Proctolin in the Brain and Ganglia of *Triatoma infestans* (Hemiptera: Reduviidae)

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ABSTRACT The distribution of proctolin in the central nervous system of the hemipteran bug, *Triatoma infestans*, was studied by immunohistochemistry using the sensitive avidin-biotin technique combined with nickel salt intensification of the reaction product. Proctolin was present in cells and fibers of the brain and ganglia. In the brain, protocerebral proctolin-immunoreactive cell bodies were found in the pars intercerebralis, the optic lobes, and the lateral soma rind. The deutocerebrum showed positive somata in relation to the antennal motor center and the tritocerebrum had intense immunoreactive fibers but few positive cells.

Proctolin-immunoreactive cell bodies of different sizes were observed in the subesophageal ganglion. Large cell bodies were found mainly rostrally and beaded positive processes were present around the ventral border of the esophageal foramen and in the rostrolateral neuropil of this ganglion.

Small- to medium-sized positive somata were found in the posterior part of the prothoracic ganglion; some of these cells were sending immunoreactive processes to the central neuropil. The meso-metathoracic-abdominal ganglionic mass showed positive cells in all the neuromeres, where some of them were large and had thick immunoreactive granules.

The results show that the labeling pattern of proctolin-like immunoreactivity in *T. i.* appears to be widespread and unique for its central nervous system. It is suggested that proctolin may serve neuroendocrine, integrative, and motor functions in the brain of *T. infestans*. J. Morphol. 240:39–47, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS: insect; central nervous system; Chagas' disease; immunohistochemistry; neuropeptides

The blood-feeding hemipteran *Triatoma infestans* is the main vector in Argentina of *Trypanosoma cruzi*, the causative agent of Chagas' disease. Despite its epidemiological importance in South American countries, there are only few reports on the morphology and histochemistry of *T. infestans* nervous system (Barth, ’75; Insauti, ’94, ’96; Villar et al., ’94).

Proctolin (PROC, H-Arg-Tyr-Leu-Pro-Thr-OH) was isolated from the hindgut of the cockroach *Periplaneta americana* (Bishop and O’Shea, ’80; Bishop et al., ’81) and is one of the first peptides whose bioactivity has been extensively studied in insects (Lange et al., ’86; Sláma et al., ’93; Goudey-Perrière et al., ’94; King et al., ’95). This pentapeptide was shown to be widely distributed among arthropod species, and has been found in the reproductive and digestive organs of *Rhodnius prolixus* (Orchard et al., ’89), another triatomine vector of Chagas' disease. PROC-immunoreactive (PROC-IR) cell bodies and fibers were recognized in the central nervous system (CNS) of *R. prolixus* using in toto preparations (Lange et al., ’88). In an attempt to further expand the knowledge on
the presence of PROC-like immunoreactivity (PROC-LI) in the CNS of triatomines, we have employed the sensitive avidin-biotin complex technique combined with nickel-salt intensification of the reaction product in T. infestans. Our results confirm the presence of PROC-LI in previously reported centers and show its localization in other hitherto unknown areas.

**MATERIALS AND METHODS**

Forty adult male T. infestans from our colony, free of T. cruzi and Blastocritidia triatomae were used in this study. The insects, originated from bugs provided by the Servicio Nacional de Chagas (Córdoba, Argentina), were reared as previously reported (Settembrini, '84; Settembrini and Tramezzani, '96). Eggs coming from this service were periodically added to the colony to avoid excessive inbreeding and to maintain high female fecundity. The bugs were fed weekly on the shaved thorax of a chicken, during 45 min under dim light.

**Dissection and fixation**

The insects were processed for immunocytochemistry according to Villar et al. ('94). Briefly, the bugs were anesthetized by chilling them on ice; the head capsules were opened and the tissues were flushed with ice cold fixative, a mixture of formalin and picric acid (4% paraformaldehyde and 0.4% picric acid; Zamboni and De Martino, '67), in 0.16M sodium phosphate buffer (PB) pH 6.9. The head and the thorax were separated from the abdomen and the dissection proceeded with the tissue immersed in fixative until the brain and the thoracic ganglia were totally excised. The nervous tissue remained in the same fixative for 6 h, then it was transferred to 0.1M PB saline (PBS, pH 7.4) containing 15% sucrose, 0.02% bacitracin (Sigma, St. Louis, MO), and 0.01% sodium azide (Merck, Darmstadt, Germany), for at least 48 h.

**Immunocytochemistry**

Complete series of 18 µm horizontal or frontal sections were made with a Microm cryostat (Zeiss; Waldorf, Germany) and processed for the avidin-biotin (ABC) technique (Hsu et al., '81). The sections were mounted on chrome-alum gelatin precoated glass slides and allowed to dry for 2 h. Then, they were washed in 0.01M PBS (2 × 10 min) and incubated overnight in a humid chamber with PROC antisera (kindly provided by Dr. P.H. Taghert, Washington University, School of Medicine, St Louis, MO). This antibody was made by immunizing rabbits against synthetic proctolin conjugated to BSA, and the animals were bled after four booster injections of this conjugate. Immunostaining of corpora cardiaca and corpora allata of grasshoppers was blocked by either preabsorbing the antibody with the pentapeptide or by incubating the sections without the primary antibody (Dr. P.H. Taghert, personal communication).

Proctolin antibody was diluted 1:600 or 1:800 in 0.01M PBS containing 0.2% (w/v) bovine serum albumin, 0.03% Triton X-100, and 0.01% sodium azide. After that, the slides were rinsed (2 × 10 min) in PBS, incubated at room temperature for 30 min in biotinylated goat anti-rabbit secondary antibodies (1:100, Vector Laboratories, Burlingame, CA), rinsed twice in PBS, and incubated during 1 h in ABC reagent (Vectastain Elite Kit, Vector Laboratories). Peroxidase activity was revealed by reaction with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) using glucose oxidase (Sigma) and nickel salts for enhancement of the reaction product (Shu et al., '88). The sections were mounted with Permount (Fluka, Buchs, Switzerland) and photographed in a Nikon Eclipse microscope with Agfapan APX 25 (Agfa Gevaert AG, Leverkusen, Germany).

**Controls**

For control purposes, parallel incubations were run with anti-PROC preabsorbed with PROC peptide 10–26 M (Sigma). Control incubations were also carried out with sections incubated with either only the primary or secondary antibodies and processed for ABC. No immunostained cells and fibers were observed when the sections were incubated with the antibody preabsorbed with the synthetic peptide or when the primary and secondary antibodies were omitted.

**RESULTS**

The general rostrocaudal distribution of immunolabeled cells and fibers is shown in frontal sections depicted in Figure 1a–g.

**Protocerebrum**

**PROC-IR cell bodies**

Protocerebral PROC-IR cells were mostly ovoid, with sizes ranging between 10–15 µm, and were usually found forming clusters.
Fig. 1. Camera lucida drawings from serial representative frontal sections of Triatoma infestans brain. Symbols represent the number of PROC-IR cell bodies: ○ = 1–5; ● = 6–10; ★ = 11–15; ★★ = more than 15. Light, medium, and dark shadowed areas represent PROC-IR fibers of, respectively, scarce, moderate, and intense densities.
In the rostral protocerebrum, a few small PROC-IR cell bodies were observed in a dorso medial position, from here on termed the pars intercerebralis (Figs. 1a, 2a) according to the terminology of Nässel and O'Shea ('87). A group of 10–15 somata were found dorsally, in the vicinity of the mushroom body calices. Also, numerous PROC-IR cell bodies were present in the soma rind of the lateral protocerebrum (Fig. 1a).

The optic lobes showed several PROC-IR cell bodies, near the lobula, surrounding the medulla and in the proximity of the lamina ganglionaris (Fig. 1b). Three PROC-IR cell clusters were found in the protocerebrum at the level of the optic lobes: two of these clusters were formed by a few cells located in the pars intercerebralis and in the ventrolateral soma rind; the third cluster, composed of several cells (10–15), was observed at the dorsal edge (Fig. 1b). More caudally, a few PROC-IR cells were placed dorsomedially and laterally, whereas 10–15 somata were found in the dorsolateral edge of the soma rind (Fig. 1c).

In the region of the midbrain, a few (7–10) PROC-IR somata were located in the pars intercerebralis and above the calices of the mushroom bodies (Figs. 1d, 2d). The lateral protocerebrum showed some scattered positive cell bodies.

The distribution of PROC-LI in the posteromedial part of the protocerebrum followed the same pattern of the preceding areas with some positive cells in the pars intercerebralis and dorsally to the calices of the mushroom bodies. The lateral soma rind had two labeled cell groups, one placed more dorsally and another one formed by fewer cells near the protocerebral-deutocerebral crest (Fig. 1e). PROC-IR cells of the posterior protocerebrum were observed in the dorsal and lateral parts of the soma rind (Figs. 1f, 2c). Caudally, only a few immunolabeled cells were placed in the dorsal and lateral fields (Fig. 1g).

PROC-IR fibers.

PROC-IR nerve fibers were found in the lateral, non-glomerular neuropil, leaving a central unstained area in the anterior protocerebrum (Figs. 1a, 2a,b). Also, a few varicose axons were seen within the area of the median bundle, and a meshwork of IR fibers was observed in the ventromedial protocerebrum (Fig. 1a).

Thick and strongly stained processes were seen in the optic lobes between the medulla and the glomerular lamina ganglionaris, whereas scarce IR fibers were detected in the latter glomerulus (Fig. 1b). In addition, varicose-positive fibers occupied the ventral protocerebral neuropil and the commissure lying below the central body complex (Fig. 1b). More caudally, dense PROC-IR axons were observed running from the optic lobe towards the unstained central body neuropil (Figs. 1c, 2e). Heavily stained fibers were found in the midline, above the central body complex. The lateral and ventromedial neuropils of the midbrain had an intense immunostaining (Figs. 1d, 2d).

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sory glomeruli were devoid of PROC-IR fibers and the antennal nerve showed no immunoreaction. The tritocerebral neuropil contained numerous immunolabeled varicose processes in most of its rostrocaudal extension (Figs. 1a–f).

Subesophageal ganglion PROC-IR cell bodies

Rostrally, several PROC-IR neuronal types were detected (Fig. 1g,h). Large cells (20–25 μm) exhibited a granular labeling pattern (Figs. 3a,4a,b). Medium-sized somata (15 μm) were seen sending processes to the neuropil of this ganglion (Fig. 4a). Some of these cells were located near the origin of the stylet nerves.

Thoracic ganglia PROC-IR cell bodies

Positive small- to medium-sized somata were found in the posterior part of the prothoracic ganglion (Fig. 4d). A few showed a granular labeling pattern in the cytoplasm.

PROC-IR somata, mostly medium sized were found in the meso-metathoracic abdominal ganglionic mass. They were generally observed forming clusters while larger ones with thick IR granules were seen scattered in the soma rind (Fig. 4c).

PROC-IR fibers in ganglia.

Beaded PROC-IR processes were observed around the ventral border of the esophageal foramen (Fig. 1g) and in the rostro-lateral neuropil (Fig. 3b) while delicate thin fibers were found in the medial part of the subesophageal ganglion (Figs. 1h, 4a). Some processes could be observed arising from the larger stained somata towards the central neuropil of the prothoracic ganglion (Fig. 4d).
In the present paper we have mapped the distribution of PROC-IR cells and fibers in *T. infestans*, expanding previous knowledge on the chemical morphology of the CNS of this insect. The distribution of PROC-LI shows great variation in the CNS among the different insect orders in which its presence has...
been reported (Orchard et al., '89). In the brain of the hemipteran bug T. infestans, the protocerebrum has the largest number of PROC-IR cells and fibers. Here, PROC-LI was observed mostly in small- to medium-sized somata that displayed an even reaction product. The pars intercerebralis and the soma rind around the mushroom body calices showed numerous IR cell bodies. These immunopositive cells resemble the median and lateral neurosecretory cells and their fibers could be the source of the PROC-LI observed in the median bundle of the protocerebrum, the nervi corpora cardiaca, and the corpora allata (not shown) as was also reported for Lymantria dispar (Davis et al., '89). Thus, in T. infestans the neurohormonal role of PROC in the control of the corpora allata activity should be investigated.

PROC-IR cells were observed in the optic medulla. These cells give origin to fibers that run towards the central body complex. It is possible that these fibers form part of the tractus opticus medialis inferioris described in Oncopeltus fasciatus (Johansson, '56). Due to the multiple connections between the central body complex and the protocerebrum (Barth, '75), PROC could serve as a biological molecule in integrative visual functions.

Of considerable interest was the presence of PROC-LI in the deutocerebrum of T. infestans. In the cricket Gryllus bimaculatus, slow and fast antennal motoneurons showing PROC-LI have been reported (Bartos et al., '94). Some of the larger positive deuto- cerebral somata described here resemble those motoneurons in size and granular labeling pattern and perhaps could serve the same function. However, further experiments combining both axonal backfillings and immunocytochemistry are required to clarify this issue. On the other hand, in other insect species, the deutocerebrum was reported as devoid of PROC-LI (Nässel and O'Shea, '87; Lange et al., '88). Differences in the methodologies applied to the nerve tissues or the higher sensitivity of the ABC technique with nickel-salt intensification of the immunoreaction product (Hsu et al., '81; Shu et al., '88) may account for these discrepancies. The antennae of T. infestans bear important receptors for orientation behavior (Di Luciano, '83, '85). The distribution pattern of PROC-LI in the deutocerebrum suggests that this pentapeptide is not involved in sensory perception, and may instead be related to neural control of antennal movements.

PROC-IR cells are more frequently placed in the lateral parts of the subesophageal ganglion rather than medially, which is in agreement with the observations in Rhodnius prolixus (Lange et al., '88). Accordingly, we observed a higher density of positive fibers in the lateral parts of this ganglion and varicose PROC-IR processes around the ventral border of the esophageal foramen. As discussed in the preceding section, variability exists on the presence or not of PROC in insect specific brain regions (Orchard et al., '89). This is the case for Aeschna cyanea, a species in which no immunoreactive proctolin somata were reported in the subesophageal ganglion (Andries et al., '91).

The meso-metathoracic-abdominal ganglionic mass has PROC-IR cells mainly in the lateral soma rind, while a few are placed in crests between the different neuromeres of this ganglion. Some of these positive cells could project to the midgut and hindgut as PROC-LI was observed in these organs (not shown). Instead, no PROC-IR fibers from these ganglionic mass were seen supplying the lateral and common oviducts where extensive proctolinergic innervation has been reported (Lange et al., '86).

In conclusion, the labeling pattern of PROC-LI appears to be widespread and unique for the nervous system of T. infestans, even though there are similarities with other insect species. Further research is needed to establish the functional role of PROC in T. infestans.

ACKNOWLEDGMENTS

The authors are indebted to Dr. P.H. Taghert (Department of Anatomy and Neurobiology, Washington University, School of Medicine, St. Louis, MO) for his generous gift of the proctolin antiserum. This work was supported by grants from Fundacion Antorchas (M. J. V.) and from Facultad de Ciencias Biomedicas, Universidad Austral. The authors thank Ms. S. Rago and Ms. D. Barreiro for their help with researching the bibliography.

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