Immunohistochemical study on peptidergic neurons containing FMRFamide in the stomatogastric nervous system of the American cockroach

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(Received 20 June 1997; Accepted 11 August 1997)

Abstract

We investigated the fine structure of Phe-Met-Arg-Phe(FMRF)amide-containing peptidergic neurons in the stomatogastric nervous system of the American cockroach by immunoelectron microscopy. Immunoreactive cell bodies were located in both the ingluvial and proventricular ganglia. Besides those ganglionic cells, several extranganglionic cell bodies within the esophageal and ingluvial nerves also contained this peptide. Immunopositive nerve fibers bearing bead-like swellings or varicosities, ran mainly along the circular muscle cells in the foregut, but no specialized neuromuscular junctions were found. The fibers contained large core vesicles (100 and 180 nm in diameter) which were thought to package this peptide, as well as small clear vesicles. These results suggest that the peptidergic neurons might have plural types of neurotransmitters, and innervate the musculature in a non-synaptic manner.

Key words: FMRFamide, stomatogastric nervous system, foregut, cockroach, immunohistochemistry

INTRODUCTION

The alimentary tract of insects consists of three parts: foregut, midgut and hindgut. The fore- and hindguts are ectoderm, and the midgut is endoderm in origin. The musculature system is comprised of striated muscle cells, unlike that of the vertebrate system. The foregut and anterior midgut are innervated by the stomatogastric nervous system which is connected with the central nervous system through the recurrent nerve via the frontal ganglion.

In the nerve fibers of the cockroach midgut, we have found various neuropeptides immunoreactive to antisera against VIP, gastrin, somatostatin, enkephalin, pancreatic polypeptide and Phe-Met-Arg-Phe(FMRF)amide (Iwanaga et al., 1981; Endo et al., 1982; Žitnán et al., 1993). Among these neuropeptides, FMRFamide-like peptides are widely distributed in the nervous system of the animal kingdom, and was originally isolated as molluscan heart-activating factor from a bivalvulous central nervous system (Price and Greenberg, 1977). It is also the same in many insect species in which the central and peripheral nervous systems have this peptide or a related one (Veenstra and Schooneveld, 1984; Carroll et al., 1986; White et al., 1986; Tsang and Orchard, 1991; Yasuyama et al., 1993; Stevenson and Pflüger, 1994; Ude and Agricola, 1995; Helle et al., 1995; Fusé et al., 1996). In the stomatogastric nervous system, localization of this peptide was reported in the wax moth (Žitnán et al., 1989) and the blowfly (Cantera and Nässel, 1991) by light microscopic immunohistochemistry. Regarding the function of FMRFamide, it was reported that in the foregut of locusts this peptide had a role as a contracting factor, but, at high concentration, it enhanced the action of 5HT as a relaxation factor and suppressed the action of proctolin as a contracting factor (Banner and Osborne, 1989).

These reports suggest that the muscle cells of the insect gut might be innervated by plural and complex nerve fibers, and some neuropeptides might serve not only as direct neurotransmitters but also as modulating factors. There is, however, little data on the fine structure of the neuromuscular junction and the location of those peptidergic nerve cell bodies. In the present study, we report the ultrastructure of peptidergic neurons containing FMRFamide in the cockroach foregut, with special reference to
the location of cell bodies and their terminal structure.

**MATERIALS AND METHODS**

The American cockroach, *Periplaneta americana*, was reared under a 12L–12D photoregime at 25°C on an artificial diet for rats (Oriental Yeast Co., MF) and water ad lib. Nymphs (within 24 h after the final moult) and adults (mainly males) were anesthetized by carbon dioxide gas and dissected in phosphate-buffered saline (PBS, 10 mM sodium phosphate, 0.9% NaCl, pH 7.2) during the light photophase. For light microscopic immunohistochemistry, the excised tissues were fixed with a mixture of 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB), pH 7.4 for 2.5 h. For immunoelectron microscopy, a final concentration of 0.2% glutaraldehyde was added to the above fixative.

For whole mount preparations, the fixed tissues were rinsed in several changes of PBS containing 0.3% Triton X-100 (TBS) for 20 h. After blocking with 5% skim milk in TBS for 1 h to reduce non-specific binding of antibodies, the tissues were incubated in rabbit anti-FMRFamide antiserum (Cambridge Research Biochemicals) diluted to 1:2,000 with TBS for 20 h at 4°C. Immunoreaction was visualized by the avidin-biotin peroxidase method (Vectastain ABC kit). In order to confirm the number of immunopositive cell bodies, ganglia from the stomatogastric nervous system of adults were embedded in paraffin and cut at 10 μm thick serial sections.

For immunoelectron microscopy, the fixed tissues were washed in several changes of 0.1 M PB, then soaked in PB containing 30% sucrose and 0.01% sodium azide, and 10 μm frozen sections were cut with a cryotome. After being mounted on chrom-alum gelatin coated slide, the sections were immunostained with anti-FMRFamide antiserum in the same manner as for whole mount preparations. After coloration by diaminobenzidine, the sections were fixed with 2.5% glutaraldehyde in 0.1 M PB for 1 h, followed by postfixation with 1% osmium tetroxide for 1 h. After dehydration in an ethanol series, gelatin capsules filled with epoxy resin (Epon 812) were put on the sections up-side-down and incubated at 60°C overnight to polymerize the resin. The epoxy blocks containing sections were mechanically detached from the glass slide, which was briefly heated in advance with a gas burner. Thin sections (50–100 nm) were cut with an ultramicrotome (Sorval MT 6000), stained with lead citrate, and examined with JEOL 100C and 1220 electron microscopes at an accelerating voltage of 80 kV.

The specificity of immunostaining was checked by the displacement of the first antiserum to normal rabbit serum.

**RESULTS**

**Light microscopic observations**

Cell bodies immunoreactive to FMRFamide were found in both the ingluvial and proventricular ganglia of the stomatogastric nervous system of the American cockroach. The ingluvial ganglion was located singly on the dorsal midline of the central foregut. This ganglion of the adult cockroach contained 30–40 immunopositive cell bodies (mean ± SD; 34.7 ± 4.15, n = 7 whole mount preparations), which were scattered throughout, especially the caudal region of the triangle-shaped ganglion (Fig. 1a). As the cell number in the whole mount preparations might be underestimated because of layering, we examined serial sections of this ganglion and counted 44 immunopositive cell bodies, although there was only one datum. These cell bodies were round in shape (about 30 μm in diameter) and their nerve fibers could not be clearly traced.

In the whole mount preparations of nymphs, we found similar numbers of immunopositive cell bodies in the ingluvial ganglion as follows: 33 and 33 cell bodies in 1st stadium nymphs; 35 and 34 in 2nd stadium; and 35 and 31 in 3rd stadium. These results indicated that the cell number of neurons containing FMRFamide was constant in the ingluvial ganglion during nymphal and adult stages. The size of the neurons and whole ganglion, however, was only half that of the adult cockroach (Fig. 2a).

A pair of proventricular ganglia were located at the lateral sides of the posterior foregut. Each ganglion of the adult cockroach contained about 20 immunopositive cell bodies (mean ± SD; 17.0 ± 1.67, n = 13 whole mount prepara-
Fig. 1. FMRFamide-immunoreactive neurons in the foregut of the adult cockroach (whole mount preparations). a: Triangle-shaped ingluvial ganglion contains 30–40 immunopositive cell bodies and numerous fibers; b: Spindle-shaped proventricular ganglion contains ~30 cell bodies in the periphery and varicose fibers in the middle; c: Extraganglionic immunopositive cell body (arrow) and varicose fibers in the ingluvial nerve between frontal and ingluvial ganglia; d: Immunopositive varicose fibers run along the circular muscle cells. bar = 100 μm.

Fig. 2. FMRFamide-immunoreactive neurons in the foregut of 1st stadium cockroach (whole mount preparations). Ingluvial ganglion (a) and proventricular ganglia (b) are small in size, but the number of positive cell bodies is similar to that of adults. bar = 100 μm.

Numerous immunopositive nerve fibers were found not only within the ganglia and thick nerve bundles (esophageal and ingluvial nerves), but also in the musculature (Fig. 1a–d). Thin nerve fibers bearing bead-like swellings, called varicosities, ran mainly in a lateral direction, suggesting that they might be associated with circular muscle cells (Fig. 1d). In the esophagus, there were only a few nerve fibers running longitudinally. We could not find the motor endplates which are generally seen in the neuromuscular junctions of the striated muscles of vertebrates.

In the midgut and hindgut, there were numerous immunopositive fibers found in the musculature (Fig. 3a, b). In the midgut, relatively thick nerve bundles ran longitudinally along the longitudinal muscle fibers, and thin ramifications ran laterally (Fig. 3a). In the hindgut, the pattern of nerve bundles was much more irregular than that of the midgut (Fig. 3b). In both the mid- and hindguts, thin immunopositive nerve fibers bore the bead-like swellings (varicosities), but we could not find cell bodies in those areas, except for the endocrine cells (paraneurons) in the midgut epithelium (Fig. 3a). As far as we could observe, there was no special relationship between the nerve fibers...
and paraneurons containing FMRFamide.

**Electron microscopic observations**

Conventional electron microscopy showed that the ingluvial ganglion of the adult cockroach has at least 4 types of nerve fibers in the neuropile, based on the size and electron density of the inclusion of vesicles (Fig. 4a, b, c). We tentatively classified and designated them as types 1 to 4. Type 1 fibers contained large dense core vesicles (150–180 nm in diameter), small electron-lucent core vesicles (75 nm) and small clear vesicles (50 nm). Type 2 had only small electron-lucent core vesicles and small clear vesicles. Type 3 had large granulous core vesicles (180 nm), medium granulous core vesicles (100 nm) and small clear vesicles. Type 4 had large electron-lucent core vesicles (120 nm) and small clear vesicles.

In addition to these vesicles, nerve fibers had mitochondria, smooth-surfaced endoplasmic reticulum, and cytoskeletons (microtubules and neurofilaments).

Immunoelectron microscopy using a pre-embedding method showed that the nerve fibers immunoreactive to FMRFamide contained 180 and 100 nm large core vesicles and small clear vesicles (Fig. 4d), indicating the fine structural characteristics of type 3. We could not find specialized neuromuscular junctions in the foregut musculature. Varicosities were found along the circular muscle cells.

**DISCUSSION**

In the present study, we demonstrated the fine structure of peptidergic neurons containing FMRFamide in the stomatogastric nervous system of the cockroach. Although it has been reported to date that several kinds of peptides regulate the functions of the insect alimentary tract (Banner and Osborne, 1989; Nachman et al., 1993), there is little related morphological data available, especially at the electron microscopic level.

According to a physiological study by Banner et al. (1989), FMRFamide plays not only a direct role in contraction, but also has roles as modulating factors of 5HT and proctolin receptors in the locust foregut. The present immunoelectron microscopic study supports the idea that this peptide may have multifunctions in the musculature system of the insect gut, because we could not find a specific relationship between the immunoreactive nerve fibers and muscle cells.

In addition, it is very interesting that FMRFamide-immunoreactive nerve fibers contained at least three morphologically different kinds of vesicles. Although the immunoreactivity appears to be diffused in the axoplasm for the pre-embedding method used, this peptide appeared to be packaged into 100 or 180 nm large core vesicles. It was consistent with the data of Ude and Agricola (1995) who classified 6 types of FMRFamide-containing granules based on
the range of density and diameter (60–90, 60–90, 120–180, 150–180, 120, 150 nm) in the lateral heart nerve of the American cockroach. In addition to those core vesicles, we found that FMRFamide-containing nerve fibers had small clear vesicles that might be generally accepted to contain small molecular neurotransmitters such as acetylcholine, glutamate, aspartate and GABA etc. These data suggest that the peptidergic neurons containing FMRFamide might have at least two or three different kinds of chemical messengers such as acetylcholine and/or amino acids, amines, and peptides. Further immuno- and enzyme-cytochemical electron microscopic studies should be carried out to demonstrate such co-localization.

The fact that no specialized neuromuscular junctions were found in our observations suggests the presence of non-synaptic regulation in the musculature system in the cockroach intestine. Helle et al. (1995) proposed that FMRFamide might be released from putative neurohemal areas such as the varicose fiber plexuses located in the superficial position of the ventral nerve cord of crickets. We also demonstrated the non-synaptic release of large core ves-
icles from various peripheral nervous systems of vertebrates (Endo, 1988 a, b; Endo et al., 1991). It is likely that the neuronal varicosities located near the muscle cells might function as a site for releasing neurotransmitters and/or neuromodulators in the stomatogastric nervous system of the cockroach.

In the cockroach midgut, FMRFamide-containing nerve fibers run relatively close to FMRFamide-containing endocrine cells in the epithelium. Those nerve fibers are thought to be extrinsic in origin, perhaps originating from the stomatogastric nervous system, because we could not find their cell bodies in the midgut. As the nerve and endocrine elements share similar bioactive substances, they might play a similar role physiologically, such as in digestion and motility, but the functional relationship between those nerve fibers and endocrine cells remains to be elucidated.

REFERENCES


