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Biochemical and Immunocytological Localization of the Neuropeptides FMRFamide, SCP_A, SCP_B, to Neurons Involved in the Regulation of Feeding in *Aplysia*

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The localization of the neuropeptide FMRFamide in the buccal ganglia and buccal muscles of *Aplysia* was studied by immunocytology and high-pressure liquid chromatography (HPLC) combined with either a sensitive bioassay or ^{35}S-methionine labeling. Immunocytology with an antiserum directed to FMRFamide stained a large number of fibers, varicosities, and neuronal somata. Two groups of stained neurons were of particular interest. One was the S cells, a group comprised of many small neurons, the majority of which were stained. HPLC of pooled labeled S cells confirmed that at least some of these neurons synthesize FMRFamide. The other group of stained neurons were in the ventral cluster, a group comprised of a small number of large neurons, many of which are motor neurons that innervate the buccal muscles involved in producing biting and swallowing movements. Several of the ventral neurons were previously shown to contain 2 other neuropeptides, the small cardioactive peptides SCP_A and SCP_B. These neurons are sufficiently large to permit HPLC analyses of the neuropeptides synthesized by individual neurons. This procedure confirmed that individual ventral neurons synthesized FMRFamide, or the SCPs, or all 3 peptides. The coexistence of FMRFamide and the SCPs in the same neuron was confirmed by simultaneous staining of sections from the buccal ganglia with a monoclonal antibody to the SCPs and an antiserum to FMRFamide. The coexistence of the 3 peptides in the same neuron was surprising in light of the observations that these peptides often have opposite biological activity. The ventral neurons are large and potentially identifiable as individuals. Thus, these neurons may be particularly useful for studying the physiological and behavioral roles of neuropeptides in generating complex behaviors.

In recent years, our understanding of chemical transmission in the nervous system has been greatly changed by the observation that a large and steadily growing number of peptides act as transmitters or modulators (Krieger, 1983). The nervous system of *Aplysia* is particularly advantageous for the functional study of neuropeptides because it is comprised of a small number of large neurons. Many of these neurons can be identified and related to well-defined sensory or motor functions. In particular, we have focused on the role of neuropeptides in the feeding system of *Aplysia*. Feeding in this animal is a relatively well-understood stereotyped behavior that nevertheless shows a significant degree of plasticity and is under the contol of a variety of motivational variables (Weiss et al., 1982).

Neurons in the paired buccal ganglia of *Aplysia* generate the cyclic motor output that drives buccal muscles to produce biting and swallowing movements (Gardner, 1971; Kupfermann, 1974; Cohen et al., 1978; Fiore and Meunier, 1979). These ganglia also provide the central innervation of the gut and salivary glands (Lloyd et al., 1985a). One class of neuropeptides, comprised of the small cardioactive peptides A and B (SCP_A, Lloyd et al., 1987; SCP_B, Morris et al., 1982), is found in particularly high concentrations in the buccal ganglia. Both the SCPs are processed from a single precursor (Mahon et al., 1985), and are co-released from individual neurons in an activity- and calcium-dependent manner (Lloyd et al., 1986). Immunocytological studies indicate that the SCPs are present in a number of motor neurons, as well as several large neurons that innervate the gut (Lloyd et al., 1985a; Lloyd, 1986). The SCPs can modulate the synaptic efficacy of motor neurons on buccal muscle (Lloyd et al., 1984). Recent reports suggest that another molluscan neuropeptide, termed FMRFamide, may also be involved in the regulation of molluscan feeding behavior (Murphy et al., 1985). FMRFamide is capable of modulating transmission at neuromuscular synapses on buccal muscles (Richmond et al., 1984) and can also inhibit spontaneously occurring gut contractions (Austen et al., 1983). We therefore investigated whether FMRFamide was present in the buccal ganglia and in individual neurons.

FMRFamide was originally isolated and characterized from the ganglia of the bivalve *Macrocallista nimbosa* (Price and Greenberg, 1977). The sequence of this peptide is moderately similar to the conserved sequence of the SCPs (Fig. 1). However, the SCPs and FMRFamide belong to different peptide classes with markedly different biological activity (Abrams et al., 1984; Lloyd et al., 1985b; Murphy et al., 1985). There is convincing evidence that authentic FMRFamide is present in the circumesophageal ganglia (comprised of the paired cerebral, pedal, and pleural ganglia) of *Aplysia brasiliana* (Lehman et al., 1984) and

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in the abdominal ganglia of *A. californica* (Schaefer et al., 1985).

FMRFamide-like immunoreactivity has been reported to be present in animals from coelenterates to mammals (e.g., Boer et al., 1984; Chronwall et al., 1984; Hooper and Marder, 1984; Triepal and Grimmelikhuijzen, 1984; Cooke and Gelperin, 1985; Kuhlman et al., 1985), but in most cases, the nature of the immunoreactive substance(s) has not been identified. Indeed, there is reason to believe that much of this immunoreactivity is not due to the presence of authentic FMRFamide (e.g., Dockray et al., 1983; O'Donohue et al., 1984; Price et al., 1985). The precise sequence of FMRFamide-like peptides is important in both the quantitative and qualitative nature of their biological actions (e.g., Greenberg and Price, 1980; Cottrell et al., 1983a). Several studies of the localization of FMRFamide-like immunoreactivity in the nervous system of *Aplysia* have been reported (Brown et al., 1985; McCaman and Ono, 1985; Schaefer et al., 1985).

In this paper, we use biochemical procedures in combination with either a bioassay or radiolabeling of peptides synthesized *in vivo* to demonstrate that authentic FMRFamide is present in the buccal ganglia of *A. californica.* We also report that FMRFamide-like immunoreactivity in the buccal ganglia is present in an extensive network of fibers and varicosities, and in a large number of small neurons, as well as in a few large neurons located in a motor neuron cluster. Labeling of the buccal ganglia with ^{35}S-methionine followed by dissection, extraction, and chromatography of single neurons confirms that genuine FMRFamide is present in large neurons. This procedure also confirms that the SCPs are synthesized by individual large neurons. Neurons that appeared to synthesize both the SCPs and FMRFamide were also found. Simultaneous immunocytology on single sections using a monoclonal antibody directed toward the SCPs and an antiserum directed toward FMRFamide confirmed that some individual neurons contain both the SCPs and FMRFamide. A preliminary report of some of these findings has appeared (Lloyd et al., 1985c).

Materials and Methods

Extraction, HPLC, and bioassay of tissue samples. Tissue samples were dissected from adult *A. californica* (150–300 gm) anesthetized by injection of isotonic $MgCl_2$ equal to 25% of their body weight. The samples were weighed, placed in a homogenizer containing 0.1 M acetic acid, and heated to 100°C for 10 min. The tissue was then homogenized and centrifuged for 10 min at 10,000 × *g*. The supernatant was filtered (0.45 μm), dried, and taken up in 100–300 μl of 0.01 M trifluoroacetic acid (TFA), and injected into the HPLC. Reverse-phase (RP)-HPLC was carried out on a DuPont C-8 column developed with a gradient from 25% CH_3CN to 50% CH_3CN over 25 min. Both the water and CH_3CN contained 0.01 M TFA. The resulting samples were dried, and the presence of FMRFamide-like material detected by a bioassay consisting of an isolated esophagus from the land snail, *Helix aspersa.* The esophagus was everted and cannulated so that test substances would flow through the inside of the preparation. Low concentrations of FMRFamide (Peninsula Labs) inhibited spontaneous peristaltic contractions. SCP_B, 10^{-8} M, was perfused through the preparation to increase the frequency and regularity of these contractions. Test samples were injected through an open T-junction several centimeters distant from the preparation. Threshold responses were seen at doses of 1 pmol or less.

^{35}S-methionine labeling of buccal ganglia. Ganglia were labeled for 20 hr at 18°C in a vial containing 1 ml *Aplysia* blood, 2.5 mM colchicine, antibiotics (penicillin, 25 units/ml; streptomycin, 25 μg/ml), and 0.2 mC/ml ^{35}S-methionine (Amersham). This solution was sterilized (0.2 μm filtered) just before use. Colchicine at this level completely blocks axonal transport as measured by transport of the SCPs into the gastroesophageal nerve (see Lloyd et al., 1985a). The labeling period was followed by a 4 hr chase period in a medium consisting of ½ filtered blood and ½ L15 culture medium (Flow Labs) to which salts were added to make it iso-osmotic with *Aplysia* blood. This chase medium contained 0.5 mM unlabeled methionine. At the end of the chase period, ganglia were homogenized and run on RP-HPLC (counter ion: TFA) as described above except the gradient was from 20 to 50% CH_3CN in 30 min. Aliquots of the resulting fractions were counted on a scintillation counter. The remainder of those fractions that had precisely the same retention time as synthetic FMRFamide were mixed with synthetic peptides and subjected to RP-HPLC in the presence of another counter ion [0.01 M heptafluorobutyric acid (HFBA)], which markedly changes both absolute and relative retention times (e.g., see Lloyd et al., 1985a), and to ion-exchange HPLC on a DuPont SCX cation-exchange column developed with a gradient from 0.2 M triethylamine acetate, pH 5.6, to 0.8 M triethylamine acetate, pH 5.6, in 25 min.

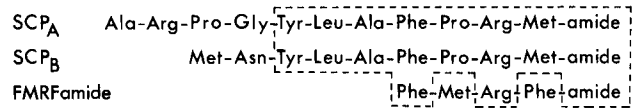

Figure 1. Sequences of SCP_A, SCP_B, and FMRFamide. The amino acid residues and the carboxyl terminal amide within the *dashed lines* are identical for each of the 3 peptides.

^{35}S-methionine labeling of individual neurons. Ganglia were labeled as described above. After the chase period, the ganglia were pinned in dishes containing artificial seawater (ASW), and individual neurons removed using a modification of the method of Ono and McCaman (1980). In brief, the ASW was replaced with several washes of a solution of 50% propylene glycol : 50% ASW at 0°C, and the connective tissue cut away from the surface of the ganglia. Individual neuronal somata were manually dissected and placed on a glass probe, which was used to deposit the cells in the base of a glass microtube containing 50 μl of 0.01 M TFA containing synthetic SCP_A (10 nmol; Sequemat Inc.), SCP_B (10 nmol; Peninsula Labs), and FMRFamide (20 nmol). This solution was filtered (0.45 μm Acro LC13, Gelman Sciences) and the filter washed with an additional 200 μl 0.01 M TFA; the combined sample subjected to RP-HPLC (counter ion: TFA) as described above, and the resulting fractions were counted in a scintillation counter.

Immunocytology for FMRFamide and the SCPs. Immunocytology was carried out using a modification of the techniques described by Goldstein et al. (1984) and Lloyd et al. (1985a). Ganglia were removed from adult animals (250–350 g) and pinned flat in Sylgard-covered dishes containing 50% isotonic $MgCl_2$: 50% ASW, and then fixed for 2 hr in 4% parformaldehyde, 0.1 M phosphate buffer (pH 7.4), 30% sucrose at 4°C. The tissue was then rinsed overnight with 0.1 M phosphate buffer, 30% sucrose. Serial 16 μm sections were cut using a cryostat, thaw-mounted onto gelatin-coated slides, and air-dried for several hours. The sections were dehydrated through a series of alcohols and rehydrated in phosphate-buffered saline containing 0.3% Triton X-100. Nonspecific sites were blocked using 1:40 dilution of normal goat serum for 1 hr at room temperature. Slides were incubated overnight at 4°C with a FMRFamide antiserum (Peninsula Labs: RAS8755), rinsed, and incubated for 2 hr in goat anti-rabbit IgG conjugated to rhodamine (Cappel). Slides were then rinsed, coverslipped in 70% glycerol, viewed with a fluorescent microscope, and photographed using Tri-X or Ektachrome film. Complete serial sections of the buccal ganglia were obtained and photographed. Immunoreactive neurons were reconstructed by tracing projected color slides. Immunostaining of ganglia whole mounts was done as described by Goldstein et al. (1984) and Lloyd et al. (1985a).

Sections for the double-labeling studies were prepared as described above and simultaneously incubated with the FMRFamide-antiserum (rabbit; Peninsula, RAS8755) and a mouse monoclonal antibody raised to SCP_B and kindly supplied by B. Masinovsky, S. Kempf, and A. O. D. Willows of the University of Washington. The sections were then rinsed and simultaneously incubated in goat anti-rabbit IgG conjugated to rhodamine and goat anti-mouse IgG conjugated to fluorescein (Cappel, 8612-0081, 1:40 dilution).

Several control experiments were done. Normal rabbit serum did not produce staining. Staining with the FMRFamide antiserum was blocked by preabsorption with 1 μM FMRFamide but not by 1 μM SCP_B. Conversely, staining with the monoclonal to SCP_B was blocked by preabsorption with 1 μM SCP_B but not 1 μM FMRFamide. The FMRFamide antiserum has been tested for cross-reactivity in radioimmunoassays by Peninsula Laboratories (p63; 1986 Catalogue). It showed limited cross-

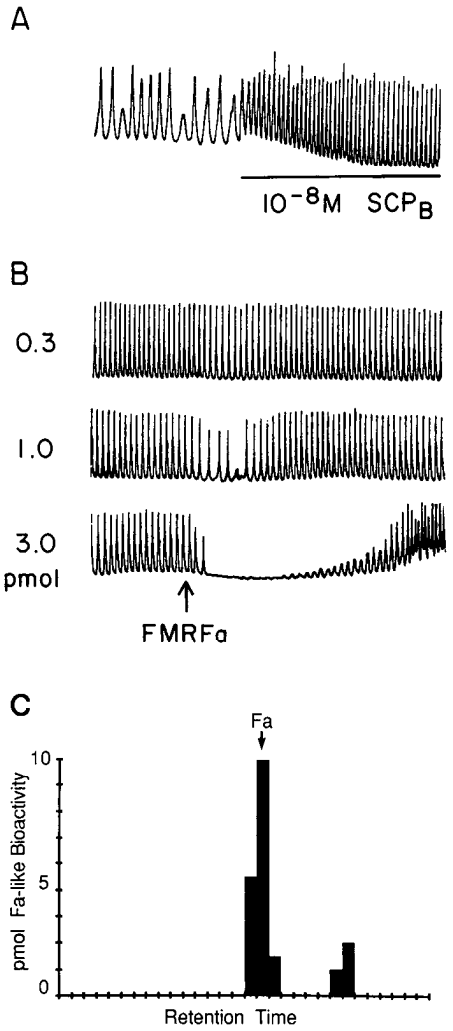

Figure 2. Bioassay for FMRFamide on an isolated snail esophagus. *A,* The regularity and frequency of peristaltic contractions were increased by perfusing the preparation with 10^{-8} M SCP$_B$ (*bar*). Each trace in *A* and *B* was 15 min in duration. *B,* Injection of low doses of FMRFamide into the perfusate inhibited the peristaltic contractions in a dose-dependent manner. SCP$_B$ was present in the perfusate throughout the assay. *C,* Profile of the FMRFamide-like bioactivity from RP-HPLC (counter ion: TFA) of an extract of buccal ganglia. The larger peak of activity had a retention time identical to synthetic FMRFamide (indicated by *arrow*). Time calibrations are 1 min.

reactivity (7%) with MRFamide, and essentially no cross-reactivity with RFamide or other peptides that terminate with -Famide such as cholecystokinin (CCK).

Results

HPLC of FMRFamide-like bioactivity from buccal ganglia extracts

The isolated everted esophagus of the land snail, *Helix,* was used to assay for FMRFamide-like bioactivity. As is the case for the *Aplysia* gut (Austen et al., 1983), low levels of FMRFamide inhibit the magnitude and frequency of spontaneously occurring peristaltic contractions of the snail esophagus. The snail preparation was used because it yielded a more sensitive and long-lasting assay than *Aplysia* gut. The sensitivity and reproducibility of the assay was further enhanced by increasing the regularity and overall rate of peristaltic contractions by perfusing the esophagus with low concentrations of SCP$_B$ (Fig. 2). Extracts

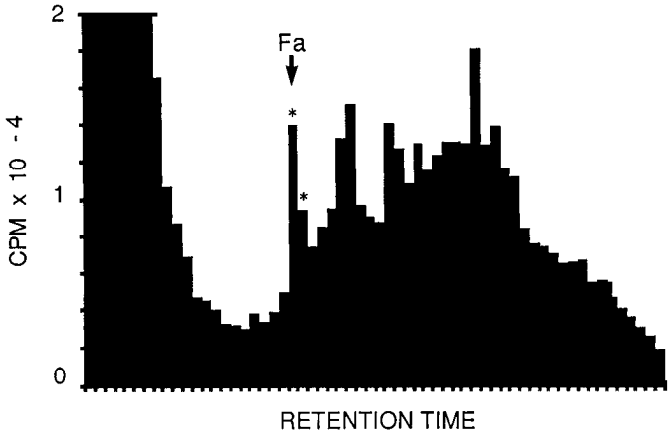

Figure 3. Profile of ^{35}S-methionine-labeled material from RP-HPLC (counter ion: TFA) of an extract of buccal ganglia. Aliquots of each sample were counted and the remainder of the 2 samples (*asterisks*) that had the same retention time as synthetic FMRFamide (*Fa*) were pooled and subjected to another mode of RP-HPLC (Fig. 4) and ion-exchange HPLC (Fig. 5). The major source of the SCPs (the esophageal cluster neurons) were dissected from the ganglia before extraction (for an unrelated experiment), thus reducing the size of the labeled SCP peaks. Time calibrations are 30 sec.

of the buccal ganglia and buccal muscle were chromatographed by RP-HPLC and the resulting fractions bioassayed. Two peaks that inhibited peristaltic activity were observed in each of these tissues (Fig. 2). One peak of bioactivity eluted with a retention time identical to that of synthetic FMRFamide, while the second peak had a retention time several minutes longer than FMRFamide. The bioactivity of the peak with shorter retention time was equal to 2000 pmol FMRFamide/gm wet weight for buccal ganglia (4 pooled ganglia) and 48 pmol/gm for pooled muscles of the buccal mass. The bioactivity of the second peak was equivalent to 375 pmol FMRFamide/gm for the ganglia and 21 pmol FMRFamide/gm for the muscles. These values are similar to the quantities previously obtained for the SCPs in the buccal ganglia and buccal muscles (Lloyd et al., 1984, 1985a).

In addition to the 2 peaks with FMRFamide-like activity, other bioactive peaks with retention times typical of peptides were observed. These included the 2 endogenous SCPs that produced an additional increase of peristaltic activity over that produced by synthetic SCP$_B$, and another major peak present in the buccal muscles with an activity that was readily distinguishable from FMRFamide and the SCPs. This finding suggests that additional, as yet unidentified, bioactive neuropeptides may be present in buccal muscles. It is possible that the FMRFamide-like bioactivity in the second peak is associated with a larger FMRFamide analog since N-terminal extended analogs of FMRFamide have been shown to be bioactive on *Aplysia* gut (Austen et al., 1983). Alternatively, the bioactivity may be due to the presence of a peptide similar to the FMRFamide-like peptide recently sequenced from *Helix* nervous tissue (Price et al., 1985).

HPLC of peptides extracted from buccal ganglia labeled with ^{35}S-methionine

Buccal ganglia labeled with ^{35}S-methionine were homogenized and chromatographed by RP-HPLC with TFA as a counter ion. A peak of radioactivity eluted with the same retention time as synthetic FMRFamide added to the extract before chromatog-

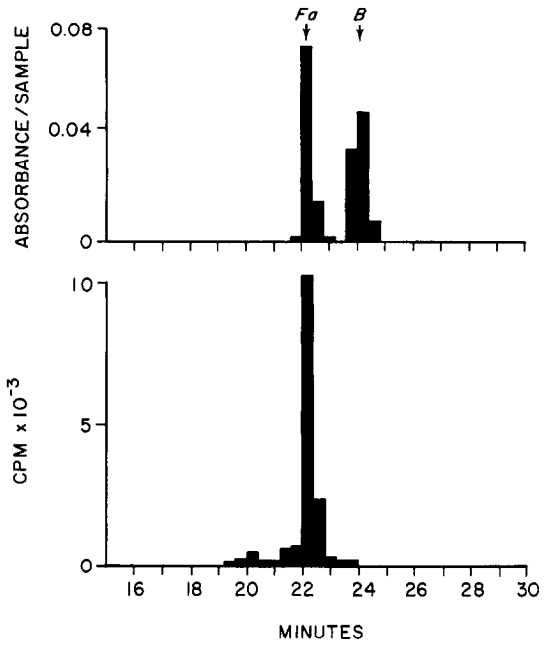

Figure 4. RP-HPLC with HFBA as a counterion of ^{35}S-methionine-labeled peak with the same retention time as FMRFamide on RP-HPLC with TFA as counter ion (aliquots of the samples marked with asterisks in Fig. 3). *Top,* Absorbance profile of synthetic FMRFamide (*Fa*) and SCP_B (*B*) cochromatographed with the labeled material (*bottom*). Note that nearly all the labeled material elutes precisely with FMRFamide. An aliquot of each sample was counted and remainder of the labeled samples with retention time of FMRFamide were used for ion-exchange HPLC (see Fig. 5).

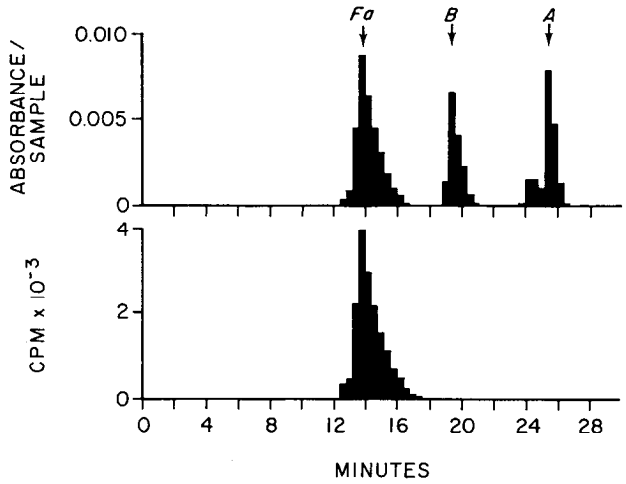

Figure 5. Ion-exchange HPLC of the labeled material with the same retention time as FMRFamide in Figure 4. *Top,* Absorbance profile of synthetic FMRFamide (*Fa*), SCP_B (*B*), and SCP_A (*A*) cochromatographed with the labeled material (*lower profile*). Note that the labeled material again had precisely the same retention time as synthetic FMRFamide.

raphy (Fig. 3). Aliquots of these samples were rechromatographed by RP-HPLC using a different counter ion (HFBA instead of TFA). This change in counterion produces large shifts in both relative and absolute retention times of peptides (Bennett et al., 1980; Lloyd et al., 1985a). Nearly all (86%) of the radioactivity that eluted in the FMRFamide peak in the first mode of RP-HPLC continued to precisely coelute with synthetic FMRFamide (Fig. 4). The remaining labeled material was dispersed, indicating that it was comprised of a number of labeled substances and not a single peptide. The third mode of chromatography was ion-exchange HPLC. Again, essentially all (97%) of the radioactivity present in the FMRFamide peak from the second mode of RP-HPLC had precisely the same retention time as synthetic FMRFamide (Fig. 5). These 3 sequential chromatography steps are sufficient to fully purify peptides from tissue extracts (Lloyd et al., 1987). These data confirm that authentic FMRFamide is present in, and synthesized by, the buccal ganglia of *Aplysia* and indicate that methionine-labeled peaks from individual buccal ganglia neurons that have the same retention time as synthetic FMRFamide on RP-HPLC with TFA as the counterion are very likely to be authentic FMRFamide (e.g., see Fig. 8).

Localization of FMRFamide-like immunoreactivity in buccal ganglia and muscle

Whole mounts of juvenile ganglia revealed extensive FMRF-like immunoreactivity in the neuropil and a number of neuronal somata. In each buccal ganglion, a large bilaterally symmetrical neuron was prominently stained (not shown).

In serial sections of adult animals, immunoreactive neurons and fibers were observed throughout the buccal ganglia (Fig. 6). In particular, roughly 75% of the small neurons of a medial cell cluster (S cells; Fiore and Meunier, 1979) and several large motor neurons of the ventral cluster were immunoreactive. Serial reconstruction of several buccal ganglia revealed that the large immunoreactive neurons in the ventral cluster were asymmetrically divided between the 2 hemiganglia. There were either 2 or 3 large stained neurons in the right ganglion to only a single stained neuron in the left ganglion. Fibers and varicosities containing FMRFamide-like immunoreactivity were observed throughout the neuropil and were particularly concentrated in the interganglionic commissure (Fig. 10*A*). Fibers were also found in the connective tissue sheath surrounding the buccal ganglia. The somata of a number of either stained or unstained neurons were surrounded by a dense ring of intensely stained varicosities (Fig. 6*B*). Finally, immunoreactive fibers and varicosities were also observed in buccal muscles (Fig. 7).

FMRFamide and the SCPs are synthesized by individual neurons in the ventral cluster of the buccal ganglia

Our immunocytological results indicated that FMRF-like peptides are present in neurons of the ventral cluster. SCP-like immunoreactivity has also been observed in individual neurons in this cluster (Lloyd et al., 1985a). The presence of peptides in these large neurons was particularly interesting, as neurons in the ventral cluster are known to provide the motor innervation for major buccal muscles (Banks, 1978; Cohen et al., 1978; E. C. Cropper, J. L. Cohen, I. Kupfermann, and K. R. Weiss, unpublished observations). In addition, the large size of these neurons permits determination of the peptides synthesized by single neurons. To ascertain if ventral cluster neurons synthesized FMRFamide or the SCPs, isolated buccal ganglia were labeled with ^{35}S-methionine (FMRFamide and both SCPs contain methionine residues; Fig. 1) in the presence of colchicine. After a chase period, individual neuronal cell bodies were dissected from the ventral cluster, extracted in the presence of excess unlabeled peptides, chromatographed by RP-HPLC (counter ion: TFA), and the resulting samples counted. Colchi-

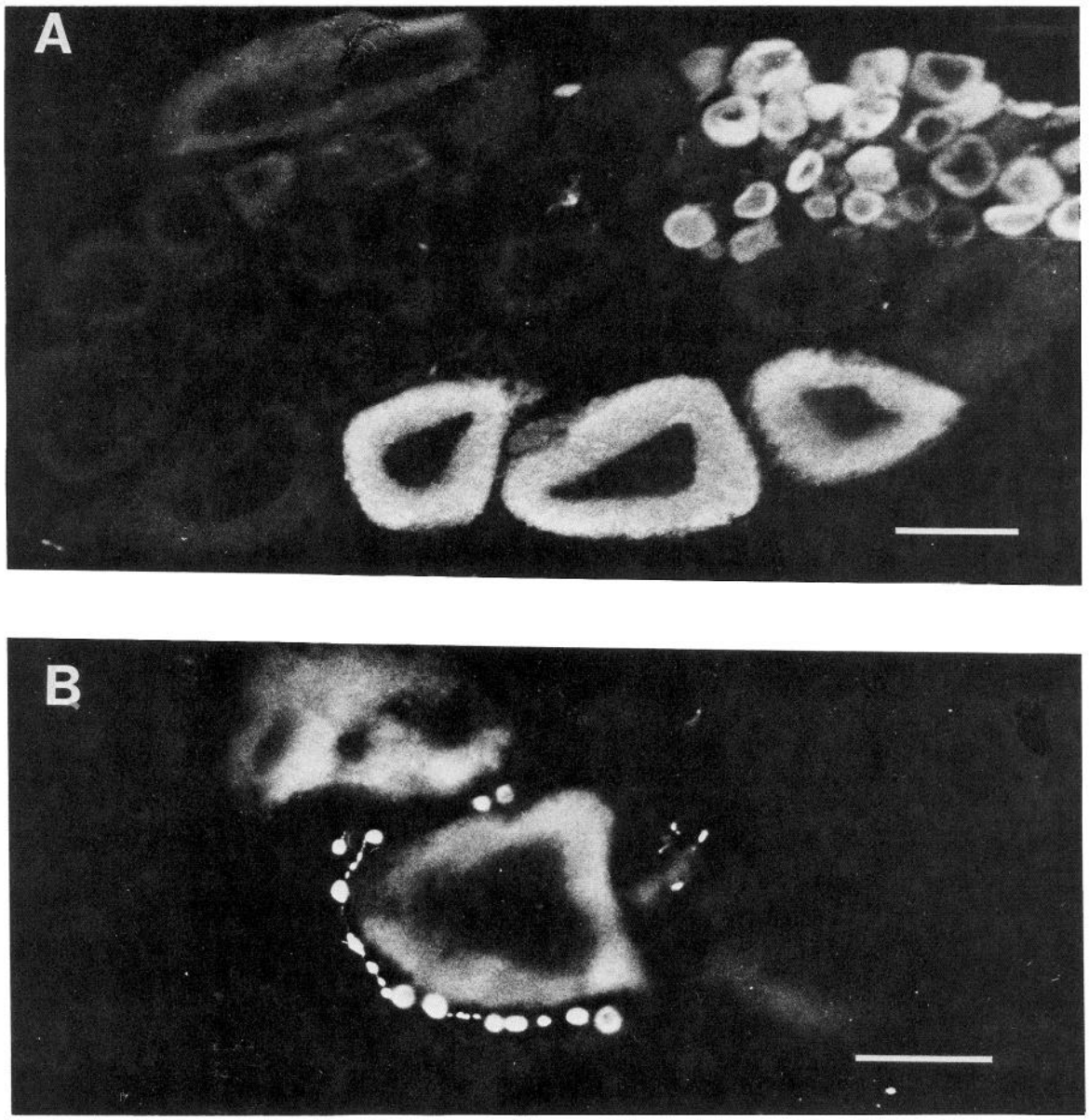

Figure 6. Low-power photomicrograph of buccal hemiganglion stained for FMRFamide. *A,* Three large ventral cluster neurons and numerous small neurons (S cells) exhibit FMRFamide-like immunoreactivity. Bar, 100 μm. *B,* FMRFamide-like immunoreactive fibers and varicosities surrounding the immunoreactive somata of a large ventral cluster neuron. Bar, 100 μm.

cine completely blocks axonal transport in *Aplysia,* as estimated by the inhibition of the transport of the SCPs into the esophageal nerve (P. Lloyd, I. Kupfermann, and K. Weiss, unpublished observations). In the present experiment, this inhibition served 2 purposes: it increased the amount of labeled peptide retained in the cell body and prevented transport of peptides from their site of synthesis to varicosities located on the cell bodies of other neurons. In the absence of colchicine, peptides synthesized in other neurons could be transported into these varicosities and would be dissected along with the cell body, resulting in significant contamination (see Ono and McCaman, 1984). Radioactive profiles from different neurons in the ventral cluster can be divided into 4 patterns (Fig. 8): (1) no incorporation of ^{35}S-methionine into small peptides, (2) incorporation into SCP_A and SCP_B, (3) incorporation into FMRFamide, and (4) incorporation into SCP_A, SCP_B, *and* FMRFamide. Three lines of evidence support our proposition that the labeled substances in these peaks are actually FMRFamide and the 2 SCPs. First is the observation that immunoreactive FMRFamide and the SCPs are present in large neurons of the ventral cluster. Second is the observation that virtually all of the methionine-labeled material from the buccal ganglia that elutes with synthetic FMRFamide in this mode of RP-HPLC is, in fact, authentic FMRFamide. Finally, neurons that synthesize 1 SCP invariably synthesize both SCPs, as would be expected from the sequence of the SCP precursor (Mahon et al., 1985).

In those neurons that did incorporate methionine into the peptides, there were other small peaks of labeled material, primarily with shorter retention times than the mature peptides. The composition of these peaks is unknown, although they may be produced during the processing of the peptide precusors. The position of these smaller peaks was reproducible and charac-

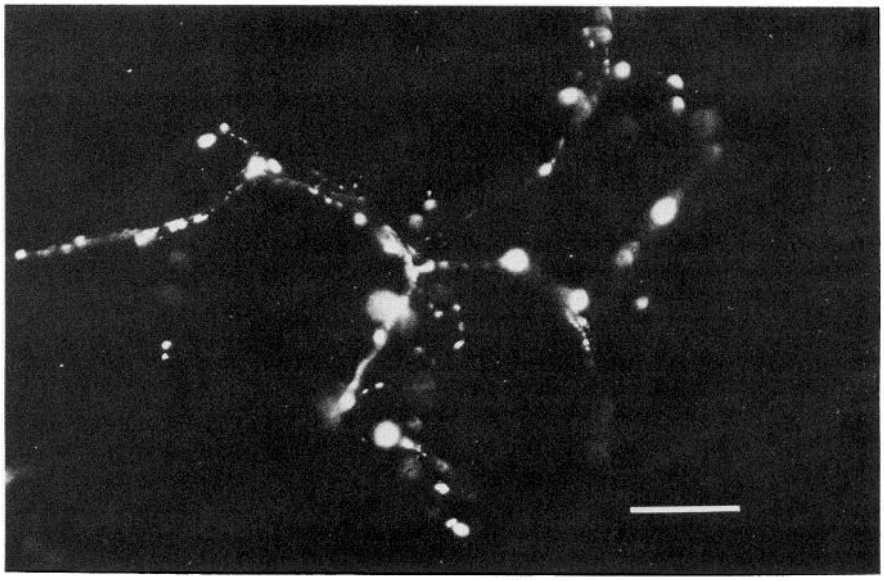

Figure 7. High-power photomicrograph of FMRFamide-like immunoreactivity in fibers and varicosities in buccal muscle. Note positive nerve fibers studded with varicosities. Bar, 30 μm.

teristic for each incorporation profile. In addition, in the FMRFamide pattern of incorporation, a small peak of label with a retention time about 150 sec longer than the major FMRFamide peak was consistently observed. The retention time of this peak is very similar to that of the minor bioactive peak observed in extracts of the buccal ganglia (Fig. 2*C*), and it is possible that the labeled and bioactive substances are the same.

In 1 pair of buccal ganglia labeled with ^{35}S-methionine, neurons that appeared to be symmetrical as judged by visual criteria such as neuron size and position in the ventral cluster were dissected and individually analyzed by RP-HPLC. Seven pairs of neurons were analyzed. Of these, 1 pair synthesized the SCPs, 1 pair synthesized both the SCPs and FMRFamide, and 2 pairs did not incorporate methionine into small peptides. The remaining neurons were asymmetrical in terms of their synthesis patterns. Two additional neurons in the right ganglion synthesized FMRFamide. No additional FMRFamide-synthesizing neurons were found in the left ganglia. One other neuron in the left ganglion synthesized the SCPs. Although not all of the neurons in the ventral cluster were analyzed, this asymmetry of neurons that synthesize FMRFamide is consistent with the asymmetry observed by immunocytology.

Although it was not feasible to confirm chemically that individual S cells synthesized FMRFamide, we did determine that the cluster as a whole does synthesize authentic FMRFamide (Fig. 9) and only very small amounts of other methionine-containing small peptides. The ratio of labeled FMRFamide to unincorporated methionine was lower than for the ventral neurons. This raises the possibility that the S cells synthesize the peptide at lower rates or that not all of the neurons within the cluster synthesize FMRFamide.

Immunocytology with a monoclonal antibody to the SCPs and antisera to FMRFamide confirms the coexistence of these peptides in individual neurons

Using a monoclonal (mouse) antibody directed toward SCP_B that also binds SCP_A but does not significantly cross-react with FMRFamide (B. Masinovsky, personal communication) and an antiserum (rabbit) directed toward FMRFamide that does not significantly cross-react with the SCPs, it was possible to stain neurons simultaneously for both the SCPs and FMRFamide. Figure 10 shows the same buccal hemiganglion section stained with FMRFamide antisera and an SCP monoclonal antibody. Intense FMRFamide-like and SCP-like immunoreactivity were colocalized to a medium-sized neuron (arrow). In addition, SCP-like immunoreactivity was present in identified neurons B1 and B2 (2 large cells in left of field), which are known to contain SCP_A and SCP_B. B1 and B2 did not stain with the FMRFamide antiserum. Several large neurons in the ventral motor neuron cluster also exhibited costaining with the FMRFamide and SCP antibodies (Fig. 11). Staining of neuron *a* was relatively specific since other neurons in the cluster showed staining for FMRFamide but not the SCPs (Fig. 11*A*) or staining for the SCPs but not FMRFamide (Fig. 11*B*). Fibers and varicosities in muscles of the buccal mass stained for both FMRFamide and the SCPs; however, as yet no example of costaining in these fibers and varicosities has been obtained.

Discussion

FMRFamide is present in individual neurons in the buccal ganglia of Aplysia

Immunocytology, using an antiserum directed toward FMRFamide, stained an extensive network of fibers and varicosities, as well as a number of neuronal cell bodies in the buccal ganglia. The immunoreactive fibers and varicosities were found throughout the neuropil, in the connective tissue sheath, and in networks of processes surrounding neuronal cell bodies. FMRFamide-immunoreactive networks surrounding cell bodies have also been observed in the pleural ganglia (Schaefer et al., 1985). The presence of neuropeptides in networks of varicosities surrounding neuronal cell bodies has been commonly observed in *Aplysia* (Scheller et al., 1984). The SCPs are an exception in that SCP-containing varicosities appear to be confined to the neuropil of ganglia (Lloyd et al., 1985a).

FMRFamide-like immunoreactivity was present in a majority of the small neurons (<50 μm in diameter) in the buccal ganglia. It is likely that many of these are proprioceptive sensory neurons (Jahan-Parwar et al., 1983). Although we have shown that the S cluster as a whole synthesizes FMRFamide, the small size and large number of neurons within the cluster precluded biochem-

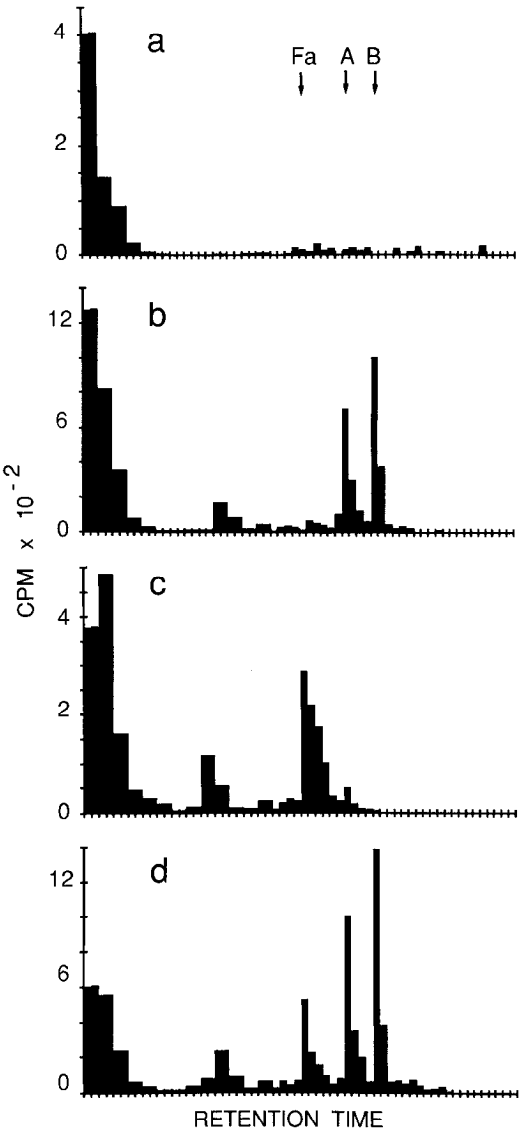

Figure 8. RP-HPLC profiles of ^{35}S-methionine-labeled material from individual neurons dissected from the ventral cluster of a buccal ganglia. Four patterns of radiolabeled profiles were observed: *a,* no incorporation of methionine into low-molecular-weight peptides; *b,* SCPs pattern, comprising 2 major labeled peaks with the same retention times as synthetic SCP_A (*A*) and SCP_B (*B*); *c,* FMRFamide pattern, comprising a major peak of label with the same retention time as synthetic FMRFamide (*Fa*); and *d,* combined SCPs and FMRFamide pattern, comprising 3 major labeled peaks with the same retention times as SCP_A, SCP_B, and FMRFamide. The large amount of label with short retention time in each pattern was largely unincorporated methionine. Time calibrations are 30 sec.

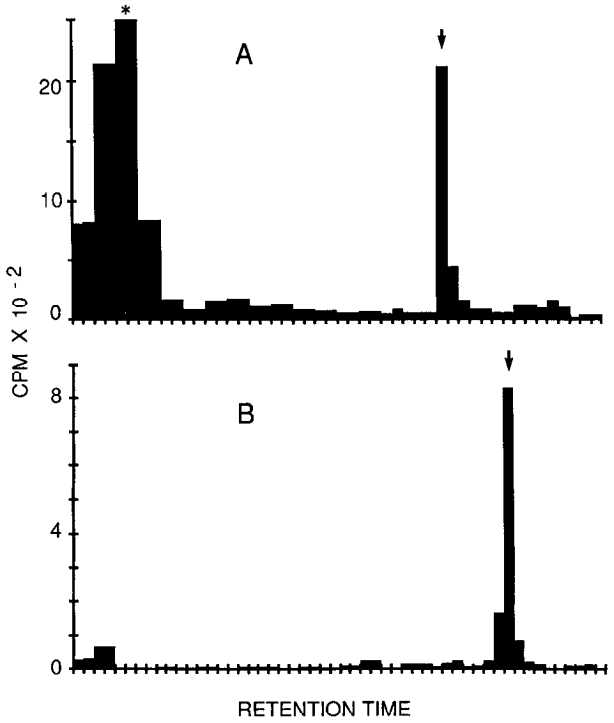

Figure 9. RP-HPLC profile of ^{35}S-methionine-labeled material from pooled S cells. *A,* RP-HPLC with TFA as counterion of an extract of S cells. The counts in the sample marked with *asterisk* is off-scale (13,610 cpm). The peak of labeled material had precisely the same retention time as cochromatographed synthetic FMRFamide (marked by *arrow*). *B,* RP-HPLC with HFBA as counterion of an aliquot of the Fa peak from *A*. Again, the Fa peak has precisely the same retention time as synthetic FMRFamide (*arrow*). These results confirm that FMRFamide was synthesized by ventral cluster neurons.

ical analyses of individual neurons. Because of the inherent limitations of immunocytology (Landis, 1985), we cannot be sure that all, or even a majority, of the neurons in this cluster contain authentic FMRFamide as opposed to antigenically related substances.

Several large neurons in the buccal ganglia also contained FMRFamide-like immunoreactivity. These neurons were located in the ventral cluster, which is primarily comprised of motor neurons that innervate muscles of the buccal mass involved in producing biting and swallowing movements. We have previously shown that several of the large neurons in this cluster contain the SCPs (Lloyd et al., 1985a). In contrast to the S cells, the large size of the neurons in the ventral cluster makes it possible to dissect and analyze the contents of individual cell bodies. Neurons in this cluster were shown to synthesize FMRFamide or SCP_A and SCP_B, confirming the immunocytological results presented here and previously (Lloyd et al., 1985a). In addition, a bilaterally symmetrical ventral cluster neuron synthesized SCP_A, SCP_B, *and* FMRFamide. Simultaneous staining of sections of the buccal ganglia with a monoclonal antibody directed toward the SCPs and an antiserum directed toward FMRFamide confirmed that both SCP-like and FMRFamide-like immunoreactivities were present in a large neuron in the ventral cluster, as well as in a few other smaller neurons. Although there are similarities in the sequences of the SCPs and FMRFamide, our controls indicate that this double staining was not due to cross-reactivity. Thus, both FMRFamide and the SCPs are synthesized by, and are present in, individual neurons in the buccal ganglia. Complementary (c) DNA clones that encode for the protein precursors of the SCPs (Mahon et al., 1985) and FMRFamide (Schaefer et al., 1985) have been sequenced. The SCP precursor contains single copies of each of the SCPs and no regions that could give rise to FMRFamide-like peptides. The FMRFamide precursor contains multiple copies of FMRFamide and no SCP-like peptides. Thus, the coexistence of the SCPs and FMRFamide in single neurons suggests that there is expression of the genes for both precursors or that there is a third gene that encodes a precusor containing copies of all 3 peptides. It will be possible to distinguish between these possibilities when the large ventral neuron has been identified as a

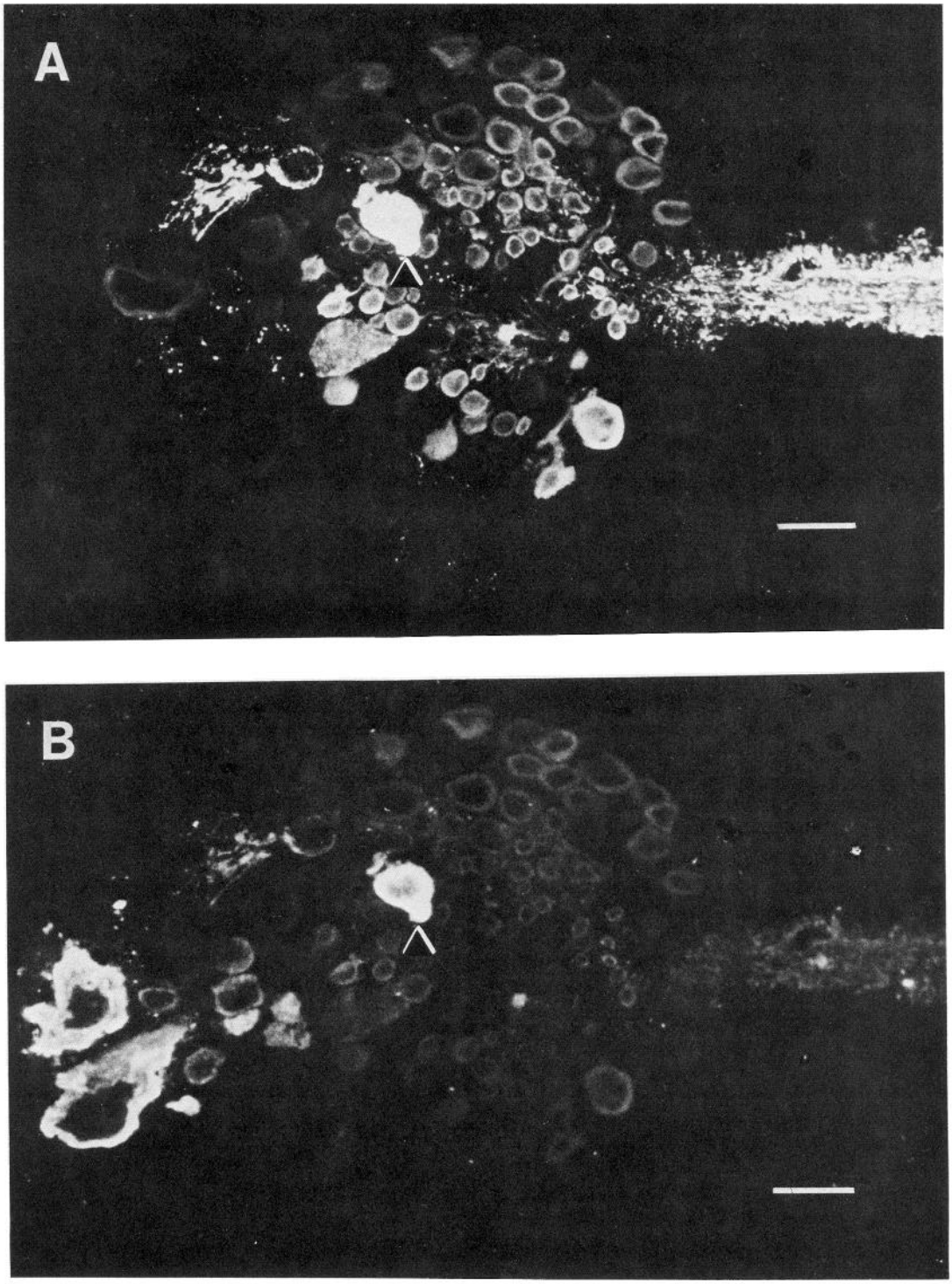

Figure 10. Low-power photomicrograph of buccal hemiganglion stained for the SCPs and FMRFamide. *A,* FMRFamide immunoreactivity. Intensely stained neuron in the middle of the ganglion (*arrowhead*) also exhibits SCP-like immunoreactivity (see below). Note the concentration of immunoreactive fibers in the commissure. Bar, 100 μm. *B,* SCPs immunoreactivity. The 2 largest immunoreactive neurons (in the left field) are known SCPs-containing neurons, B1 and B2 (Lloyd et al., 1985a). Note the intensely stained neuron (*arrowhead*) that also showed FMRFamide immunoreactivity. The great majority of cells and fibers, however, exhibit only SCPs or FMRFamide immunoreactivity. Bar, 100 μm.

unique individual. A recent preliminary report indicates that the nervous system of nudibranch molluscs may also contain neurons that show immunoreactivity for both FMRFamide and SCP_B (Longley and Longley, 1985).

FMRFamide was originally purified and sequenced from bivalve nervous tissue (Price and Greenberg, 1977). The HPLC bioassay procedure indicated that extracts of the buccal ganglia and buccal muscles of the gastropod *A. californica* contain a substance with the biological activity and retention time of synthetic FMRFamide. Buccal ganglia incorporated radioactive methionine into a peptide that had precisely the same retention time as synthetic FMRFamide in 2 different modes of RP-HPLC

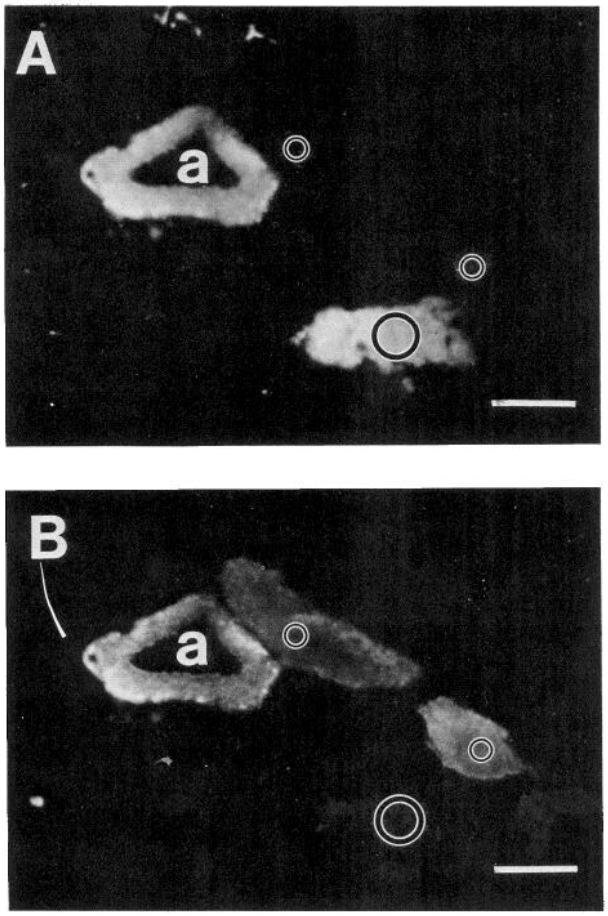

Figure 11. Photomicrograph of FMRFamide and the SCPs immunoreactivity in a ventral cluster neuron (*a*). *Large circle* indicates position of a neuron that exhibits immunoreactivity for FMRFamide but not the SCPs. *Small circles* indicate positions of neurons exhibiting immunoreactivity for the SCPs but not FMRFamide. *A,* FMRFamide immunoreactivity. *B,* SCPs immunoreactivity. Bars, 100 μm.

and in ion-exchange HPLC. Combining these results with those of the immunocytology decribed above, it is extremely likely that the buccal ganglia contain and synthesize authentic FMRFamide. Authentic FMRFamide has also been shown to be present in the circumesophageal ganglia (cerebral, pleural, and pedal ganglia) of *A. brasiliana* (Lehman et al., 1984) and in the abdominal ganglia of *A. californica* (Schaefer et al., 1985). Thus, the peptide sequence of FMRFamide has been conserved between 2 molluscan classes: gastropods and bivalves. However, other FMRFamide-like peptides are present in gastropod nervous tissue (Price et al., 1985), and these peptides often cross-react with antisera raised to FMRFamide. It is crucial to characterize precisely the nature of the FMRFamide-like peptides in each system because they may have markedly different bioactivity than FMRFamide itself (Cottrell et al., 1983a).

Functional roles of the SCPs and FMRFamide in the ventral cluster neurons

Most of the large neurons in the ventral cluster are motor neurons that produce buccal muscle contractions with short latency. Where their actions on buccal muscles have been analyzed, the SCPs and FMRFamide do not directly produce contractions but modulate the efficacy of motor input to the muscles (Lloyd et al., 1984; Richmond et al., 1984). Therefore, it is possible that the ventral neurons containing both the SCPs and FMRFamide may also contain a conventional excitatory transmitter in addition to the 3 peptides. Although coexistence of neuropeptides with conventional transmitters in vertebrate neurons has received the most attention (Hökfelt et al., 1984), the coexistence of neuropeptides from different classes has also been observed in a number of studies (for recent references, see Reiner et al., 1985). The conclusion that emerges from these studies and our own results is that an individual neuron can utilize a great variety of transmitters or modulatory substances.

Although somewhat similar in sequences, FMRFamide and the SCPs belong to 2 distinct peptide families and often have opposite actions in *Aplysia.* For example, the SCPs facilitate synaptic transmission between sensory and motor neurons in the abdominal ganglia, enhance the efficacy of neuromuscular transmission in a particular buccal muscle (the ARC), and increase the frequency of peristaltic contractions in the gut. In contrast, FMRFamide is inhibitory in each of these systems (Abrams et al., 1984; Lloyd et al., 1984; Weiss et al., 1984; P. Lloyd, I. Kupfermann, and K. Weiss, unpublished observations). The primary source of the SCPs in the buccal ganglia are the esophageal clusters, which include giant neurons B1 and B2 and are involved in the regulation of gut activity (Lloyd et al., 1985a). FMRFamide may be present in a number of small neurons and may act as a sensory transmitter or modulator. In addition, both the SCPs and FMRFamide are present in large neurons in the ventral cluster of the buccal ganglia. It is possible that the ventral cluster neurons that contain the peptides use them as excitatory neuromuscular transmitters. In particular, such a role has been proposed for FMRFamide or related peptides in other molluscs (Painter, 1982; Cottrell et al., 1983b). However, a more likely possibility for neurons of the ventral cluster is that the SCPs and FMRFamide are modulatory agents that coexist with conventional excitatory transmitters. Both the SCPs and FMRFamide have been shown to be potent modulators of the contractile activity of several buccal muscles in *Aplysia* (Lloyd et al., 1984; Richmond et al., 1984; K. Weiss, P. Lloyd, and I. Kupfermann, unpublished observation). In addition, FMRFamide or FMRF-like substances have been implicated in the modulation of the contractile activity of gill muscles in *Aplysia* (Weiss et al., 1984).

Determination of the physiological roles of the peptides in ventral cluster neurons must await the unequivocal identification of individual neurons and correlation of the physiological actions of the neuron with its peptide content. It is attractive to speculate, however, that the neuropeptides are released from terminals of the motor neurons to modulate the efficacy of the same neurons on their target muscle. This would constitute a form of homosynaptic modulation in a system where heterosynaptic modulation has already been well described (Weiss et al., 1978). Evidence has been presented that suggests that this type of homosynaptic modulation involving co-release does occur at neuromuscular synapses in insects (Adams and O'Shea, 1983; O'Shea and Schaffer, 1985).

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