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Peptidergic modulation of patterned motor activity in identified neurons of *Helisoma*

(Phe-Met-Arg-Phe-NH_2/small cardioactive peptide B/molluscan feeding/snail)

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ABSTRACT **The neuroactive peptides SCP_B (small cardioactive peptide B) and FMRFamide (Phe-Met-Arg-Phe-NH_2), both originally isolated from molluscs, have potent modulatory effects upon the production of patterned motor activity in identified neurons (e.g., B5 and B19) in the buccal ganglia of the snail *Helisoma*. Such patterned motor activity has previously been shown to underlie feeding behavior. Micromolar concentrations of SCP_B initiate patterned motor activity in quiescent ganglia and increase the rate of activity in ganglia that are spontaneously active. Micromolar concentrations of FMRFamide inhibit patterned motor activity in *Helisoma* buccal ganglia, and 10 μM FMRFamide completely suppresses such activity. In addition, there are both anti-SCP_B- and anti-FMRFamide-immunoreactive neurons in *Helisoma* buccal ganglia. Our results suggest that peptides may play a prominent role in the regulation of feeding behavior in *Helisoma*.**

Neuroactive peptides appear to be ubiquitous. Immunocytochemical staining, for example, has revealed "peptidergic neurons" throughout the metazoa. In addition, neuromodulatory effects of peptides have been implicated in a variety of systems (1–3). However, there are relatively few systems in which peptidergic regulation of a discrete behavior appears amenable to physiological and anatomical analyses at the level of identified neurons. Certain invertebrate systems (4–6), and especially neuronal systems controlling feeding in gastropod molluscs, may allow such analyses (7, 8).

Several aspects of the neural basis of feeding in the snail *Helisoma trivolvis* have been described. Cyclical activity of many identified effector neurons and elements of the central pattern generator has been characterized (9–11). In addition, the monoamines serotonin (12) and dopamine (13) have been shown to activate or modulate such patterned motor activity (PMA) associated with feeding and generated by neurons in the buccal ganglia. As a first step in characterizing the potential roles of neuroactive peptides in regulating feeding behavior in *Helisoma*, we report here that two neuroactive peptides, originally discovered in molluscs, have profound effects on the production of PMA (which apparently represents "fictive feeding") by buccal ganglion neurons. Small cardioactive peptide B (SCP_B, Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2; ref. 14) has stimulatory effects upon the generation of PMA that resemble, at least superficially, those described for serotonin (5-hydroxytryptamine) and dopamine. In contrast, FMRFamide (Phe-Met-Arg-Phe-NH_2; ref. 15) inhibits and, at a sufficient concentration (10^{-5} M), effectively suppresses PMA in the buccal ganglia. SCP has been indicated, by chromatographic techniques, to occur in *Helisoma* central nervous system (see table 1 in ref. 14). We show here that the buccal ganglion of *Helisoma* contains both neurons with SCP_B-like immunoreactivity and neurons with anti-FMRFamide immunoreactivity.

MATERIALS AND METHODS

Albino specimens of *Helisoma trivolvis* were used; they were obtained from stocks maintained in the laboratory of A. G. M. Bulloch (University of Calgary) and derived from the Oregon Red stock of S. B. Kater's laboratory (University of Iowa). The dissection, the physiological saline, and the electrophysiological recording and display techniques have been described (16, 17). For these experiments, either isolated buccal ganglia or buccal ganglia attached to the central ring of ganglia via the cerebrobuccal connectives were used. There was no detectable difference in the effects of peptides upon PMA whether or not the central ganglia were attached, but the rate of spontaneous PMA varies considerably and tends to be higher in isolated buccal ganglia. Once it had been determined that SCP_B was excitatory and FMRFamide was inhibitory, relatively quiescent preparations were used for SCP_B experiments and relatively active preparations were used for FMRFamide experiments. The synthetic peptides, SCP_B (Peninsula Laboratories, San Carlos, CA) and FMRFamide (Sigma), were used. Saline volume (<0.5 ml) in the recording chamber was maintained by suction from the surface, and solutions were changed by perfusing 4–10 ml of normal physiological saline or peptide solutions through the chamber.

PMA was monitored by intracellular recordings from identified neuron B5, which innervates the gut (18), and/or B19, a supralateral radular tensor muscle motoneuron (9). These neurons are the preferred monitors of PMA because they are two of the largest neurons in the buccal ganglia and they show distinctive electrophysiological activity throughout each feeding rasp or cycle of PMA. They generate bursts of action potentials during the protraction phase of the cycle (9, 19) and, during the retraction phase (when retractor motoneurons generate bursts of action potentials), neurons B5 and B19 receive a large, compound, inhibitory postsynaptic potential (IPSP). When the central pattern generator is sporadically active or cycling at a low rate, the frequency of action potentials in the retractor motoneuron bursts can be variable, and action potentials may occur during the interburst interval. Under these conditions the delineation of retractor bursts can be somewhat problematical. In contrast, even when "feeding cycles" occur at low rates, the large IPSPs of neurons B5 and B19 remain distinctive, reflecting the rate of activity in the central pattern generator.

The effects of peptides upon PMA were quantified by counting the number of these large IPSPs occurring during three defined intervals: immediately before and after appli-

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Abbreviations: FMRFamide, Phe-Met-Arg-Phe-NH_2; IPSP, inhibitory postsynaptic potential; PMA, patterned motor activity; SCP_B, small cardioactive peptide B.

cation of the peptide-containing saline solution and immediately after replacement of the peptide solution with normal saline. For each experiment, the number of large IPSPs in normal saline was taken as the control value for cycles of PMA. Since the control rate of spontaneous PMA was variable, the data were normalized by calculating the percentage of the control value for the intervals in peptide solution and after wash with normal saline. The percentage of control for each of these intervals in all of the experiments was then averaged, and the average percentage of control was plotted. The intervals used in FMRFamide experiments were 1 min. However, there were often no spontaneous cycles of PMA in the minute prior to SCP_B application (relatively quiescent preparations). Therefore, 3-min intervals were used for the SCP_B experiments, so that the percentage of control values could be calculated. The significance of the peptidergic effects was confirmed by a one-tailed *t* test.

For immunocytochemical staining, ganglia, connectives, and nerves were dissected in saline and pinned onto Sylgard in small Petri dishes. They were treated for up to 30 min (depending upon the size of the donor animal) at 20°C with 0.5–1.0% protease (Sigma type VI) in saline, rinsed briefly in saline, and fixed overnight at 4–8°C in buffered picrate/paraformaldehyde (20). They were washed 3–5 times (1 hr each) in saline at 20°C and then peptides were localized by conventional immunofluorescence methods (21). Adaptations for thick specimens included longer incubation times (up to 2 days at 20°C for primary antiserum) and the addition of 0.1% NaN_3 to the primary antiserum to prevent growth of protease-generating microorganisms (31). The best staining of neuronal somata was obtained in ganglia from small animals (4-mm vertical shell diameter) treated with protease for 5–10 min. We used a rabbit polyclonal antiserum to FMRFamide (provided by T. L. O'Donohue and J. F. Bishop; no. 232, refs. 22 and 23) and a mouse monoclonal antiserum (provided by S. Kempf, B. Masinovsky, and A. O. D. Willows) to SCP_B, visualized with fluorescein- or rhodamine-labeled, affinity-purified goat anti-rabbit or anti-mouse IgG. Characterization of the antiserum to FMRFamide used in this study showed it to be highly specific for the COOH-terminal amino acid sequence, -Arg-Phe-NH_2 (22). A characterization of the monoclonal antibody to SCP_B used in this study is forthcoming (B. Masinovsky, S. Kempf, J. Calloway, and A. O. D. Willows; personal communication). Specificity in *Helisoma* was monitored by staining with antisera preabsorbed overnight at 4–8°C with 10^{-4}–10^{-6} M synthetic peptide.

RESULTS

SCP_B and FMRFamide have antagonistic effects upon the production of PMA in buccal ganglion neurons. The inhibitory effects of FMRFamide and the excitatory effects of SCP_B upon PMA are exemplified in Fig. 1. In the case shown, 10^{-6} M FMRFamide completely suppressed cyclical PMA and, in addition, caused a large hyperpolarization of neuron B5. However, the concentration of FMRFamide required to suppress PMA varied for ganglia from different animals. In some ganglia PMA was inhibited relatively weakly by 10^{-6} M but was totally suppressed by 10^{-5} M FMRFamide. The effects of 10^{-6} M and 10^{-5} M FMRFamide were quantified as indicated in *Methods*. FMRFamide at 10^{-6} M reduced the cyclical PMA rate to one-third of the control rate (mean ± SEM = 33.8 ± 7.3%, $n = 21$; $P < 0.001$; Fig. 2*A*). Upon washing with normal saline, the PMA rate returned to normal. The higher concentration of FMRFamide (10^{-5} M) completely suppressed PMA (Fig. 2*B*, average percentage of control rate = 1.2% ± 1.2%; $n = 11$; $P < 0.001$). In one experiment, two cycles of PMA occurred after the addition of 10^{-5} M FMRFamide. In 10 of the 11 experiments, no PMA was seen in the interval following application of 10^{-5} M FMRFamide. PMA again returned to control levels upon return to normal saline. Thus, FMRFamide dramatically and reversibly inhibits PMA in *Helisoma* buccal ganglia.

The effects of SCP_B on PMA closely parallel those of 5-hydroxytryptamine (12). SCP_B (10^{-6} M) consistently initiated PMA in quiescent buccal ganglia (Fig. 3) and increased

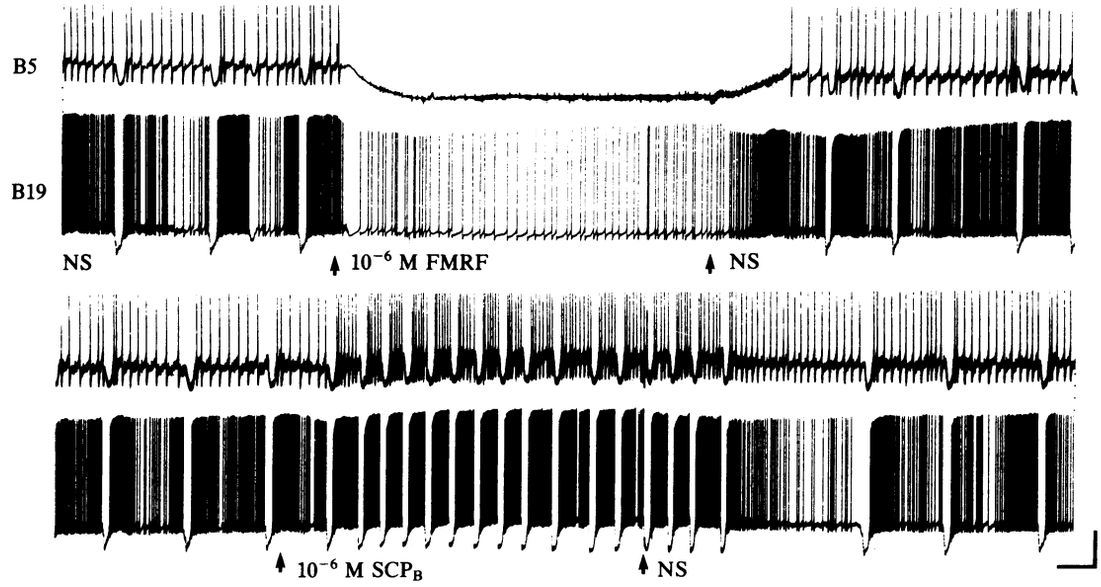

FIG. 1. Inhibition of PMA by FMRFamide, and stimulation of PMA by SCP_B. Simultaneous recordings from neurons B5 and B19 are shown. Upper and lower pairs of traces are continuous. Spontaneous cycles of PMA (typified by the large compound IPSPs) were occurring in normal saline (NS). At the time represented by first arrow, 10^{-6} M FMRFamide was applied, and it completely blocked the cyclical PMA. It also greatly hyperpolarized neuron B5. PMA recovered upon wash with NS. Application of 10^{-6} M SCP_B (third arrow) greatly increased the frequency of cyclical PMA, as well as the frequency of action potentials within a "burst." Action potentials of neuron B5 are clipped. Calibrations: 20 mV, 10 sec.

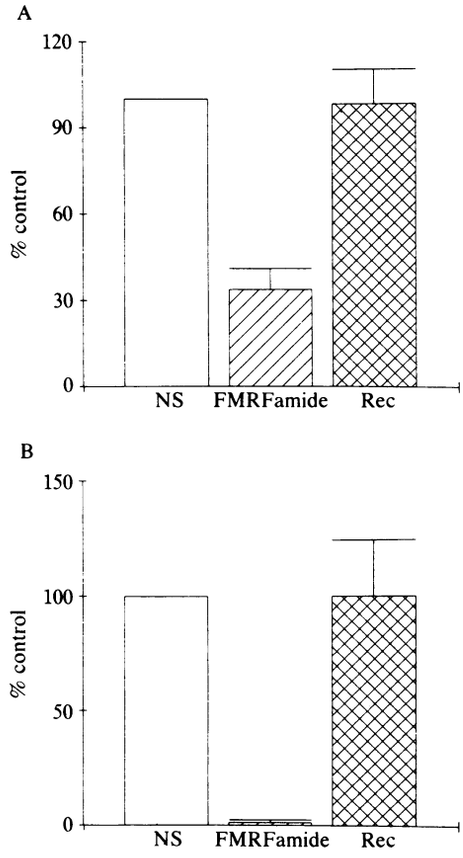

FIG. 2. Effect of FMRFamide upon the rate of cyclical PMA. (*A*) The average percentage of the control rate in normal saline (NS, open bar) is plotted for the interval immediately after 10^{-6} M FMRFamide application (hatched bar; mean ± SEM = 33.8% ± 7.3%, n = 21 experiments in 10 different animals) and again for the recovery interval (Rec) in normal saline (cross-hatched bar; mean ± SEM = 98.2% ± 12.1%, n = 21 experiments in 10 different animals). See *Methods* for the protocol for quantifying the rate of PMA. (*B*) In 10^{-5} M FMRFamide the average percentage of the control rate of PMA was reduced to 1.2% ± 1.2% (n = 11 experiments in 6 animals). Upon return to normal saline, the rate of PMA recovered but showed considerable variability (mean ± SEM p = 100.2% ± 24.4%). In some experiments, the recovery rate of PMA was greater than the original control rate.

the rate of PMA in active preparations approximately 10-fold (Fig. 4; average % control = 1047.9% ± 404.9%; $P < 0.05$).

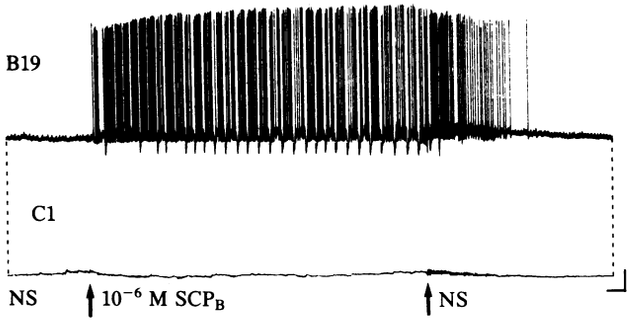

FIG. 3. SCP_B can activate PMA independently of the serotonergic neuron C1. Simultaneous recordings from neurons B19 and C1 are shown. Both neurons were quiescent in normal saline (NS). Addition of SCP_B (10^{-6} M) (first arrow) activated PMA in neuron B19 but had no effect on neuron C1. When SCP_B was removed (second arrow), PMA subsided. Calibrations: 10 sec, 20 mV upper trace, 12 mV lower trace.

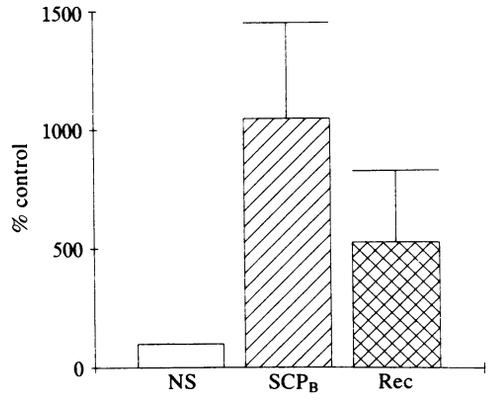

FIG. 4. The effect of SCP_B on the rate of cyclical PMA. Application of 10^{-6} M SCP_B increased the PMA rate to 1047.9% ± 404.9% of control values (n = 8 experiments in 7 animals). Three-minute intervals were used for PMA measurements and the PMA rate remained elevated during the recovery (Rec) interval immediately following removal of SCP_B (average % of control rate = 527% ± 300.5%, n = 8 experiments in 7 animals).

There was some residual stimulatory effect upon SCP_B-induced PMA after return to normal saline. This effect is also reminiscent of the slight lag between removal of bath-applied 5-hydroxytryptamine and cessation of PMA (12).

The similarity of the effects of SCP_B and 5-hydroxytryptamine upon PMA might suggest that one of these agents acts by stimulating the release of the other. Since the serotonergic input to the buccal ganglia is known to arise from the identified cerebral neuron C1 (12, 24, 25), we were able to obtain a partial answer to this question. SCP_B can activate PMA monitored in neuron B19 while having no effect upon the spiking activity of neuron C1 (Fig. 3). Thus, 5-hydroxytryptamine may yet act by stimulating SCP_B-containing neurons, but SCP_B can surely activate PMA without stimulating the serotonergic system.

If the pharmacological results are to have physiological significance, there must be neuronal somata and/or processes containing FMRFamide and SCP_B (or closely related peptides) in the buccal ganglia. For a first indication of their presence, we performed indirect immunofluorescence staining of buccal ganglia with antibodies to FMRFamide and SCP_B. On the ventral surface of each of the paired buccal ganglia there is a single neuron with FMRFamide-like immunoreactivity that stains very brightly, and usually 2–4 other somata that are less immunoreactive (Fig. 5). On the dorsal surface there is a large medial cluster of about 30 small neurons with FMRFamide-like immunoreactivity, several of which lie between identified neurons B5 and B19 (Fig. 5). There are usually 7 or 8 pairs of neurons showing SCP_B-like immunoreactivity on the dorsal surfaces and an additional 4 or 5 pairs on the ventral surfaces of the buccal ganglia (Fig. 6). Liquid preabsorptions of antisera with 10^{-6}–10^{-4} M concentrations of the respective synthetic peptides reduced but did not eliminate immunoreactive staining. Thus FMRFamide-like and SCP_B-like immunoreactivity has been localized in identifiable candidate regulatory buccal neurons, and the synthetic peptides have inhibitory or excitatory pharmacological effects on PMA in the buccal ganglia.

DISCUSSION

To the best of our knowledge, neither FMRFamide nor SCP_B-immunoreactive peptides have been characterized biochemically in *Helisoma*. However, analysis of the FMRFamide-like peptides in a wide variety of other molluscs has shown that true FMRFamide is present in every case,

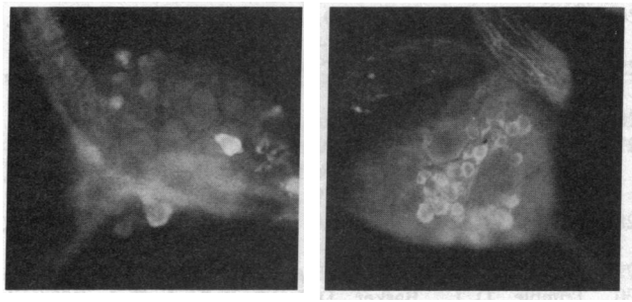

FIG. 5. FMRFamide-like immunoreactivity in the buccal ganglia. (*Left*) Ventral view of FMRFamide-like immunofluorescence in the left buccal ganglion. There is a single brightly stained neuron in the posteromedial part of the ganglion and three or four more lightly stained neurons in the ganglion. (×110.) (*Right*) Dorsal view of FMRFamide-like immunofluorescence in the left buccal ganglion (different preparation from that shown in *Left*). A large cluster of about 30 small FMRFamide-like neurons lies in the medial part of the ganglion. Several of these lie between the large identified neurons B5 and B19. This location should facilitate their physiological characterization. (×145.)

usually accompanied by one or two NH_2-terminally extended homologs (26). In any event, the limited sequence homology of FMRFamide and SCP_B (-Phe-Xaa-Arg-Yaa-NH_2) appears to be of no functional consequence in this system, since the actions of exogenous FMRFamide were inhibitory and those of SCP_B were stimulatory for PMA. Immunocytochemical staining, furthermore, localized FMRFamide-like and SCP_B-like immunoreactivity to discrete populations of neurons (Figs. 5 and 6), although a few neurons in the cerebral, parietal, and visceral ganglia were stained with both antisera (unpublished data). It seems probable at this point that *Helisoma* may contain one or more members each of the FMRFamide and SCP families of peptides.

The decision to feed or not to feed must depend on a number of intrinsic and extrinsic stimuli. One might expect these stimuli to impinge upon the "neural pattern generator" underlying feeding motor activity via a variety of regulatory and modulatory pathways. We suggest that at least two peptides (SCP_B-like and FMRFamide-like) and at least two monoamines (5-hydroxytryptamine and dopamine) may be involved in the regulation of PMA in the buccal ganglia (i.e., in the "decision to feed") in *Helisoma*. 5-Hydroxytryptamine has diverse effects related to feeding in a variety of gastropods (27), and there are preliminary reports that dopamine (28) and SCP_B (7) can stimulate PMA in other gastropods.

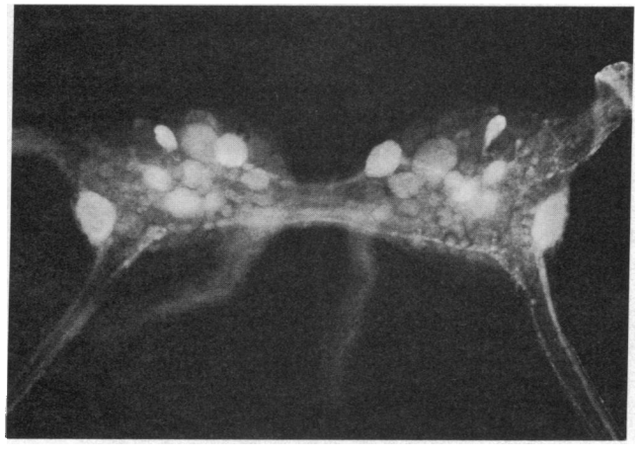

FIG. 6. Dorsal view of SCP_B-like immunofluorescence in the paired buccal ganglia. There are usually seven or eight pairs of neurons on the dorsal surface that show SCP_B-like immunoreactivity. Some pairs consistently stain more brightly than others. The four bright, but unfocused, somata seen here in the central areas of the ganglia are actually located on the ventral surface, and they have axons in the posterior buccal nerves (see ref. 8). (×125.)

The precise physiological roles of these neuroactive substances in the decision to feed in *Helisoma* remain unclear. For instance, tonic activity in the serotonergic neuron C1 usually activates PMA underlying feeding, but it does not always do so (12, 25) and, as shown here, action potentials in neuron C1 are unnecessary for SCP_B-evoked PMA expression. Thus, activation of the serotonergic system is neither always necessary nor always sufficient for initiating PMA in the buccal ganglia, and its role in particular behavioral and environmental contexts is uncertain. Similarly, the physiological roles of FMRFamide and SCP_B (or related peptides) and of dopamine in the regulation of feeding remain to be defined. Important considerations include (*i*) the extent to which the PMA evoked by 5-hydroxytryptamine, dopamine, and SCP_B may vary qualitatively and (*ii*) the possibility that these neuroactive substances may evoke slightly different forms of ingestive behavior (9) or even egestive behavior (29). In any event, the demonstrated (5-hydroxytryptamine; refs. 12, 24, and 25; dopamine; ref. 30) or inferred (FMRFamide and SCP_B; Figs. 5 and 6 and ref. 14) presence of these neuroactive substances in *Helisoma* neurons, combined with marked pharmacological effects upon buccal ganglion PMA, suggests that they may work, in parallel or together, to regulate feeding in *Helisoma*. Thus, this system affords a promising opportunity to elucidate, at the level of identifiable neurons, the interactions among monoaminergic and peptidergic systems regulating a discrete behavior.

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