Neural Modulation of Gut Motility by Myomodulin Peptides and Acetylcholine in the Snail *Lymnaea*

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Perry, Stephen J., Volko A. Straub, György Kemenes, Niovi Santama, Belinda M. Worster, Julian F. Burke, and Paul R. Benjamin. Neural modulation of gut motility by myomodulin peptides and acetylcholine in the snail *Lymnaea*. *J. Neurophysiol.* 79: 2460–2474, 1998. Families of peptide neuromodulators are believed to play important roles in neural networks that control behaviors. Here, we investigate the expression and role of one such group of modulators, the myomodulins, in the feeding system of *Lymnaea stagnalis*. Using a combination of in situ hybridization and antibody staining, expression of the myomodulin gene was confirmed in a number of identified behaviorally significant neuronal types, including the paired B2 motor neurons. The B2 cells were shown to project axons to the proesophagus, where they modulate foregut contractile activity. The presence of the five myomodulin peptide structures was confirmed in the B2 cells, the proesophagus, and the intervening nerve by mass spectrometry. Using a sensitive cell culture assay, evidence that the B2 cells are cholinergic also is presented. Application of four of the five myomodulin peptides to the isolated foregut increased both contraction frequency and tonus, whereas the main effect of acetylcholine (ACh) application was a large tonal contraction. The fifth myomodulin peptide (pQIPMLRLamide) appeared to have little or no effect on gut motility. Coapplication of all five myomodulin peptides gave a greater increase in tonus than that produced by the peptides applied individually, suggesting that corelease of the peptides onto the gut would produce an enhanced response. The combined effects that the myomodulin peptides and ACh have on foregut motility can mimic the main actions of B2 cell stimulation.

**INTRODUCTION**

One of the important advantages of using invertebrate nervous systems for studying the cellular basis of behavior is the ability to identify specific neurons within defined neural networks. In mollusks, these cells are often large enough for their functions to be deduced and their transmitter contents to be biochemically defined. The neural network controlling feeding behavior in the pond snail *Lymnaea stagnalis* uses a large variety of transmitter molecules to produce and modulate rhythmic feeding in response to food. Although the roles of small molecule transmitters [e.g., acetylcholine (ACh) and serotonin] in this system have been defined partly (Yeoman et al. 1993, 1994), no functional analysis of the role of neuropeptides has been attempted. In a preliminary analysis, several peptide families, including those related to the myomodulins, have been localized by immunocytochemistry to the highly identifiable neurons of the feeding circuit (Santama et al. 1994a). Previously, we have shown that there are likely to be five members of the myomodulin peptide family in *Lymnaea*, predicted from the sequence of the gene (Kellett et al. 1996). Four members of this family (PMSMLRLa, SMSMLRLa, SLSMLRLa, and GLQMLRLa) have been isolated from *Lymnaea* CNS extracts and sequenced (Santama et al. 1994b; van Golen et al. 1996), and two (PMSMLRLa and GLQMLRLa) have been detected in nervous tissue by mass spectrometry (Kellett et al. 1996).

Some of the nine myomodulin peptides found in the sea hare *Aplysia californica*, have been shown to modify the contractile characteristics of a defined muscle involved in feeding as a consequence of second-messenger–mediated ion channel modulation (reviewed in Brezina et al. 1994a,b; 1995; Weiss et al. 1992). The structurally different peptides appear to control the same ion channels to differing degrees but act in concert to increase the size and relaxation rate of ACh-induced contractions. In *Lymnaea*, similar effects on the penis retractor muscle have been demonstrated for PMSMLRLa (Li et al. 1994c) and three other myomodulin peptides (van Golen et al. 1996), indicating that this family of peptides shares common properties within these two divergent mollusks.

Here we analyze for the first time the myomodulin peptides within the feeding system of *Lymnaea*. In situ hybridization revealed that a number of neurons involved in the generation of feeding behavior express the gene. Peptide expression was confirmed in one of these, the paired B2 motor neuron, by mass spectrometric analysis, and evidence indicating that it is cholinergic also is presented. The peripheral target tissue of the B2 cells was identified as the proesophagus by dye injection and by measurement of their modulatory effects on spontaneous gut contractions. Application of the individual myomodulin peptides to the isolated esophagus produced increases in tissue tonus and the frequency of contractions, but the activities of each of the peptides varied greatly. The peptides produced a greater increase in tonus when applied together rather than individually, suggesting that corelease would produce an enhanced response, whereas ACh also induced gut contractions, although its main effect was on tonus. A combination of ACh and the myomodulin peptides can mimic the main effects that B2 stimulation has on gut contractile activity.

**METHODS**

Wild *L. stagnalis*, collected from freshwater ponds, were supplied by Blades Biological (Edenbridge, UK) and laboratory-
reared specimens from the Department of Biology at the Vrije Universiteit, Amsterdam. The snails were maintained at 20°C in 40-l aquaria in drip-fed tap water, kept under a 12 h:12 h light:dark cycle and fed on washed lettuce ad libitum. Chemicals were purchased from Sigma, UK, unless otherwise stated.

**Detection of myomodulin mRNA in the CNS and buccal ganglia by reverse transcription PCR (RT-PCR)**

Ring ganglia [whole snail CNS excluding buccal ganglia] and buccal ganglia alone were isolated, and total cellular RNA was extracted (RNAeasy columns, QIAGEN, following the manufacturer’s instructions.) Reverse transcription from random hexamers of 2.5 μg of RNA from each sample was carried out using Superscript II MMLV reverse transcriptase [GIBCO-BRL, Paisley, UK]; reaction conditions used: 10 μl reaction volume, manufacturer’s first strand synthesis buffer, 200 μM each dNTP, 20 U RNAsin (Promega, Southampton, UK) 500 μg/ml random hexamer (Promega), 100 U enzyme; 42°C 45 min, 90°C 2 min. Identical reaction conditions omitting the enzyme were set up to ensure against contaminating DNA. Amplification of 145 bp of the myomodulin coding region spanning the intron splice site (position 645 in the myomodulin cDNA sequence; see Kellett et al. 1996) was performed using the entire volume of the reverse transcription reactions and primers designed for sequences 548–568 bp (GATCTGGTCTCTCGTTGAGA) and 692–670 bp (TGTCTGGTCAATTGTAAGTCT) using TaqSupreme enzyme [Igi Fermenta; reaction conditions used: 50 μl volume, 50 mM tris (hydroxymethyl)aminomethane (Tris)-HCl, pH 9.1, 16 mM ammonium sulfate, 3.5 mM MgCl₂, 150 μg/ml bovine serum albumin, 200 μM each dNTP, 50 μM each primer, 1 U enzyme; 94°C for 30 s, 63°C for 30 s, 68°C for 30 s, 20 cycles]. Products from 5 μl of each PCR were separated on a 1.5% agarose gel, blotted onto BioTran nylon filter (ICN, Thame, UK) and hybridized to the random primer-labeled 861-bp EcoRI fragment of myomodulin cDNA clone pMM52 (Kellett et al. 1996).

**In situ hybridization**

Probe labeling, tissue isolation, treatment, and hybridization were performed using the protocols previously described by Kellett et al. (1996). Oligonucleotide probes were designed in the coding region of the myomodulin gene (P25: GAAGCTCGTCCACGTCTCGAGTA) and exon I of the FMRFamide neuropeptide gene (TCTAACAGACCTCAGAATTTTG; positions 196–175 in Kellett et al. 1994) were labeled with digoxigenin-11-dUTP by 3’ tailing. Hybridization was visualized by labeling with antidigoxigenin/alkaline phosphatase followed by treatment with the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT).

**Immunocytochemistry**

Alternate sections of isolated CNS were prepared as described in Bright et al. (1993). All slides were treated in an identical manner up to and including dewatering, but half of the slides were then processed for immunocytochemistry using the method described in Burke et al. (1992). Labeling was carried out using a 100-fold dilution of the primary antisera [rabbit antimyomodulin C (A. californica), described in Miller et al. 1991] and visualized by treatment with horseradish peroxidase-conjugated anti-rabbit/mouse Ig (Dako) and 0.5 mg/ml diamobenidine in 0.01% vol/vol H₂O₂, 50 mM Tris base, 150 mM NaCl, pH 6.0.

Isolation of identified B2 motor neurons, nervous and gut tissue for mass spectrometric analysis of their peptide contents

B2 motor neurons were identified on the basis of their size and position within the buccal ganglia and isolated by the method described in Kellett et al. (1996). The cells were placed directly in the well of a mass spectrometer target, excess saline was removed by aspiration, and 1 μl of matrix solution [10 mg/ml dithioerythritol (DHB) in 0.1% wt/vol trifluoroacetic acid] was pipetted onto the cell and allowed to dry. Small lengths (~1 mm) of the dorsobuccal nerves and the proesophagus were removed and placed on a mass spectrometer target in 0.5 μl 10 mg/ml DHB.

Samples were analyzed on a Micromass matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS). Short laser bursts (3 ns, 337 nm wavelength) were used to ionize the peptides, which were focused using a reflectron system to enhance the resolution of the mass peaks.

**Measurement of pharmacological effects of transmitters on the proesophagus**

A 1-cm section of the most anterior region of the esophagus was isolated and immediately immersed in normal N-2-hydroxyethylpiperazine-N’2-ethanesulfonic acid (HEPES)-buffered saline [NS (in mM): 24 NaCl, 2 KCl, 4 CaCl₂, 0.1 NaH₂PO₄, 35 NaOH, and 55 HEPES and 833 μM D-glucose] within the chamber of an organ bath. The tissue was suspended between a pin in the base of the chamber and an isotonic force-displacement transducer (Grass FT03C, Quincy, MA) with suture silk and tungsten hooks. The tissue was superfused with NS (2 ml/min) for 2 h under moderate tension (~0.5 g) before experiments were started. Excess NS was removed from the chamber by a suction pump, keeping the volume of the bath at 1.7 ml. All peptides and transmitters were injected into the chamber at 10 times the molar concentration (dissolved in 170 μl of NS) at the point where the fresh saline entered the chamber (flow rate 2 ml/min), thus ensuring rapid mixing. Recordings were made on a Gould chart recorder for 1–3 min before thorough washing with NS (10 ml/min for 2–5 min). A recovery period of 3–5 min of perfusion at 2 ml/min was allowed between experiments.

**Dye injection of the B2 motor neuron**

Whole snail CNS were dissected with the esophagus, buccal mass, salivary glands, and all connecting nerves left intact. The buccal mass was cut along the ventral surface to allow pinning out with the nerves from the buccal ganglia visible. The preparation was superfused with zero calcium saline [containing (in mM) 2 KCl, 18 MgCl₂, 0.1 NaH₂PO₄, 35 NaOH, 50 HEPES, and 2 ethylene glycol-bis-(β-aminoethyl ether)-N,N',N''-tetraacetic acid and 833 μM D-glucose] for 30 min before injection was attempted. The B2 motor neurons were injected with 3% 5(6)-carboxyfluorescein (5CF) for 30–45 min with ~0.5 to ~1 nA current pulses of 0.5 s duration every second. After filling, the preparation was transferred to a small silicone elastomer (Sylgard) block and immersed in 10 mM probenecid (to prevent diffusion of dye from the cell), overnight at 4°C. The preparation then was transferred to 50% vol/vol glycerol in zero calcium saline in a cavity slide and viewed under ultraviolet light on a Zeiss Axioshot microscope.
Measurement of esophageal contractions during manipulation of B2 motor neuron activity

Gut-CNS preparations consisting of the proesophagus attached to the buccal ganglia by the paired dorsobuccal nerves were set up in a Sylgard-lined dish and constantly superfused with NS. The CNS was secured to the Sylgard base by a number of straight and U-shaped tungsten pins to facilitate simultaneous muscle movement and electrophysiological recordings from the B2 cells. A U-shaped pin was used to secure the proximal section of the proesophagus to the dish while a small tungsten hook attached to suture silk was passed through the distal end and looped over a force-displacement transducer (Grass FT03C, Quincy, MA). The transducer was connected to a Neurolog NL107 recorder amplifier (Digiform, Welwyn Garden City, UK) to record movements of the proesophagus. For intracellular recordings from the B2 motor neurons, electrodes were drawn from 2-mm capillary tubing (Clark Electromedical, Reading, UK) and filled with 4 M KCl giving tip resistances of 30–80 MΩ. Signals were amplified with Neurolog NL102 amplifiers, and these, together with signals from the NL107 recorder, were displayed on an oscilloscope (Gould 1604, Gould Instrument Systems, Hainault, UK), a chart recorder (Gould TA240S) and a DAT recorder (Biologic DTR-1801, Biologic Science Instruments, Claix, France). The B2 cells were activated or inactivated intracellularly by the application of steady depolarizing or hyperpolarizing currents (~0.5 nA) lasting for 20–24 s.

Pharmacological demonstration of ACh content of the B2 motor neuron

The techniques for isolation of B2 neurons and preparation of the cell extract were adapted from Yeoman et al. (1993) to include a protease-treatment step. The isolated CNS was incubated in a mixture of trypsin (0.67 mg/ml) and collagenase/dispase (Boehringer Mannheim, Lewes, UK, 1.33 mg/ml) in defined media (DM: 33 ml Leibowitz L-15 media, 27 ml NS, containing 10 mg L-[-l-]glutamine, 5.4 mg D-[-l-]glucose; volume made up to 100 ml with cell culture-grade water, pH 7.9) for 30 min at room temperature. After a further 10 min incubation in soya bean trypsin inhibitor (1 mg/ml in DM), the brain was incubated in 50% vol/vol propylene glycol/NS containing 1 µM eserine to fix cells and prevent enzymatic degradation of ACh. Cell bodies were isolated by gentle suction with a fire-polished micropipette (tip diameter ~150 µm) prepared from 1.5 mm Sigmaocte-treated glass tubing (Clark Electromedical Instruments, GC150T-10). B2 neurons were transferred into individual wells of a 72-well microtitre plate filled with eserine saline (1 µM in NS). Twenty B2 neurons were combined in a total volume of 20 µl eserine saline, and their intracellular contents were released into solution by repeated heating to 60°C and freezing in liquid nitrogen. An aliquot of the extract (2 µl) was incubated with a solution of torpedo choline esterase (1 µl containing 56 U) for 15 min at room temperature to degrade all ACh present in the extract. In a control aliquot, B2 extract (2 µl) was treated with heat-inactivated torpedo choline esterase (90°C for 15 min). Samples of both preparations were then loaded into micropipettes for application to cultured B4 neurons.

Isolation and culture of Lymnaea B2 and B4 neurons

After protease treatment as described above, the CNS was pinned out in a dissection dish filled with high-osmolality DM (30 mM glucose in DM). B4 and B2 neurons were identified visually, and their cell bodies exposed by mechanically disrupting the inner connective tissue. The cell bodies were isolated with suction micropipettes, transferred onto poly-L-lysine–coated culture dishes containing a 1:1 mixture of conditioned medium (DM incubated with 2 snail CNS/ml for 3 days, filter sterilized) and DM and cultured at 20°C for ≤5 days.

Electrophysiological recording from cultured neurons

Culture dishes containing B4 neurons that had grown extensive new processes in cell culture were superfused with NS at a flow rate of ~1 ml/min for ≥30 min before the experiment to remove all culture medium. The superfusion was maintained throughout the experiment. Cell bodies were impaled with microelectrodes filled with saturated potassium sulfate (tip resistance 25–30 MΩ), and standard single and two-electrode intracellular recording techniques using an Axoclamp 2B amplifier were performed. Experimental and control extracts of B2 cells and ACh at various concentrations (see RESULTS) were pressure applied to the cell soma from separate micropipettes using a picospritzer. The cholinergic antagonists hexamethonium chloride (HMT, 1 mM) and phenyltrimethyl-ammonium chloride (PTMA, 1 mM) were added to the superfusion system to block ACh receptor sites when necessary.

RESULTS

Detection of myomodulin mRNA in the feeding system of Lymnaea

It was shown in a previous study that the myomodulin gene is transcribed in the CNS of Lymnaea and that the expression appears to occur specifically in neurons (Kellett et al. 1996). However, no direct evidence for the expression of the gene in ganglia containing feeding network neurons was presented. To do this, RNA was extracted specifically from the buccal ganglia where the main feeding circuitry is located and then subjected to reverse transcription–PCR to amplify a fragment of the myomodulin gene. Agarose gel separation and hybridization with part of the myomodulin gene confirmed that a 145-bp PCR product from the myomodulin gene was produced from the buccal ganglion RNA (Fig. 1A). An identical analysis of RNA extracted from the rest of the brain amplified the same 145-bp PCR product, confirming myomodulin gene expression in this tissue as well. Primer sequences were chosen that would amplify a fragment of this size spanning the intron boundary, as predicted from the genomic sequence of the myomodulin gene. In this way, contamination of the samples with genomic DNA would not produce a fragment of the desired size, eliminating the possibility of falsely predicting gene expression. Control reactions that were not treated with the reverse transcriptase enzyme were also negative, confirming that the fragment was amplified from templates originating from reverse transcribed RNA.

In situ and antibody analysis of myomodulin gene expression in the feeding system of Lymnaea

The previous interpretation of myomodulin expression in neurons of the Lymnaea feeding network using a polyclonal antibody (Santama et al. 1994a) was equivocal because of the cross-reactivity of the antibody to a structurally related peptide (EFLRamide) processed from the precursor of the FMRFamide gene (Santama et al. 1994b). In situ hybridization using an oligonucleotide with a sequence complementary to part of the coding region of the myomodulin gene allowed a more specific analysis of myomodulin expression (myomodulin in situ alone, n = 4 preparations) that then
was compared with myomodulin antibody (Ab) expression in alternative sections from the same brain (n = 2). To confirm the presence or absence of FMRFamide gene expression in specific neurons, alternate sections were prepared and analyzed with either the myomodulin in situ probe or one complementary to exon I of the FMRFamide gene (n = 2). Exon I encodes a common hydrophobic leader sequence present on both alternatively spliced mRNA variants of the FMRFamide gene (Kellett et al. 1994).

Figure 1B summarizes the distribution of myomodulin expressing cells in the buccal ganglia revealed by in situ analysis of the myomodulin gene. In every cell in which in situ analysis showed expression of the myomodulin gene, it also showed Ab staining (e.g., Fig. 1, C, D, I, and J) although sometimes
the Ab staining was weak. Conversely, no cells that stained with the Ab were negative with the in situ myomodulin probe. Analysis of alternate sections stained for myomodulin and FMRFamide gene expression revealed no coexpression of these genes in the buccal ganglia (Fig. 1, E and F). The FMRFamide gene is known to be expressed in one or two pairs of small cells in the buccal ganglia (Bright et al. 1995), and this was confirmed in the present study. This was not examined in detail in cerebral ganglion feeding neurons apart from in the cerebral giant cells (CGCs) where again myomodulin in situ expression was found to be absent in the absence of Exon I coexpression (not shown).

Identified feeding neurons that were myomodulin in situ/Ab positive included the B1 and B2 cells that form bilaterally symmetrical pairs in left and right buccal ganglia. The B1s are considered to be salivary gland motor neurons (Benjamin et al. 1979), and the B2s are motor neurons for the gut (see further text). The B2 cells are the most striking cells to be stained with the in situ probe for myomodulin and are observed readily in sections in comparison with the B1 cells that stain much more lightly (Fig. 1E). A third pair of individually identifiable cells that stain lightly with both myomodulin probes are the modulatory serotonergic neurons of the feeding network, the CGCs (Fig. 1, I and J); these large cells are located in the anterior lobes of both cerebral ganglia (McCrohan and Benjamin 1980). Among the smaller myomodulin expressing cells of the buccal ganglia (Fig. 1, C and E) are three or four heavily staining cells (20–40 µm diam) lying close to the B1 and B2 cells on the dorsal surface of both buccal ganglia. One of these only occurred on the left or right side and probably corresponds to the modulatory cell called the slow oscillator (SO) shown to be myomodulin Ab positive in the previous study (Santama et al. 1994a). Other cells known to occur in this region are members of the feeding central pattern generator circuit known as the N1 lateral (N1L) cells (Yeoman et al. 1995). There are a symmetrical pair of myomodulin staining cells on the posterior dorsal surface of each of the buccal ganglia that could be members of the B4CL, but other members of the B4CL/B8/B10 motor neuron cluster do not appear to be stained. The largest members of the left and right B4 clusters, the B4 cells, can be identified positively but did not express the myomodulin gene. Another symmetrical pair of motor neurons that can be identified positively, but also did not express the myomodulin gene, were the B3 cells. This was unexpected as the previous study suggested that they were immunoreactive to the myomodulin antibody (Santama et al. 1994a). This result could not be repeated here. More ventrally located in situ/Ab myomodulin positive cells are a pair of heavily staining lateral cells (50 µm cell body diameter) that lie beneath the B1 cells (Fig. 1G) and a heterogeneous ventral cluster (6–8 cells on each side) that vary in size (40–100 µm cell body diameter) and density of staining (Fig. 1H). These cannot be positively recognized as corresponding with any previous identified cells of the feeding network.

**B2 is a gut motor neuron that modulates prooesophageal motility**

The prominent expression of the myomodulin gene in the B2 cells of the buccal ganglia suggested an important physiological role for these peptides. However, before this could be investigated further it was necessary to determine the function of the B2 cells. An earlier study (Benjamin et al. 1979) suggested that the B2 cells might be motor neurons for the gut but there was only preliminary anatomic evidence for this hypothesis. Here, axonal projections of the B2s were mapped by intracellular dye injection to determine their putative peripheral target (Fig. 2A). Axons were shown to leave the buccal ganglia via the ipsilateral dorsobuccal nerve, and split into all of its major branches (summarized in Fig. 2B). Minor projections into the contralateral buccal ganglion and into the contralateral dorsobuccal nerve were also visible, although these were always very faint. Contralateral projections became indistinct before leaving the distal end of the nerve. Processes from small projections of the dorsobuccal nerve arborized over much of the surface of the ipsilateral part of the proesophagus, with both B2 cells together providing innervation of the whole circumference of the tissue (not shown). Some of these axons could be seen to enter the tissue itself, suggesting local points of innervation. At the anterior end of the proesophagus processes were visible that appeared to fold back and under the foregut to its junction with the buccal mass. Along the edge of the proesophagus axons also could be seen to project to the ventral surface, suggesting that the degree of arborization observed on the dorsal surface occurs over the entire surface of the foregut.

More posteriorly projecting axons also could be followed for some distance along the surface of the esophagus, eventually becoming indistinct at the boundary of the pro- and postesophagus. It was assumed these axons continued toward more posterior targets in the foregut but could not be visualized because of the long distance from the site of injection (~1 cm) and the increasing opacity of the surrounding tissue.

The proesophagus was confirmed as a peripheral target of the B2 motor neurons by measuring the effect that stimulating and inhibiting B2 spike activity had on spontaneous esophageal muscle contractions. In two preparations where both the proesophagus and the B2 cells showed strong spontaneous activity, simultaneous suppression of firing of both B2s by injection of hyperpolarizing current resulted in a decrease in tonus of the muscle, the amplitude, and number of contractions. An example of this is shown in Fig. 2C where, after a delay of ~4 s, injection of hyperpolarizing current into both B2s led to a progressive relaxation of the muscle and an eventual cessation of strong rhythmic contractions. This effect was reversed completely after cessation of current injection. A quantitative analysis of one such experiment showed that the mean number of large contractions in a 20-s period immediately before hyperpolarization was 7.33 ± 0.33 (mean ± SE), and this was significantly reduced to 3.67 ± 0.88 during the 20-s period immediately after the onset of B2 hyperpolarization (3 injections of hyperpolarizing current, P < 0.03, paired t-test). This same level of significance was obtained by repetition of the experiment in another preparation. Increased firing of the B2s immediately after the release of hyperpolarization was accompanied by a rapid increase in underlying gut tonus and enlarged individual contractions. However, both the contraction size and frequency returned to their preinhibition levels after 8–10 s, again corresponding to a return to normal firing patterns.
MYOMODULINS AND ACETYLCHOLINE CONTROL GUT MOTILITY

FIG. 2. A: carboxyfluorescein dye injection into a B2 motor neuron reveals processes leaving the buccal ganglion (B.G.) by the ipsilateral dorsobuccal nerve (DBN) and arborizing over the surface of the proesophagus (Proes.). Fine axonal projections to the contralateral buccal ganglion and DBN were also visible. Scale bar, 100 µm. B: diagram summarizing the morphology of the B2 motor neuron as revealed by fluorescent dye injection (Oes, esophagus). C: suppression of B2 cell spiking eliminates gut contractile activity. Simultaneous recording of a strongly contracting esophagus (Oes) and both B2 motor neurons (B2L and B2R) firing in a gut-CNS preparation. Hyperpolarization of both B2 cells (arrows on/off) completely suppressed B2 spiking and resulted in decreases in gut tonus (marked with descending arrow), contraction rate, and amplitude. Release of B2 cells from hyperpolarization resulted in a restoration of normal gut contractile activity. Hyperpolarization was achieved by current passage through the recording electrode. At this gain the traces were clipped by the chart recorder, and, although the level of hyperpolarization could not be quantified, it was sufficient to eliminate impulses in both cells, as monitored on an oscilloscope set at a lower gain than that of the chart recorder. D: activation of B2 cell spiking initiates gut contraction. Depolarization of a single B2 motor neuron (arrows on/off) in a semi-intact preparation where the esophagus shows no intrinsic contractile activity, induces large rhythmic contractions. Gut returns to a quiescent state after depolarization of the B2 ceases.

by the motor neurons. In two other preparations where the proesophagus was completely quiescent, left and right B2s showed a low and variable firing rate (mean 1.5 spikes/s). Here, activation of a B2 cell to a higher firing rate (9 spikes/s) by injection of depolarizing current, clearly increased the tonus of the muscle and triggered large rhythmic contractions (Fig. 2D). There was no simple relationship between the pattern of B2 spike activity and individual muscle contractions. Instead, the gut began slow rhythmic contractions 3–5 s after B2 depolarization was begun, and these were maintained during the period of neuronal stimulation. Analysis of three replicate B2 depolarizations from one experiment showed the mean number of contractions increased from 0 in a 20-s period immediately before the onset of current injection to 5.3 ± 0.3 during the 20-s period immediately after injection was initiated. There was also a marked increase in the tonus of the gut during B2 depolarization, and both this and the contractions ceased shortly after depolariza-
tion was terminated. It appears, then, that B2 motor neuron activity can modulate the intrinsic contractile activity of the foregut.

Myomodulin peptides are present in the B2 motor neurons, the dorsobuccal nerve, and the proesophagus

It was important to confirm the myomodulin content of the B2 cells and to determine the specific types of myomodulin-related peptides that were present. These then could be synthesized and used for pharmacological studies to investigate the modulatory roles of the peptides on gut motility. MALDI-TOF mass spectrometry (MS) was considered to be the optimal method because the masses of the peptides from single *Lymnaea* neurons can be determined accurately (Li et al. 1994a,b) and compared with those predicted from the deduced amino acid sequence encoded by the myomodulin gene (Kellett et al. 1996) or already isolated from the *Lymnaea* CNS (Santama et al. 1994b; van Golen et al. 1996). Individual B2 motor neurons (n = 3) were dissected from the buccal ganglia, and their peptide content was analyzed. Mass peaks of 819 Da (SLSMLRLamide + H⁺), 837 Da (SMSMLRLamide + H⁺), 847 Da (PMSMLRLamide + H⁺), and 854 Da (pQIPMLRLamide + H⁺) were detected, corresponding to protonated average isotopic masses of four of the five myomodulin peptides predicted from the deduced amino acid sequence of the precursor protein (Fig. 3A) (Kellett et al. 1996). The fifth myomodulin peptide, GLQMLRLamide, was not detected, probably because its concentration within the cell was below the sensitivity threshold for detection. The existence of GLQMLRLamide in *Lymnaea* has been confirmed by biochemical purification and sequencing from brain extracts (van Golen et al. 1996). In addition, its presence in nervous tissue has been detected by MALDI-MS (Kellett et al. 1996).

The majority of axons of the B2 motor neurons project along the dorsobuccal nerves (Fig. 2, A and B). As predicted, MALDI-MS analysis of these nerves (Fig. 3B) revealed the presence of all the myomodulin peptides that were detectable in the B2 cells as well as a mass peak of 830 Da corresponding to the protonated fifth peptide, GLQMLRLamide. Analysis of the peptide content of the proesophagus again revealed mass peaks for all five myomodulin peptides (Fig. 3C), consistent with its innervation by the B2 cells. Other mass peaks also were detected in the proesophagus, presumably indicating the localization of other peptides in the tissue (not shown), possibly derived from axons of other neurons that project to the gut.

Evidence for ACh as a cotransmitter in the B2 motor neurons

Small molecule transmitters often are colocalized with neuropeptides in molluscan neurons, and ACh is a candidate cotransmitter for the B2s as it is believed to be present in the homologous large gut motor neurons of *Aplysia* (Lloyd et al. 1988) and *Helisoma* (Haydon 1989). To assess the transmitter content of the B2 neuron, cultured *Lymnaea* B4 motor neurons were used as an ACh assay system because they respond to applied ACh and have been previously shown to receive cholinergic inhibitory synaptic inputs from feeding interneurons (i.e., SO, N1) in the intact nervous system (Elliott and Kemenes 1992; Yeoman et al. 1993). Furthermore, their easy identification and isolation from the intact nervous system makes them ideal for cell culture. In cell culture, individual B4 neurons readily start to grow new processes within the first 12 h after isolation and continue to extend for a period of 3–4 days.

Initial tests using 1-s pulses of ACh demonstrated that B4 neurons retain their sensitivity to ACh in culture and respond with a characteristic hyperpolarization at resting membrane potentials. Consistent responses of this type were elicited by all ACh pipette concentrations tested ranging from 100 μM down to 0.1 μM, but 1 μM concentrations normally were applied. Altering the membrane potential by injecting hyper-
FIG. 4. Effects of acetylcholine (ACh) and B2 extract on individual cultured B4 neurons. Ai: change in \( V_m \) recorded from a cultured B4 neuron in response to the application of 1-s pulses of ACh (1 \( \mu \)M), recorded using single electrode current clamp. Before application, \( V_m \) was set at various values by current injection. The amplitude of the inhibition reduces and eventually reverses at more negative \( V_m \) values. Aii: currents elicited by 1-s pulses of ACh recorded under 2 electrode voltage clamp (TEVC) from a different cultured B4 neuron at holding potentials between \(-90\) and \(-60\) mV. Relationship between holding membrane potential (\( V_m \)). Inset: peak amplitude of the current response (\( I \); the reversal potential is \( \pm 85 \) mV in this example). Bi: change in \( V_m \) recorded from a cultured B4 neuron in response to 1-s pulses of B2 extract (equivalent of 1 B2 cell/\( \mu \)l of extract) under the same conditions as described for Ai and using the same neuron. Similar to the response to ACh, the amplitude of the inhibition reduces and eventually reverses at more negative values of \( V_m \). Bii: currents elicited by B2 extract recorded under TEVC from a different B4 neuron under identical conditions as used in Aii. Reversal potential is measured in the insert as \(-83\) mV.

Polarizing current into the cell body led to a decrease in the response amplitude and eventual reversal to a depolarizing response (Fig. 4Ai). These experiments used a single electrode for voltage recording and current injection, but two electrodes in bridge or voltage clamp mode were used to study the membrane potential dependence of the ACh response in more detail. With these techniques, it was possible to establish the relationship between membrane potential and the amplitude of the current induced by ACh application and calculate an average reversal potential for the response of \(-85 \pm 1\) mV (\( n = 4 \), Fig. 4Aii). These results demonstrated that cultured B4 neurons specifically respond to ACh and can be used as a sensitive assay system for the presence of ACh.

The application of short pulses of B2 extract to the surface of individually cultured B4 neurons by a pressure pipette caused a hyperpolarization of the membrane potential (Fig. 4B) with features similar to the response to ACh. The amplitude of the fast inhibition was comparable with responses elicited by 0.1–1 \( \mu \)M ACh (Fig. 4, Ai and Bi) applied to the same neurons. When alternating pulses of B2 extract and ACh were applied, it was observed that both responses reverse at an almost identical membrane potential. With two electrode voltage-clamp techniques, the mean reversal potential for the response elicited by application of B2 extract was measured as \(-87 \pm 4\) mV (\( n = 2 \); Fig. 4Bii). This value corresponds well with the reversal potential for the ACh response (\(-85 \pm 1\) mV) and supports the hypothesis that the hyperpolarization observed after the application of B2 extract is due to ACh.

The hypothesis was tested further by incubating B2 extract with choline esterase to enzymatically deplete any ACh present in the extract. As predicted, B2 extract pretreated in this way did not elicit an inhibitory response in B4 neurons.
In contrast, a control preparation of B2 extract incubated with a choline esterase solution that had been heated to 90°C to denature the choline esterase, caused responses comparable to those recorded after the application of untreated B2 extract (n = 4, Fig. 5Ai).

Furthermore, the effect of the ACh antagonist HMT on the response of B4 neurons to both B2 extract and ACh was studied (Fig. 5B). HMT is believed to be a specific blocker of excitatory cholinergic responses in the pond snail (Elliott and Kemenes 1992; Yeoman et al. 1993). However, in cell culture, superfusion of HMT completely blocked the inhibitory effect of local application of B2 extract and almost completely the effect of 1 μM ACh (n = 4). Experiments using the alternative cholinergic antagonist PTMA also demonstrated a reversible blocking of B4 cell inhibition induced by both ACh and B2 extract (n = 4, data not shown).

Further experiments involving the coculture of B2 and B4 neurons showed that B2 cells could form inhibitory chemical synapses with B4 cells. After 2–4 days in culture, 53% (n = 32) of B4 motor neurons showed 1:1 inhibitory postsynaptic potentials (IPSPs) after B2 action potentials (Fig. 6). These 1:1 IPSPs resemble the response of B4 neurons to ACh described above. Furthermore, reversible blocking of these IPSPs by HMT superfusion confirmed their cholinergic nature (Fig. 6, B and C).

An electrotonic connection also was seen to occur in 59% (n = 32) of the B2-B4 pairs tested. This caused a weak general excitation of the B4 cell when the B2 cell was depolarized during HMT superfusion (Fig. 6B). This probably provided a component of the synaptic response of the B4 cell after B2 spiking in NS (Fig. 6, A and C) but cannot be responsible for the main hyperpolarizing component of the response that was blocked by HMT.

The ability of B2 cells to form inhibitory synaptic connections with B4 cells in culture that can be blocked by cholinergic antagonists provides further strong evidence that ACh is a cotransmitter with the myomodulins in B2 neurons.

**Pharmacological actions of Lymnaea myomodulins on the isolated proesophagus**

Mass spectrometric analysis had confirmed the presence of four of the five myomodulin peptides within the B2 motor neurons and the presence of all five of the peptides in the proesophagus (see Fig. 3). As proesophageal contractions are modulated by B2 firing activity (e.g., Fig. 2, C and D), it was possible that the release of myomodulin peptides by the B2 cells was responsible for the observed modulation. To test this hypothesis, the synthetic myomodulin peptides were applied to preparations of the isolated proesophagus while recording longitudinal gut contractile activity. The peptide QIPMLRLamide was synthesized as the pyroglutamyl form (pQIPMLRLamide) as this is a common posttranslational modification of amino-terminal glutamine residues in molluscan neuropeptides. In addition, the modified form of the peptide was detected by MALDI-TOF MS in the B2 neurons, the dorsobuccal nerve and the proesophagus itself (Fig. 3), indicating this to be the predominant form of the peptide in vivo.

Pieces of proesophagus tissue were suspended in the organ bath for 4 ± 2 h after isolation, after which they usually showed spontaneous contractile activity of varying frequency [mean 4.17 ± 1.1 (SD) contractions/min] and amplitude [mean 0.105 ± 0.034 (SD) g]. The effect of application of each peptide was measured by recording changes in contractile frequency, amplitude, and tissue tonus at final bath concentrations between 1 nM and 10 μM (Fig. 7). A delay of 5–15 s after addition of the peptides to the bath was observed before responses were detected, suggesting either the peptides had to diffuse some way into the tissue to their site.
of action, or they were eliciting their effects via a relatively slow signal transduction pathway. Four of the five peptides (PMSMLRLa, SLSMLRLa, SMSMLRLa, and GLQMLRLa) produced qualitatively similar effects: dose-dependent increases in the tonus of the tissue and the frequency of contractions (Fig. 7, A–D). At high concentrations, the three most active peptides (PMSMLRLa, SMSMLRLa and SLSMLRLa) induced large changes in tonus, causing strong sustained contractions and preventing changes in the amplitude of individual contractions from being measured. The fifth peptide, pQIPMLRLa, displayed little or no activity at the concentrations at which it was tested (Fig. 7E). In a third of the preparations, a small increase in contraction frequency was observed but with no accompanying alteration in the tonus.

Quantitatively, the peptides differed in their potency considerably. The most active peptide, PMSMLRLa, produced significant changes in frequency at 10 nM and in tonus at 100 nM [n = 12, Δ frequency +67 ± 16% (mean ± SE), P < 0.001, Δtonus + 0.015 ± 0.007 (SE) g, P < 0.05]. No activity was observed for SLSMLRLa > 100 nM, with significant effects at this concentration (n = 8, Δ frequency +106 ± 34%, P < 0.05, Δtonus + 0.038 ± 0.014 g, P < 0.05). SMSMLRLa produced some effects on tonus and frequency at 100 nM, and significant effects at 1 µM (n = 8, Δ frequency 133 ± 18%, P < 0.001, Δtonus 0.041 ± 0.008 g, P < 0.001), whereas GLQMLRLa only produced significant effects at 10 µM (Δ frequency 130 ± 63%, P < 0.05, Δtonus 0.12 ± 0.026 g, P < 0.01). The least active peptide produced small variable increases in contraction frequency at a concentration of 10 µM, but no significant changes were observed at the concentrations tested (n = 6, Δ frequency +46 ± 25%, P < 0.13). This gave an activity series of PMSMLRLa > SLSMLRLa = SMSMLRLa > GLQMLRLa > pQIPMLRLa, based on the threshold of activity concentrations of each peptide.

The activity of the most potent of the peptides, PMSMLRLa, then was compared with that of a mixture of all five peptides in the ratio predicted by their copy numbers encoded within the myomodulin precursor protein (Kellett et al. 1996). This ratio (9 copies of PMSMLRLa: 2 copies SLSMLRLa: 1 copy each of the remaining three peptides) was hypothesized to be similar to that found within neurons expressing the myomodulin gene, including the B2 motor neuron, and if released in similar proportions, mimic the effect produced by physiological release of the peptides onto the esophagus (Fig. 8). Measurement of induced changes in contraction frequency from concentrations of 1 nM to 10 µM of total peptide (0.65 nM and 6.5 µM PMSMLRLa in the peptide mixture) revealed no significant differences in the effect between PMSMLRLa alone and the mixture. However, Fig. 8C shows that the peptide cocktail had significantly greater activity with respect to alterations in esophageal tonus than PMSMLRLa alone. The cocktail showed a lower activity threshold (10 nM rather than 100 nM) as well as a greater overall increase at all concentrations above this (100 nM: 0.1 ± 0.021 g; 1 µM: 0.095 ± 0.02 g). This represents a sixfold increase at 100 nM and a twofold increase at 1 µM, with respect to the effect of PMSMLRLa at these concentrations.

When a comparison of the responses of gut muscle contractility to the application of the myomodulin peptides is made with those to B2 stimulation, it is interesting to note several similarities. First, both stimuli are excitatory, leading to a sustained increase in tonus and in contraction frequency and amplitude. In both cases, the magnitude of the changes in contractile activity tended to increase with the concentration of peptide applied or with the rate of B2 firing (compare Fig. 2D with Fig. 8). The increases observed were reversible.
within a short time after washout of the peptide or hyperpolarization of the cell. Both stimuli also can modulate an intrinsic contractile activity as well as initiate contractions in quiescent preparations (Fig. 2D and see further text). These observations are all compatible with the B2 motor neuron releasing myomodulin peptides onto the proesophagus in an activity-dependent manner, and these peptides modulating gut contractile activity.

**Joint effects of myomodulin peptides and ACh on gut muscle activity**

ACh has been demonstrated to be colocalized with the myomodulin peptides in the B2 motor neurons (see previous text). In *Aplysia* and *Tritonia*, it also has been demonstrated that ACh has powerful fast stimulatory effects on the gut (Ajimal and Ram 1981; Lloyd and Willows 1988; Lloyd et al. 1988) and thus may be a major neurotransmitter in the cells equivalent to the B2 motor neurons of *Lymnaea*. In light of these data, possible interactions between the myomodulins and ACh activity in the esophagus were investigated. First, ACh alone was applied at concentrations from 1 nM to 1 μM. At concentrations of ≥10 nM, the main effect of ACh was a large dose-dependent increase in tonus (Fig. 9A). The frequency of contractions also appeared to increase by 60 ± 20% (mean ± SE) above the basal rate at all concentrations that elicited a response (n = 12). After washing, the preparations underwent a period of inhibition of contractile activity, lasting ≤3 min, before contractions returned to the level observed before application.

Measurements of the effects of application of both the myomodulin cocktail and ACh together on spontaneously contracting preparations were then made to mimic possible corelease. At low (threshold) concentrations of peptides and ACh (<100 nM), no significant changes in contractile activity were observed, and at higher concentrations, the overwhelming increase in tonus caused by ACh masked any possible modulatory effects of corelease. However, on three quiescent preparations, in which the esophagus showed no spontaneous contractile activity, at threshold concentrations (100 nM in the example in Fig. 9B) of ACh, small increases in tonus and no increase in contraction frequency were recorded (Fig. 9Bi). Application of the myomodulin peptides alone produced small increases in tonus and some arrhythmic contractions (Fig. 9Bii), but on corelease of peptides and ACh, a larger increase in tonus was observed (Fig. 9, B, iii–v, and C), indicating an additive effect. This effect was not significantly different.
MYOMODULINS AND ACETYLCHOLINE CONTROL GUT MOTILITY

A well-defined collection of neurons involved in the production and modulation of feeding behavior. Comparison of this map to that derived by immunocytochemistry, both here and in a previous study (Santama et al. 1994a), has confirmed expression of the myomodulin gene in a number of identified neurons in addition to other cells of unknown function. Of special interest are the modulatory CGCs, the SO cell, and the B1 and B2 motor neurons. Another identified cell type, the B3 motor neuron, was reported previously to be myomodulin antibody positive (Santama et al. 1994a), however, this result could not be repeated here. The cells also failed to stain by in situ hybridization, suggesting that they do not express the myomodulin peptide gene and that the faint immunostaining originally detected was an artifact of the whole-mount staining procedure used.

All of the in situ-positive–identified neurons offered potential sites at which to study central and peripheral roles for the myomodulin neuropeptides. Because of their ease of identification and the high level at which they appear to express the myomodulin gene, the B2 motor neurons were chosen in this initial study as a suitable model to allow the characterization of a function for the myomodulins in *Lymnaea* feeding. A previous study had suggested that the B2 cells were foregut motor neurons and therefore they were potentially the *Lymnaea* homologues of the *Helisoma* B5 cells and the *Aplysia* B2 cells (Benjamin et al. 1979). Fluorescent dye-filling of the B2 motor neurons determined that the proesophagus was their peripheral target and revealed an almost identical anatomy to the B5 motor neuron in the closely related mollusc *Helisoma* (Berdan et al. 1989), lending support to the hypothesis that the B2 cells are indeed the *Lymnaea* homologues of the B5s.

Measurement of the effects that manipulation of B2 motor neuron activity had on esophageal muscle revealed no simple relationship between B2 firing and muscle contractions. Instead, a general increase in muscle tonus, amplitude, and rate of rhythmic contractions was observed during depolarization of the B2 cells, whereas hyperpolarization of both cells simultaneously produced the opposite effect. A wide range of variability in intrinsic B2 and gut activity was observed in the preparations, and this made it difficult to quantify the data. However, gut contractions always accompanied B2 cell firing, and strong rhythmic contractions of the gut were initiated by B2 cell stimulation in quiescent preparations. Studies of gut motor neurons in other mollusks also have revealed that manipulation of motor neuron activity had on esophageal muscle revealed no simple relationship between B2 firing and muscle contractions. Instead, a general increase in muscle tonus, amplitude, and rate of rhythmic contractions was observed during depolarization of the B2 cells, whereas hyperpolarization of both cells simultaneously produced the opposite effect. A wide range of variability in intrinsic B2 and gut activity was observed in the preparations, and this made it difficult to quantify the data. However, gut contractions always accompanied B2 cell firing, and strong rhythmic contractions of the gut were initiated by B2 cell stimulation in quiescent preparations. Studies of gut motor neurons in other mollusks also have revealed that manipulation of motor neuron activity produces changes in the contractile properties of the foregut (Lloyd and Willows 1988; Lloyd et al. 1988). However, our experiments studying the contractile properties of isolated foregut preparations revealed the presence of intrinsic rhythmic contractile properties, suggesting that central neurons are not required to produce rhythmic contractions (Figs. 7–9), although we cannot rule out the possibility that the terminals of central neurons are still active. It appears, therefore, that motor neurons with centrally located soma play a modulatory role in the control of gut motility.

In this study, we have investigated a role for a family of neuropeptides, the myomodulins, in a defined neural network in *Lymnaea*. The presence of myomodulin gene transcripts in the buccal ganglia was confirmed by RTPCR, and their specific neuronal localization mapped by in situ hybridization. Examination of this expression pattern has identified myomodulin gene transcripts in a number of neurons previously known to function within the feeding neural network, from the sum of the increase seen for application of each separately, suggesting that little or no modulation of the effect of ACh by the myomodulin peptides occurs.

**DISCUSSION**

In this study, we have investigated a role for a family of neuropeptides, the myomodulins, in a defined neural network in *Lymnaea*. The presence of myomodulin gene transcripts in the buccal ganglia was confirmed by RTPCR, and their specific neuronal localization mapped by in situ hybridization. Examination of this expression pattern has identified myomodulin gene transcripts in a number of neurons previously known to function within the feeding neural network.
FIG. 9. A: effects of ACh application to the isolated esophagus. ACh was applied for 2 min at each of the concentrations shown, revealing a large dose-dependent increase in tissue tonus and an increase in contraction frequency. After washout, contractile activity underwent a period of inhibition before returning to levels comparable with those recorded before application of ACh. B: Coapplication of ACh and myomodulin peptide mix to a quiescent foregut preparation. Threshold concentrations of ACh (Bi) and myomodulins (Bii) applied separately produced small increases in tissue tonus (ACh and myomodulins) and contraction frequency (myomodulins only). Coapplication (Biii) produced a large tonal contraction, which was eliminated rapidly on washout. Large tonal contraction also was observed on application of myomodulins to the preparation pretreated with ACh (Biv) and with ACh after pretreatment with myomodulins (Bv). C: analysis of the effect on tonus of coapplication of ACh and myomodulins to the foregut. Coapplication of ACh and myomodulins (MMs) induces a larger tonal contraction than either applied alone (compare the 1st and 2nd columns with the 3rd), the result of summation of their individual effects (compare the 3rd column with the 4th).

(Kellett et al. 1996) produced large changes in longitudinal contraction frequency and tonus with varying thresholds of activity; the fifth predicted peptide produced little or no effect. Although the peptides are very similar in structure, they do appear to vary in their potency by several orders of magnitude, with pQIPMLRLamide displaying almost no activity at the concentrations tested. Application of the peptides as a mixture produced a qualitatively similar response as the peptides applied individually. However, a significantly greater change in tonus was observed, suggesting the different peptides released from the same cell can facilitate each other’s actions, a phenomenon observed in a number of other cases of peptide corelease (e.g., egg-laying hormones in Lymnaea) (Ter Maat et al. 1988). It was shown from work in both Lymnaea and Aplysia that the various isoforms of myomodulin peptides affect ion channel activity in subtly different manners and that the overall effect of coapplication of the peptides is a combination of these responses (Brezina et al. 1994a,b, 1995; van Golen et al. 1996). A similar mechanism could underlie the observed facilitatory effect of coapplication on the Lymnaea gut.

The response of the proesophagus to myomodulin peptide
application was similar to that produced by B2 stimulation, indicating that some component of the B2-induced response could be evoked by the release of myomodulin peptides by the B2 cells. Other transmitter substances also may be present within the B2 motor neurons, including the small cardi-oactive peptides that were localized to the B2 cells by immuno-nostaining (Santama et al. 1994a), and small molecule trans-mitters, for instance, ACh. Here we investigated the possibility of ACh being a cotransmitter as it has been sug-gested that the homologous neurons in Aplysia and Helisoma are cholinergic (Haydon 1989; Lloyd et al. 1988). A sensitive ACh assay system was developed here that uses B4 motor neurons grown in culture to detect the presence of ACh. B4 cells maintain their inhibitory response to ACh when in culture, and a comparable hyperpolarization also was produced by application of B2 cellular extract. Both the response to ACh and B2 extract were blocked or reduced by the cholinergic antagonists HMT and PTMA, and treatment of the B2 cell extract with the ACh-metabolizing en-zyme, choline esterase, abolished its hyperpolarizing effect on the B4 cell. Hyperpolarization induced by both ACh and B2 extract displayed the same mean reversal potential of about –87 mV, indicating that the responses were identical and presumably mediated by the same ionic current. Activity-dependent release of ACh from the B2 also was demon-strated when cholinergic synapses between cocultured B2 and B4 cells were formed, again blockable by HMT. Interest-ingly, the ability of HMT to block ACh inhibition of the B4 cells conflicts with two previous studies in which only PTMA, methylxylcho line, and d-tubocurarine blocked inhibitory ACh responses (Elliott et al. 1992; Yeoman et al. 1993). This discrepancy may be the result of more thorough antagonist penetration in culture experiments than in isolated brain preparations as, in the present study, HMT was applied for 30 min at 1 mM before testing, whereas in the studies using isolated preparations, HMT was applied for less time and at lower concentrations. It is also unclear in one of the studies (Elliott et al. 1992) whether recordings were made from the B4 retractor motor neuron used here or from one of a number of B4 cluster neurons. It is possible that ACh-induced hyperpolarization in some or all of the B4 cluster neurons is mediated by a different ACh receptor to that present in the B4 motor neurons, one displaying HMT insen-sitivity.

Previous examinations of the functions of the myomodulin peptides have shown that the peptides often are coreleased with ACh and modulate its effects on specific muscles, such as the accessory radula closer muscle of Aplysia (Cropper et al. 1987) and the penis retractor muscle of Lymnaea (Li et al. 1994c; van Golen et al. 1996). The presence of both ACh and the myomodulins in the B2 motor neurons demonstrated here, suggested such a function also may exist for the peptides in the control of foregut motility. ACh application to the isolated foregut induced qualitatively similar effects as the myomodulin peptides: a large tonal contraction accompanied by an increase in the rate of rhythmic contrac-tions, unlike in the preparations mentioned above where the peptides failed to elicit a response alone but modulated the effects of other transmitters or of electrical stimulation. Be-cause of the difficulty of comparing peptide and ACh-in-duced effects on rhythmically contracting preparations, it was only possible to study the effects of coapplication on a small number of preparations displaying no intrinsic contractile activity. In these experiments, under specific conditions where threshold concentrations of ACh and the myomodulin peptides were coapplied, the effects appeared to be purely additive, displaying no facilitation of the response to either transmitter. Because foregut tissue is a complex mixture of different muscle fiber types, neurons, and connective tissues, the myomodulin peptides and ACh may function as modula-tors or transmitters at several different sites. Indeed, the fact that B2 motor neurons do not appear to form monosynaptic excitatory connections with muscle fibers (evident from the lack of a simple 1:1 correlation between B2 firing and muscle contraction) suggests that this is likely to be the case. Further investigation into the exact sites of myomodulin peptide action await to be made.

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