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Neuropeptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH): Immunological detection and neuronal localization in insect central nervous system

(peptide neurotransmitter/identified neurons)

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ABSTRACT **Proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH) is a pentapeptide first extracted from cockroaches. It is known to have many neurohormonal effects and has been associated with specific, identified cockroach neurons. We have produced proctolin antisera and report here on their application in detecting proctolin-like immunoreactivity (PLI) in the cockroach central nervous system. Radioimmunoassay, capable of detecting 50 fmol of proctolin, was used to quantify the distribution of PLI. Highest concentrations were detected in the genital ganglia and lowest in the cerebral ganglia. Immunohistochemistry on the cockroach central nervous system demonstrated that PLI is localized to neurons. Neurons stained by using immunohistochemistry were widespread in the ganglia. Cell bodies were found to be in constant positions from animal to animal and to occur in bilaterally symmetrical pairs. These neurons are potentially identifiable.**

It is now widely accepted that peptides may act as neurotransmitters, but precisely how they act is unknown. Vertebrate preparations have been prominent in the study of peptide neurobiology (1, 2). Invertebrates, in which single neurons can be individually characterized physiologically, morphologically, and biochemically, have provided useful model preparations in which to study a variety of neurophysiological processes (3, 4). It is likely that they also will be useful in extending our understanding of the function of neuropeptides. Some neurons in invertebrates can be recognized uniquely in different individuals of the same species. Therefore, neuron function can be studied on a cellular level by the accumulation of information from precisely homologous neurons. The localization of a neuropeptide to specific, identified neurons is a necessary prelude to the development of preparations in which neuropeptide function can be studied *in vivo* on the cellular level.

Proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH) is one of the few invertebrate peptides of known sequence. It was first extracted from cockroaches (5–7). Because it is highly bioactive in a number of invertebrate visceral, skeletal, and heart muscle preparations, it has been proposed as a neurotransmitter (5, 8–12). However, the presence of proctolin in neurons has only recently been biochemically detected in a single identified cockroach neuron (13).

In the present study, we report on the production of two proctolin antisera. These have been used to determine the distribution of proctolin-like immunoreactivity (PLI) in the cockroach central nervous system and to localize neurons that may contain proctolin. Neurons identified by this approach can be individually assayed biochemically as a prelude to a cellular investigation of neuropeptide action in a relatively simple nervous system.

MATERIALS AND METHODS

Adult specimens, both male and female, of the large American cockroach (*Periplaneta americana;* Carolina Biological Supply, Burlington, NC) were used. Authentic proctolin for immunization was obtained from Sigma. Enkephalins were a gift of S. Wilkinson, Wellcome Research Laboratories (Beckenham, Kent, England). Gut bombesin was a gift of J. Rivier, Salk Institute (La Jolla, CA). Other peptides were obtained from Peninsula Laboratories (San Carlos, CA).

Preparation of Anti-Proctolin Antisera. Antigenic conjugates of proctolin with bovine serum albumin were prepared by adding 5 μl of 8% (wt/vol) glutaraldehyde to 200 μl of 0.1 M sodium phosphate (pH 7.4) containing 0.5 mg of proctolin and 1 mg of albumin. The conjugate was mixed with 0.5 ml of 0.1 M sodium phosphate (pH 7.4) and emulsified with an equal vol of Freund's complete adjuvant. It was injected subcutaneously into a female New Zealand White rabbit at multiple sites. Each month the rabbit was bled and boosted with half this quantity of proctolin conjugate, which was emulsified with 0.5 ml of Freund's incomplete adjuvant and 0.5 ml of 0.1 M sodium phosphate (pH 7.4). Twenty rabbits were immunized in this manner.

Radioiodination. To 10 μl of 0.1 M sodium borate (pH 10), 2 μl of proctolin solution (0.5 mg of proctolin per ml of 1% HOAc) was added. To this, 0.5 mCi of carrier-free ^{125}I (New England Nuclear) and 5 μl of dilute sodium hypochlorite (15 μl NaOCl in 10 ml of 0.1 M sodium borate, pH 10) were introduced and agitated for 15 sec. Twenty microliters of metabisulfide solution (10 mg/ml in 0.1 M sodium borate, pH 10) was added for 2 min and then 20 μl of NaI solution (100 mg/ml in 60% HOAc) was added for 2 min. Finally, 50 μl of 0.25 M sodium phosphate, pH 7.6/0.3% bovine serum albumin/0.1% sodium azide was added. The reaction mixture was immediately applied to a Sephadex G-10 column, prepared in a siliconized 14.5-cm Pasteur pipette, previously eluted with 1 ml of the sodium phosphate buffer and 2 ml of 10% (vol/vol) acetic acid. The column was eluted with 10% acetic acid. The ^{125}I-labeled proctolin (^{125}I-proctolin) was eluted in the void volume.

Radioimmunoassay. Radioimmunoassays (RIAs) were carried out in 0.25 M sodium phosphate, pH 7.6/0.3% albumin/0.1% sodium azide. RIAs contained 100 μl of anti-proctolin antiserum at a final dilution of 1:400, 50 μl of ^{125}I-proctolin (10,000

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Abbreviations: PLI, proctolin-like immunoreactivity; RIA radioimmunoassay.

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cpm) and 50 μl of proctolin standard or tissue extract. Samples were incubated at 4°C for 12 hr. After this incubation, 100 μl of dextran-coated charcoal was added and the samples were centrifuged. Finally, 200 μl of the sample supernatants were removed and counted in a γ-scintillation counter. Any alterations of this procedure are noted in the figure legends.

Extraction of Cockroach Tissues. The central ganglia of the cockroach central nervous system and nonneuronal tissue (fat-body tissue) were dissected from living specimens, weighed, and then homogenized (30:1, wt/vol) in 2 M acetic acid. The homogenate was centrifuged, the supernatant was adjusted to pH 7.0 and recentrifuged; the second supernatant was dried at 80°C. The dried sample was desalted by dissolving it in methanol; the methanol-soluble fraction was dried and dissolved in the RIA buffer. Recovery of ^{125}I-proctolin was found to be 80%. Reported RIA values have been corrected for extraction losses.

Immunohistochemistry. Central nervous system ganglia were fixed in 4% (vol/vol) buffered formalin, dehydrated, embedded in paraffin, sectioned at 6 μm, and mounted on gelatin-coated slides. Proctolin antiserum no. 9 was diluted 1:400 and incubated overnight with bovine serum albumin (3.3 mg/ml) prior to its application to the sections. Immunoreactivity to the proctolin antiserum was visualized by using the peroxidase-antiperoxidase technique (14). Controls consisted of adding proctolin or [Leu^5]enkephalin (0.5 mg/ml) to the incubation solution to test for specificity of proctolin staining. Nonimmune rabbit serum also was tested. Serial sections were separated so that adjacent 6-μm sections were exposed to anti-proctolin antiserum and to proctolin-blocked and enkephalin-blocked anti-proctolin antiserum. Some animals were injected with 0.05 ml of 0.3% colchicine in physiological saline (140 mM NaCl/5 mM KCl/5 mM $CaCl_2$/4 mM $NaHCO_3$/1 mM $MgCl_2$/5 mM Trehalose/100 mM sucrose/5 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, pH 7.2) 2 days prior to fixation for immunocytochemistry.

RESULTS

Several specific proctolin antisera were developed. They differed markedly in both their sensitivity and titer. The following results are based on antisera produced by two rabbits (nos. 9 and 13). Fig. 1 shows RIA displacement curves for these two antisera. Both antisera are diluted 1:400 (final dilution). Antiserum 9, although far less sensitive and of lower titer compared to antiserum 13, is the only one so far developed that can be used for immunocytochemistry. Antiserum 13 is the most sensitive and, therefore, has been employed in RIA of the cockroach extracts. The specificity of the proctolin antisera was determined by checking for crossreactivity with the following 12 neuropeptides: bombesin, gut bombesin, bradykinin, eledoisin, β-endorphin, [D-Ala^2, D-Leu^5]enkephalin, [Leu^5]enkephalin, [Met^5]enkephalin, kassinin, somatostatin, substance P, and xenopsin. Less than 17% of the maximum number of counts could be displaced by addition of 50 pmol of these peptides. None of these neuropeptides crossreacted appreciably with the proctolin antisera. This is important because, although these are vertebrate peptides, recent immunological evidence suggests the presence of similar peptides in invertebrate nervous systems. Tissue extracts of cockroach central nervous system ganglia contained PLI when assayed with antiserum 13. Displacement curves produced by serial dilutions of tissue extracts were parallel to those produced by dilutions of authentic proctolin. The displacement of bound ^{125}I-proctolin produced by ganglia extracts is susceptible to destruction by proteolytic enzymes such as protease (1 mg/ml; Sigma), as is the displacement produced by proctolin (Fig. 2).

The distribution of PLI was determined by RIA performed on pooled individual segment ganglia and abdominal fat-body tissue (Fig. 3). Abdominal fat-body tissue extracts produced measurable but relatively low levels of PLI compared to the levels found in the central nervous system. However, this specific association between higher proctolin levels and the neural tissue was not uniform throughout the central nervous system. For example, the cerebral ganglia (brain) contain relatively low levels of immunoreactivity, and the terminal or genital ganglia contain the highest levels. The genital ganglion is a compound

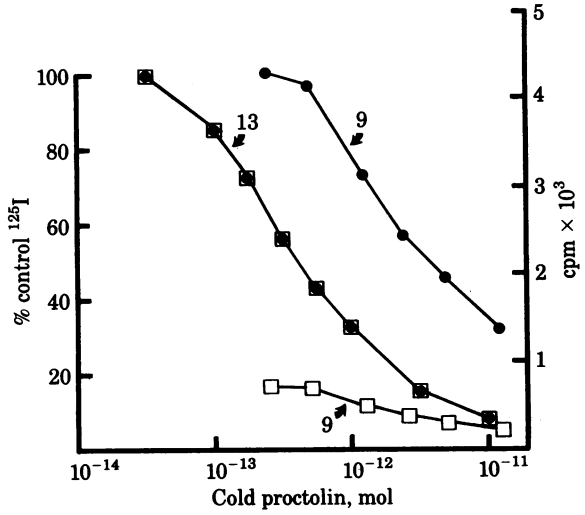

FIG. 1. Inhibition of binding ^{125}I-proctolin to two antisera (1:400 final dilution) by unlabeled proctolin. Points are means of duplicate incubations. □, Absolute number of cpm bound; ●, percentage of maximum counts bound.

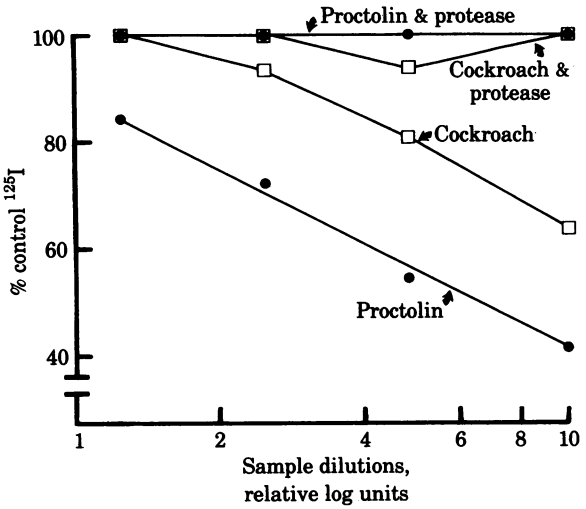

FIG. 2. RIA of cockroach central nervous system tissue extracts and authentic proctolin with protease and inactivated protease treatment. Thoracic, abdominal, and terminal ganglia were collected from 19 animals, homogenized, and prepared as described. This sample was divided into two halves to which either protease (1 mg/ml) or inactivated protease (1 mg/ml) was added. (Protease was inactivated by heating to 100°C for 1 hr and then centrifuging to remove the precipitate.) In addition, each of two tubes containing authentic proctolin (2×10^{-8} M) was treated with protease or inactivated protease (same concentrations as for tissue). All solutions were incubated for 3 hr at 37°C. They then were heated to 100°C for 1 hr to inactivate remaining protease and centrifuged. Dilutions of each sample in duplicate were assayed with antiserum 13. Points are means of duplicate determinations.

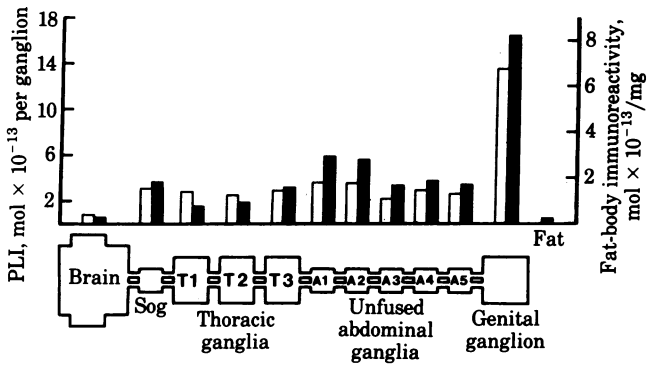

FIG. 3. Distribution of PLI in the cockroach central nervous system as determined by RIA with antiserum 13. Distribution is expressed per ganglion (□) and per mg of wet weight (■). A schematic diagram of the cockroach segmental ganglia is shown below the histogram. Sog, sub-oesophaegeal ganglion. A determination of immunoreactivity in fat-body tissue is shown on the right.

or fused ganglion, representing the fusion of five embryonic abdominal segmental ganglia. Therefore, it is perhaps not surprising that the apparent level of proctolin in this ganglion is about 4.5 times the mean level in the unfused abdominal ganglia. However, the elevated levels of PLI in the terminal ganglion may not fully account for ganglia fusion because the compound terminal ganglion is only approximately 1.4 times the mass of an unfused abdominal ganglion without connectives. Therefore, PLI expressed per mg of tissue (Fig. 3) shows that it is highly concentrated in the terminal ganglion.

PLI appears from our results to be concentrated in certain regions of the central nervous system. In an attempt to characterize more precisely the distribution and location of PLI in the central nervous system, we have employed antiserum 9 in immunocytochemistry. Results of these experiments show that PLI is localized to neurons of the central nervous system, providing further evidence that proctolin is a neurotransmitter.

Figs. 4 and 5 show immunoreactive monopolar neuronal somata, axons, and varicosities in the second unfused abdominal ganglion. Staining was not seen in glial elements of the central nervous system. No staining occurs when the sections were incubated with a nonimmune primary sera. Furthermore, visualization of cell bodies, axons, and varicosities is blocked in control experiments in which the proctolin antiserum is preincubated with proctolin but is not blocked by pre-incubation with enkephalin. Fig. 4 *D–F* show three adjacent serial sections of the same neuron that were used in the experimental and two control incubations. The complete inhibition of staining caused by proctolin and lack of inhibition by [Leu^5]enkephalin is good evidence for staining specificity for proctolin-like antigens.

The number of cell bodies per ganglion that stained with the anti-serum was positively correlated with the amount of PLI detected by RIA. For example, unfused abdominal ganglia contained about 20 stained neurons, and the fused terminal ganglion contained about 80 stained neurons. These numbers are approximately proportional to the PLI in these ganglia as determined by RIA. This fact combined with the absence of staining in nonneuronal elements of the central nervous system suggests that all the PLI measured by RIA of central nervous system tissue can be accounted for by neuronally localized proctolin or similar peptides.

Stained somata were widely dispersed in the ganglia. They were found in dorsal, ventral, and lateral regions. However, the total number of stained cell bodies was low, accounting for about 5% of the neurons of the abdominal ganglia. Although some stained cell bodies appeared clustered, many appeared widely separated from one another.

Some stained cell bodies could be recognized from preparation to preparation by their constant position. In addition, stained neurons frequently occurred in bilaterally symmetrical pairs (Fig. 5). These two features suggest that some of the staining neurons may be individually identifiable and, therefore, suitable for study at the cellular level.

Somata of immunoreactive neurons could be seen using antiserum 9 without pretreatment of animals with colchicine. How-

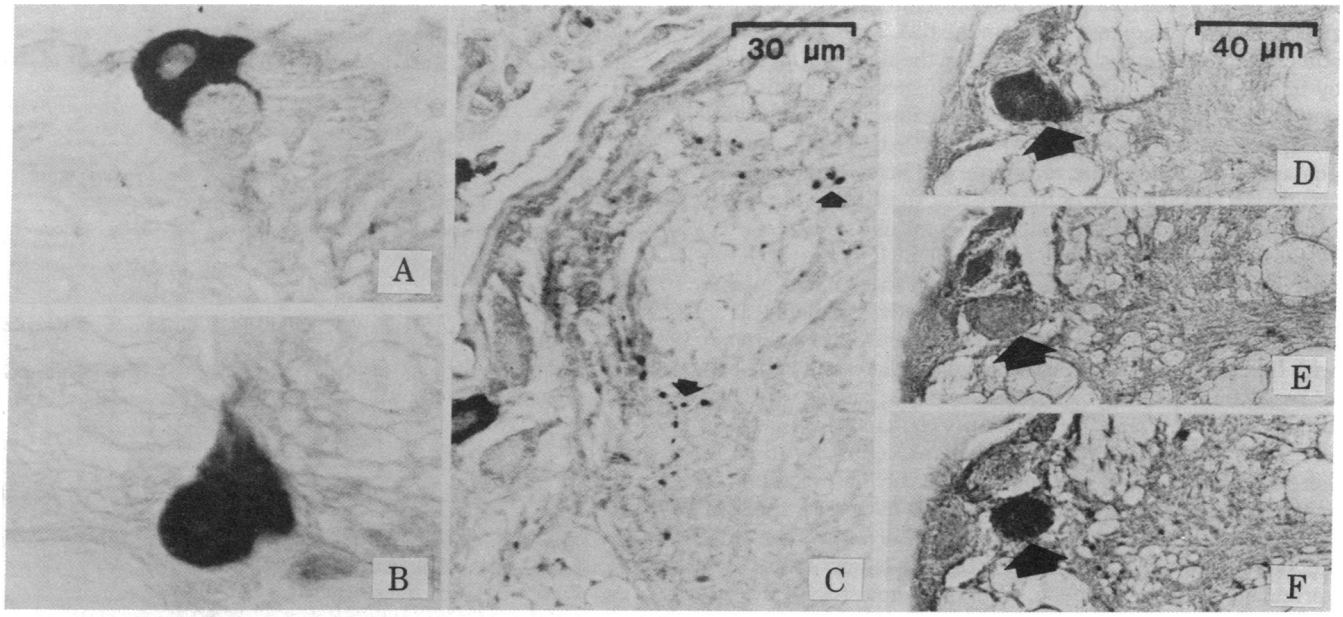

FIG. 4. PLI in neurons in the second abdominal ganglion of the cockroach. (*A*) Dorsal cell. (*B*) Two ventral cells. (*C*) Numerous varicosities. (*D–F*) Consecutive serial sections through a lateral cell in the cockroach second abdominal ganglion which shows PLI (*D*). The cell body is arrowed in each section. The staining is inhibited when the proctolin antiserum is preincubated with proctolin (*E*). The staining is not reduced by pre-incubation of the antiserum with [Leu^5]enkephalin (*F*).

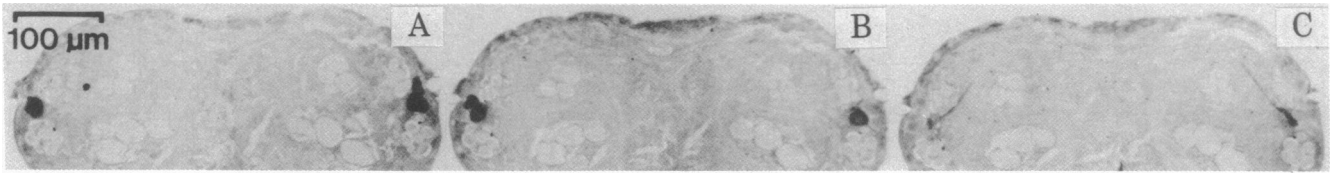

FIG. 5. Consecutive serial sections showing two pairs of bilaterally symmetrical abdominal ganglion cells which have PLI. Axons of one pair of cells are shown in *C*.

ever, colchicine pretreatment revealed additional immunoreactive somata and could enhance the staining of neurons whose visualization does not require colchicine. Colchicine appeared to have little effect on the staining of varicosities and, furthermore, varicosities stained more readily than cell bodies with lower concentrations of antiserum (1:1000 compared to 1:400). This suggests that the varicosities contain high concentrations of PLI and may be release sites. Axons, particularly in colchicine-treated animals, can be traced through the neuropil and into peripherally projecting nerve roots and connectives between ganglia.

DISCUSSION

This report describes the detection of PLI by RIA and immunohistochemistry in the cockroach. The two antisera employed have rather different characteristics. Antiserum 13 is of relatively high titer and can be used for RIA. Under our standard assay conditions, 50 fmol of proctolin can be detected. This antiserum does not produce satisfactory staining when used in immunohistochemistry. On the other hand, antiserum 9, which has lower titer and sensitivity in RIA, produces specific staining for proctolin when used in immunohistochemistry. It is not clear why staining with antiserum 13 is less successful; however, it is frequently observed that the ability of an antiserum to identify an antigen in immunohistochemistry does not necessarily correlate with its ability to function in an RIA (15).

Several pieces of evidence suggest that both the RIA and immunohistochemistry procedures are specific for proctolin-like peptides. For example, authentic proctolin and PLI can be destroyed by protease treatment, and authentic proctolin and PLI give parallel displacement curves in the RIA. Moreover, several other neuropeptides did not compete in the RIA. PLI identified by immunhistochemistry also appeared specific. In this case, staining for PLI could be blocked by preabsorption of the antiserum with proctolin but not with [Leu^5]enkephalin, another pentapeptide found in the vertebrate and invertebrate nervous system (16, 17). In spite of the fact that the RIA and immunohistochemistry procedures used different antisera, there is a close agreement between the results obtained by the two methods. Thus, the terminal abdominal ganglion contains approximately 4 times the number of immunoreactive cell bodies as are contained in an unfused abdominal ganglion. This corresponds with the difference between the ganglia as determined by RIA.

Based on immunocytochemical evidence alone, we cannot be sure that the stained neurons truly contain proctolin. However, two observations suggest that the procedures we describe here will be useful in locating and identifying proctolin-containing neurons. First, proctolin is known to be associated with the cockroach proctodeal and stomatodeal nerves, which innervate the gut (5). These nerves originate in nerve roots of the terminal or genital ganglion. Our immunohistological procedures reveal axons projecting from the terminal ganglion in appropriate peripheral nerve roots and the presence of neuronal somata within the ganglion. It is likely, therefore, that included among the stained cells are the proctolin-containing neurons that are thought to innervate the viscera. Second, proctolin has been shown to be associated with an identified, bilaterally symmetrical pair of neuronal cell bodies in the cockroach abdominal ganglia (13). Our immunological procedures reveal bilaterally symmetrical pairs of neurons in these ganglia, one of which may correspond to the proctolin-containing neuron identified by O'Shea and Adams. Establishing that antiserum 9 stains proctolin-containing neurons will require the combination of immunocytochemistry with biochemical methods directed at individual neurons. Chemical identification at the single-cell level is made possible by the ability to study repeatedly the same identified neuron in different individuals. Immunocytochemistry will allow rapid screening for additional identifiable proctolin-containing neurons and will help in developing model systems to investigate the function of characterized peptidergic neurons on the cellular level.

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