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ORIGINAL ARTICLE

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Regulation of pharyngeal motility by FMRFamide and related peptides in the medicinal leech, *Hirudo medicinalis*

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Abstract The medicinal leech possesses FMRFamide-like immunoreactivity in neural processes and somata associated with the pharynx and pharyngeal ganglia. The pharynx possessed about 25 immunoreactive somata; about half of the approximately 20 neurons of the pharyngeal ganglia were immunoreactive. We provide brief descriptions of several neurons located in the first neuromere of the subesophageal ganglion involved in controlling pharyngeal motility. Double-labeling experiments indicate that one of these cells, named Swallow neuron 1 (SW1), contains a FMRFamide-like peptide. Stimulation of SW1 caused the mouth to open and the pharynx to dilate. Upon termination of SW1 stimulation, the mouth closed, and a peristaltic wave progressed from the mouth down the length of the pharynx. Stimulation of SW1 did not produce 1:1 postsynaptic potentials in pharyngeal muscle cells. Thus, SW1 is apparently not a motor neuron. The pharynx responded to application of FMRFamide and related peptides by producing a series of 20- to 35-s phasic contractions superimposed upon an increase in basal tonus. The peptide-induced response was quantified by measuring increases in basal tonus, peak tension, and integrated area. Although there were some differences in the order of potency depending upon which parameter was considered, the approximate order of potency of RFamide peptides tested was: pQDPFLRFamide ≥ FMRFamide ≈ YGGFMRFamide ≥ YMRFamide ≈ FLRFamide ≈ GGKYMRFamide ≈ YLRFamide > leucomyosuppressin ≈ perisulfakinin. Except for differences in potency, each of the RFamide peptides produced similar contractile waveforms. FMRFamide-induced responses were reduced by the protein kinase C inhibitor bisindolylmaleimide I (10 μM), the nonspecific protein kinase inhibitor H-7 (50 μM), and were increased by the protein phosphatase inhibitor okadaic acid (1 μM). However, the FMRFamide-induced response was unaffected by the protein kinase A inhibitor H-89 (1 μM), the phosphodiesterase inhibitor theophylline (1 mM), the phospholipase A_2 inhibitor OBAA (0.1 μM) or the cation channel blocker amiloride (100 μM).

Key words Gut · Feeding · Second messenger · Central pattern generator · Muscle

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Introduction

The medicinal leech, *Hirudo medicinalis*, is a sanguivorous predator. Previous work on the control of feeding in *Hirudo* has concentrated on the role of serotonin (reviewed by Lent et al. 1989). However, the roles of other neurotransmitters as well as the neuronal circuitry underlying feeding in the leech are largely unknown. In general, animal guts are responsive to a wide variety of neurotransmitters, particularly neuropeptides. There is no reason to suspect that the leech gut should be an exception to this rule. In fact, it was recently shown that the pharynx is responsive to acetylcholine through activation of a nicotinic receptor (O'Gara, Abbasi et al., in press). It seems likely that neurotransmitters aside from serotonin and acetylcholine have important roles in

controlling gut motility in the leech. A number of FMRFamide-related peptides have been isolated from *Hirudo* (Evans et al. 1991), and FMRFamide-like immunoreactivity is associated with the foregut of the related leech species *Haemopis marmorata* (Flanagan and Zipser 1986). Since FMRFamide-related peptides (RFamide peptides) have frequently been shown to affect gut motility (see below), the FMRFamide-related peptides are excellent candidates for substances that might be involved in controlling leech gut motility.

Peptides with a C-terminal RFamide are active on the guts of many animals. Depending on the peptide, animal, and region of the gut examined, RFamide peptides can have excitatory or inhibitory effects. For example, FMRFamide is excitatory to the clam rectum and has both excitatory and inhibitory effects on different regions of earthworm foregut (Doble and Greenberg 1982; Ukena et al. 1996). FMRFamide inhibits contractions or causes relaxation of the polychaete esophagus, mollusc gizzard and crop, *Limulus* midgut, and locust foregut (Krajniak et al. 1992; Austin et al. 1983; Krajniak et al. 1989; Groome et al. 1992; Banner and Osborne 1989). One group of RFamide peptides, the myosuppressins (leucomyosuppressin, neomyosuppressin, Schisto-FLRFamide, MasFLRFamide I), are decapeptides having inhibitory actions on arthropod guts (Holman et al. 1986; Robb et al. 1989; Fónagy et al. 1992; Fujisawa et al. 1993). However, the related heptapeptides MasFLRFamide II and III have excitatory actions on the adult *Manduca* ileum (Kingan et al. 1996).

RFamide peptides are known to exist in the leech. Evans et al. (1991) isolated five RFamide peptides from the leech, *H. medicinalis*: FMRFamide, FLRFamide, YMRFamide, YLRFamide, and GGKYMRFamide. A functional role for some of these peptides has been demonstrated in the leech, where they cause contractions of longitudinal body wall muscle and heart muscle as well as modulate the neuronal circuit controlling the heart (Kuhlman et al. 1985b; Norris and Calabrese 1987, 1990; Simon et al. 1992, 1994).

In this report, we demonstrate the sensitivity of the leech pharynx to a number of RFamide peptides. We also examine a number of second messenger systems for their potential role in mediating FMRFamide-induced effects. In addition, we document some general characteristics of neurons involved in controlling pharyngeal contractions, and show that at least one of these neurons contains an RFamide-like peptide. Some of the results presented here have been previously reported in abstract form (O'Gara 1990).

Materials and methods

Medicinal leeches, *H. medicinalis*, were obtained from commercial suppliers (Leeches U.S.A., Westbury, N.Y. or ZAUG, Biebertal, Germany) and maintained in aquaria at about 18 °C on a 12 h light:12 h dark photoperiod. Animals maintained in the laboratory for longer than 4 months were fed bovine blood.

Immunocytochemistry

Tissues were fixed in Zamboni's fixative at 4 °C for at least 24 h. Fixative was removed from the tissue by several changes of phosphate-buffered saline containing 1% Triton X-100 (PBS-T) over 2 h. Autofluorescence was reduced by incubating the tissue for 30 min in either 1% sodium borohydride in distilled water or a saturated solution of lithium carbonate in 70% ethanol. To reduce nonspecific staining, the nerve cords were incubated in 20% normal goat serum in PBS-T for 4 h. The tissue was incubated for 12 h at room temperature in a polyclonal antibody raised against FMRFamide [DiaSorin (formally Incstar), Stillwater, Minn.] at a dilution of 1:500 in PBS-T. The antibody has a strong requirement for an amiditated C-terminal phenylalanine. The primary antibody was visualized using a goat anti-rabbit antibody conjugated to one of the following: rhodamine (Oreganon Teknika, West Chester, Pa.), Texas Red, or FITC (Accurate Chemical, Westbury, N.Y.). The secondary antibody was diluted 1:100 in PBS-T and incubated with the tissue for 4 h at room temperature. The tissue was stored and mounted in 0.05 M TRIS buffer prior to viewing with a fluorescence microscope. Staining was eliminated by preincubation of the primary antibody with FMRFamide (250 µg/ml; approximately 0.4 mM).

Dissection and electrophysiology

For pharynx – head ganglia preparations, leeches were anesthetized with ice-cold saline and pinned ventral side down in a frozen wax-bottomed dissection dish. A dorsal midline incision was used to expose the pharynx. The pharynx was freed from its connections to the body wall by severing the extrinsic radial muscles. The ventral radial muscles were severed by first cutting through the crop posterior to the posterior sphincter of the pharynx, and then severing the remaining radial muscles on the ventral side of the pharynx as the tissue was elevated using forceps. The supraesophageal ganglion, the subesophageal ganglion, and the first two segmental ganglia along with the pharynx and mouth were removed from the animal as a unit. The nerve cord was pinned ventral side up in a small Sylgard-lined dish (Dow-Corning, Midland, Mich.), and the pharynx was reflected anteriorly so that it was not under the nerve cord. Movements of the pharynx were monitored using an isometric force transducer (FORT-10, WPI, Sarasota, Fl.) or by the movement artifacts produced when a microelectrode was placed in contact with the surface of the pharynx. The subesophageal ganglion was desheathed using fine scissors and illuminated during the experiment by a dark-field condenser to allow viewing of the neuronal somata within the ganglion. The preparation was continuously superfused with saline at a rate of 0.5 ml/min. Microelectrodes for electrophysiology were filled with either 3 M KCl or 4 M potassium acetate, 20 mM KCl and had resistances of 20–35 MΩ. Electrodes for dye injection were filled with 3% Lucifer yellow in 1 M LiCl. Dye was ejected from the electrodes by passing-5 nA of current for 15–30 min. Some of the ganglia with dye-injected cells were processed for FMRFamide-like immunocytochemistry as noted above.

Isolated pharynx preparation

The initial dissection was similar to that described above for electrophysiology. However, the supraesophageal and subesophageal ganglia were not removed from the animal. Once the pharynx was freed from the surrounding tissue, it was removed from the leech by transecting the pharynx just posterior to where it passes through the circumesophageal connectives. A short length of the pharynx, from the mouth to the dorsal surface of the head ganglia, was not removed from the leech. The pharyngeal (or accessory) ganglia (Hanke 1948; Sawyer 1986), which are embedded in the surface of the pharynx at the level of the circumesophageal connectives, were not removed from the animal with the isolated pharynx. Microsurgical needles, bent into a hook shape and attached to 8-0 monofilament nylon suture material (Ethicon 2808G, Sommerville,

N.J.), were inserted through each end of the pharynx. One nylon suture was anchored to the bottom of a small perfusion tissue bath (volume 0.2 ml), while the suture attached to the other end of the pharynx was attached to an isometric force transducer (FORT-10). This physical arrangement most effectively monitored longitudinal contractions of the pharynx; however, circular contractions were also recorded by the transducer (as judged by simultaneous visual observation of the pharynx). The pharynx was placed under approximately 20–25 mN of tension and allowed to relax for approximately 1 h. Most pharynxes were initially quiescent, but exhibited spontaneous contractions well before the end of the 1-h relaxation period.

The output of the force transducer was fed into a transducer interface (Transbridge, WPI, Sarasota, Fl. or ETH-200, CB Sciences, Dover, N.H.), whose output was fed into a computer-based data acquisition system (CODAS or WINDAQ, Dataq Instruments, Akron, Ohio). The signal from the force transducer was digitized at 50 samples/s and recorded to disk. Subsequent data analysis of the records was performed using the playback software of the data acquisition system and Advanced CODAS software (Dataq Instruments).

The tissue bath was continuously perfused with saline through an inlet at the bottom of the chamber at a rate of 1 ml/min; saline was removed from the top of the chamber by suction. The application of control or experimental salines was controlled by a valve attached to reservoirs containing each of the salines. An air bubble was introduced into the perfusion line to indicate the beginning and end of each treatment. Each application of peptide-containing saline was 1 ml in volume and approximately 1 min in duration.

Experiments conducted to examine the possible role of second messenger systems in mediating FMRFamide-induced responses used the following protocol. Following the initial relaxation period, the pharynx was exposed to a 1-ml aliquot (approximate duration 1 min) of either 0.1 or 1 μM FMRFamide and the response noted. The pharynx was continuously washed in normal saline for 1–2 h after the FMRFamide application. Drugs applied to affect second messenger systems were than applied to the preparation for 10–30 min prior to a 1-ml aliquot of FMRFamide and the drug. When it was necessary to dissolve a drug in DMSO or ethanol, during the control trial the pharynx was exposed to the concentration (and duration) of solvent used to dissolve the drug.

Statistics were performed using SigmaStat 2.0 (SPSS Inc, Chicago, Ill.). Values are presented as mean ± SE if the data were normally distributed (Kolmogorov-Smirnov test with Lilliefors' correction). If the data were not normally distributed, the data are presented as the median (25th percentile, 75th percentile). Control and drug-treated trials were compared using paired *t*-tests; however, when the data were not normally distributed, they were compared using a Wilcoxon signed rank test.

Drugs and saline

During the dissection and experiment, the preparation was superfused with a physiological saline containing 115 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM HEPES buffer, and brought to pH 7.4 with NaOH. Experiments were conducted at room temperature (21–25 °C). Peptides or drugs were purchased from the following vendors: FMRFamide, FLRFamide, pQDPFLRFamide, amiloride (*N*-amidino-3,5-diamino-6-chloropyrazinecarboxamide hydrochloride), and theophylline (Sigma, St. Louis, Mo.); YMRFamide and YGGFMRFamide (Peninsula Laboratories, Belmont, Calif.); leucomyosuppressin (Peninsula or Bachem Bioscience, Philadelphia, Pa.); perisulfakinin (Bachem); H-89 {N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; Seikagaku America, Ijamsville, Md.} H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride] and okadaic acid sodium (RBI, Natick, Mass.); OBAA [3-(4-octadecyl)-benzoylacrylic acid; Biomol, Plymouth Meeting, Pa.]; bisindolylmaleimide I (BIM) {2-[1-(3-dimethylaminopropyl)-1H-indol-3-3-yl]-3-(1H-indol-3-yl)maleimide, HCl; Calbiochem, La Jolla, CA USA}. The peptides YLRFamide and GGKYMRFamide were custom synthesized by Genemed Synthesis (South San Francisco, Calif.). Each of the peptides was dissolved in distilled water and frozen in small aliquots. The aliquot was thawed and diluted with leech saline just prior to use. Perisulfakinin stock solutions were dissolved and frozen in 0.01% sodium metabisulfite to reduce oxidation of the peptide. H-89 was prepared as a 10 mM stock solution dissolved in DMSO and OBAA was prepared as a 10 mM stock solution dissolved in ethanol. Both drugs were diluted to their final concentration in saline just prior to the experiment.

Results

Presence of FMRFamide-like immunoreactivity in cells associated with the pharynx

The serosal surface of the pharynx of the medicinal leech possesses numerous cells and cellular processes that stain for FMRFamide-like immunoreactivity (Fig. 1A). We classified these cells as neurons based on their morphology. There was an average of 24.3 ± 4.27 somata present on the surface of the pharynx ($n = 10$). Processes extended from the somata, usually along the anterior-posterior axis of the pharynx. A few cells extended processes around the circular axis of the pharynx. Regardless of orientation, processes branched only a few

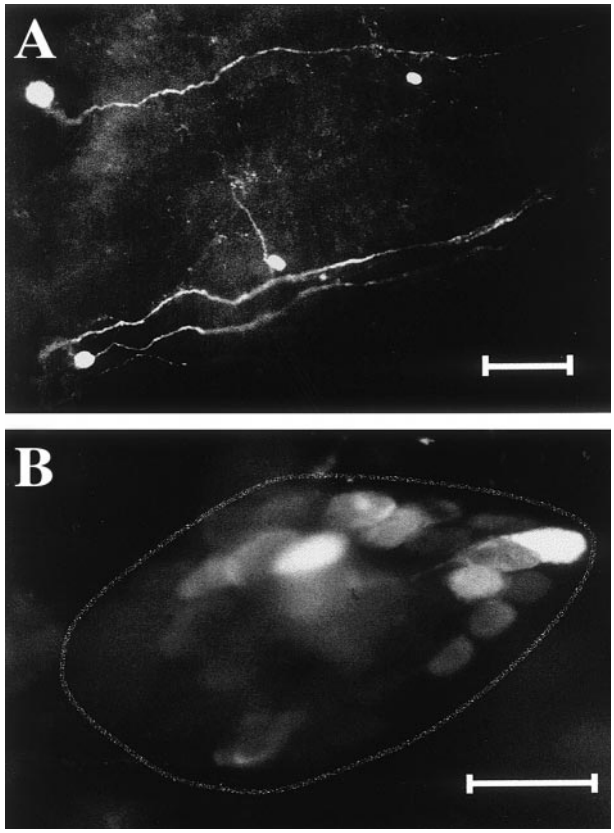


Fig. 1A, B FMRFamide-like peptides are present in neural processes and somata associated with the pharynx and in neurons of the pharyngeal ganglia. **A** FMRFamide-like immunoreactivity in neurons associated with the pharynx. Most processes extend along the longitudinal axis of the pharynx (*across the figure*). **B** FMRFamide-like immunoreactivity occurs in approximately half of the neuronal somata in the pharyngeal ganglia. The *stippled line* indicates the approximate borders of the pharyngeal ganglion. *Bars* A, B 100 μm

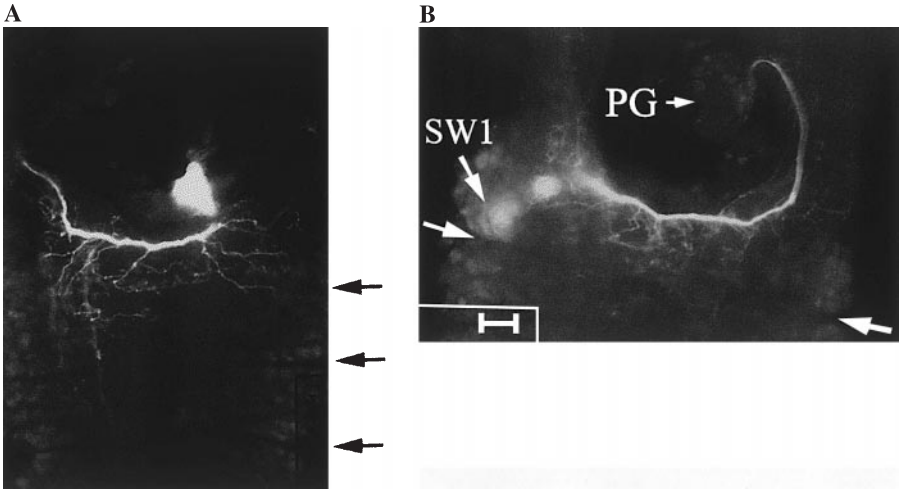
times, and branching beyond the secondary level was rare. Many processes had varicosities sparsely distributed along their length. Somata were more prevalent in the posterior half of the pharynx, especially near the posterior sphincter (the posterior sphincter is sometimes called the esophagus; e.g., Michel and DeVillez 1978). The distribution of FMRFamide-like immunoreactive processes on the pharynx did not show obvious regional differences. Each pharynx possessed a number of FMRFamide-like immunoreactive processes that were not obviously attached to a soma. However, it was difficult to determine how many of the processes were not connected to a pharyngeal soma. About half of the approximately 20 cells in the pharyngeal ganglia were immunoreactive for a FMRFamide-like substance (Fig. 1B). The pharyngeal ganglia each contained 9.67 $\pm$ 1.15 FMRFamide-like immunoreactive somata (n = 12). The axons of at least some of the FMRFamide-like immunoreactive somata within the pharyngeal ganglia appear to project into the central nervous system (CNS) through the stomatogastric nerve.

Fig. 2A,B Typical anatomy of neurons in the subesophageal ganglion involved in control of pharyngeal contractions. **A** Dorsal view of a neuron filled with Lucifer yellow that, when stimulated, caused a maintained longitudinal contraction of the pharynx. The soma is on the ventral surface and is out of focus in the photograph. The axon of the cell extends contralaterally from the soma and enters the circumesophageal connective. *Arrows* indicate the divisions between the four neuromeres of the subesophageal ganglion. Note that one process of the cell extends into the third neuromere. This cell possesses the longest extensions of processes within the subesophageal ganglion of all known cells that cause pharyngeal contractions. **B** Dorsal view of a Lucifer yellow fill of Swallow Neuron 1 (*SW*1). The soma of SW1 is located on the ventral surface of the ganglion, and is therefore out of focus. The position of the SW1 soma is indicated by the *labeled arrow*. SW1 possesses many of same anatomical characteristics of the cell in **A**. However, almost all the processes of SW1 are restricted to the first neuromere of the subesophageal ganglion. The *unlabeled arrows* delimit the border between the first and second neuromere of the subesophageal ganglion (*PG* pharyngeal ganglion, *SW1* position of SW1 soma). *Bar* (for both panels) 100 μm

Presence of FMRFamide-like immunoreactivity in a neuron that caused swallowing movements of the pharynx

We performed a search for neurons involved in controlling movements of the pharynx and found a number of such cells. All cells causing pharyngeal movements shared a number of morphological characteristics (Fig. 2). The somata were located on the ventral surface of the first neuromere of the subesophageal ganglion. The axons of these cells projected dorsally from the soma until they nearly reached the dorsal surface of the ganglion. The axon then crossed the midline at the extreme anterior margin of the subesophageal ganglion and extended into the contralateral circumesophageal connective. The axon left the CNS through the stomatogastric (stomatodaeal) nerve (Hanke 1948; Sawyer 1986) and entered a pharyngeal ganglion. Using Lucifer yellow fills of these neurons, we were not able to see axons extending beyond the pharyngeal ganglia. Within the subesophageal ganglion, most cells branched profusely within the first neuromere. Cells frequently sent processes into the second neuromere and into both circumesophageal connectives. One cell was seen to extend a process as far posteriorly as the third neuromere of the subesophageal ganglion (Fig. 2A). Few varicosities were present on the processes extending from these cells. We have never saw processes extending into the supraesophageal ganglion or leaving the CNS through any nerve other than stomatogastric nerve. We recorded data from several neurons that caused pharyngeal contractions besides those described below. Stimulation of these cells caused either a sustained longitudinal or a circular contraction of the pharynx. However, we were not sure of the exact number of such cells, and have not yet performed extensive characterization of any of these neurons.

We have confirmed previous findings (Lent and Dickinson 1984; O'Gara et al., 1999) that stimulation of the large lateral serotonin-containing cells (LL cells) causes repetitive movements of the pharynx (not shown).



To determine if the LL cells contained an FMRFamide-like peptide, we filled five LL cells with Lucifer yellow and subsequently processed the head ganglia for FMRFamide-like immunocytochemistry. LL cells are frequently identifiable due to their position and size within the ganglion so that these cells can be identified without dye injection. No FMRFamide-like immunoreactivity was observed in the five dye-injected LL cells or in numerous unlabeled LL cells.

With the exception of LL cells, whose stimulation produced repetitive contractions, stimulation of most other cells that caused pharyngeal movements produced either a sustained circular or longitudinal contraction of the pharynx for the duration of depolarizing current injection (not shown). However, stimulation of one particular cell produced complex movements of the pharynx. The soma of this cell was approximately 50 μm in diameter and was often the second largest cell in the neuromere (after the LL cell). The soma was located adjacent to the LL cell and was just lateral and posterior to the LL cell in most preparations. The anatomy of the cell (Fig. 2B) was similar to the general description above and to that of the LL cell (Lent and Dickinson 1984). Specifically, the cell possessed a ventral soma near the lateral margin of the first neuromere; the axon projected dorsally from the soma and crossed the midline near the anteriormost margin of the ganglion. The axon then entered the contralateral circumesophageal connective, and exited the CNS via the stomatogastric nerve. Almost all processes of this cell were confined to the first neuromere of the subesophageal ganglion and the circumesophageal connectives. The spike-generating zone of the cell was apparently a considerable electrical distance from the soma since action potentials were usually about 1 mV in amplitude. When the cell was directly stimulated by current injection, the mouth and pharynx dilated, and the pharynx slightly shortened (Fig. 3). Upon termination of current injection, the mouth closed and a peristaltic wave was initiated at the mouth that progressed to the posterior end of the pharynx. It is our impression that fluid would be drawn into the pharynx during stimulation of the cell and swallowed at the conclusion of stimulation. Because of the actions of this cell, we designate the cell as Swallow Neuron 1 (SW1).

The long electrical distance between the soma, spike-initiation zone and input regions of the cell make analysis of its synaptic inputs difficult. As is true of most other bilaterally paired neurons in the leech, the pair of SW1 somata were electrically coupled. SW1 was excited (as judged by a small depolarization or increase in spiking activity) by stimulation of most if not all sensory neurons in the subesophageal ganglion (not shown). We did not attempt to determine if the synaptic connections from the sensory neurons were mono- or polysynaptic. Stimulation of the sensory neurons usually caused a vigorous contraction of the pharynx. Pharyngeal contractions initiated by sensory stimulation are probably mediated by cells in addition to SW1 since the sensory neurons have synaptic outputs onto many neurons.

To examine the role of SW1 in controlling pharyngeal contractions, we recorded from muscle cells of the pharynx during SW1 stimulation (Fig. 4). The radial muscles extend between the pharynx and body wall of the animal, and their contraction would lead to dilation of the pharynx. In isolated head ganglia-pharynx preparations, we recorded from the stumps of severed radial muscles while stimulating SW1 (Fig. 4A). During SW1 stimulation, a series of excitatory postsynaptic potentials

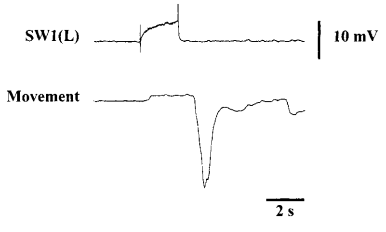


Fig. 3 Complex contraction of the pharynx induced by SW1 stimulation. When SW1 was depolarized by current injection, the pharynx and mouth dilated, and the pharynx shortened (indicated by the *upward deflection* of the *bottom trace*). Upon termination of SW1 stimulation, the mouth closed and a peristaltic wave was initiated at the mouth that progressed the length of the pharynx (indicated by the *downward deflection*). Movement of the pharynx was detected by a microelectrode placed in contact with the pharyngeal surface. In this and subsequent figures, the designation of (*L*) or (*R*) indicates that the cell soma was on the right or left side of the ganglion, respectively

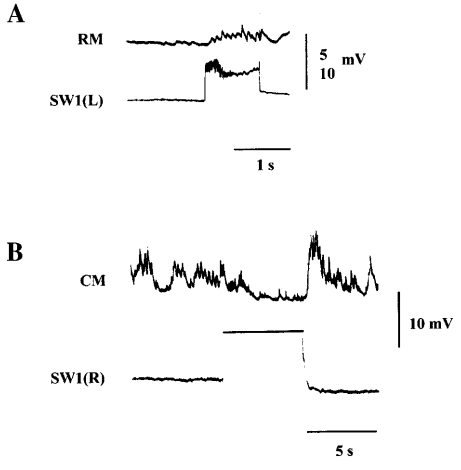


Fig. 4A, B Stimulation of SW1 does not produce direct effects in pharyngeal muscle cells. **A** Top trace (*RM*) is an intracellular recording from the cut stump of a radial muscle located near the mouth. Stimulation of SW1 causes a series of depolarizing excitatory postsynaptic potentials in the muscle. However, there is not a 1:1 correlation between spikes in SW1 and EPSPs in the radial muscle. *Scale*: *Top* 5 mV, *bottom* 10 mV. **B** Top trace (*CM*) is an intracellular recording from a circular muscle cell near the center of the anterior-posterior axis of the pharynx. During SW1 stimulation, spontaneous EPSPs in the circular muscle were reduced and the cell hyperpolarized. At the termination of SW1 stimulation, a peristaltic wave was initiated. When the peristaltic wave reached the recording site, a large compound EPSP was recorded in the circular muscle cell. The intracellular amplifier output saturated during current injection in **B**

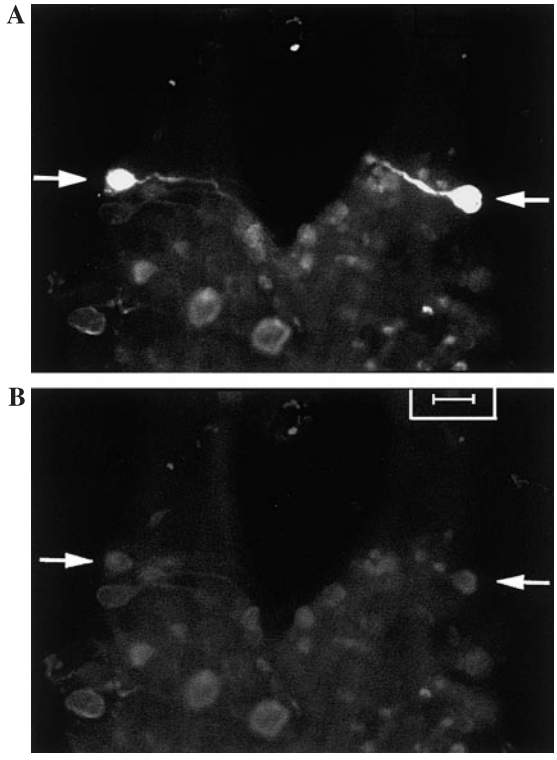


Fig. 5A, B SW1 contains an RFamide-like peptide. **A** Two SW1 cells were injected with Lucifer yellow (*arrows*). The tissue was subsequently processed for FMRFamide-like immunocytochemistry and viewed with filters for rhodamine fluorescence (**B**). Cells SW1 (*arrows*) both contain an immunoreactive peptide. Cells visible in **A**, aside from SW1, are due to bleed through of rhodamine fluorescence through the filter used for Lucifer yellow fluorescence. *Bar* 100 μm

(EPSPs) were induced in the radial muscles. These EPSPs were not correlated in a 1:1 fashion with spikes in SW1. Recordings from circular muscle cells of the pharynx indicated that spontaneous EPSP activity was reduced during SW1 stimulation (Fig. 4B). A burst of EPSP activity occurred at the end of SW1 stimulation as the peristaltic wave reached that region of the pharynx. The lack of any 1:1 correlation between spikes in SW1 and postsynaptic potentials in pharyngeal muscle cells indicates that SW1 is probably not a motor neuron, but is more likely to be a higher order neuron involved in pharyngeal control.

We filled ten SW1 cells with Lucifer yellow and then processed the tissue for FMRFamide-like immunocytochemistry. In seven of ten injected cells, FMRFamide-like immunoreactivity was clearly present (Fig. 5). In two of the remaining three cells, weak immunoreactivity may have been present. Thus, SW1 contains an FMRFamide-like peptide.

Response of the isolated pharynx to FMRFamide and other RFamide peptides

We examined the responses of the isolated pharynx (minus the CNS) to RFamide peptides by removing the

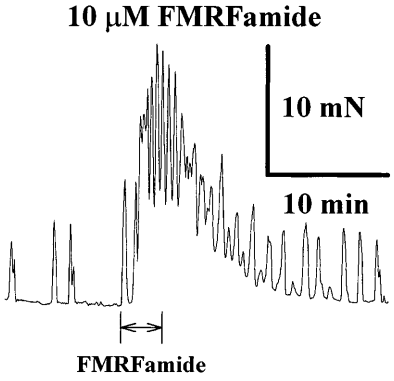


Fig. 6 Contractile response of the pharynx elicited by 10 μM FMRFamide. An approximately 1-min application of FMRFamide causes a series of phasic contractions superimposed on an increase in basal tonus. Responses of the pharynx to other RFamide peptides are similar in form

pharynx from the animal, and mounting it in an organ chamber such that pharyngeal contractions could be monitored with a force transducer. When the pharynx was exposed to a 1-min application of FMRFamide, a concentration-dependent complex contractile response was produced (Fig. 6). The response consisted of an elevation in basal tonus upon which a series of phasic contractions was superimposed, whose individual durations were 20–35 s. We quantified the peptide-induced responses by measuring the maximal increase in basal tonus, the peak tension, and the integrated area under

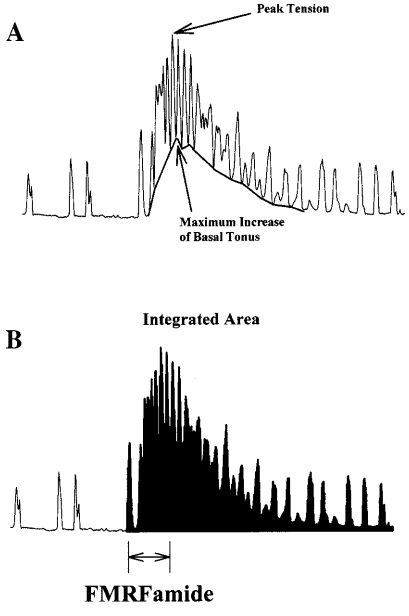


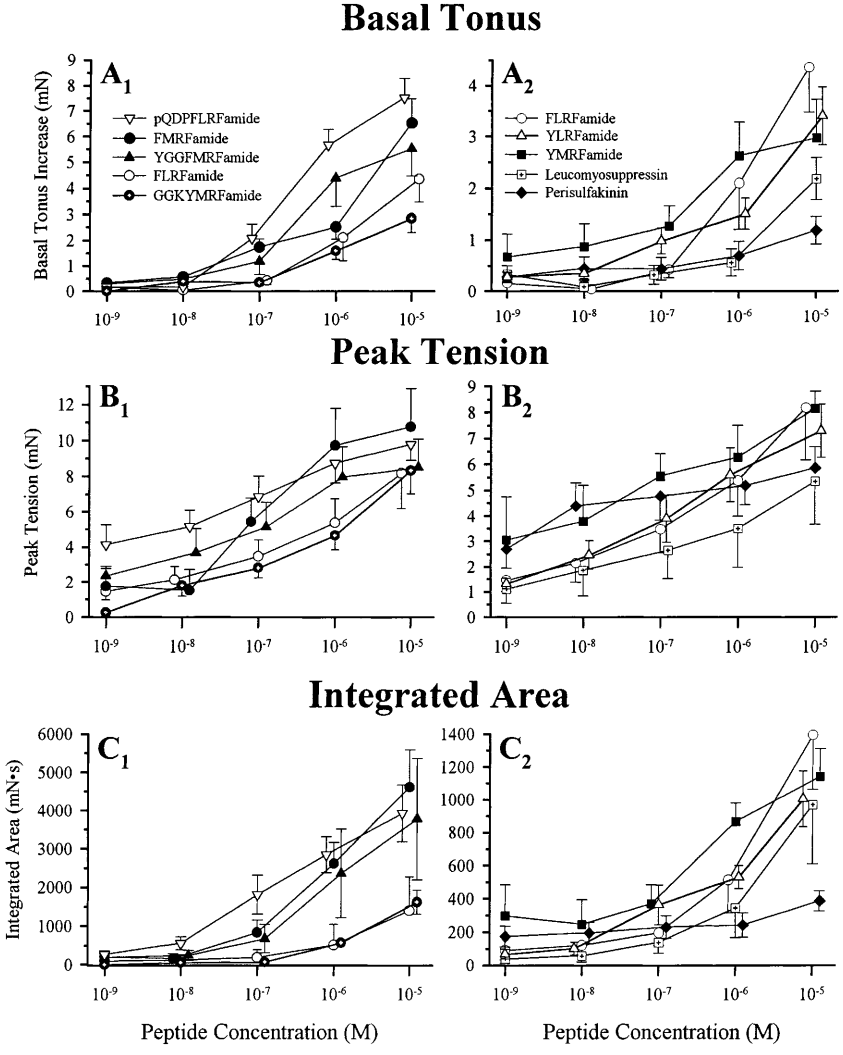
Fig. 7A, B Parameters measured to quantify the response of the pharynx to RFamide peptides. **A** Peak tension is the greatest tension produced in response to the peptide. The maximal increase in basal tonus is measured as the highest valley between phasic contractions. **B** Integrated area is measured as the area under the contraction curve from when the peptide was administered until the basal tonus has returned to baseline

the contraction curve (Fig. 7). We measured both the peak tension of the phasic contractions and the maximal increase in basal tonus since, for at least some neurotransmitters; these responses are pharmacologically separable (O'Gara et al., 1999). Integrated area is sensitive to changes in the duration of a response as well as the frequency of phasic contractions.

Concentration-response relationships for basal tonus, peak tension, and integrated area are plotted in Fig. 8. The amplitude of each parameter increased with higher concentrations of FMRFamide up to at least 10 μM. However, peak tension may have reached a plateau beginning at 1 μM. Peptide concentrations higher than 10 μM were not tested.

We examined the activity of a number of RFamide peptides upon the pharynx. We chose to examine the four RFamide peptides known to be present in *Hirudo* (in addition to FMRFamide), FLRFamide, YLRFamide, YMRFamide, and GGKYMRFamide (Evans et al. 1991); two heptapeptides, YGGFMRFamide and pQDPFLRFamide, since heptapeptides and tetrapeptides are reported to activate distinct receptors in *Helix* (Cottrell and Davies 1987; Payza et al. 1989; Cottrell 1997); leucomyosuppressin (pQDVDHVFLRFamide), an RFamide peptide with inhibitory actions upon insect visceral muscles (Holman et al. 1986); and perisulfakinin

Fig. 8A–C Concentration-response curves of the pharynx to a number of RFamide peptides. For each measure (basal tonus, peak tension, integrated area), the data are split between two graphs (e.g., $\mathbf{A}_1$ and $\mathbf{A}_2$). Data plotted in the *left graph* are of the higher potency peptides, while the data plotted in the *right graph* are of lower potency peptides. Note the different *vertical axis* scales used in the two graphs. FLRFamide (*open circles*) is plotted in both graphs for comparative purposes. **A** Maximal increase in basal tonus. **B** Peak tension. The values for peak tension at low concentrations often indicate the presence of a "spontaneous" phasic contraction occurring shortly after the peptide application. The amplitudes of such contractions are reported here since there was no way of determining if the phasic contraction was induced by the peptide application or if the contraction was truly spontaneous. **C** Integrated area. In each graph, some data points have been displaced horizontally for clarity. Data points clustered around a particular tick mark on the *x*-axis are derived from identical peptide concentrations. When error bars are not visible, the SE is smaller than the symbol. Number of preparations examined for each peptide: pQDPFLRFamide (7), FMRFamide (7 for peak tension and integrated area; 14 for basal tonus), YGGFMRFamide (9), FLRFamide (7), YMRFamide (7), YLRFamide (12), GGKYMRFamide (10), leucomyosuppressin (10), and perisulfakinin (12)



(EQFDDY$_{(SO_3)}$GHMRFamide), an RFamide peptide that also shows sequence homologies to the gastrin/cholecystokinin peptide family (Veenstra 1989). We chose to examine the actions of several of these peptides (FMRFamide, FLRFamide, YMRFamide, pQDPFLRFamide) because they also have been tested on leech longitudinal body wall muscle (Norris and Calabrese 1990); thus, allowing comparison of the body wall and pharyngeal RFamide receptors.

Each of the tested RFamide peptides was active on the pharynx, and the waveform of the contractile response was similar to that described for FMRFamide (Fig. 6). However, there were large differences in the potency of the various peptides in inducing the contractile response (Fig. 8). When integrated area is considered (Fig. 8C), the relative potency of the various RFamide peptides separated out into two groups: a higher potency group, composed of FMRFamide, YGGFMRFamide, and pQDPFLRFamide (Fig. 8C$_1$), and a lower potency group, composed of FLRFamide, YMRFamide, GGKYMRFamide, YLRFamide, leucomyosuppressin, and perisulfakinin (Fig. 8C$_2$). The same approximate order of potency was found in the data for basal tonus (pQDPFLRFamide $\geq$ FMRFamide $\geq$ YGGFMRFamide $\geq$ FLRFamide $\approx$ YMRFamide $\geq$ GGKYMRFamide $\geq$ YLRFamide $>$ leucomyosuppressin $>$ perisulfakinin). However, the clear distinction between the higher and lower potency groups present in the integrated area data was not as evident for basal tonus. When the data for peak tension are considered, leucomyosuppressin and perisulfakinin were less potent when applied at high concentrations than the other peptides. The approximate order of potency for peak tension was: FMRFamide $\approx$ pQDPFLRFamide $\geq$ YGGFMRFamide $\approx$ FLRFamide $\approx$ YMRFamide $\approx$GGKYMRFamide $\geq$ YLRFamide $>$ perisulfakinin $\approx$leucomyosuppressin. However, measurements of peak tension were not as good at distinguishing between the various peptides as were measurements of basal tonus or integrated area.

Amiloride does not block the FMRFamide-induced response

FMRFamide can directly gate a Na^+ channel that is blocked by amiloride (Cottrell 1997). Application of 100 μM amiloride did not alter the response induced by subsequent application of 1 μM FMRFamide (n = 5; Table 1).

Effects of manipulating second messenger systems on FMRFamide-induced responses

We attempted to determine if the FMRFamide-induced responses were mediated through a particular second messenger system. We examined a number of drugs known to affect second messenger systems for their effects on the FMRFamide-induced response.

Bisindolylmaleimide I (BIM) is a potent and selective inhibitor of protein kinase C (Toullec et al. 1991). Application of BIM reduced the FMRFamide-induced increase in basal tonus and integrated area (Table 2). However, BIM had no effect on FMRFamide-induced peak tension.

H-7 is a nonspecific protein kinase inhibitor; however, it has been reported to have some specificity for protein kinase C (Kawamoto and Hidaka 1984; Hidaka et al. 1984; Conn et al. 1989; Boulis and Davis 1990). Application of H-7 produced a reduction in the FMRFamide-induced increase in basal tonus (Table 2). However, H-7 did not affect the FMRFamide-induced peak tension or integrated area.

Okadaic acid is a potent inhibitor of protein phosphatase 1 and 2A. Application of okadaic acid caused a significant increase in FMRFamide-induced peak tension (Table 2). The FMRFamide-induced increase in basal tonus or integrated area was unaffected by okadaic acid. In a few preparations, application of okadaic acid alone appeared to increase the frequency of "spontaneous" phasic contractions.

The cAMP second messenger system is unlikely to be involved in the FMRFamide-induced response since inhibition of phosphodiesterase by theophylline or inhibition of protein kinase A by H-89 did not alter these responses (Table 2). For the theophylline experiments, we used two concentrations of theophylline and FMRFamide. Our first experiments used a theophylline concentration of 0.1 mM and a FMRFamide concentration of 1 μM. These experiments did not show any effect of theophylline. If cAMP were involved in the FMRFamide-induced response, we would have expected theophylline to increase the response. However, if the FMRFamide-induced responses were near the maximum possible response (due to near maximal stimulation of the cAMP system), any increase in cAMP levels due to theophylline treatment might have been ineffectual. To examine this possibility, we reduced the FMRFamide concentration to 0.1 μM and increased the theophylline concentration to 1 mM. Under these conditions, theophylline again failed to augment the FMRFamide-induced response (Table 2). Many of the actions of the cAMP second messenger system are

Table 1 Effect of 100 μM amiloride on pharyngeal contractions induced by 1 μM FMRFamide

	Control	Amiloride	t value
Basal tonus increase (mN)	2.69 ± 0.42	2.47 ± 0.54	t = 0.40, P = 0.71
Peak tension (mN)	3.27 ± 0.50	3.74 ± 0.75	t = −1.47, P = 0.22
Integrated area (mN·s)	986.84 ± 182.36	816.05 ± 181.42	t = 1.31, P = 0.26

Table 2 Effects of second messenger system manipulations on FMRFamide-induced contractions of the pharynx

Target	Drug (concentration) FMRFamide [concentration]	Basal tonus (mN)			Peak tension (mN)			Integrated area (mN·s)		
		Control	Drug	Value of statistic	Control	Drug	Value of statistic	Control	Drug	Value of statistic
Protein kinase C	BIM (10 μM) FMRFamide [1 μM] $n = 13$	2.29 ± 0.41	1.93 ± 0.46	$t = 2.41$ $p = 0.033$*	2.80 ± 0.48	3.38 ± 0.66	$t = -1.66$ $p = 0.12$	881.90 ± 196.01	550.11 ± 125.01	$t = 2.35$ $p = 0.037$*
Protein kinase C Protein kinase A	H-7 (50 μM) FMRFamide [1 μM] $n = 8$	3.23 ± 0.61	2.38 ± 0.50	$t = 3.24$ $p = 0.01$*	4.74 ± 0.60	4.08 ± 0.63	$t = 1.80$ $p = 0.12$	743.66 ± 146.65	752.53 ± 207.77	$t = -0.13$ $p = 0.91$
Protein phosphatases	Okadaic acid (1 μM) FMRFamide [1 μM] $n = 10$	0.81 ± 0.29	0.98 ± 0.37	$t = -0.98$ $p = 0.35$	3.25 ± 0.74	4.25 ± 0.81	$t = -6.02$ $p < 0.001$*	184.35 ± 65.23	233.65 ± 81.46	$t = -2.03$ $p = 0.07$
Phosphodiesterase	Theophylline (0.1 mM) FMRFamide [1 μM] $n = 5$	3.23 ± 0.70	3.21 ± 0.92	$t = 0.05$ $p = 0.96$	4.85 ± 1.03	5.30 ± 1.07	$t = -0.51$ $p = 0.63$	1517.28 ± 493.08	1517.29 ± 394.33	$t = -0.02$ $p = 0.99$
	Theophylline (1 mM) FMRFamide [0.1 μM] $n = 6$	0.75 ± 0.25	1.01 ± 0.26	$t = -1.11$ $p = 0.32$	2.48 (1.11, 2.87)	2.91 (1.99, 3.14)	$W = 19.00$ $p = 0.06$	182.30 ± 56.27	263.35 ± 87.89	$t = -1.40$ $p = 0.22$
Protein kinase A	H-89 (1 μM) FMRFamide [0.1 μM] $n = 9$	0.48 ± 0.22	0.68 ± 0.21	$t = -0.59$ $p = 0.57$	4.29 ± 0.92	5.39 ± 0.91	$t = -1.89$ $p = 0.10$	197.95 ± 50.73	238.77 ± 48.99	$t = -0.86$ $p = 0.42$
	H-89 (1 μM) FMRFamide [1 μM] $n = 6$	3.05 ± 0.68	3.27 ± 0.81	$t = -0.08$ $p = 0.43$	5.92 (2.97, 7.07)	5.37 (3.07, 7.12)	$W = 7.00$ $p = 0.56$	1120.96 ± 204.52	1139.09 ± 258.16	$t = -0.20$ $p = 0.85$
Phospholipase A_2	OBAA (0.1 μM) FMRFamide [1 μM] $n = 5$	2.26 ± 0.21	2.72 ± 0.21	$t = -1.35$ $p = 0.24$	4.03 ± 0.13	4.97 ± 0.43	$t = -2.37$ $p = 0.08$	906.59 ± 200.48	1157.66 ± 98.38	$t = -1.45$ $p = 0.22$

* Statistically significant ($p < 0.05$)

mediated via protein kinase A. To examine the potential role of protein kinase A in mediating the FMRFamide-induced response, we inhibited protein kinase A with H-89 in the presence of two concentrations of FMRFamide (0.1 and 1 μM). As was true of theophylline, H-89 did not significantly alter the FMRFamide-induced responses (Table 2).

We also did not find evidence for the involvement of the arachidonic acid pathway since FMRFamide-induced responses were unaffected by inhibition of phospholipase A_2 by OBAA (Table 2).

Discussion

The immunocytochemical data presented here demonstrates the presence of an RFamide-like peptide or peptides in neurons associated with the pharynx and in a neuron within the subesophageal ganglion involved in the control of swallowing movements. In addition, RFamide peptides induce contractions of the pharynx. Thus, it is likely that RFamide peptides play an important role in the regulation of pharyngeal motility in the leech.

Histological findings

Most of the FMRFamide-like immunoreactive processes on the pharynx of *Hirudo* run along the longitudinal axis of the organ (Fig. 1A). This suggests that RFamide peptides may be more important in regulating longitudinal contractions of the pharynx than circular contractions. The organization of the FMRFamide-immunoreactive processes on the pharynx of *Haemopis marmorata* (Flanagan and Zipser 1986) is somewhat different in that the FMRFamide-like immunoreactive possesses are more likely to project orthogonal to the longitudinal axis of the organ. These differences in neural organization may be due to differences in the feeding behavior of the two species. *Hirudo* is sanguivorous and ingests liquid, while *Haemopis* is carnivorous and ingests solid food. These differences in feeding habits have led to modifications of the head ganglia and stomatogastric nervous system between the two species (Hanke 1948). In comparison to these leech species, the polychaete gut also possesses a few FMRFamide-immunoreactive somata; however, their exact number and location have not been characterized (Krajniak and Greenberg 1992). In contrast, the other major class of annelids, the oligochaetes, apparently lack FMRFamide-immunoreactive somata associated with the foregut (Fujii et al. 1989; Reglödi et al. 1997). However, these earthworms do have FMRFamide-immunoreactive neural processes associated with the foregut. The orientation of the FMRFamide-immunoreactive processes in the oligochaetes and the polychaetes has not been described.

All neurons discovered within the CNS that cause pharyngeal contractions have a number of similar anatomical features (Fig. 2). After the axon exits the CNS, we were not able to follow the axons beyond the pharyngeal ganglia. It is not clear if the reason that dye was not seen beyond the pharyngeal ganglia is because these neurons terminate within the pharyngeal ganglia, or if the dye simply failed to migrate beyond the pharyngeal ganglion in axons that actually extend to the pharynx. The use of a lipophilic dye (such as DiI), which can be used to delineate the anatomy of individual neurons over long distances, may answer this question.

We found that the pharyngeal ganglia contained FMRFamide-immunoreactive somata (Fig. 1B). The function of the pharyngeal ganglia is unknown; however, based purely on anatomical studies, Hanke (1948) found that nerves from the pharyngeal ganglia innervated both the jaw muscles and the pharynx. Our failure to trace the axons of the neurons (from the subesophageal ganglion) that caused pharyngeal contractions beyond the pharyngeal ganglia may indicate that these neurons synapse upon neurons within the pharyngeal ganglia, which in turn cause pharyngeal movement. Unfortunately, the anatomical arrangement of the stomatogastric nervous system makes physiological study of the pharyngeal ganglia very difficult.

Swallow neuron 1

We have provided a brief description of SW1, a neuron that could have an important role in controlling pharyngeal peristalsis during feeding. Stimulation of SW1 induces the occurrence of EPSPs in the extrinsic radial muscles of the pharynx (Fig. 4A). However, SW1 is apparently not directly responsible for these EPSPs since there was not a 1:1 correlation between action potentials in SW1 and the EPSPs in pharyngeal muscle cells. While it is possible that we simply failed to penetrate muscle cells innervated by SW1, we do not believe that this is likely given the extensive search we made for such cells. It is also possible that the lack of 1:1 postsynaptic potentials could be explained if the pharyngeal muscle cells are ''unitary'' muscles such as found in vertebrate gut (Prosser 1991). In a unitary muscle, the motor innervation modulates the endogenous rhythmicity of the muscle without producing conventional postsynaptic potentials. However, examples of unitary muscles in the invertebrates are rare. Therefore, we believe that the lack of any apparent direct effect of SW1 upon pharyngeal muscle cells suggests that SW1 is a high order neuron involved in controlling pharyngeal motility. It is likely that the intrinsic neurons of the pharynx have a vital role in generating swallowing movements, as may the pharyngeal ganglia neurons. Cell SW1 may act as a command element for these neurons, which in turn may directly control the pharyngeal muscles.

Our double-labeling experiments indicate that SW1 contains an RFamide-like peptide (Fig. 5). A question that remains is if the RFamide peptide is the sole neurotransmitter of SW1, since RFamide-like peptides are frequently colocalized with other neurotransmitters. In the leech alone, RFamide peptides have been colocalized with (or with substances resembling) acetylcholine, SCP_B, oxytocin, octopamine, myomodulin, and cholecystokinin (CCK) (Norris and Calabrese 1987; Evans and Calabrese 1989; Salzet et al. 1993; Gilchrist et al. 1995; Keating and Sahley 1995; our unpublished observations). At least some of these substances are involved in feeding in the leech. The leech pharynx possesses immunoreactive processes for SCP_B and is sensitive to the peptide at high concentrations (O'Gara 1990). The pharynx is also sensitive to acetylcholine and possesses a nicotinic receptor (O'Gara et al., in press). While this study shows that the pharynx is sensitive to perisulfakinin [which has structural similarity to both CCK and FMRFamide (Dockray 1989); Fig. 8], it is insensitive to CCK-8 (unpublished).

Effects of different RFamide peptides on the pharynx

In many animal groups, several different RFamide receptors exist. For example, molluscs possess at least four different RFamide receptors, whose actions are mediated through second messenger systems or direct activation of a ligand-gated ion channel (Walker 1992; Cottrell 1993, 1997). A comparison of the actions of several of the RFamide peptides on the pharynx and the longitudinal body wall muscles (Norris and Calabrese 1990) suggests that the leech possesses at least two RFamide receptors. On the leech longitudinal body wall muscle, FLRFamide and YMRFamide were approximately equipotent with FMRFamide. However, on the pharynx, FLRFamide and YMRFamide were much less potent than was FMRFamide (Fig. 8). The presence of at least two RFamide receptors in the leech is also suggested by the study of Sahley et al. (1993) on the Retzius neurons. The Retzius neurons depolarize and rhythmically burst in response to FMRFamide application. Application of some RFamide agonists produced depolarization without bursting, while other agonists produced bursting without depolarization.

Effects of manipulating second messenger systems on FMRFamide-induced responses

The results of this study suggest that the actions of FMRFamide on the leech pharynx were at least partially mediated via protein kinase C (Table 2). To our knowledge, this is the only direct linkage of the actions of FMRFamide with protein kinase C. However, a long-lasting synaptic enhancement in crayfish muscle induced by DRNFLRFamide is dependent on protein kinase C (Friedrich et al. 1998). In addition, there are several instances where the effects of RFamide peptides are apparently mediated by inositol triphosphate (IP_3) (Falconer et al. 1993; Nelson and Huddart 1994; Pivovarov et al. 1995; Huddart and Hill 1996). However, the IP_3-dependent inhibitory actions of SKPYMRFamide on an acetylcholine-induced response in *Helix* were not mediated via protein kinase C (Pivovarov et al. 1995). The results of Falconer et al. (1993) are especially interesting when compared to the results of the present study. Application of FMRFamide to the *Helix* tentacle retractor muscle induces a series of phasic contractions superimposed upon an increase in basal tonus; a response which is qualitatively similar to the actions of the RFamide peptides on the leech pharynx (Fig. 6). Falconer et al. (1993) speculate that the FMRFamide-induced phasic contractions of the tentacle retractor muscle may be due to oscillatory release of Ca^{2+} from intracellular stores; an action often induced by agents that cause hydrolysis of inositol lipids (reviewed by Berridge et al. 1988). Although the actions of FMRFamide upon both the tentacle retractor muscle and the leech pharynx are qualitatively similar, the pharmacological profile of the two RFamide receptors is not identical. In the leech pharynx, FMRFamide and pQDPFLRFamide produce qualitatively similar contractile responses [although pQDPFLRFamide is usually more potent than FMRFamide (Fig. 8)]. However, in the *Helix* tentacle retractor, pQDPFLRFamide induces a relaxation of the muscle and does not increase IP_3 levels (Falconer et al. 1993).

The pharynx of *Hirudo* is sensitive to at least four neurotransmitters: the RFamides, serotonin (Lent and Dickinson 1984; O'Gara et al., 1999), SCP_B (O'Gara 1990) and acetylcholine (O'Gara et al., in press). However, the ways in which the animal uses each of these neurotransmitters in controlling pharyngeal motility remain to be elucidated. The guts of all animals examined to date are sensitive to a variety of neurotransmitters. The broader question remains as to why the control of gut motility requires several neurotransmitter systems, which on the surface, appear to be at least partially redundant. Although one can speculate that different neurotransmitters mediate different functional gut behaviors, such issues have been rarely addressed directly.

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