regulated by a small set of higher-order interneurons that project to the ganglia. In crustacea, there is considerable evidence that the effects of a number of the higher-order interneurons are produced by the release of peptide neuromodulators (Harris-Warrick and Marder 1991). In the snail *Lymnaea*, immunocytochemical and pharmacological evidence suggests that one identified cerebral-buccal interneuron (CBI) may use the peptide APGWamide as a neuromodulator (McCrohan and Croll 1997). In *Aplysia* it was demonstrated that the cerebral ganglion, which contains the cell bodies of CBIs (Rosen et al. 1991), synthesizes and transports myomodulin and a number of other neuropeptides to the buccal ganglion (Lloyd 1989). We have attempted to characterize those CBIs in *Aplysia* that use modulatory peptides as transmitters or cotransmitters. The current paper focuses on the previously identified molluscan peptide myomodulin. Preliminary reports of some of these findings have appeared in abstract form (Hurwitz et al. 1996; Xin et al. 1996a).

**METHODS**

**Animals**

Approximately 200 wild-type *Aplysia californica* weighing 200–300 g (Marinus, Long Beach, CA) were used in this study. The animals were maintained at 14–16°C in holding tanks containing aerated, filtered artificial sea water (ASW) for 3–6 days before being used for experiments.

**Preparations**

Animals were immobilized by injecting isotonic magnesium chloride at 25% (vol/wt) body weight, and dissection was performed in ASW composed of (in mM) 460 NaCl, 10 KCl, 11 CaCl2, 55 MgCl2 , and 5 NaHCO3 containing 25% isotonic magnesium chloride. Three types of preparations were used in the experiments: isolated head ganglia, reduced preparations, and semi-intact preparations.

The isolated head ganglia preparation consisted of the cerebral, buccal, and pedal-pleural ganglia. The ganglia were pinned to a silicone elastomer (Sylgard) floor of a recording chamber containing ASW. The reduced preparations consisted of the head ganglia and buccal mass, which was connected to the buccal ganglion by buccal nerves. Specifically we used this preparation to investigate the effect of the cerebral-to-buccal interneurons 8 and 9 (CBI-8/9) on I7, a radula opener muscle (Evans et al. 1996). The I7 muscle was set up as...
previously described (Evans et al. 1996). In brief, we pinned the buccal mass and head ganglia to a clear Sylgard floor of a recording chamber containing fresh ASW with the cerebral and pedal-pleural ganglia pinned dorsal side up, the buccal ganglion pinned rostral surface up, and the buccal mass ventral side up. The chamber floor had stages at two levels. The buccal mass was placed on the lower stage so that the whole buccal mass could be immersed in ASW. The head ganglia were placed on the higher stage just beneath the surface of the ASW so that they could be better visualized. The collostylar cap, to which all the radula opener muscles are attached, was cut from the radula sac. The cap was pinned to the chamber floor, and the cut end of an I7 muscle was pinned for intracellular recording. The cut end was attached with a thread to an isotonic transducer (Harvard Bioscience) the output of which was proportional to the length of the muscle.

The semi-intact preparations consisted of head ganglia and parts of the head, including the mouth, lips, and anterior tentacles connected to the head ganglia by anterior tentacular, and upper and lower labial nerves. Also included was the cephalic artery, which supplies the mouth, lips, and tentacles. The branches of the cephalic artery that go to the foot and buccal mass were ligated, and the artery was cannulated. Fresh ASW was pumped into the artery at a rate of 0.5 ml/min to perfuse the tissue and to simulate the hydrokeleton of head structures. The preparations were set in a clear Lucite recording chamber consisting of two compartments containing ASW. The head ganglia were pinned in one compartment in the same way as described earlier for the isolated ganglia preparation. The mouth, lips, anterior tentacles, and the cephalic artery were set in the second compartment. The second compartment was deeper than the first, so that the tissue could be immersed completely in the ASW. A suction tube for the outflow was set in the compartment to control the fluid level. The isolated head could be presented with mechanical or chemical stimuli. Mechanical stimuli were provided by the tip of a heat-sealed glass Pasteur pipette. Combined chemomechanical stimuli consisted of pieces of moistened dried-seaweed (Laver, Vega Trading, New York, NY) that were applied to the lips or tentacles with a fine, blunt forceps. A pure chemical stimulus consisted of a seaweed extract solution, which was applied by a 1 ml syringe and slowly injected into the ASW near one side of the lip and tentacle region (Susswein et al. 1978). The partition between the two compartments contained fine grooves that allowed the peripheral nerves to pass through. The grooves were filled with petroleum jelly (Vaseline) to maintain a watertight seal between the two compartments.

**Electrophysiology**

All in vitro experiments were carried out at room temperature (19–21°C). For the intracellular recording and stimulation, neurons were impaled with double-barreled microelectrodes that were made of thin-walled glass (World Precision Instruments, FL) and contained 2 M potassium acetate. The electrodes were flow-beveled so that their impedances ranged from 10 to 15 MΩ. To identify neurons and examine their morphologies, the potassium acetate in the stimulating electrode was replaced by a solution of 3% 5(6)-carboxyfluorescein dye (Kodak) in 0.1 M potassium citrate, titrated to pH 8.0 with KOH (Rao et al. 1986). These electrodes were beveled so that the impedance of the electrode containing the dye was 15–20 MΩ and the impedance of the potassium acetate electrode was 10–15 MΩ. To test for the monosynapticity of connections, the threshold for action potential generation was raised by bathing the ganglia in a high-divalent cation sea water solution. Unless otherwise specified, this solution contained three times the normal concentration of calcium and magnesium (final concentration, 30 mM Ca²⁺ and 150 mM Mg²⁺).

For the extracellular recording or stimulation of nerves, the cut end of a nerve (or connective) was drawn into small-diameter polyethylene suction electrode. Nerve recordings were made with AC amplifiers (A-M Systems), and electrical stimulation of the nerves was provided by a Grass 88 stimulator. The numbering of the nerves follows that of Gardner (1971).

**Morphology**

To determine the size, shapes, and destination of processes of individual identified neurons, cells were filled with 3% 5(6)-carboxyfluorescein dye. Successful intracellular labeling was achieved by iontophoretic ejection of the dye for 15–30 min, followed by a 48-h incubation period at 4°C to allow the dye to fully fill the processes. To reduce active transport of the dye from the cells during incubation, the bathing solution included 1 mM probenecid final concentration (Steinberg et al. 1987). The living ganglia were cleared in 50% glycerol in ASW; the fluorescence was visualized with a Nikon fluorescence microscope, and the labeled cell body with its processes was photographed.

**Biocytin back-fill**

The cut end of the cerebral-to-buccal connective (CBC) attached to the cerebral ganglion, was sucked into a polyethylene tube and the ASW in the tube was replaced with a 5% biocytin aqueous solution (Molecular Probes, biocytin in 50 mM NaHCO₃, pH 8.0). The ganglia then were incubated in ASW with 1 mM probenecid at 4°C for 2 days. After a brief wash in ASW, the ganglia were fixed in 4% paraformaldehyde at room temperature for 2 h. The ganglia were washed in 0.1 M phosphate buffered saline (PBS, pH 7.6) four times, each time for 30 min at 4°C. The preparation was permeabilized by overnight incubation in 0.1 M PBS containing 2% Triton X-100 at 4°C with gentle shaking and then was incubated in 50 μg/ml streptavidin Bodipy FL conjugate (Molecular Probes), in PBS Triton at 4°C for 12–24 h. After washing the ganglion in 0.1 M PBS for 2–3 days, it was cleared in PBS/glycerol(1:6), and viewed and photographed with a Nikon fluorescent microscope.

**Double-labeling of cells with biocytin and immunocytochemistry for myomodulin**

Cerebral-buccal interneurons first were back-filled with biocytin as described above. After incubation in the strepavidin Bodipy FL, the ganglia were preincubated for 2 h at room temperature in PBS containing 1% normal goat serum (NGS; Miles Science, Naperville, IL) to reduce nonspecific primary antibody binding. Ganglia then were incubated in myomodulin primary antisera (1:500 in PBS-NGS) for 2 days at 4°C, followed by washing for 24 h in several changes of PBS. The ganglia then were incubated in a secondary antibody solution (1:50, goat anti-rabbit IgG rhodamine-conjugated Fab fragment; Cappel, Malvern, PA). The ganglia were washed with several changes of PBS/Triton X-100 overnight, and had a final wash (24–48 h) in PBS without Triton X-100. Ganglia were cleared in a 1:6 dilution of phosphate buffer in glycerol, mounted on depression slides with Aqua-Poly Mount (Polyscience, Warrington, PA), and examined with a Nikon microscope equipped with filter packs for viewing rhodamine or fluoresceine/Bodipy FL epifluorescence. A similar methodology has been published previously (Li and Chase 1995).

**Double-labeling of cells with Lucifer yellow and immunocytochemistry for myomodulin**

Hypermolarizing current was used to eject 5% Lucifer yellow into cells that were identified by electrophysiological criteria and position. The ganglia were fixed at room temperature for 2 h in 4% paraformaldehyde, 0.1 M PBS (pH 7.4), 30% sucrose and then washed overnight with 0.1 M PBS, 30% sucrose at 4°C. The immunocytochemistry was carried out using a modification of previously described techniques (Lloyd et al. 1985; Miller et al. 1991). The fixed and washed ganglia were preincubated at room temperature for 2 h with 1% NGS
in 0.1 M PBS to reduce nonspecific binding of primary antibody. The ganglia then were incubated in rabbit antiserotonin serum (Sigma) diluted 1/500 in PBS containing 1% NGS for 2 days at 4°C, washed in PBS at 4°C for 1 day, and incubated at 4°C for 1 day in goat anti-rabbit IgG rhodamine-conjugated Fab fragment diluted 1/50 (Cappel). Finally, the ganglia were washed in PBS again at 4°C for 1 day, then mounted on slides and coverslipped with Aqua-Poly Mount, viewed under a Nikon microscope equipped for epifluorescence, and photographed with Tri-X film.

Six ganglia were used for controls. No staining was observed when the primary (n = 3) or secondary (n = 3) antibody was omitted, and it was shown previously that staining was abolished when the myomodulin antibody was preincubated with myomodulin (Miller et al. 1991).

**DRUG APPLICATION** To evoke buccal motor programs, the cerebral and buccal ganglia were placed in separate chambers and carbachol (1 mM) was applied to the cerebral ganglion or to local regions of the cerebral ganglion. Local application was accomplished by filling a broken microelectrode (tip diameter around 10 μ) with the carbachol solution and placing the tip of the electrode within 10–50 μ from a particular cell or region of the ganglion (Gapon and Kupfermann 1996).

**HPLC OF CONTENTS OF CBI-8/9** The presence of authentic Myomodulin A in CBI-8/9 was established using reverse phase high-pressure liquid chromatography (RP-HPLC) using techniques similar to those that were described previously (Cropper et al. 1991). In brief, CBI-8/9 first were identified electrophysiologically and injected with a vital stain (1% fast green), so that the cells could be dissected individually. The ganglion then was incubated with 35S-methionine (0.5 mCi per ganglion) for 24 h, and 17 individual cells were removed and pooled. The contents of the cells together with authentic synthetic myomodulin A then were purified sequentially by HPLC under two gradient conditions using an Aquapore RP-300 column. In the first step of purification, solvent A was 0.015 M trifluoroacetic acid (TFA) and water, solvent B was 0.015 M TFA and acetonitrile, and gradients were 5% B for 10 min and 5–40% B in 35 min. In the second step of chromatography, solvent A was 0.01 M heptafluorobutyric acid (HFBA) and water, solvent B was 0.01 M HFBA and acetonitrile, and the gradient was 10–50% B in 40 min. In the first step of chromatography, the radioactivity of a 10% aliquot of each fraction was determined. The remainder of the fraction that contained authentic Myomodulin A (MMA), as determined by optical absorption, then was used in the second step of chromatography.

**RESULTS**

*Biocytin back-fill and immunocytochemical double labeling*

As an initial step in identifying myomodulin-containing CBIs, we performed immunocytochemistry on ganglia that first
were backfilled with biocytin applied to the cerebral-buccal connective. Fifteen preparations were backfilled. In nine preparations, a relatively large number of cerebral cells were stained in a roughly similar distribution. The biocytin backfill, which can be combined with immunocytochemistry, appears to label cells at least as well as the previously used cobalt chloride (Rosen et al. 1991). Filled cells were prominent in the M, G, E, J, and K clusters (Fig. 1, A and B). With the possible exception of dye-coupled cells or cells with very small axons, the backfilled cells presumably define the total population of cerebral cells that send an axon to the cerebral-buccal connective. By convention, sensory neurons and the metacerebral cells (MCCs), which have peripheral axons, are not classified as cerebral-buccal interneurons even if they make synaptic connections to buccal cells. Previous studies have shown that the J and K clusters largely or exclusively consist of the cell bodies of sensory neurons (Nagahama and Takata 1988; Rosen et al. 1979, 1982). It was estimated that four to eight of the sensory neurons send an axon into the cerebral-buccal connective, and on the basis of their position and size, these appear to be have been labeled in the current back-fills. If these cells are excluded, there appear to be 13 pairs of candidate CBIs distributed in the M, G, and E clusters (Fig. 1B). We cannot, however, completely exclude the possibility that one or two of the cells in the J and K clusters are CBIs rather than sensory neurons.

The nine preparations that exhibited extensive back-filling were processed for immunostaining for myomodulin. In all nine preparations (right CBC backfill in 4 preparations and left backfill in 5 preparations), three cells were positive for both myomodulin and biocytin (Fig. 2, A and B). One of the double-labeled cells was located on the ventral surface of the cerebral ganglion, within the M cluster, just next to CBI-2 (Rosen et al. 1991), and has been termed CBI-12 (Hurwitz et al. 1999). The two other cells were located on the dorsal surface of the cerebral ganglion in the crotch between the anterior tentacle nerve and the CBC. The two cells had similar shapes and size and were always adjacent to one another. These two cells were named CBI-8 and CBI-9 and are designated CBI-8/9 because currently they are virtually indistinguishable.

**Identification of CBI-8 and -9**

Exploration of the anterior region of the E cluster revealed that it contained a pair of CBIs in the position of the pair of cells that exhibited myomodulin staining. The neurons usually did not fire in the absence of imposed currents unless the buccal ganglion exhibited spontaneous or evoked buccal motor programs. Both of their cell bodies are oval-shaped and have little pigment. Carboxyfluorescein dye-fills confirmed that these two cells are almost identical in their morphology. Each sends a single axon to the buccal ganglion via the ipsilateral CBC, and the process courses toward the buccal commissure but it either terminates in the ipsilateral ganglion or contralateral processes could not be resolved. In the cerebral ganglion, the cells exhibit fine processes projecting toward the M cluster region. Typically, two main processes extend a short distance into the anterior tentacular nerve and upper labial nerve (Fig. 3A). A striking characteristic of the two cells is that when one cell was injected with carboxyfluorescein dye, the other cell typically also exhibited fluorescence. In our experience, dye coupling is rare in *Aplysia,* even for cells that exhibit strong electrical coupling. To determine the incidence of dye coupling between CBI-8 and CBI-9, for 10 pairs of cells we injected dye into one cell of the pair for 60 min. In 6 of the 10 preparations, we observed dye in both cells (Fig. 3B). It does not appear as if the coupling occurs from soma to soma because no contact between the somata could be observed. Thus the coupling may occur through distal processes that may have variable diameters or distances from the cell bodies. There were no obvious gross structural differences, however, between pairs of cells that were dye coupled and pairs that were not coupled.

The dye-coupling results suggested that the cells were likely to be coupled electrically, and indeed the cells exhibited electrical coupling with a coupling ratio of ~1:3. As shown in Fig. 3C, although CBI-8 and -9 are coupled strongly, firing one CBI-8/9 does not bring the other to fire. CBI-8 and -9 also were found to be weakly (1:30 to 1:50 coupling ratios) electrically coupled to CBI-12 (Fig. 4D), a CBI the cell body of which is located in the M cluster (Hurwitz et al. 1999).

**CBI-8 and -9 synthesize myomodulin**

The contents of the CBI-8/9 cells that were identified electrophysiologically and marked with fast green dye contained a...
substance that coeluted with authentic MMa under the first condition of counterion gradients (Fig. 5A) and also coeluted with authentic MMa during the rechromatography under different conditions (Fig. 5B). Thus the cells appear to synthesize MMa. Although these experiments used pooled CBI-8 and -9 cells, all indications, including immunocytochemistry (see next section), suggest that the cells are virtually identical.

Labeling of CBI-8/9 with Lucifer yellow and immunocytochemistry for myomodulin

To confirm that CBI-8 and -9 are myomodulin immunopositive, we electrophysiologically identified them on the basis of their synaptic inputs (see subsequent sections) and by recording their extracellular axon activity in the CBC. We then labeled them with Lucifer yellow and stained for myomodulin. In each of 10 preparations, the Lucifer-labeled CBI-8 and -9 (Fig. 6A) showed strong positive reactions to the myomodulin antibody (Fig. 6B). In six control preparations (1st antibody replaced by NGS in 3 preparations and 2nd antibody replaced by NGS in 3 preparations), CBI-8 and -9 did not show any positive staining (data not shown).

Inputs to CBI-8/9

In addition to electrical coupling potentials from the firing of CBI-12 and from the paired CBI-8/9, described earlier, CBI-8/9 also receive inputs from various other sources, including the lips, tentacles, buccal mass, and other central neurons. We used a semi-intact preparation to study the inputs that CBI-8/9 receives from the lips and anterior tentacle region. When the lip was contacted with a glass pipette or a piece of moistened seaweed, the cells exhibited an initial inhibition, followed by weak excitation that outlasted the stimulus presentation (Fig. 7A). Under the conditions of these experiments, the inputs from the lip and anterior tentacles were weak. There were no differences between stimulation applied either to the lip or to the anterior tentacle, and there was no difference between the response to a pure mechanical stimulus (glass rod) or a combined mechanical and chemical stimulus (contact with a piece of moistened seaweed). In the absence of mechanical stimulation, application of an extract of sea weed did not evoke a response in CBI-8/9.

We next examined whether CBI-8/9 receives input from various neurons that have been shown to be involved in the appetitive phases of feeding. Elsewhere (Hurwitz et al. 1999) we reported that C-PR, a neuron involved in the head-lifting and neck lengthening phase of appetitive feeding, produces a slow excitation in CBI-8/9. We found that CC5, a multifunctional neuron that is involved in neck shortening during both appetitive and consummatory feeding behaviors (Xin et al. 1996b), produces a slow inhibition in CBI-8/9 (Fig. 7B).

The fact that C-PR excites CBI-8/9 and CC5 inhibits CBI-
8/9 suggested that CBI-8/9 may be active during forward movements of the head or buccal mass. Because previous results have shown that application of the cholinergic agonist carbachol elicits a robust ingestive-like program when applied to the cerebral ganglion (Susswein et al. 1996), we examined the activity of CBI-8/9 during buccal motor programs by applying carbachol (CCh, 1 mM) to the cerebral ganglion. Local application of CCh directly to the cell body of CBI-8/9 excited the cells (Fig. 7C) but failed to evoke a robust buccal program. We found, however, that when a buccal program was evoked by application of CCh to the M cluster region, CBI-8/9 weakly fired in phase with the program (Fig. 8A). A rhythmic buccal program also can be elicited by the application of the muscarinic agonist, pilocarpine, to the cerebral ganglion although the pilocarpine-induced program is different from the CCh-induced program (Susswein et al. 1996), presumably because it does not activate the identical receptors that are activated by CCh. It also has been shown (Susswein et al. 1996) that CBI-2, a command-like neuron that evokes an ingestive buccal program is not involved in the pilocarpine-induced

![FIG. 4. Excitatory coupling between CBI-8/9 and CBI-12 (n = 3). A: depolarization and firing of CBI-12 produced small depolarizations in CBI-8/9. An evoked buccal motor program is evident in the recording from buccal nerve 3 (BN3). B: fast sweep speed revealed that CBI-12 spikes were one for one with the excitatory postsynaptic potentials (EPSPs) in CBI-8/9. C: depolarization and firing of CBI-8/9 evoked a slow depolarization in CBI-12, whereas depolarization of CBI-12 evoked an initial depolarization of CBI-8/9 followed by fast depolarizations linked to spikes in CBI-12. D: hyperpolarizing pulses injected into either CBI-12 (D1) or CBI-8 (D2) were associated with small hyperpolarizations in the other cell with a decrement in the range of 30–50 to 1. Data shown in C and D suggest that CBI-8/9 is coupled electrically to CBI-12.

![FIG. 5. Reverse phase high-pressure liquid chromatography (RP-HPLC) confirms that CBI-8/9 contain authentic myomodulin. Cells were identified electrophysiologically and marked with fast green dye. Ganglia then were incubated with 35S-methionine, and the cell contents sequentially chromatographed by HPLC in the presence of authentic myomodulin (MMa), under 2 conditions with different counterions (see METHODS). A: radioactivity (counts per minute, CPM) of each fraction measured in 10% aliquots of the fractions that eluted under the 1st set of conditions (see METHODS). B: 2nd step of rechromatography using the remainder of the fraction that coeluted at the same time as authentic MMs during the 1st step of purification. Peak of radioactivity coeluted again with that authentic MMs.](http://jn.physiology.org/)
program, suggesting that the pilocarpine program is induced by activation of CBIs other than CBI-2. We found that pilocarpine (1 mM) applied directly onto the cell body of CBI-8/9 failed to excite the cell and in fact, evoked a hyperpolarization (Fig. 9A). The effect of pilocarpine on CBI-8/9 may be direct because it was not blocked by a high-magnesium and low-calcium sea water solution (Fig. 9B).

We also examined whether CBI-8/9 receives inputs due to firing of C2, a histaminergic mechanosensory cell in the E cluster of the cerebral ganglion (Weiss et al. 1986). C2 has been suggested to be involved in the control of feeding because it is activated by the mechanical properties of seaweed applied to the perioral zone of the animal (Weiss et al. 1986). When CBI-8/9 was at resting potential, firing C2 did not produce a significant depolarization of the cell (Fig. 10A); but when CBI-8/9 was depolarized 5 mV by injection of constant depolarizing current, C2 evoked a distinct but small depolarization (Fig. 10B). When CBI-8/9 was depolarized 10 mV from rest, firing of C2 produced spiking in the cell. The connection of C2 to CBI-8/9 may be polysynaptic because the excitatory effect of C2 on CBI-8/9 could be blocked by a high divalent solution (data not shown).

Firing of CBI-8/9 can generate rhythmic activity in buccal nerves but does not typically evoke an organized motor program

To examine the outputs of CBI-8/9 and its effect on the buccal mass, we recorded from buccal nerves while the cells were directly fired. As previously mentioned, CBI-8/9 usually does not fire spontaneously and has little or no spontaneous excitatory or inhibitory postsynaptic potentials (EPSPs or IPSPs). In one-half of the preparations, it was not possible to produce a sustained firing of the cell by means of constant depolarizing current. In those instances in which the cell could be fired tonically, we observed substantial rhythmic activity in buccal nerve 3 (BN3). In addition to a large spike that occurred in relatively brief bursts, BN3 also exhibited a smaller spike that had a relatively long duration burst and did not appear to be in phase with the briefer bursts. Less activity was seen in buccal nerve 2 (BN2), and unlike what is seen during typical rhythmic buccal motor programs, the activity observed was often tonic or only weakly rhythmic (Fig. 11A). Thus although activity of CBI-8/9 recruited bursts of firing of several buccal neurons, it did not appear that it evoked a well organized and coordinated buccal motor program such as that elicited by some CBIs such as CBI-2 (Church and Lloyd 1994; Rosen et al. 1991). In some instances in which CBI-8/9 was fired, it appeared that extracellular recordings from the contralateral buccal nerve 2 exhibited a very small spike that was one for one with the spikes in CBI-8/9. This was observed in 3 of 10 preparations, and it is not clear whether it represents a field potential recording of a spike in the contralateral process of...
CBI-8/9 or whether it reflects the existence of a process that extends at least partially into the contralateral nerve 2. As previously mentioned, dye-fills have failed to reveal any processes entering buccal nerves but do not completely rule out the possible existence of fine processes that cannot be visualized.

When CBI-8/9 was fired in the presence of the buccal ganglion, the contralateral CBI-8/9 exhibited only very slight signs of phasic activity (Fig. 11B). If, however, the contralateral CBI-8/9 was depolarized close to its threshold, weak but distinct rhythmic excitatory input could be revealed (Fig. 11C).

CBI-8/9 firing can alter a CCh-induced buccal program

During a CCh-induced program, when CBI-8/9 was fired at 15–20 Hz (Fig. 8A, middle), the ongoing program appeared to show relatively subtle changes. The burst rate of the ongoing buccal program was slowed, and there was an increase in the firing of one or more medium-sized units recorded in BN 2, resulting in an increase of activity during the relatively quiet periods between bursts. In addition, units that were associated with the largest extracellular spikes in the radula nerve and nerve 2 appeared to be inhibited. By contrast the distinctive but behaviorally undefined program that was elicited by applying pilocarpine to the cerebral ganglion also induced a buccal motor program in which CBI-8/9 activity was recruited. Firing of CBI-8/9 at a high rate by intracellular current did not have an obvious effect on the program (Fig. 8B).

CBI-8/9 elicits rhythmic protraction and opening of the radula

To further explore a possible role of CBI-8/9 in feeding, we used a reduced preparation in which the buccal mass and its innervation from the buccal ganglion was included with the other head ganglia. CBI-8/9 was impaled and fired by intracellular injection of depolarizing current. It was observed that firing of CBI-8/9 did not evoke any movements that were reminiscent of normal organized feeding or rejection behaviors, but it caused obvious radula opening-like movements that were associated with some protraction of the radula. As previously reported (Evans et al. 1996), radula opening is evoked by buccal muscle 17 and the associated muscles 18, 19, and 110. We therefore measured the length of 17 while CBI-8/9 was fired. Firing of CBI-8/9 was associated with a robust and reliable contraction of the 17-110 muscles (Fig. 12A). The latency for the contraction ranged from 1 to 3 s. In trials in which CBI-8/9 was fired in repeated bursts, each burst produced a rapid contraction of the muscles (Fig. 12B).
Previous studies have identified buccal neuron 48 (Church and Lloyd 1994; Evans et al. 1996) as a motor neuron that contracts I7 and the associated muscles. We found a neuron located in the position of B48 that when excited contracted the I7-I10 muscles and that was excited by firing of CBI-8/9. To confirm that this neuron was B48, we impaled the neuron and obtained intracellular recordings from the I7 muscle. Firing of CBI-8/9 produced a burst of firing in B48 (Fig. 13). With continued firing of CBI-8/9, B48 went into a burst mode. Extracellular recording from buccal nerve 3 indicated that during the bursting of B48 some other units also fired, but the bursting was not associated with substantial in-phase bursting of a substantial number of other neurons as occurs during organized feeding programs. It thus appears as if CBI-8/9 activity, rather than directly firing B48, may promote an endogenous bursting property of the cell.
To determine if the contraction of the I7 muscle that occurs when CBI-8/9 is fired is due to the firing of B48, simultaneous intracellular recordings were obtained from CBI-8/9, B48, and the I7 muscle. With B48 slightly depolarized, a burst of firing of B48 evoked excitatory junction potentials (EJPs) in the I7 muscle (Fig. 14, left). The EJPs in the muscle were associated with a contraction, and previous findings (Evans et al. 1996) indicate that a relatively low rate of firing of B48 can evoke contractions of the I7 muscle. When CBI-8/9 was fired, a burst of firing of B48 again evoked one-for-one EJPs in the muscle (Fig. 14, middle). When, however, CBI-8/9 was fired while B48 was hyperpolarized to prevent its firing, no EJPs were associated with CBI-8/9 firing (Fig. 14, right).

The connection of CBI-8/9 to B48 was tested in the presence of a high divalent sea water solution (3 times normal magnesium calcium). Although it was difficult to fire CBI-8/9 at a high rate in the high divalent cation solution, the slow EPSP produced by CBI-8/9 in B48 persisted (Fig. 15), indicating that the excitation, at least in part, may be monosynaptic.

CBI-8/9 evokes synaptic input to various buccal neurons other than B48 including B8

Because firing of CBI-8/9 excites a radula opener motor neuron, we examined its effect on the paired B8 (a and b) motor neurons, which are involved in an antagonistic movement (radula closing) (Church and Lloyd 1994; Morton and Chiel 1993a). During firing of CBI-8/9, B8 exhibited tonic inhibition that appeared to be associated with an increase of the rate of occurrence of IPSPs (Fig. 16). During the inhibition of B8, the radula opener motor neuron, B48, exhibited bursty activity, and the recording from the radula nerve showed that in addition to the spike from B8 another neuron is evident that fires in phase with B48. Although when CBI-8/9 was fired at least two in-phase neurons became active, this does not necessarily indicate that CBI-8/9 was evoking a buccal motor program. In several other experiments we have encountered neurons near B48 that are coupled electrically to B48 and that send an axon into the radula nerve. Thus the burst of firing of B48 may directly excite one of these neurons rather than both neurons being driven by rhythmic interneurons that fire as part of a buccal program.

In addition to inputs to identified cells we have found that firing of CBI-8/9 evoked inhibition or excitation in a small number of unidentified neurons located primarily on the caudal surface of the buccal ganglion. Their approximate position in the ganglion is indicated in Fig. 17. Unidentified neurons located close to B8 were inhibited. Extracellular recordings from nerves showed that a small number of neurons in addition to B48 fire rhythmically when CBI-8/9 is fired continuously (see, for example, the unit in the radula nerve, in phase with firing of B48 shown in Fig. 16). Furthermore, a small number of unidentified neurons in the general vicinity of B61 (Fig. 17) were excited when CBI-8/9 was fired. Direct firing of the cells whose activity was affected by CBI-8/9 typically produced small movements of the buccal mass musculature, suggesting that they may be buccal motor neurons.

The effects of CBI-8/9 suggested that its activity is associated with the promotion of radula opening concomitantly with suppression of radula closing. Because there is evidence that
the phase of radula opening relative to that of radula protraction provides a means of distinguishing ingestive from egestive programs (Morton and Chiel 1993a), we examined the effect of CBI-8/9 on protractor motor neuron B61. We found a pattern of in-phase radula opening and protraction, which is consistent with ingestive movements. Specifically, firing CBI-8/9 evoked excitatory input to protractor motor neuron B61 (Fig. 18A). Depolarization of B61 did not produce reciprocal synaptic input into CBI-8/9 (Fig. 18B). Interestingly even though CBI-8/9 and B61 are situated in different ganglia, strong hyperpolarizing pulses injected into CBI-8/9 were accompanied by a small but distinct hyperpolarization in B61 (Fig. 18C), suggesting that the cells might be coupled electrically through a pathway involving the CBI-8/9 axon that travels in the cerebral-buccal connective.

**Fig. 15.** Excitation of B48 produced by CBI-8/9 persists in a solution that raises the threshold of interneurons (n = 3). A: firing of CBI-8/9 produced slow excitation and spikes in B48. B: slow excitation (and reduced spikes) persisted in a solution containing high divalent cations (3 × Ca\(^{2+}\), 3 × Mg\(^{2+}\)).

**Fig. 16.** Firing of CBI-8/9 evokes inhibition of B8, a radula closer motor neuron, and excitation of B48, a radula opener neuron (n = 4). B48 was excited and fired in a burst pattern, whereas identified neuron B8 exhibited tonic inhibition. Inhibition of B8 persisted for several seconds after the firing of CBI-8/9 was terminated. To accentuate the inhibition, B8 was fired slowly by means of tonic depolarizing current. Firing of CBI-8/9 evoked a burst of spikes in a unit that could be recorded extracellularly from the radula nerve (RN). Bursts in the radula nerve were in phase with B48 bursting, but the individual spikes were not linked one to one with the spikes of B48, which does not send an axon into the radula nerve. Unit in the radula nerve, which was inhibited, is due to the firing of the axon of B8.

**DISCUSSION**

The combination of biocytin back-filling of a cerebral-buccal connective with myomodulin immunocytochemistry of the cerebral ganglion provided an effective means for specifically locating candidate cerebral-buccal interneurons that may synthesize myomodulin. The back-fills obtained with biocytin appeared to be equal or superior to the cobalt or nickel backfills routinely used in our laboratory. In addition to the MCC and sensory cells of the J and K mechanoafferent clusters, the back-fills revealed a consistent bilateral pattern of 13 filled cells located in the dorsal or ventral surface of the cerebral ganglion at the origin of either the cerebral-buccal connectives or tentacular nerves. Almost all of these cells have been identified as cerebral-buccal-interneurons (Hurwitz et al. 1997; Perrins and Weiss 1998; Rosen et al. 1991). It is possible that a few other CBIs may be present in the J or K clusters, but it is likely that the total population CBIs does not consist of substantially >13 pairs of cells. This figure is roughly similar to the number of CBIs reported for diverse gastropod mollusks, including the land slug Limax (Delaney and Gelperin 1990a), the carnivorous mollusc *Pleurobranchaea* (Gillette et al. 1982), and the pond snail *Lymnaea* (McCrohan and Kyriakides 1989).

The combined biocytin back-fill and immunocytochemistry revealed that three of the CBIs in *Aplysia* are myomodulin positive. In the present paper, we describe some of the basic features of two of these cells, CBI-8 and CBI-9. Further characterization of the third myomodulin positive neuron, CBI-12 is presented in Hurwitz et al. (1999). CBI-8 and CBI-9 are virtually identical and consequently are referred to as CBI-8/9. The cells appear to form a twin pair, similar to the paired buccal neurons B4/5 (Gardner 1971), B31/32 (Susswein and Byrne 1988), B61/62 (Hurwitz et al. 1994), and cerebral neurons CBI-5/6 (Perrins and Weiss 1998). As is the case of the other examples of twin pairs, the somata of the two CBI-8/9 cells are adjacent to one another but do not appear to be in contact although the cells are coupled electrically. CBI-8 and -9 have morphologies similar to one another and appear to share putative transmitters/modulators. Both cells receive slow excitatory inputs from the appetitive feeding command-like neuron, C-PR (Hurwitz et al. 1996, 1999). The similarity of the two CBI-8/9 cells to one another does not preclude the possibility that they might have some differences.

Although CBI-8/9 are similar to one another, their properties define them in many respects as quite different from other CBIs. For example, except for CBI-5/6, the other CBIs do not come in twin pairs. Another distinctive feature of CBI-8/9 is their total absence of spontaneous fast inhibitory or excitatory synaptic input.

One characteristic feature of the CBI-8/9 cells is that they are myomodulin immunopositive, and the only other myomodulin positive CBI is CBI-12. The three myomodulin-containing cells are coupled electrically, suggesting that they may be part of a functional system. Although CBI-12 is similar to CBI-8/9 in that it apparently does not evoke a full-blown, buccal motor program, the reported effects of firing of CBI-12 appear to be quite distinct from those of CBI-8/9 (Hurwitz et al. 1999). Further information about the normal activity and effects of these cells is needed to confirm the hypothesis that they are related functionally.
The crustacean stomatogastric ganglion (STG) has been extensively shown to receive interganglionic input from cells that contain a variety of modulators, including a large number of peptides (Harris-Warrick and Marder 1991). Interestingly in the stomatogastric ganglion, these modulators are not synthesized by any of the cells intrinsic to the stomatogastric ganglion. By contrast, myomodulin is synthesized by neurons in the buccal ganglion (Brezina et al. 1995; Cropper et al. 1991; Miller et al. 1991, 1993). Some of the buccal myomodulin-containing neurons have been shown to be motor neurons, and they release myomodulin in the periphery (Vilim et al. 1996). It has not been shown, however, that the myomodulin-containing buccal neurons release myomodulin within the ganglion. A second neuromodulator in \textit{Aplysia}, dopamine, is present both in a CBI (CBI-1) as well as in buccal ganglion interneurons (Rathouz and Kirk 1988; Rosen et al. 1991; Teyke et al. 1993; Tritt et al. 1983). Thus the observation that modulators used by neurons outside a ganglion are not also used by neurons within the ganglion may not reflect a general principle but rather could simply be an outcome of the fact that the nervous system has available a relatively large number of neuromodulators, which operate in a system that contains a relatively few total neurons.

In the stomatogastric ganglion, some peptides and peptide-containing interneurons appear to drive specific patterns of STG programs (Bartos and Nusbaum 1997; Fan et al. 1997; Harris-Warrick and Marder 1991; Simmers et al. 1995). The available data on CBI-8/9 suggest that activity of these cells does not drive a fully organized and functional program but rather appears to evoke components of programs. Unlike what is seen during normal feeding programs (Morton and Chiel 1993b) or programs driven by CBIs such as CBI-2 (Rosen et al. 1991), relatively few buccal neurons appear to be excited or inhibited when CBI-8/9 is fired. Furthermore, unlike what typically is seen during “normal” programs, some buccal neurons exhibited continuous or long-lasting inhibition or excitation while CBI-8/9 was fired and some appeared to fire with no fixed phase relationship to other neurons. We currently do not know how B-8/9 fires during normal feeding programs, but it is quite possible that at its normal rate it is incapable of evoking rhythmic activity but instead largely modulates rhythmic activity that is driven by other neurons.

The cerebral ganglion communicates with the buccal ganglion via only \textit{\textasciitilde}13 interneurons. The current results identify two of these cells. Our data provide information about the inputs and outputs of CBI-8/9 and have shown that they contain a bioactive peptide; but the findings do not permit a definitive conclusion about the precise function of the cells. We have not observed substantial synaptic input to the cells when the lip or anterior tentacle is contacted with tactile or chemical stimuli, suggesting that CBI-8/9 may not be importantly involved in the initiation of feeding behavior. Bursts of firing of CBI-8/9 are highly effective in evoking contraction of one specific set of muscles, the radula opener muscles, and in this respect it appears to be functioning as a ’’simple’’ premotor or ’’modulatory’’ neuron for B48. CBI-8/9 also inhibits radula closer neurons (B8a/b) and excites a radula protractor neuron (B61). Thus CBI-8/9 may be involved in eliciting the coordinated protraction/opening component of ingestive responses. The pattern of synaptic effects evoked by CBI-8/9 is consistent with the observations that exogenous application of myomodulin to the buccal ganglion appears to promote radula protraction and suppress radula retraction phases of buccal programs (Perrins and Weiss 1997).

Our findings support the idea that the CBIs form a relatively small but extremely diverse population of interneurons. Some neurons, such as CBI-3, do not initiate any activity in the buccal ganglion and may instead play an inhibitory or sculpting role in ongoing programs. Tonic firing of other CBIs, such as CBI-2 and CBI-4, is highly effective in eliciting...
continuous rhythmic buccal programs that bear similarities to normal programs and are reflected in extensive rhythmic activity that is conveyed out to the periphery by way of all the major buccal nerves. Finally, cells such as CBI-8/9 may function as premotor neurons that drive the activity of a small number of motor neurons and interneurons. Alternatively, although direct firing of CBI-8/9 can evoke motor neuron firing, during actual feeding CBI-8/9 may not initiate or drive responses but may serve purely modulatory functions, acting primarily as extrinsic modulators (Cropper et al. 1987; Katz and Frost 1996) such as the metacerebral cell (Rosen et al. 1989), that optimize the parameters of the buccal circuitry to adapt that circuitry to changing needs associated with the different modes in which the circuitry functions (see Kupfermann et al. 1997).

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