Small cardioactive peptide-like immunoreactivity and its colocalization with FMRFamide-like immunoreactivity in the central nervous system of the leech *Hirudo medicinalis*

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**Summary.** The distributions of small cardioactive peptide (SCP)- and FMRFamide-like immunoreactivities in the central nervous system of the medicinal leech *Hirudo medicinalis* were studied. A subset of neurons in the segmental ganglia and brains was immunoreactive to an antibody directed against SCPb. Immunoreactive cell bodies were regionally distributed throughout the nerve cord, and occurred both as bilaterally paired and unpaired neurons. The majority of the unpaired cells displayed a tendency to alternate from side to side in adjacent ganglia. A small number of neurons were immunoreactive only in a minority of nerve cords investigated. Intracellular injections of Lucifer yellow dye and subsequent processing for immunocytochemistry revealed SCP-like immunoreactivity in heart modulatory neurons but not in heart motor neurons. FMRFamide-like immunoreactivity was also detected in cell bodies throughout the central nervous system. A subset of neurons contained both SCP- and FMRFamide-like immunoreactivities; others stained for only one or the other antigen. These data suggest that an antigen distinct from FMRFamide is responsible for at least part of the SCP-like immunoreactivity. This antigen likely bears some homology to the carboxyl terminal of SCPa and SCPb.

**Key words:** Antigen localization – FMRFamide-like immunoreactivity – Immunocytochemistry – Invertebrate ganglia – Small cardioactive peptide-like immunoreactivity – *Hirudo medicinalis* (Annelida)

The small cardioactive peptides (SCPs) were originally described as cardioactive substances in gastropods (Lloyd 1978, 1982). SCPa and SCPb were isolated from the sea slug *Aplysia* (Morris et al. 1982; Lloyd et al. 1987b), and found to be amidated peptides of eleven and nine amino acids, respectively; they share the same carboxyl terminal residues. The SCPs are excitatory on *Aplysia* heart (Lloyd et al. 1985b), gut (Lloyd 1986), and buccal muscles (Lloyd et al. 1984), and modulate a number of neuronal currents (Abrams et al. 1984; Ocurr and Byrne 1985; Acosta-Urquidi 1988) in this animal. The actions of the two SCPs are indistinguishable from one another, and are indistinguishable from those of serotonin (Lloyd et al. 1984; Lloyd 1986). These actions of the SCPs in *Aplysia* are mediated, at least in part, through cAMP (Abrams et al. 1984; Ocurr and Byrne 1985; Lloyd et al. 1985b).

SCP-like peptides have now been localized immunocytochemically in other molluscs (Kempf et al. 1987; Masinovsky et al. 1988); crustaceans (Callaway et al. 1987; Masinovsky et al. 1988); the glossopterid leech *Helobdella triserialis* (Shankland and Martindale 1987a, b); and insects (Masinovsky et al. 1988). To date, the identities of these peptides have not been determined, although authentic SCPb is apparently present in the marine gastropod *Tritonia diomedeae* (Lloyd et al. 1983).

FMRFamide (Phe-Met-Arg-Phe-NH2), another molluscan cardioexcitatory peptide, was isolated from the clam *Macrocallista nimboana* (Price and Greenberg 1977). FMRFamide is widely distributed among molluscs (Greenberg et al. 1985). Many peptides sharing the carboxyl terminal sequence RFamide have been described in several different phyla and, in some cases, isolated and sequenced (see Joosse 1987). This growing list includes the discovery of an RFamide peptide in the leech *Hirudo medicinalis* (Kuhlman et al. 1985a; Li and Calabrese 1987). Some fifty neurons per ganglion show FMRFamide-like immunoreactivity including several identified motor neurons (Kuhlman et al. 1985a; Norris and Calabrese 1987). Li and Calabrese (1987) showed that approximately 85% of FMRFamide-like immunoreactivity extractable from leech nerve cords co-migrates with authentic FMRFamide or its oxidation products on two different HPLC solvent systems, leading these workers to conclude that leech CNS contains true FMRFamide.

SCP- and FMRFamide-like immunoreactivities have been colocalized in neurons of crustaceans (Callaway et al. 1987), *Helobdella* (MQ Martindale and M Shankland, personal communication), and *Aplysia* (Lloyd et al. 1987a). Individual neurons in *Aplysia* synthesize SCPa, SCPb, and FMRFamide (Lloyd et al. 1987a).

The leech nervous system is an accessible preparation for studying neuropetptides. Each segmental ganglion contains approximately 400 neurons (Macagno 1980), many of which are identified and have known physiological roles (Muller et al. 1981). The ganglion is subdivided into six packets of cell bodies by giant glial cells, there being two anterolateral, two ventromedial, and two posterolateral packets. The anterior brain is divided into sub- and suprasophageal areas, with the subesophageal being formed from four fused rostral neuromeres, while the posterior

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brain is formed from seven fused caudal neuronomes. Immunoreactive neurons and processes can be viewed in whole mounts of the ganglia.

In this report we describe the distribution of SCP-like immunoreactivity in the central nervous system of Hirudo, and compare the extent of its colocalization with FMRFamide-like immunoreactivity. A minor aspect of this research was to compare the neuronal distribution of FMRFamide-like immunoreactivity described by Kuhlmann et al. (1985a) with that determined with a different FMRFamide antiserum (Marder et al. 1987).

Materials and methods

Adult leeches Hirudo medicinalis were purchased from Ricarimpex or Blutegel Import und Versand, and kept at 15°C in artificial pond water. They were occasionally fed defibrinated bovine blood (Carolina Biological).

Isolated nerve cords or heart tubes were used for immunocytochemical experiments. Whole nerve cords or nerve cord sections were removed from the animal and pinned out in clear Sylgard-coated Petri dishes filled with standard leech saline (Müller et al. 1981). Lateral heart tube sections were removed and pinned at both ends in Sylgard-coated petri dishes filled with saline. Connective tissue surrounding the heart tubes was then removed. For some cell identification experiments the glial sheath surrounding the ganglion was removed with fine dissecting scissors and forceps to facilitate cell body penetration with microelectrodes.

Tissue was fixed in modified Zamboni's fixative (pH 7.4) (Stefanini et al. 1967) or 4% paraformaldehyde for 2–10 h. When paraformaldehyde was used, removal of the glial sheath was necessary for good antibody penetrability.

A monoclonal antibody directed against the C-terminal of SCPB (Masinovsky et al. 1988) was raised in mouse and generously donated by A.O. Dennis Willows. Antiserum specific for the C-terminal of FMRFamide was raised in rabbit; this antiserum, termed 671C, was raised in our laboratory and characterized by Marder et al. (1987). Antiserum were diluted in 0.4% Triton X-100 (Sigma) 0.025% sodium azide (Sigma) in 0.1 M phosphate buffer (PBTA) to give dilutions of 1:50 (SCP antibody) and 1:500 (FMRFamide antiserum). Ganglia were incubated 14–20 h at 4°C. For double-labeling experiments using both primary antiserum, ganglia were incubated separately with each antiserum at the concentrations stated.

Visualization of the primary antibody was accomplished by two methods. Secondary antibodies, goat anti-rabbit IgG or goat anti-mouse IgG (Organon Teknika), conjugated to fluorescein isothiocyanate or rhodamine were used for the indirect immunofluorescence technique (Coons 1958). In the second method, biotinylated secondary antiserum, goat antimouse IgG or swine anti-rabbit IgG (Dako Quick Staining Kit), were applied and then further processed by application of avidin conjugated to horseradish peroxidase (HRP) and developed with diaminobenzidine (DAB)/H2O2. All secondary antiserum were diluted 1:100 in PBTA; ganglia were incubated for 10 h in secondary antiserum. For double-labeling experiments using both primary antiserum, rhodamine-conjugated goat anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG antiserum were applied simultaneously at the concentrations stated.

Preabsorption controls were performed by incubation of each primary antiserum with either synthetic SCPB (Peninsula) or FMRFamide (Bachem) for 2 h prior to application. Peptides were combined with the antisera at working dilutions to give desired final concentrations. Preabsorption of the primary SCP antibody with synthetic SCPB (5 x 10^-6 M) blocked all specific staining, whereas preabsorption with synthetic FMRFamide (1.7 x 10^-5 M) did not. Preabsorption with higher concentrations of FMRFamide (1.7 x 10^-4 M), however, did eliminate all specific staining. FMRFamide-like immunoreactivity was completely blocked by preabsorption with synthetic FMRFamide (8.8 x 10^-6 M), but was unaffected by SCPB (8.8 x 10^-6 M). Deletion of primary or secondary antiserum eliminated all specific staining.

For identification of immunoreactive cells the double-labeling procedures of Li and Calabrese (1985) were used. Electrode tips were filled with 5% Lucifer yellow dye (Stewart 1978), and the shanks of the electrodes were backfilled with 100 mM LiCl. Dye was injected into identified cell bodies by iontophoresis from intracellular electrodes prior to fixation and immunocytochemical processing. Neurons were identified on the basis of soma position, morphology, and electrical activity recorded with 4 M potassium acetate electrodes prior to Lucifer yellow injection.

For physiological experiments, sections of body wall containing lateral heart tubes were dissected along with ganglia 2–8 in a connected chain. The hearts were left innervated by anterior and posterior nerve roots from one side of ganglion 6. This preparation was pinned out in a Sylgard-coated petri dish and bathed in normal leech saline solution.

Peptides were dissolved in leech saline solution and superfused onto the preparation (see Norris and Calabrese 1987). Heart tension was monitored using a force transducer (Grass FT30B) attached to one end of the heart tube with a small hook and a human hair.

Results

Distribution of SCP-like immunoreactivity

Neuronal cell bodies and processes throughout the central nervous system displayed SCP-like immunoreactivity (SLI). Immunoreactive cell bodies and processes were found in all 21 segmental ganglia, as well as both anterior and posterior brains. Each segmental ganglion contained 5–20 immunoreactive neurons. Visualization of the primary antibody with either indirect immunofluorescence (rhodamine-conjugated secondary antiserum) or DAB reaction product (biotinylated secondary antiserum/peroxidase-conjugated avidin) gave similar distributions of immunoreactive cells. The latter method gave a higher signal-to-background ratio. A number of cell bodies stained inconsistently. Only in a minority (less than 20%) of nerve cords were these neurons detected, and they varied widely in staining intensity.

Bilaterally paired immunoreactive neurons. Several immunoreactive neurons occurred as bilateral pairs. Only one such pair was found in all 21 segmental ganglia; these cells were relatively small (approximate diameter 20 μm) and were located on the dorsal posterior surface of each ganglion near the ganglionic margin (Figs. 1, 5B).

Seven bilaterally paired immunoreactive neurons were found regionally distributed throughout the nerve cord. The only dorsal neurons of this description were located in ganglia 2 and 3 in the posterior cell packet near the lateral
nerve roots (Fig. 5B). A ventral immunoreactive paired neuron was present in the posteromedia1 cell packet of ganglia 2–19 (Figs. 2, 3, 5A). A small paired neuron lay near this neuron in ganglia 6 and 7 (Fig. 5A). The large (aproximate diameter 40 μm) paired neuron in the anterolateral cell packet of ganglion 21 (unnamed cells of Fig. 3B) was found only in ganglion 21.

Three other ventral paired neurons were found only in ganglia 5 and 6. The largest of the three was located near the ganglionic margin in the anterolateral cell packet (Fig. 2B), and the second was found medial to the first (Fig. 2B). A large immunoreactive neuron was present in the posterolateral cell packet near the lateral nerve roots in these ganglia (Fig. 2B).

Unpaired immunoreactive neurons. All other consistently staining SCP-like immunoreactive neurons in the segmental ganglia were ventral unpaired cells, many of which displayed a tendency to alternate from side to side in adjacent ganglia along the nerve cord. Alternating neurons located in medial cell packets alternate sides within the packet; those in lateral packets alternate between contralateral packets in adjacent ganglia. Neurons were tested for alternation by scoring the number of times a neuron was found on the opposite side in adjacent ganglia and the number of times the neuron was found on the same side in adjacent ganglia. Neurons not clearly on one side or the other were not scored. The tendency for alternation was expressed as the percentage of total neurons scored that were found on the opposite side in adjacent ganglia (Table 1).

![Fig. 1. HRP preparations showing typical dorsal staining patterns for small cardioactive peptide (SCP)-like immunoreactivity in segmental ganglia. Both ganglia are viewed from the dorsal surface. Drawings in part 2 are the same ganglia as in part 1, with dorsal immunoreactive cell bodies indicated. Consistently staining immunoreactive cell bodies are solid in part 2; cells shown in outline stained inconsistently. Ganglion numbers are indicated in the lower left corners of part 1. Scale bars: 100 μm](image)

| Table 1. Unpaired or alternating SCP-like immunoreactive neurons in the leech* |
|---------------------------------|---|---|---|---|
|                                | N | O  | S  | %ALT |
| AAS                             | 7 | 25 | 9  | 74  |
| AAS20–21                        | 11| 9  | 2  | 82  |
| CAS                             | 15| 37 | 8  | 82  |
| LAS                             | 4 | 14 | 2  | 88  |
| MUS                             | 8 | 11 | 17 | 39  |
| PAS                             | 10| 123| 21 | 85  |

* N lists the number of nerve cords in which observations were made. O denotes the number of times the cell was located on the opposite side in two adjacent ganglia. S denotes the number of times the cell was located on the same side in two adjacent ganglia. %ALT expresses O/O+S as a percentage. Chi-squared test gives a probability of random side-to-side distribution of <0.05 for all categories except MUS, for which 0.30>P>0.20. (RAS neurons are not included in this list, as they were detected only in ganglion 1.)

Ganglion 1 contained only one unpaired immunoreactive neuron, located near the ganglionic margin in the anterolateral cell packet (Fig. 2A), and it appeared on either left or right sides with apparently equal probability. This cell possibly corresponds to the rostral alternating SCP-like immunoreactive neuron described in Helobdella triseriata (Shankland and Martindale 1987a, b), and thus will be referred to as the RAS neuron. The posteromedia1 packet of ganglia 2–21 contained an unpaired ventral neuron
(Figs. 2, 3) which displayed a strong tendency to alternate from side to side in adjacent ganglia (Table 1). Due to its position within the ganglion, this neuron is named the posterior alternating SCP-like immunoreactive (PAS) neuron. The anteromedial packets of ganglia 2–12 contained a somewhat larger unpaired immunoreactive cell body, which also tended to alternate in adjacent ganglia (Fig. 2B). These cells are called the anterior alternating SCP-like immunoreactive (AAS) neurons due to their anterior location within the ganglion. Ganglia 20 and 21 each contained an alternating neuron in a position similar to that of the AAS neuron (Fig. 3B). These cells may or may not be homologous to the AAS neurons, and will be designated AAS20 and AAS21 (Table 1).

One prominent series of alternating immunoreactive cells is present in ganglia 18–21 (Figs. 3, 5A). These large neurons have axons that ascend in the contralateral interganglionic connective (data not shown). These cells are similar in location and distribution to the caudal alternating SCP-like immunoreactive neurons of Helobdella (Shankland and Martindale 1987a, b), and therefore are called CAS neurons. The designations RAS and CAS refer to location within the nerve cord, whereas all other names given to unpaired neurons found in this study refer to a neuron's position within the ganglion.

Inconsistently immunoreactive neurons. Some apparently immunoreactive neurons stained inconsistently from one nerve
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The small cardioactive peptides (SCPs) were originally described as cardioactive substances in gastropods (Lloyd 1978, 1982). SCP and SCP were isolated from the sea slug Aplysia (Morrison et al. 1982; Lloyd et al. 1987b), and found to be amidated peptides of eleven and nine amino acids, respectively; they share the same seven carboxy terminal residues. The SCPS are excitatory on Aplysia heart (Lloyd et al. 1985b), gut (Lloyd 1986), and buccal muscles (Lloyd et al. 1984), and modulate a number of neuronal currents (Abrams et al. 1984; Ocorr and Byrne 1985; Acosta-Urquidi 1988) in this animal. The actions of the two SCPS are indistinguishable from one another, and are indistinguishable from those of serotonin (Lloyd et al. 1984).

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Lloyd 1986). These actions of the SCPS in Aplysia are mediated, at least in part, through cAMP (Abrams et al. 1984; Ocorr and Byrne 1985; Lloyd et al. 1985b).

SCP-like peptides have now been localized immunocytochemically in other molluscs (Kemp et al. 1987; Masinovsky et al. 1988); crustaceans (Callaway et al. 1987; Masinovsky et al. 1988); the glossopodid leech Helobdella tsierensis (Shankland and Martindale 1987a, b); and insects (Masinovsky et al. 1988). To date, the identities of these peptides have not been determined, although authentic SCP is apparently present in the marine gastropod Tritonia diomedea (Lloyd et al. 1988).

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One prominent series of alternating immunoreactive cells is present in ganglia 18–21 (Figs. 3, 5A). These large neurons have axons that ascend in the contralateral interganglionic connective (data not shown). These cells are similar in location and distribution to the caudal alternating SCP-like immunoreactive neurons of Helobdella (Shankland and Martindale 1987a, b), and therefore are called CAS neurons. The designations RAS and CAS refer to location within the nerve cord, whereas all other names given to unpaired neurons found in this study refer to a neuron's position within the ganglion.

Inconsistently immunoreactive neurons. Some apparently immunoreactive neurons stained inconsistently from one nerve
cord to another (i.e., in less than 20% of the preparations stained). When these cells stained, the intensity of staining also varied. As noted in the previous section, all segmental ganglia contained a bilaterally paired immunoreactive neuron in the dorsal posterolateral cell packet. Occasionally, more than one bilaterally paired cell stained in this location, there being two additional immunoreactive cells in ganglia 5–10, and one additional cell in ganglia 11–17, 20, and 21 (Fig. 5B). No additional cells stained in this location in ganglia 1–4, 18, or 19. Another inconsistently staining paired neuron was located in the anterolateral packet of ganglia 1–17 along the lateral ganglionic margin of the dor-

Fig. 3. HRP preparations showing typical staining patterns for SCP-like immunoreactivity in posterior ganglia. Both ganglia are viewed from the ventral surface. Drawings in part 2 are the same ganglia as part 1, with ventral immunoreactive cell bodies indicated. Unnamed cell pair in part B2 was found only in ganglion 21. Cells labeled are as follows: AAS21 possible correlate to anterior alternating SCP-like immunoreactive neuron; CAS caudal alternating SCP-like immunoreactive neuron; PAS posterior alternating SCP-like immunoreactive neuron. Ganglion numbers are indicated in the lower left corners of part 1. Scale bars: 100 μm

Fig. 4. Typical SCP-like immunoreactivity in anterior and posterior brains. A Anterior brain, dorsal surface. B Posterior brain, ventral surface. Note the two large unpaired neurons located along the midline of the posterior brain. Arrows indicate nerve tracts. Scale bars: 100 μm
Fig. 5. Schematic representation of the leech nerve cord, showing typical pattern of SCP-like immunoreactivity in neuronal somata, as constructed from camera lucida drawings of HRP preparations. Cell bodies of consistently staining neurons are solid; inconsistently staining immunoreactive neurons are shown in outline. Nerve cords are oriented with anterior to the left; ganglion numbers are indicated. Dashed lines indicate outlines of cell packets. Abbreviations are those described in Figs. 2 and 3. A Ventrally located immunoreactive neurons. B Dorsally located immunoreactive neurons.
Fig. 6. Identification of SCP-like immunoreactive neurons by intracellular injection of Lucifer yellow dye and further processing for SLI with rhodamine-conjugated secondary antiserum. A1 Both HA (heart modulatory) neurons in ganglion 5 were injected with Lucifer yellow and photographed using optical filters for viewing fluorescein. A2 Same ganglion as A1, viewed using optical filters for viewing rhodamine, showing SCP-like immunoreactive cell bodies. HA cell bodies are indicated by arrowheads. B1 The right HE neuron in ganglion 8 was injected with Lucifer yellow and photographed under fluorescein optics. B2 Same ganglion as B1, viewed under rhodamine optics, showing SCP-like immunoreactive cell bodies. Arrowhead in B2 indicates the position of the unlabeled HE cell body. Scale bars: 100 μm.

Ganglionic surface (Figs. 1A, 5B). Two additional immunoreactive paired neurons sometimes stained in the anterior part of the ventral anteromedial packet of ganglia 2 and 3 (Fig. 5A). All other inconsistently staining neurons were unpaired and located on the ventral surface of the ganglia. The inconsistently staining unpaired neurons were regionally distributed along the nerve cord. One of these neurons was seen in the ventral posterolateral packet of ganglia 8–18 (Figs. 2C, 5A). This neuron alternated from side to side in adjacent ganglia (Table 1). Due to its position within the ganglion, this neuron is named the lateral alternating SCP-like immunoreactive (LAS) neuron. The other unpaired ventral cell was located in the anteromedial packet of ganglia 7–14, posterior to the AAS neuron (Figs. 2C, 5A). No tendency to alternate in adjacent ganglia was seen (Table 1), and therefore the cell is named the medial unpaired SCP-like immunoreactive (MUS) neuron.

Immunoreactive neurons were observed in both head and tail brains. Numerous cell bodies stained on the ventral and dorsal surfaces in both the supra- and subesophageal areas of the head brain (Fig. 4A). Most appeared to occur as bilateral pairs, with the majority being located on the dorsal surface. The tail brain, on the other hand, contained only a small number of immunoreactive neurons (Fig. 4B). Two cells on the ventral surface of the posterior brain were immunoreactive; both were located along the midline in the two most anterior neuromeres. The dorsal surface contained a series of bilaterally paired cells, apparently iterated in the six most anterior of the seven fused neuromeres that comprise the posterior brain. A second less intensely stained
bilaterally paired cell was also present in most of these neuromeres (data not shown).

In addition to cell bodies, immunoreactive neuronal processes were seen throughout the leech central nervous system. The neuropilar staining was concentrated mainly in the dorsal region of the central nervous system (CNS), and formed two parallel tracts which ran throughout the length of the ventral nerve cord. These tracts were particularly prominent in the tail brain, and they also extended into the head brain, where they met as one transverse tract in the suprasphageal ganglion (Fig. 4).

**Identification of immunoreactive neurons**

The large immunoreactive paired neurons found in ganglia 5 and 6 along the anterolateral margin of the ganglion are similar in size and position to the heart modulatory (HA) neurons (Calabrese and Maranto 1984). To determine if these immunoreactive neurons are the HA neurons, we physiologically identified the HA neurons and labeled them by injection of Lucifer yellow dye. These labeled preparations were subsequently processed immunocytochemically for SCP-like immunoreactivity using rhodamine-conjugated secondary antiserum. Colocalization of the two fluorescent markers demonstrated that the HA neurons are SCP-like immunoreactive (Fig. 6A). As a control for the double-labeling method, the heart motor (HE) neurons were similarly labeled and processed and found not to contain detectable SCP-like antigens (Fig. 6B).

**Distribution of FMRFamide-like immunoreactivity**

Approximately 50 cells in a typical segmental ganglion (more than 50 for ganglia 5 and 6, see below) stained for FMRFamide-like immunoreactivity (FLI) with antiserum 671C (Fig. 7A, B). FMRFamide-like immunoreactivity was detected in neuronal cell bodies and processes throughout the CNS. Visualization of the primary antiserum (671C) achieved with either indirect immunofluorescence (fluorescein- or rhodamine-conjugated secondary antiserum) or DAB reaction product (biotinylated secondary antiserum/peroxidase-conjugated avidin) gave comparable results. The
The staining pattern observed is very similar to that previously obtained using anti-FMRPamide antisera 231 (O’Donohue et al. 1984; Kuhlman et al. 1985a; Norris and Calabrese 1987), although some differences exist.

Neurons previously identified as containing FLI with antisera 231 include the HA, IIE, L, AP, RPE, LPE, AE, 204 cells, and several dorsal motor neurons (Kuhlman et al. 1985a; Norris and Calabrese 1987). In the present study, most of these neurons were not positively identified as immunoreactive by dye injection and subsequent processing for FMRPamide-like immunoreactivity. Size, relative position within the ganglion, and distribution along the nerve cord, however, strongly indicate that these neurons are also immunoreactive to antiseraum 671C.

There are several immunoreactive neurons in the distribution of FLI determined here with 671C that do not appear in the distribution determined with antisera 231. In ganglia 5 and 6, a large number of small immunoreactive neurons distributed over both ventral and dorsal surfaces stained with antiseraum 671C (Fig. 7C, D). In addition, antiseraum 671C indicated the presence of FMRPamide-like an-
tigens in the alternating and unpaired SCP-like immunoreactive neurons reported in this study.

**Colocalization of SCP-like and FMRFamide-like immunoreactivity**

A number of neurons contained both SCP- and FMRFamide-like immunoreactivities. We demonstrated that the HA neuron, which contains FMRFamide or a very closely related peptide (Kuhlman et al. 1985a; Li and Calabrese 1987), also exhibits SCP-like immunoreactivity, whereas the HE neuron, which also contains FMRFamide-like immunoreactivity (Kuhlman et al. 1985a), does not contain SCP-like immunoreactivity. Other neurons might also contain SCP- and FMRFamide-like antigens. To determine the extent of colocalization of SCP- and FMRFamide-like immunoreactivities present in the leech CNS we performed double-labeling experiments with both the SCP and FMRFamide antisera. Most of the SCP-like immunoreactive neurons were FMRFamide-like immunoreactive, whereas the majority of FMRFamide-like immunoreactive neurons did not contain detectable amounts of SCP-like antigen. As shown in Fig. 8, most of the ventral SCP-like neurons also contained FMRFamide-like antigens. The dorsal anterior SCP-like immunoreactive cell pair that stained inconsistently with the anti-SCP antibody stained very intensely with antisera to SCP (Fig. 7B).

A few SCP-like immunoreactive neurons were not FMRFamide-like immunoreactive. The only ventral SCP-like immunoreactive neurons that were not FMRFamide-like immunoreactive are the paired ventral cells located medially to the HAs in ganglia 5 and 6 (Fig. 2B). The paired SCP-like immunoreactive cells located dorsally in every ganglion likewise did not contain detectable levels of FMRFamide-like antigens. The one or two paired posterior cells that inconsistently stained for SCP-like antigens on the dorsal surface in some ganglia (see above), stained consistently for FMRFamide-like antigens.

**Bioactivity of SCP**

Two types of neurons innervate the animal's hearts, HE motor neurons and HA modulatory neurons; both show FMRFamide-like immunoreactivity (Kuhlman et al. 1985a), and have modulatory effects on heart muscles that are mimicked by bath-applied FMRFamide (Kuhlman et al. 1985b). Since HA neurons also contain SLI, we tested the action of bath-applied SCP on the heart both alone and in conjunction with FMRFamide. Bath application of SCP (1 × 10^{-6} M) onto an innervated heart preparation caused no significant change in the heartbeat rate or basal tension (Fig. 9C), whereas similar application of FMRFamide (1 × 10^{-5} M) increased each of these parameters (Fig. 9A). Moreover, the response to application of both the peptides (1 × 10^{-6} M) together was similar to the response to FMRFamide alone (Fig. 9B).

**Discussion**

A limited number of neurons in the leech CNS are immunoreactive to a monoclonal antibody raised against molluscan SCP. this immunoreactivity is localized to a subset of the neurons in each ganglion. SCP-like immunoreactivity (SLI) is not detected when the SCP antibody is preabsorbed with SCP, but is detected when the primary antibody is preabsorbed with a similar concentration of FMRFamide. Since their original description as cardioactive factors in the snail *Helix* (Lloyd 1978), the small cardioactive peptides or similar molecules have been detected in a growing list of invertebrate species (see Lloyd et al. 1985a, 1985; Masinovsky et al. 1988).

The molecular structure of the SCP-like antigen detected here appears to be distinct from both SCP and SCP. In preliminary experiments, leech CNS extracts separated on a reverse phase high performance liquid chromatograph showed no recoverable bioactivity on the SCP-sensitive *Helix* heart corresponding to SCP or SCP. but did contain substances that comigrated with FMRFamide, acetylcho-
line, and serotonin (PE Lloyd, personal communication). SLI in *Hirudo* is likely due to a peptide with some similarity to the C-terminal hexamer common to SCPa and SCPb (Lloyd 1986), since this hexamer contains the antigenic determinant recognized by the SCP antibody (Masinovsky et al. 1988).

SCP- and FMRFamide-like immunoreactivities are partially colocalized in leech CNS neurons and appear to be due, at least in part, to different antigens. SLI is colocalized with FMRFamide-like immunoreactivity (FLI) in many neurons, but, unlike crab CNS neurons (Callaway et al. 1987), where all SCP-like immunoreactive neurons also contain FLI, a subset of SCP-like immunoreactive neurons in leech are not FMRFamide-like immunoreactive. Likewise, many leech neurons contain FLI but not SLI. Masinovsky et al. (1988) have shown that FMRFamide exhibits a low binding affinity for the SCP antibody in ELISA assay, and Lloyd et al. (1987a) saw no crossreactivity of FMRFamide with the SCP antibody in immunocytochemical preabsorption control experiments. The double-labeling experiments described in this study using both FMRFamide and SCP antisera also suggest the presence of at least two antigenic species. These data suggest that some, if not all, SCP-like immunoreactive neurons in leech contain an antigen different from FMRFamide-like antigens. Lloyd et al. (1987a) have localized SCPs and FMRFamide in *Aplysia* neurons, and Callaway et al. (1987) have suggested the presence of distinct SCP- and FMRFamide-like antigens in crustacean neurons.

Some neurons stained inconsistently for SLI. Two possible explanations for this variability are suggested. First, in response to internal or external cues, levels of SLI may be changing during adulthood in some or all of the inconsistently staining neurons detected in this study. Second, the neurons that are inconsistently labeled by immunocytochemical means may not be present in all nerve cords. Callaway et al. (1987) have suggested similar possibilities to explain variability in immunoreactivity.

A number of different SCP-like immunoreactive neurons alternate from side to side in adjacent ganglia. Similar alternating patterns have been described in neurons of *Helobdella* (Shankland and Martindale 1987a, b) and postero-medial serotonin-containing (PMS) neurons of *Hirudo* (Macagno and Stewart 1987).

FMRFamide antisera 231 and 671C apparently recognize and bind to similar epitopes, which include the carboxyl terminal Arg-Phe-amide sequence (O'Donohue et al. 1984; Marder et al. 1987; Li and Calabrese 1987). Antiserum 671C, however, is somewhat less specific than antisera 231. As determined by relative binding affinities in radioimmunoassay, antisera 671C appears to be less sensitive to substitutions at the Met position of FMRFamide (O'Donohue et al. 1984; Li and Calabrese 1987; Marder et al. 1987), and thus will probably recognize a broader range of FMRFamide-like peptides than antisera 231. The less specific nature of antisera 671C is supported by the present finding that 671C indicates the presence of FLI in some neurons not found to be FMRFamide-like immunoreactive as determined with antisera 231.

We conclude that an antigen different from the SCPs and FMRFamide is present in individual neurons of the leech. This antigen likely possesses some sequence homology with the carboxy-terminal hexamer common to both SCPs.

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