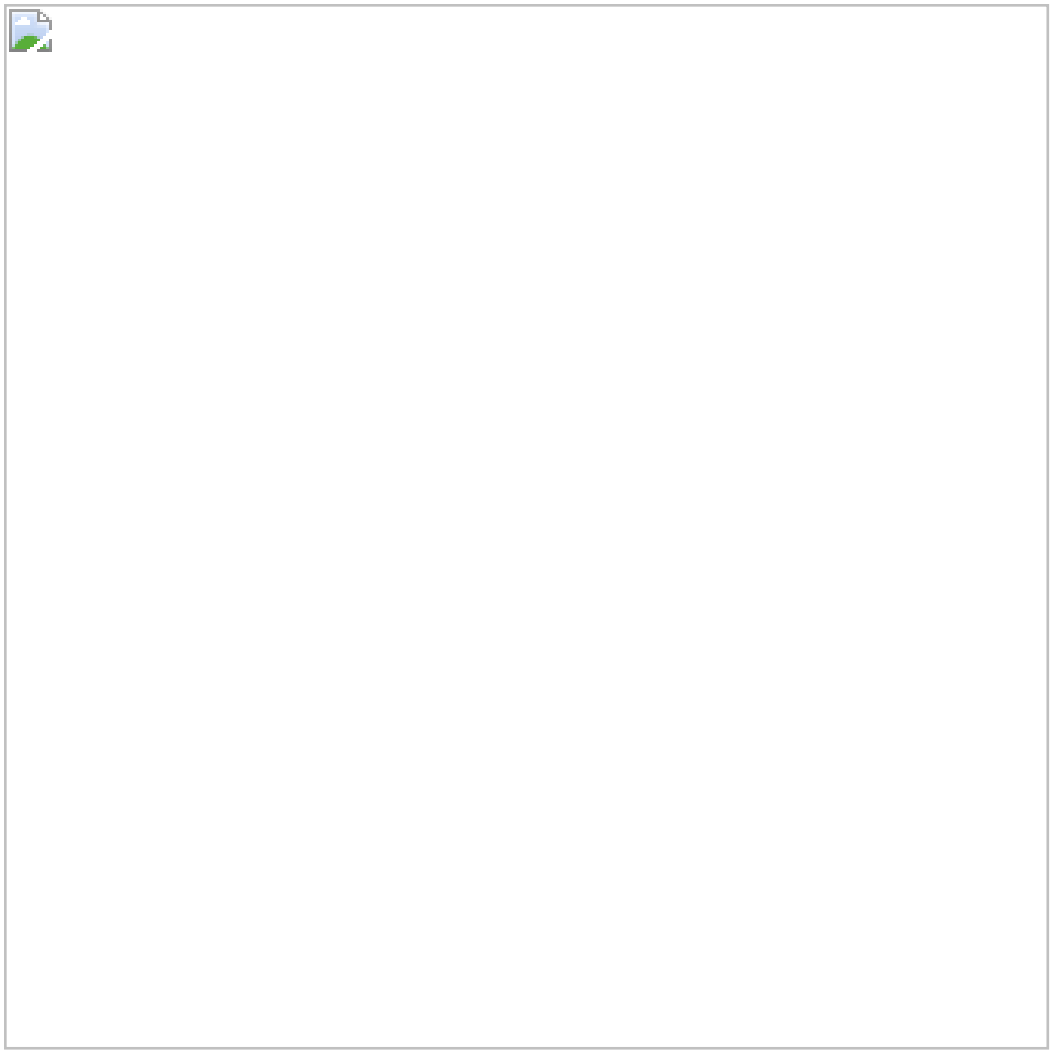

Localization of RFamide-like Immunoreactivity in the Visceral Organs and Peripheral Neurosecretory Cells Related to the Terminal Abdominal Ganglion in the Cricket, Gryllus bimaculatus

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Localization of RFamide-like Immunoreactivity in the Visceral Organs and Peripheral Neurosecretory Cells Related to the Terminal Abdominal Ganglion in the Cricket, *Gryllus bimaculatus*

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ABSTRACT—The distribution of RFamide-like immunoreactivity was examined in the terminal abdominal ganglion (TAG)-related peripheral nervous system, and in the visceral tissues of the female cricket, *Gryllus bimaculatus*. In the TAG, most of the RFamide-like immunoreactive (RFaLI) neurons were bilaterally paired, and were found in the neuromeres of the 7th to the 10/11th segment. The remaining neurons were found in the midline region. These included anterior unpaired neurons, a cluster of neurons in the posterior end, and ventral paired neurons in the 9th segment. Immunocytochemistry combined with Lucifer Yellow back-filling indicated that the immunoreactive paired medial neurons innervated the rectum. In the visceral tissues innervated by the TAG, RFaLI fibers were seen in the oviducts and rectum, while the spermathecal duct and spermatheca did not exhibit any immunoreactivity. In the peripheral nervous system, RFaLI peripheral neurosecretory cells (PNCs) were found on the fifth and seventh segmental nerve roots of the TAG in both sexes. The perikaryon of the PNCs contained several immunoreactive large electron-dense granular vesicles. Intracellular dye injection showed that the PNCs probably form neurohaemal-release sites for RFamide-like peptide. The PNCs did not project any processes into the TAG.

INTRODUCTION

The molluscan cardioexcitatory peptide FMRFamide [33] has been found in many phyla [3], and is now considered a member of the growing family of FMRFamide-related peptides (FaRPs) [34]. The FaRPs in insects have been shown to have modulatory effects upon a variety of visceral and skeletal muscles [2, 7, 8, 23, 32]. Immunocytochemical studies have localized FMRFamide-like immunoreactivity not only in neuronal cell bodies [15, 26, 27, 45, 47-49], but also in neurosecretory cells and neurohaemal areas [5, 10, 19, 25, 29, 39, 45, 46], suggesting that FaRPs may play a neuroendocrine role in insects.

Insect reproductive organs and alimentary canal provide useful model systems for the examination of neural and hormonal control of insect visceral muscles. The oviduct muscles in the locust [21, 30] and cockroach [40, 42] have been intensively studied with regard to their innervation and pharmacological properties in response to various neuroactive substances. The oviducts of these insects receive polyneuronal innervation from both octopamine-containing and proctolin-containing neurons [22, 30, 40]. Recently, a second peptidergic innervation, FMRF-like immunoreactive innervation, has been demonstrated in locust oviducts [12, 23, 37]. It has been reported that not only serotonin and proctolin but also RFamide-like peptide is involved in neural control of the male cricket accessory gland [17, 52, 54, 55]. With regard to the alimentary canal, dual peptidergic innervation by both proctolin and FMRFamide has been immunocytochemically demonstrated in the blowfly hindgut [4]. In the isolated locust foregut, FMRFamide was reported to potentiate the relaxation induced by serotonin and to inhibit the contraction caused by proctolin [2]. In contrast, in the locust oviduct, this peptide increases the frequency and amplitude of myogenic contractions and also increases basal tension [12, 32]. These observations suggest that multiple peptidergic innervation plays an important role in the regulation or modulation of contractile activity in insect visceral muscles.

Our recent study indicated that the cricket oviduct system is innervated by eight pairs of motor neurons, nine dorsal unpaired median (DUM) neurons and four ventral unpaired median (VUM) neurons [6], which run through a pair of the second nerve roots of the terminal abdominal ganglion [50]. The immunocytochemical study of female cricket reproductive tissues is still rare, in contrast to the extensive studies in the locust and other insects. In this paper, we examine the distribution of RFamide-like immunoreactivity in the TAG and visceral tissues, including the oviduct, spermathecal duct, and rectum, all of which receive innervation from the TAG, in female crickets. Furthermore, we describe novel RFamide-like immunoreactive peripheral neurosecretory cells (PNCs) which are attached to the dorsal nerves of the TAG.

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MATERIALS AND METHODS

Animals

The terminal abdominal ganglion (TAG), visceral tissues (oviduct, spermathecal duct, spermatheca, and rectum) and TAG-related peripheral nervous system of adult female crickets (*Gryllus bimaculatus*) were used in this study. The rectum and TAG-related peripheral nervous system of males were also used to examine any sexual differences in immunoreactivity. The animals were obtained from a laboratory culture reared under a 12 hr light-12 hr dark cycle at 28°C.

Immunocytochemistry

Antisera: The rabbit anti-FMRFamide antiserum (Cambridge Research Biochemicals) used for this experiment is specific to the C-terminal (RFamide), and a positive reaction to this antiserum demonstrates the existence of RFamide-like peptides. Antiserum was diluted 1:2000 in phosphate-buffered saline (PBS, pH 7.4) with 0.5% Triton X-100 and 0.5% bovine serum albumin.

Light and electron microscopic immunocytochemistry: The tissue was fixed for 6–12 hr in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C. Light microscopic immunocytochemistry for paraffin sections and whole mount preparations was applied using the avidin-biotin-peroxidase complex (ABC, Vector Lab.). For electron microscopic immunocytochemistry, the post-embedding protein A-gold method was used. Detection of RFamide-like immunoreactivity and the absorption test for control of specificity were performed as previously described [55].

Double-labeling: Lucifer Yellow back-filling and RFamide immunocytochemistry were combined to identify RFamide-like immunoreactive (RFaLI) neurons innervating the visceral tissues. The proximal cut end of the nerve concerned was plunged into a glass microelectrode with a broken tip (filled with 5 % Lucifer Yellow (Sigma) in distilled water) for 20 hr at 4°C, and then the TAG was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 15 hr at 4°C, followed by washing in PBS. Subsequently, for whole-mount immunocytochemistry, the TAG was incubated in the primary antiserum for 72 hr at 4°C. After washing in PBS, immunoreactivity was revealed by treatment with a 1:50 dilution of rhodamine-conjugated swine anti-rabbit IgG (Dako) for 24 hr at 4°C. The TAG was washed in PBS and mounted in a mixture of PBS and glycerin (1:1).

Back-fillings

Nickel back-filling of the peripheral neurosecretory cells, PNCs, on the segmental nerve root of the TAG was performed by overnight infusion of nickel chloride (3% in distilled water) at 4°C *via* the proximal cut end of the nerve root along which the PNCs extended their axons toward the periphery. The back-filled tissue was processed to precipitate the nickel according to Sakai and Yamaguchi [38], and then silver-intensified according to the procedure of Bacon and Altman [1]. The tissue was cleared with methyl salicylate, and then viewed in a whole-mount preparation.

Differential back-filling was also performed to characterize the RFaLI neurons innervating the visceral tissues. Each of the proximal cut ends of the paired segmental nerve roots in question was plunged into a thin capillary filled with 3% nickel chloride and cobalt chloride solution, respectively, for 20 hr at 4°C. The back-filled tissue was processed to precipitate the metal ions according to Sakai and Yamaguchi [38], and then cleared and viewed as described above.

Intracellular recording and dye injection

The cell bodies of RFaLI-PNCs located on the nerve root were impaled with a glass microelectrode filled with 4% Lucifer Yellow (20–50 MΩ). After physiological recordings, the cells were iontophoretically filled with the dye by applying hyperpolarizing current for several minutes. The TAG was isolated together with the nerve root containing the dye-injected axons of PNCs, and fixed in 4% formaldehyde in phosphate buffer for 20 min. After dehydration in alcohol and clearing with methyl salicylate, the nerve root and TAG were viewed in a whole-mount preparation.

RESULTS

Distribution of RFamide-like immunoreactive neurons in the female TAG

The TAG is composed of five fused neuromeres originating in embryonic abdominal ganglia (the 7th, 8th, 9th, 10th and rudimentary 11th neuromere; A7, A8, A9 and A10/11) [31]. From this ganglion arise eight pairs of segmental nerve roots (R1-R8) and two median nerves (Fig. 1). The odd-numbered nerve roots are the dorsal nerve roots, and the even-numbered nerve roots are the ventral ones [50]. In the female, the second pair of nerve roots (R2s) innervates the oviduct, and the fourth pair (R4s) innervates the spermathecal duct and spermatheca [44]. The paired nerve branches

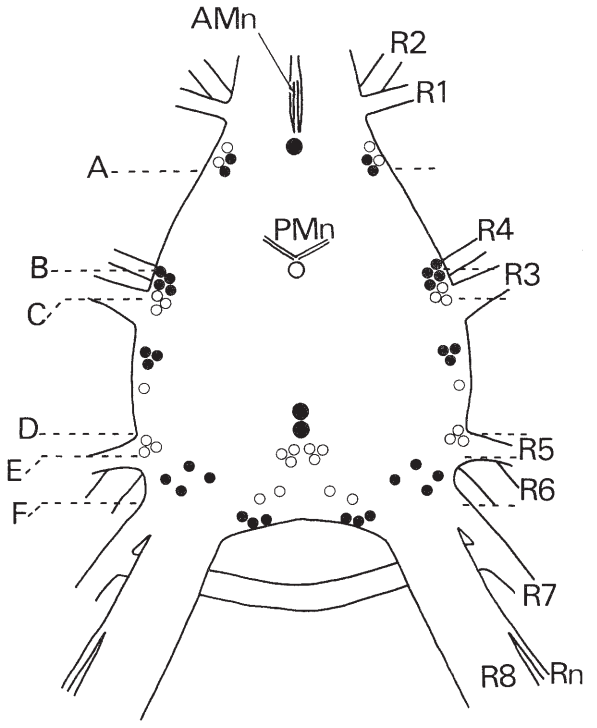

FIG. 1. Diagram showing the distribution of RFamide-like immunoreactive cell bodies within the female TAG. Dorsal view: ● ventral, ○ dorsal. The number and location of the immunoreactive cell bodies were adapted from nine whole-mount preparations. The dotted lines lettered A to F on the left indicate the respective planes of the sections shown in Figure 2 A-F. AMn, anterior median nerve; PMn, paired median nerve; R1-R8, first-eighth nerve roots; Rn, rectal nerve.

(rectal nerves) arising from the 8th nerve roots (R8s) innervate the hindgut musculature in both sexes.

Immunocytochemical staining of whole-mount TAGs revealed a variable number of RFaLI somata, but identifiable somata were found in exactly the same region in different preparations. The intensity of the immunoreaction also varied between different preparations. Figure 1 summarizes the distribution of over 60 RFaLI neurons which were stained consistently. Most of the RFaLI neurons were symmetrically located in the dorso-lateral and ventro-lateral regions in each neuromere (A7-A10/11) of the TAG (Fig. 1). The paired neurons ranged in size from about 10 to 20 μm in diameter (Fig. 2). The remaining RFaLI neurons were found along the midline of the TAG. The anterior-most immunoreactive medial neuron in A7 was located ventrally, and was about 40 μm in diameter. The dorsal medial RFaLI neuron was located near the site where the median nerves arise, and had a diameter of about 30 μm (Fig. 2B). These two medial neurons showed moderate or weak immunoreactivity (Fig. 2B). In the posterior region (A9) of the TAG,

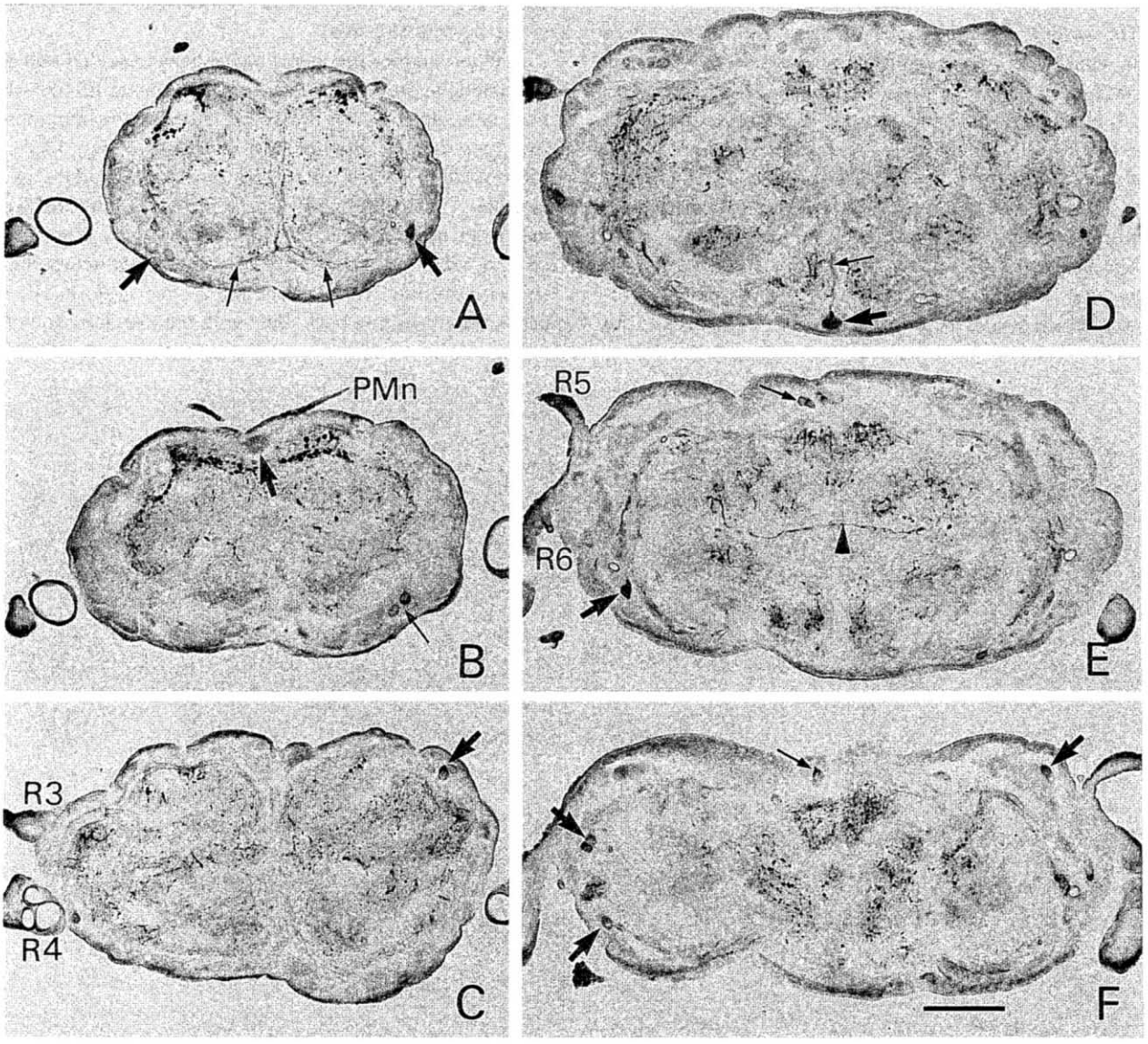

FIG. 2. Cross-sections (12 μm) through the TAG stained with anti-RFamide antiserum. A is the most anterior section, and F is the most posterior section. The planes of the sections are shown in Fig. 1. (A) A section at the level of neuromere A7. The ventro-lateral cell bodies (thick arrows) and fibers displaying a 'J'-formed trajectory (thin arrows) show immunoreactivity. (B, C) Sections at the level of neuromere A8. B shows the immunoreactive ventro-lateral (thin arrow) and dorso-medial (thick arrow) cell bodies. The median nerves (PMn) are also stained. C indicates the dorso-lateral immunoreactive cell bodies (thick arrow). (D, E) Sections at the level of neuromere A9. D shows the immunoreactive ventro-medial cell body (thick arrow) projecting a stained axon dorsally (thin arrow). E shows the stained ventro-lateral (thick arrow) and dorso-medial (thin arrow) cell bodies. A nerve fiber within the commissure (arrow head) is also stained. (F) A section at the level of neuromere A10/11. Both of the lateral cell bodies (thick arrows) and the dorso-medial cell body (thin arrow) show immunoreactivity. Note the extensive immunoreactivity in the neuropil regions throughout the ganglion. R3-R6, third-sixth nerve roots. Bar, 100 μm.

two ventrally located somata showed strong RFamide-like immunoreactivity (Figs. 2D and 5A). These neurons were about 30 μm in diameter. In the caudal region of the TAG, 6 to 8 small RFaLI somata (about 10 μm in diameter) formed a cluster in the dorsal midline (Fig. 2E). The anti-RFamide antiserum also specifically stained some transverse axon tracts (Fig. 2A) and commissures (Fig. 2E). Fiber profiles with strong immunoreactivity were also seen in the extensive neuropil regions of the TAG (Fig. 2).

RFamide-like immunoreactivity in the female reproductive tissue

The oviduct system of the cricket, like that in other insects, consists of a pair of lateral oviducts, and a common oviduct formed by the fusion of these lateral oviducts. This tissue is innervated by a pair of nerve branches arising from R2 of the TAG. RFaLI fibers were found in both of the lateral and the common oviducts (Fig. 3A, B). A dense plexus of strongly immunoreactive varicose fibers surrounded the entire surface of the oviducts.

The spermathecal duct is a highly convoluted duct about 30 mm long. This duct is connected to the pouched spermatheca, which acts as a sperm reservoir. These tissues are surrounded by circularly arranged muscle fibers [51], and receive innervation from the TAG through a pair of nerve branches of the fourth nerve roots [44]. No RFamide-like immunoreactivity was detected in these tissues with our experimental procedure (Fig. 3C, D).

RFamide-like immunoreactivity in the rectum

The posterior hindgut, including the rectum, is innervated by nerve fibers from the TAG through a pair of nerve branches (rectal nerves) arising from R8. In the musculature of the rectum in both males and females, the RFaLI varicose fibers formed a plexus, which is less dense than that in the oviduct (Fig. 4B).

Whole-mount immunocytochemistry revealed two RFaLI somata on the ventral midline in neuromere A9 in both the male and female TAGs (Fig. 5A), and also one immunoreactive fiber running through each rectal nerve (Fig. 4A). We refer to these RFaLI neurons in the ventral midline as VM neurons. To confirm whether or not VM neurons are involved in the RFamide-like immunoreactivity found in the rectum, double-labeling which combined immunocytochemistry with Lucifer Yellow back-filling was performed. The preparation was first back-filled with the dye from one of the

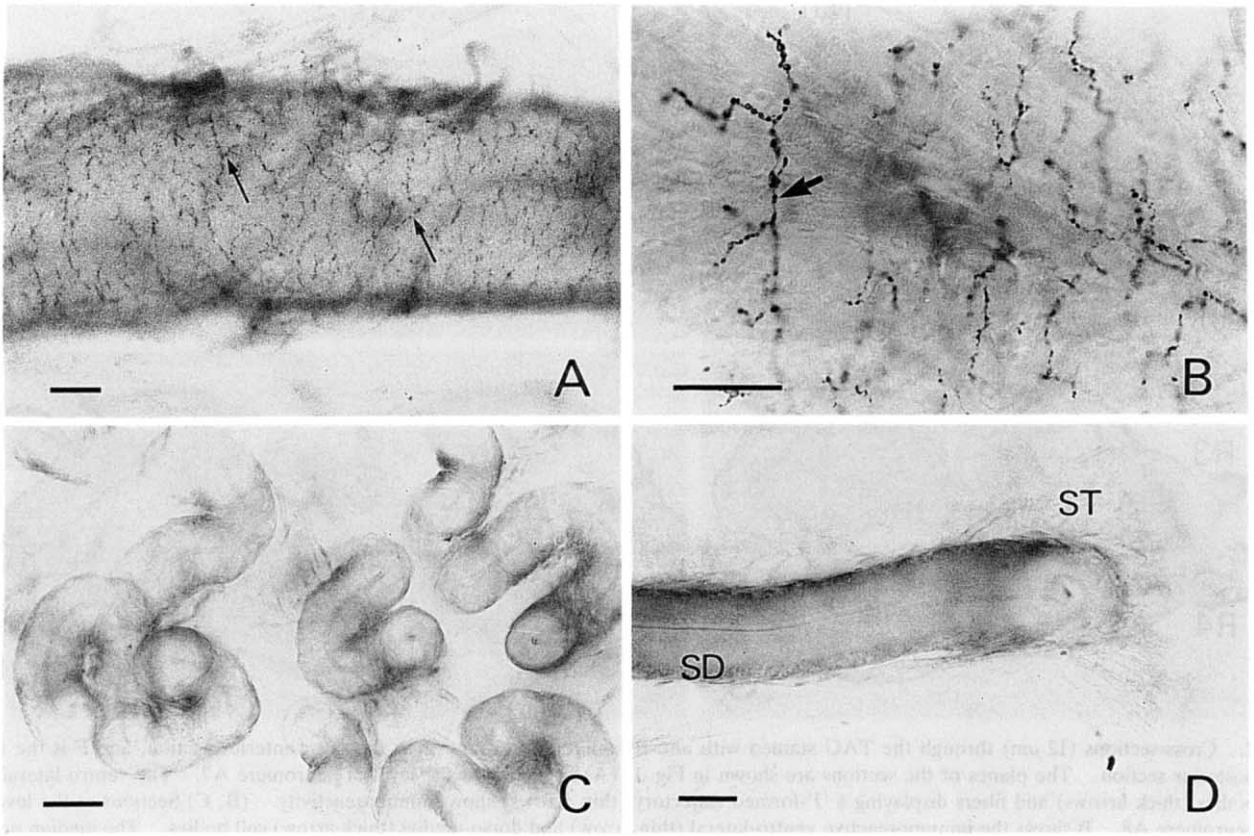

FIG. 3. RFamide-like immunoreactivity in whole-mount preparations of the oviduct, spermathecal duct, and spermatheca. (A) Lateral view of the lateral oviduct showing a plexus of immunoreactive varicose fibers (thin arrows) surrounding the entire surface of the duct. (B) A magnified view of the immunoreactive fibers in the lateral oviduct. Note the meshes composed of varicose fibers (thick arrow). (C) The median region of the spermathecal duct where the duct is highly convoluted.(D) The distal region of the spermathecal duct (SD) near the junction with the spermatheca (ST), where the duct passes straight along the surface of the spermatheca. No immunoreactive staining was detected in either the spermathecal duct or spermatheca. Bar, 50 μm.

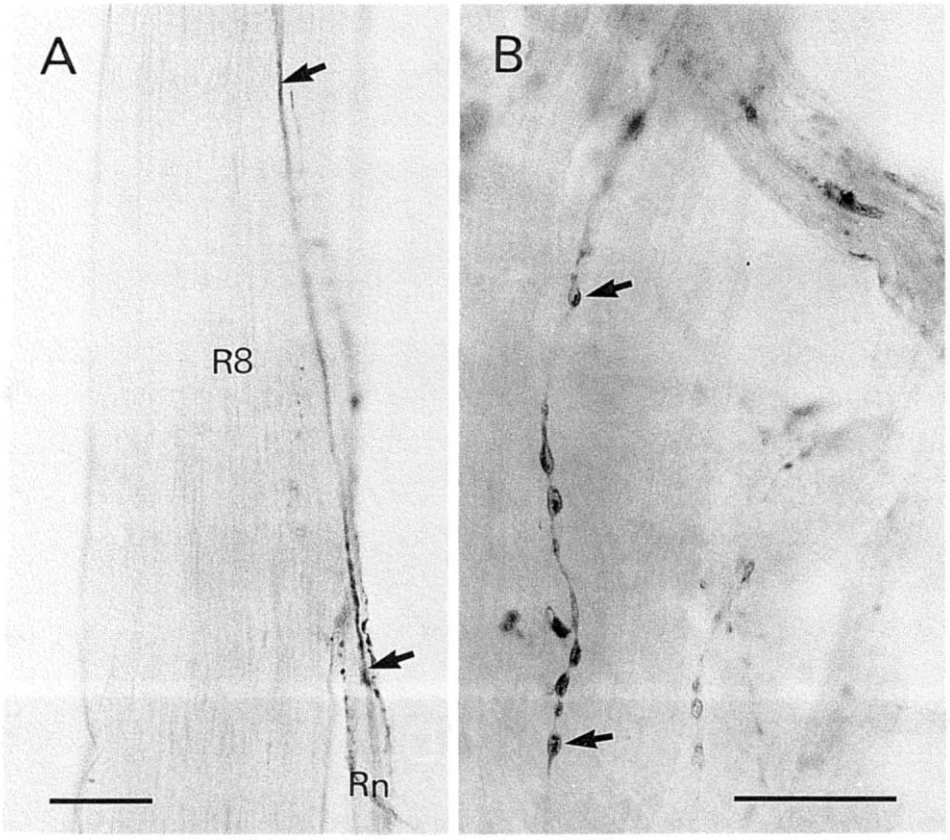

FIG. 4. RFamide-like immunoreactivity in whole-mount preparations of the rectal nerve and the rectum. (A) The immunoreactive fiber (arrows) running into the rectal nerve (Rn) from the TAG *via* the eighth nerve root (R8). (B) Immunoreactive varicose fibers in the rectum, which arise from a branch of the rectal nerve. Bar, 50 μm.

rectal nerves, and then stained with anti-RFamide antiserum. The result showed that the neuron stained by back-filled Lucifer Yellow in the ventro-midline of A9 (Fig. 5C) was one of the two RFaLI-VM neurons (Fig. 5D). Simultaneous back-filling from the left and right rectal nerves with nickel and cobalt, respectively, resulted in differential staining of the neurons corresponding to the VM neurons: one appeared blue (stained by Ni^{2+}) and the other was yellow (stained by Co^{2+}) (Fig. 5B). These results indicate that the VM neurons in the midline of neuromere A9 of the TAG are not ventral unpaired median neurons, but rather are paired neurons, each of which monolaterally projects the RFaLI fiber to the visceral muscle of the rectum.

RFamide-like immunoreactive peripheral neurosecretory cells on the segmental nerve roots of the TAG

Two peripheral neurosecretory cells, PNCs, were attached to each dorsal nerve root (R1, R3, R5 and R7) spreading from the TAG in both sexes. These PNCs were close together and located in the vicinity of the first bifurcation of the nerve root. Their size, shape and exact position varied greatly among preparations. The anti-RFamide antiserum revealed varicose immunoreactive fibers in the neural sheath of all of the dorsal nerve roots (Fig. 6), but not in the ventral nerves (R2, R4, R6 and R8). In addition to these immunoreactive varicose fibers, the PNCs on R5 (PNCs-R5) and R7 (PNCs-R7) exhibited strong RFamide-like immunoreactivity, while those on R1 (PNCs-R1) and R3 (PNCs-R3) did not (Fig. 6). PNCs-R5 and -R7 projected immunoreactive thick axons along the surface of the nerve root toward the periphery. Electron microscopic observation of the PNCs-R5 showed that the perikaryon contained numerous ribosomes and a large number of Golgi bodies associated with electron-dense granular vesicles with a diameter of about 140 nm (Fig. 7). RFamide-like immunoreactivity was restricted to the granular vesicles (Fig. 7). These observations indicate that the RFaLI cells on R5 and R7 of TAG are neurosecretory cells which produce the RFamide-like peptide. The materials contained in PNCs-R1 and PNCs-R3 were not characterized in the present study (data not shown).

Retrograde staining from the second nerve branch of R5 (R5Br2), along which the PNCs-R5 project their axons, revealed a fine process projecting from one of the PNCs-R5 into the thin link nerve connecting R5 with R6 (Fig. 9). To further examine the morphology and physiological properties of the RFaLI-PNCs, intracellular recording and dye-injection with Lucifer Yellow were performed. The experiments were mainly carried out on the PNCs-R5, because they were more accessible than the PNCs-R7. Intracellular recordings

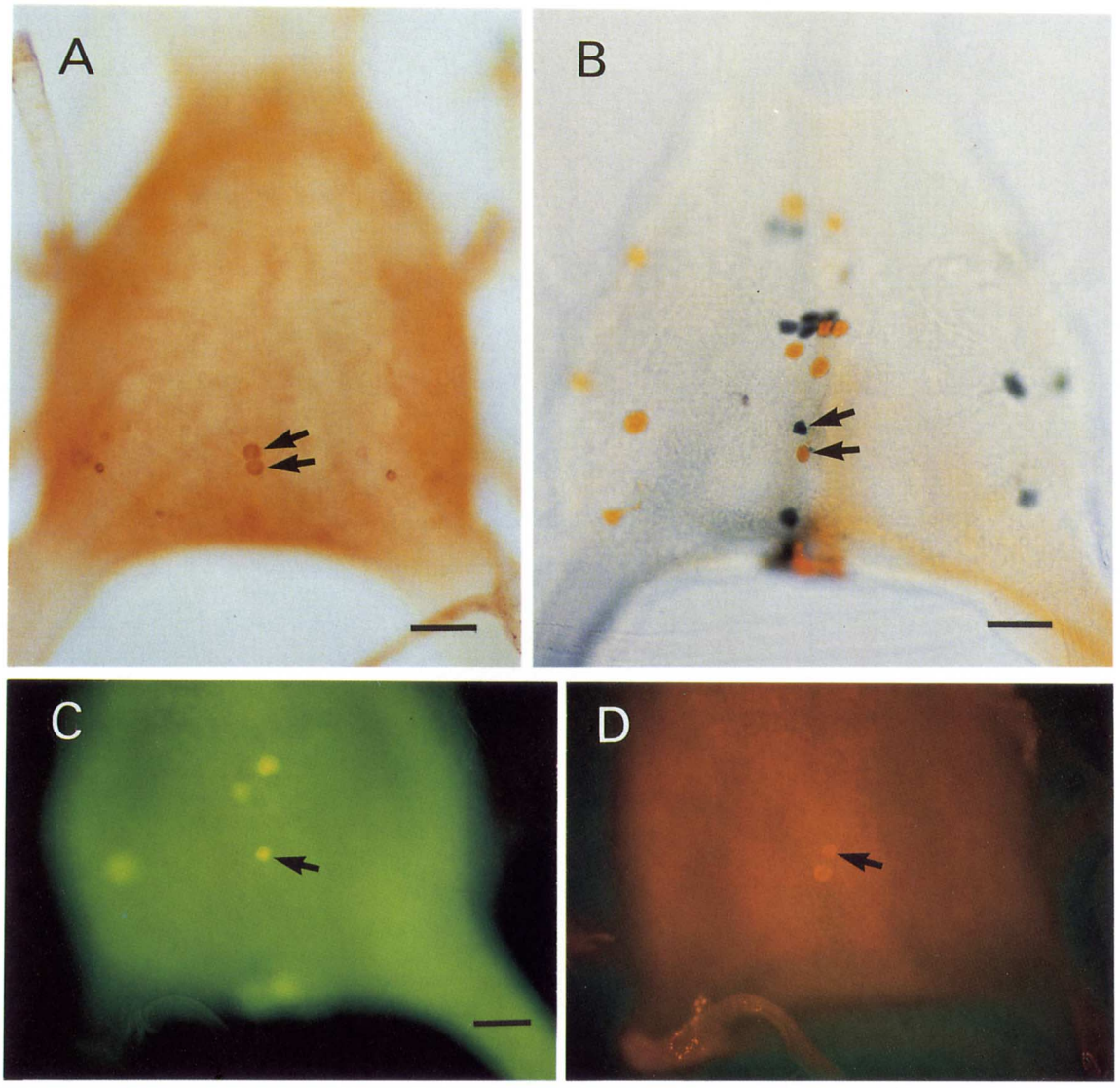

FIG. 5. Ventral views of whole-mount preparations of the female TAG. (A) A preparation stained by the ABC method, showing two RFamide-like immunoreactive cell bodies (arrows) in the ventro-medial region of neuromere A9. (B) A preparation stained by the differential back-filling technique. The right and left rectal nerves were back-filled with nickel and cobalt, respectively. Arrows indicate the two differentially stained cell bodies (blue and yellow) corresponding to the RFamide-like immunoreactive cell bodies shown in A. (C, D) A preparation stained by the double-labeling technique which combined an immunofluorescence method using rhodamine-conjugated secondary antibody with Lucifer Yellow back-filling. C shows the cell bodies stained by Lucifer Yellow. D indicates the RFamide-like immunoreactive cell bodies in the ventro-medial region of neuromere A9. Note that one of the two immunoreactive cell bodies (arrow in D) is stained with back-filled Lucifer Yellow (arrow in C). Bar, 100 μm.

from the cell bodies revealed spontaneous action potentials at a frequency of 2–10 Hz. These action potentials had long-lasting after-potentials, an amplitude of 20–40 mV, and a duration (measured at half of the peak amplitude) of 3–4 msec (Fig. 8). The long duration of the action potentials may be an electro-physiological characteristic of neurosecretory cells [20]. Intracellularly stained thick axons of the PNCs-R5 ran toward the periphery along the R5Br2 (Fig. 10). Some of the stained axons ended in the neural sheath of the R5Br2, with a swollen appearance on the way to the nerve branch (Fig. 10A, B). This was usually observed in axons from the proximal PNCs-R5. In another case, the stained axons were traced to the vicinity of the heart (Fig. 10C, D). This often occurred in axons from the distal PNCs-R5. Unfortunately, we could not confirm whether the stained axon of the latter PNCs-R5 reached the heart. Immunocytochemistry using anti-RFamide antiserum revealed RFaLI varicose fibers on the cardiac and alary muscles of the heart (data not shown). However, the present study did not clarify whether PNCs-R5 play a role in regulating the activity of the heart.

Intracellular dye-injection showed several fine neural processes arising from the somata of PNCs-R5. The stained fine processes extended distally along the first nerve branch of

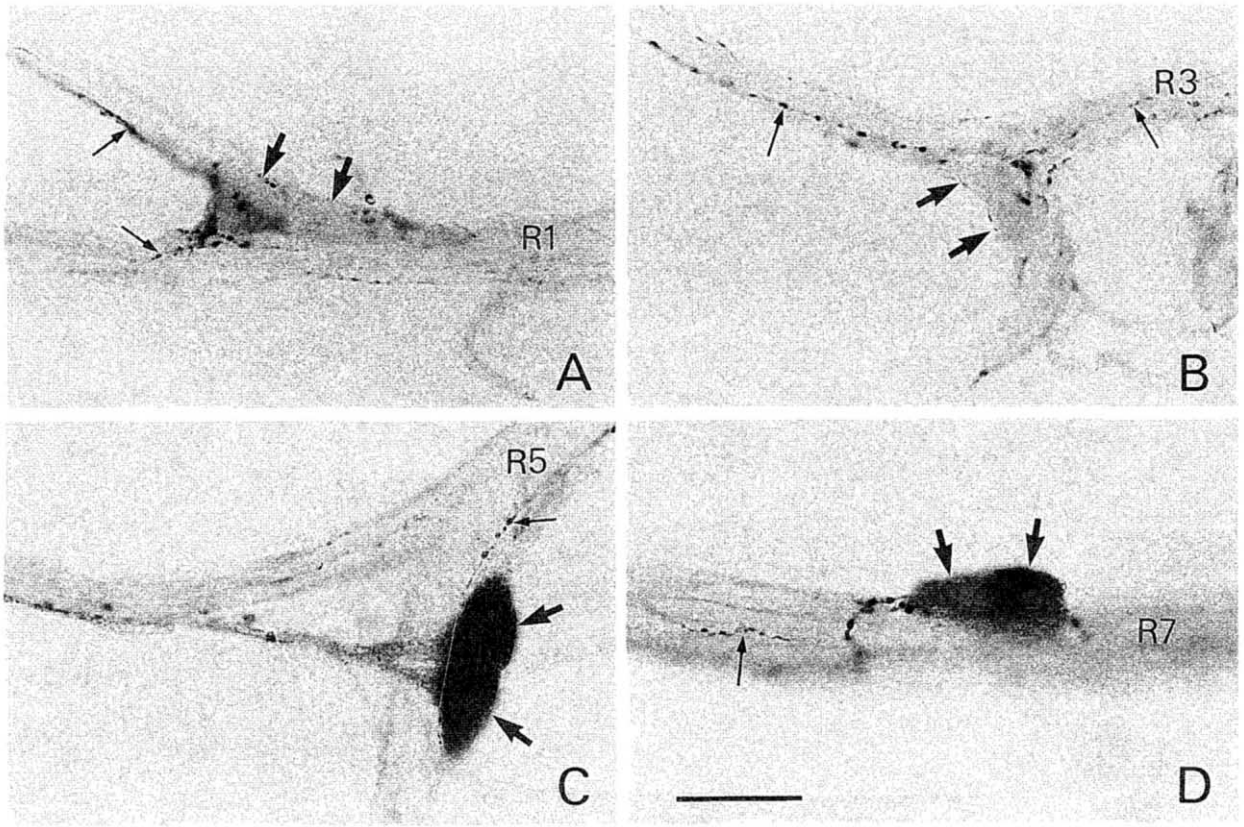

FIG. 6. RFamide-like immunoreactivity in whole-mount preparations of the dorsal nerve roots of the TAG. Two peripheral neurons (thick arrows) occur in the vicinity of the first bifurcation of the nerve roots. Of these peripheral neurons, the cell bodies attached to the fifth (R5 in C) and the seventh (R7 in D) nerve roots showed strong immunoreactivity, while those on the first (R1 in A) and third (R3 in B) nerve roots did not. Note that immunoreactive varicose fibers (thin arrows) are seen in the neural sheaths of R1 (A) and R3 (B), as well as in those of R5 (C) and R7 (D). Bar, 50 μm.

R5 (R5Br1), along the thin link nerve connecting R5 with R6 (Fig. 10E) as revealed by retrograde staining (Fig. 9), or proximally along R5 toward the TAG (Fig. 10D). However, the stained processes from the PNCs did not project into the TAG. These observations suggest that the PNCs may form widely dispersed peripheral release areas along the dorsal nerves for RFamide-like peptides. The origins of the RFamide-like immunoreactive fibers in nerve roots R1 and R3 (Fig. 6A, B), on which the PNCs did not exhibit any apparent immunoreactivity, are still unknown. These fibers may originate from the neurosecretory cells in the TAG. In addition to the paired lateral nerve roots (R1-R8), the paired median nerves arising from the dorsal surface of the TAG showed strong RFamide-like immunoreactivity (Fig. 2B).

DISCUSSION

Location of RFamide-like immunoreactive neurons in the abdominal ganglia

Several studies have examined the distribution of FMRFamide-like immunoreactive neurons in insect nervous systems [3, 5, 10, 15, 28, 36, 47, 48]. Most of these studies have been confined to the brain, thoracic ganglia and retrocerebral endocrine system. Only a few studies have described immunoreactivity in the abdominal ganglia, including the fused thoraco-abdominal ganglion [13, 24, 26, 27, 45, 49]. In the locust, *Schistocerca*, bovine pancreatic polypeptide/FMRFamide-like immunoreactive cells lie along the midline of the abdominal ganglia, and no bilaterally paired neurons are found in the ganglia, including the TAG [26]. In contrast, in *Locusta*, both of the bilateral and the medial bovine pancreatic polypeptide/FMRFamide-like immunoreactive neuron groups are found in the unfused abdominal ganglion [13]. Bilaterally symmetric and medially located immunoreactive neurons are also seen in the fly thoraco-abdominal ganglia [24], as well as in the mesothoracic ganglionic mass in *Rhodnius* [45]. The present study showed in the female cricket TAG that over 60 RFaLI neurons are located along the midline and bilaterally in the neuromeres (Figs. 1 and 2). Descriptions of RFaLI neurons in the TAG are still rare in hemimetabolous insects.

Distribution of RFamide-like immunoreactive fibers in visceral tissues

The locust oviduct has been shown to contain FMRFamide-like peptides [37]. Studies on the effects of FaRPs on

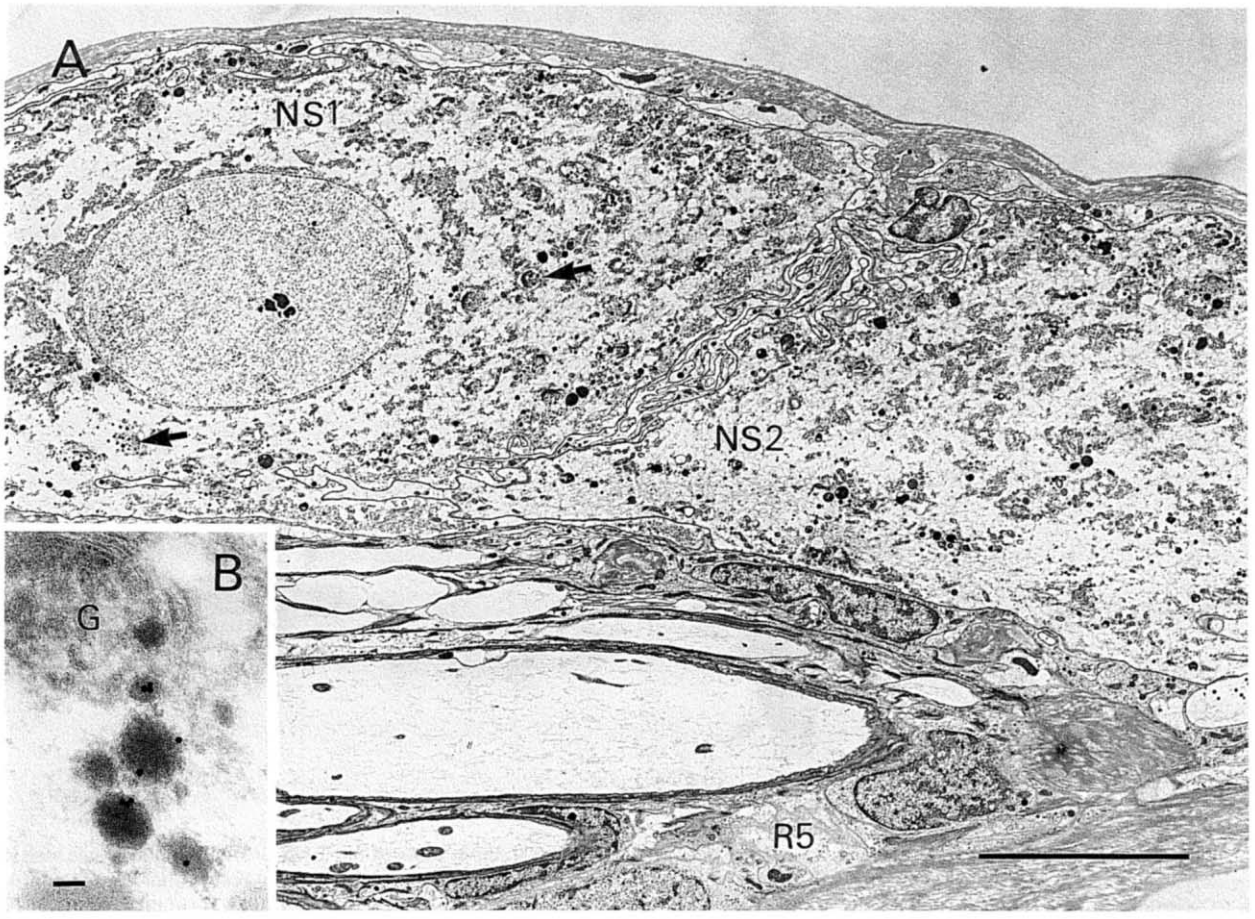

FIG. 7. (A) An electron microphotograph of the two peripheral neurosecretory cells (NS1 and NS2) on the fifth nerve root (R5) of the TAG. The adjacent cytoplasmic membranes of the two cells are infolded. In the perikaryon, Golgi bodies (arrows) and numerous free ribosomes are prominent. Bar, 10 μm. (B) RFamide-like immunoreactive vesicles associated with the Golgi body (G) in the perikaryon of the peripheral neurosecretory cell. Immunogold labeling was restricted to the vesicles. Bar, 0.1 μm.

FIG. 8. Endogenous action potentials recorded from the RFamide-like immunoreactive peripheral neurosecretory cell located on the fifth nerve root of the TAG. Note the long duration and long-lasting hyperpolarizing after-potential.

contraction of the locust oviduct have indicated that FMRF-amide has an excitatory effect on myogenic contraction and increases basal tension [12, 32]. In the present study, we found a dense plexus of RFaLI varicose fibers surrounding the cricket oviduct (Fig. 3A, B). FMRFamide has an excitatory effect on spontaneous contraction of the cricket oviduct (Chen and Yamaguchi, in preparation). These facts suggest that FaRPs probably act as locally-released neuromodulators in the cricket oviduct, as well as in the locust oviduct.

The present study did not identify which neurons project their RFaLI axons to the oviduct. Whole-mount immunocytochemistry revealed a dorsal and a ventral medial neuron in neuromere A7, from which the nerve roots (R2s) innervating the oviduct arise, in addition to bilaterally paired neurons (Fig. 1). The dorsal medial RFaLI neurons located close to the region where the paired median nerves leave the TAG may contribute to the immunoreactivity in the median nerves. Further studies using double-labeling with immunocytochemistry and back-filling are required to identify the RFaLI neurons which innervate the cricket oviduct.

Hustert and Topel [16] have shown in the cricket that serotonin-like immunoreactive fibers innervate the proximal region of the spermathecal duct. We have also briefly described in the cricket [53] that serotonin-like immunoreactive fibers are distributed in the proximal region of the spermathecal duct, while proctolin-like immunoreactivity is found extensively throughout the entire length of this duct. In the present experiment, we did not detect RFamide-like peptides in the spermatheca or spermathecal duct.

The hindgut of the blowfly has been reported to be

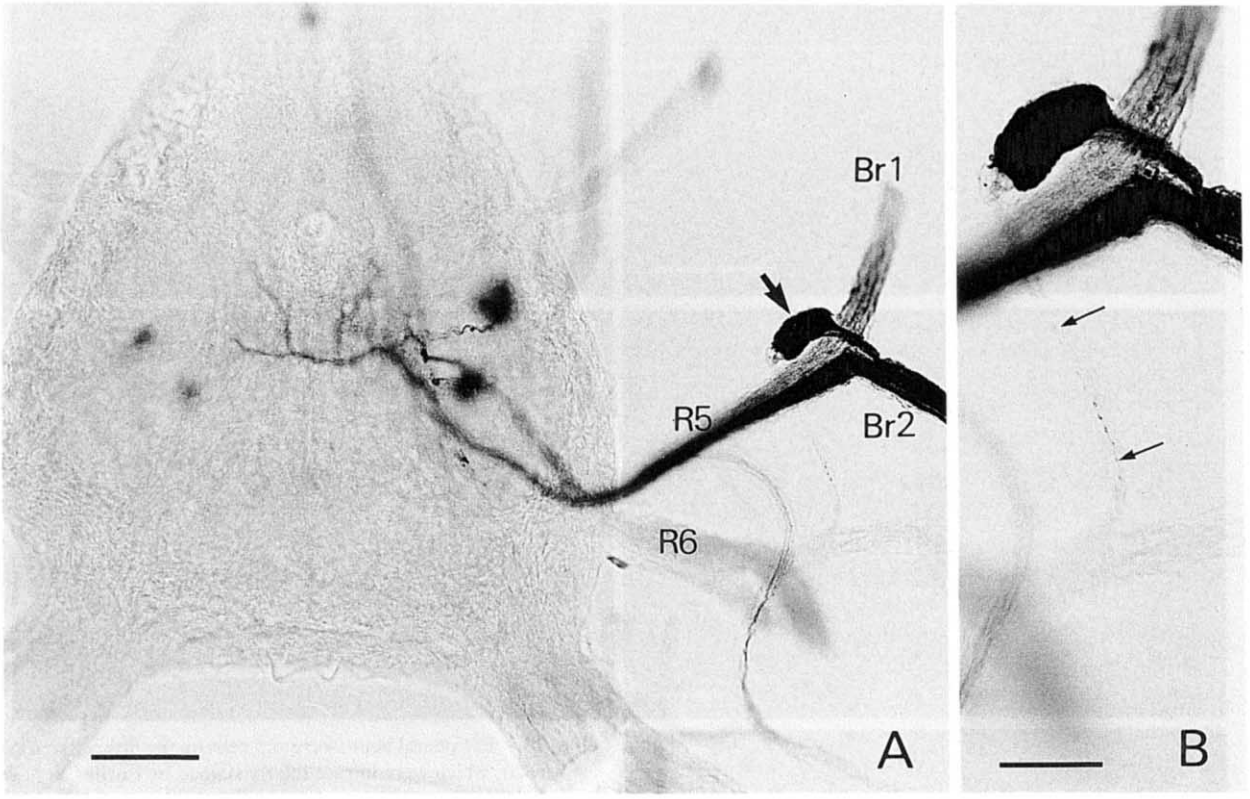

FIG. 9. A dorsal view of a whole-mount preparation of the TAG, which was back-filled with nickel through nerve branch 2 (Br2) of the right fifth nerve root (R5). (A) Two cell bodies (thick arrow) attached to R5 were stained, as well as several cell bodies within the ganglion. Bar, 100 μm. (B) A magnified view of a fine neural process (thin arrows) arising from one of the stained cell bodies and running to the sixth nerve root (R6) *via* the link nerve connecting R5 to R6. Br1, nerve branch 1 of the fifth nerve root. Bar, 50 μm.

innervated by two different sets of neurons displaying proctolin- and FMRFamide-like immunoreactivity in the fused abdominal ganglion [4]. Our immunocytochemical results reveal a plexus of RFaLI fibers in the rectum (Fig. 4B). These fibers were demonstrated to originate from the paired ventral medial neurons in neuromere A9 of the TAG (Fig. 5). Concerning innervation of the cricket hindgut, Klemm *et al.* [18] reported that serotonin-like immunoreactive neurons located medially at the posterior end of the TAG project axons to the hindgut *via* the rectal nerve. Proctolin-like immunoreactivity has been reported in the cockroach hindgut [9], though it is unclear whether proctolin is present in the cricket hindgut. These observations imply the dual peptidergic innervation of the hindgut of hemimetabolous insects, and also suggest a possible interaction among serotonin, proctolin and FMRFamide in the regulation or modulation of the hindgut, as has been described in the locust foregut: FMRFamide causes contraction of the foregut, inhibits proctolin-induced contraction, and potentiates serotonin-induced relaxation [2].

RFamide-like immunoreactive peripheral neurosecretory cells

In several insects, FMRFamide-like immunoreactivity has been identified in median neurosecretory cells in the cephalic ganglia as well as in the corpora cardiaca [3, 5, 10, 15, 28, 45, 47], suggesting that FaRPs may function as neurohormones through their release into the general circulation. Peripheral neurohaemal areas where FMRFamide-like immunoreactive varicose fibers form plexuses have also been described in the neural sheath of the ganglia [24, 29, 46], in that of the thoracic median or paired nerves [27, 46], and in that of the abdominal paired nerve [25, 45]. In most cases, the immunoreactivity has been attributed to neurons in the central nervous system.

In the present study, RFaLI peripheral neurosecretory cells were located on the segmental nerve roots: two cell bodies were found in each dorsal nerve, and the cell bodies on R5 and R7 exhibited RFamide-like immunoreactivity (Fig. 6). Electron microscopic and electrophysiological studies demonstrated that the PNCs-R5 produce RFamide-like peptides, and that they project their main axons toward the heart along the dorsal nerve root, but not to the TAG (Fig. 10). Application of FMRFamide to the cricket heart results in an increase in the frequency of spontaneous contraction (Chen, unpublished data). RFamide-like peptides in the identified PNCs may be released as local neurohormones to modulate the activity of visceral muscles such as the heart and alimentary canal.

Some peptides are known to occur in PNCs, e.g., glucagon in the abdominal latero-ventral PNCs in the link

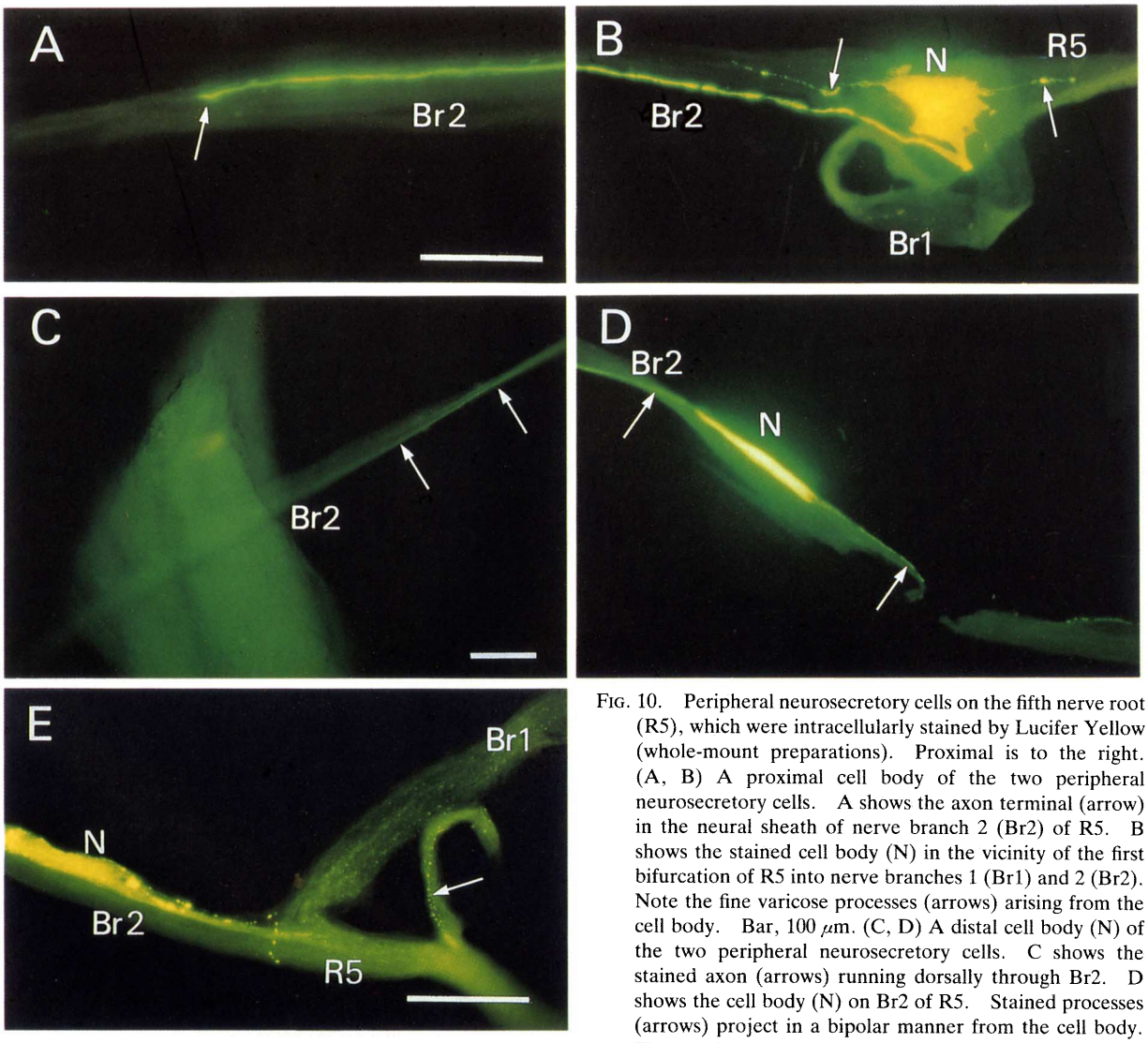

FIG. 10. Peripheral neurosecretory cells on the fifth nerve root (R5), which were intracellularly stained by Lucifer Yellow (whole-mount preparations). Proximal is to the right. (A, B) A proximal cell body of the two peripheral neurosecretory cells. A shows the axon terminal (arrow) in the neural sheath of nerve branch 2 (Br2) of R5. B shows the stained cell body (N) in the vicinity of the first bifurcation of R5 into nerve branches 1 (Br1) and 2 (Br2). Note the fine varicose processes (arrows) arising from the cell body. Bar, 100 μm. (C, D) A distal cell body (N) of the two peripheral neurosecretory cells. C shows the stained axon (arrows) running dorsally through Br2. D shows the cell body (N) on Br2 of R5. Stained processes (arrows) project in a bipolar manner from the cell body. The proximally projecting process (indicated by the right arrow) did not reach the TAG. Note that the shape of the cell bodies varied between individuals. Bar, 100 μm. (E) Stained neural processes arising from the cell body (N). Varicose processes are seen in the neural sheath of R5, Br1, and in the link nerve (arrow) connecting R5 to the sixth nerve root of the TAG. Bar, 100 μm.

nerve region of the locust, the cockroach and the stick insect [35], bursicon in the PNCs in the neck region of the cricket [14], and bovine pancreatic polypeptide in the PNCs in the neurohaemal area of the locust thoracic transverse nerve [27]. We applied an antiserum against glucagon to characterize PNCs-R1 and PNCs-R3, but no staining was detected in any of the PNCs associated with the dorsal nerve roots (Yasuyama, unpublished observation). The neurosecretory materials produced by PNCs-R1 and PNCs-R3 were not identified in the present study. PNCs-R5 were positively stained with neutral red (Yasuyama, unpublished observation), which selectively stains monoamine-containing neurons [11, 43]. This suggests that RFaLI-PNCs might also contain the biogenic amine octopamine. Stevenson and Pflüger [41] reported the colocalization of octopamine and FaRP in the locust DUM heart-1, which projects to the heart and associated alary muscles.

The antiserum we used revealed varicose immunoreactive fibers in the neural sheath of all of the dorsal nerves, in addition to PNCs-R5 and -R7 (Fig. 6), while immunoreactive fibers were not seen in the ventral nerves of the TAG. However, we could not determine the origins of the immunoreactivity in the dorsal nerves, R1 and R3, which are not associated with immunoreactive PNCs. Some of the paired RFaLI neurons located in the TAG (Figs. 1 and 2) may contribute to the immunoreactivity found in these nerve roots.

In summary, the present study showed that RFamide-like immunoreactivity is distributed in 60 or more cell bodies as well as in the neuropil regions of the TAG, and in several peripheral sites, including the oviduct, rectum and abdominal peripheral neurosecretory cells. These observations indi-

cate that this RFamide-like peptide probably has a variety of different roles: it may act as a neurotransmitter substance for interneurons in the TAG, as a neuromodulator substance in the innervation of visceral tissues, and as a neurohormone released from peripheral neurosecretory cells.

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