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Localization of corazonin in the nervous system of the cockroach *Periplaneta americana*

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Abstract. Antisera raised to the cardioactive peptide corazonin were used to localize immunoreactive cells in the nervous system of the American cockroach. Sera obtained after the seventh booster injection were sufficiently specific to be used for immunocytology. They recognized a subset of 10 lateral neurosecretory cells in the protocerebrum that project to, and arborize and terminate in the ipsilateral corpus cardiacum. They also reacted with bilateral neurons in each of the thoracic and abdominal neuromeres, a single dorsal unpaired median neuron in the suboesophageal ganglion, an interneuron in each optic lobe, and other neurons at the base of the optic lobe, in the tritocerebrum and deutocerebrum. The presence of corazonin in the abdominal neurons and the lateral neurosecretory cells was confirmed by HPLC fractionation of extracts of the abdominal ganglia, brains and retrocerebral complexes, followed by determination of corazonin by ELISA, which revealed in each tissue a single immunoreactive peak co-eluting with corazonin in two different HPLC systems. Antisera obtained after the first three booster injections recognized a large number of neuroendocrine cells and neurons in the brain and the abdominal nerve cord. However, the sera from the two rabbits reacted largely with different cells, indicating that the majority of this immunoreactivity was due to cross-reactivity. These results indicate that the production of highly specific antisera to some neuropeptides may require a considerable number of booster injections.

Key words: Immunoreactivity – Corazonin – Cardioactive peptide, insect – Immunocytology – Cross-reactivity – DUM neuron – *Periplaneta americana* (Insecta)

Introduction

The anatomy of the insect neurosecretory system has been studied extensively using classical neurosecretory stains (for review, see Rowell 1977), backfillings from neurohaemal organs (e.g., Mason 1973; Nijhout 1975; Rademakers 1977; Taghert and Truman 1982; Copenhaver and Truman 1986), and, more recently, immunocytochemistry with antisera to vertebrate peptides (e.g., Veenstra et al. 1985; Homberg et al. 1991). Although few insect neurohormones have been fully identified, the number of chemically characterized insect neuropeptides is growing rapidly (Holman et al. 1990). The arthropod peptide family characterized by adipokinetic hormone (AKH) and red pigment concentrating hormone (RPCH) is now especially well characterized in insects (Orchard 1987; Gäde 1990). In insects the members of this peptide family appear to be synthesized exclusively in the glandular cells of the corpus cardiacum (Schooneveld et al. 1987). They stimulate the release of energy substrates from the fat body, and in the American cockroach also increase the rate of heart contraction (Baumann and Gersch 1982; Scarborough et al. 1984).

Corazonin (pGlu-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn-amide), another neuropeptide that has been isolated from the American cockroach, is present in the corpora cardiaca in an amount large enough to suggest that it is involved in the regulation of heart contraction (Veenstra 1989). Although corazonin is not a member of the AKH/RPCH family, it does show structural similarity to some of the members of this peptide family (Veenstra 1989). It, therefore, is of interest to know whether corazonin also is synthesized by the glandular cells of the corpus cardiacum or synthesized elsewhere in the nervous system. We describe here the localization of this neuropeptide within the nervous system of the cockroach *Periplaneta americana*.

Materials and methods

Histology

For paraffin embedding, tissues were dissected in 0.9% NaCl and fixed overnight in GPA (1 vol. of 25% glutaraldehyde, 3 volumes of a saturated aqueous solution of picric acid and 1% acetic acid;

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Boer et al. 1979) at room temperature. Sections of 8 µm were immunocytologically stained with the peroxidase-anti-peroxidase method of Sternberger (1979), using 3,3′ diamino-benzidine as substrate for the peroxidase. Incubation with the corazonin antisera was performed overnight at dilutions ranging from 1:500 to 1:2000, depending on the particular serum.

For whole-mount immunofluorescence the procedure of Davis et al. (1989) was followed. Nervous systems were dissected in a saline solution and fixed overnight in 4% paraformaldehyde in phosphate buffer (pH 7.4) at 4° C, after which the tissues were washed in phosphate-buffered saline containing 0.5% Triton X-100 (PBST). Incubation in the corazonin antisera, diluted 1:2000 in PBST, was performed at 4° C for 2 days. After washing, the tissues were incubated in rhodamine-conjugated goat anti-rabbit serum (Boehringer Mannheim, Germany, Indianapolis, Ind.) diluted 1:200.

Preabsorption of the antisera overnight at 4° C with 10 nmol corazonin in the working dilution of the antiserum abolished all immunoreactivity. This was also true for the early bleedings, which yielded unspecific antisera.

Antisera

Antisera to corazonin were raised in two rabbits (CZ-1 and CZ-2) over a time span of almost 2 years using a conjugate prepared by coupling corazonin to bovine serum albumin (BSA) with 1,5-difluoro-2,4-dinitrobenzene. Booster injections were administered 6, 12, 18, 30, 37, 43, 49, 64, 70, 76, 83 and 90 weeks after the initial injection. Blood was collected before the first injection (pre-immune serum) and 7 days after each booster injection (Veenstra 1991). Antisera that were tested in immunocytology are those obtained after the 1st, 2nd, 3rd, 4th, 7th and 12th boosters.

HPLC and ELISA of tissue extracts

Tissues were dissected in 0.9% NaCl, stored in a glass homogenizer on dry ice and homogenized in 1 ml of Bennett's mixture [1% NaCl, 5% formic acid, 1% trifluoroacetic acid (TFA) in 1 M HCl (Bennett et al. 1981)], centrifuged and the supernatants loaded on previously activated and equilibrated C_{18} Sep-Paks (Waters Associates, Mass.). The Sep-Paks were eluted with 5 ml water and 5 ml 10% acetonitrile (both containing 0.1% TFA) and corazonin-immunoreactive material was then eluted with 4 ml of 65% acetonitrile containing 0.1% TFA. This material was either lyophilized with 100 µg of radio-immunoassay grade BSA or concentrated and further separated using high performance liquid chromatography (HPLC), on a Beckman C_{18} column and precolumn. A gradient between water (A) and 65% acetonitrile (B) was used, with either 0.1% heptafluorobutyric acid (HFBA) or TFA as pairing ions. The column was eluted isocratically at a flow rate of 1 ml/min for 20 min at 20% B followed by a linear gradient over 40 min to 50% B. The column eluant was simultaneously monitored at 214 and 280 nm. Aliquots of 1 min fractions were assayed in the corazonin-ELISA after lyophilization with 100 µg radio-immunoassay grade BSA as described (Veenstra 1991).

Results

Histology

Sections through the retrocerebral complexes were used to screen antisera. Initially no obvious differences between antisera of the rabbits were found, except that antisera from rabbit CZ-1 could be diluted twofold further to obtain a positive reaction. Antisera from both rabbits labelled neurosecretory processes in the corpora cardiaca. However, when the brain and the ventral nerve cord were studied, the immunoreactivity of the two antisera was strikingly different and the background staining observed with the antisera obtained after these initial boosters was high. Antisera from rabbit CZ-1 recognized cells that were not immunoreactive with antisera from rabbit CZ-2 (Fig. 1) and vice versa.

Since the antisera from the two rabbits obtained after the first three booster injections did not recognize the same cells, it was not clear which, if any, of the immunoreactive cells were producing corazonin. A quantitative immunoassay for corazonin was therefore developed to test the immunocytological results. As antisera obtained after the first three booster injections were not useful in ELISA, the immununization of the rabbits was continued (Veenstra 1991). When the sera obtained after the seventh booster were tested for immunocytology, their specificity had changed dramatically; the antisera from both rabbits showed identical immunocytological specificity, recognized a subgroup of the lateral neu-

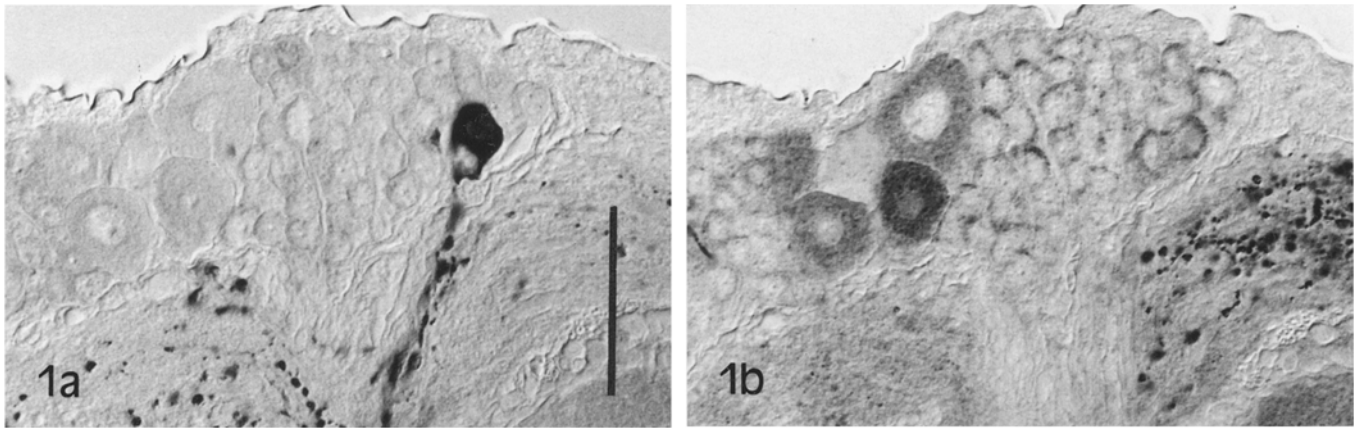

Fig. 1. Adjacent sections through the protocerebrum stained with antisera obtained after the third boosters from rabbit CZ-1 (**a**) and rabbit CZ-2 (**b**). Note that the neuron stained in (**a**) is not stained in (**b**), and also that different areas of neuropile are immunoreactive. *Scale bar:* 50 µm

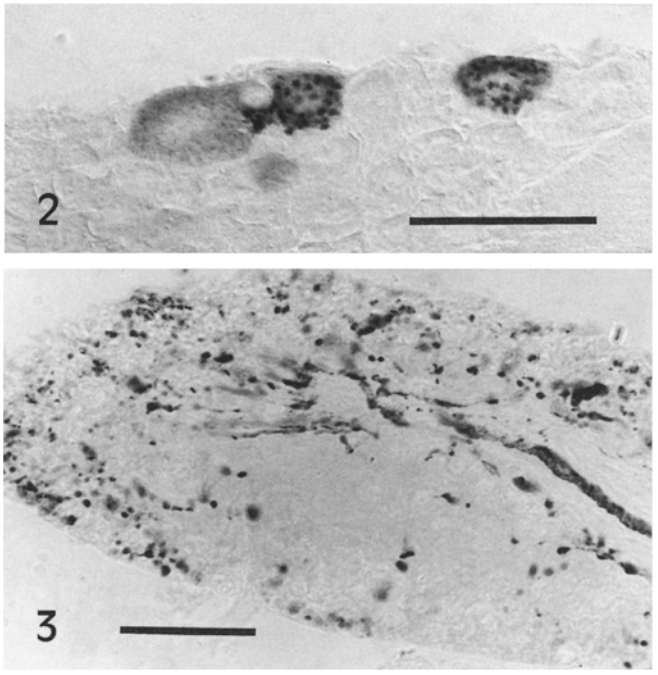

Fig. 2. Corazonin-immunoreactive lateral neurosecretory cells in sectioned material; note the different staining intensities of the cells. *Scale bar:* 50 μm

Fig. 3. Corazonin-immunoreactive processes in sections of the corpus cardiacum. *Scale bar:* 50 μm

rosecretory cells of the brain and their projections to terminals in the corpora cardiaca (Figs. 2, 3), and recognized several other neurons as well. After additional booster injections the titers of the antisera and their affinity for corazonin were further improved, as measured by ELISA (Veenstra 1991), but no differences were observed in the immunocytological characteristics of the antisera obtained between the seventh and twelfth booster. Antisera obtained after the seventh booster, therefore, were used for routine work.

In sectioned material the staining intensity of neurosecretory cells of the pars lateralis was quite variable. The cytoplasm of some cells stained only very weakly, and these cells could only be recognized in the absence of any background staining. In others the cytoplasm contained several immunoreactive granules, while the smallest cells within a group had a virtually homogeneously darkly stained cytoplasm; these various types were present within the same cell group (Figs. 2, 6). The

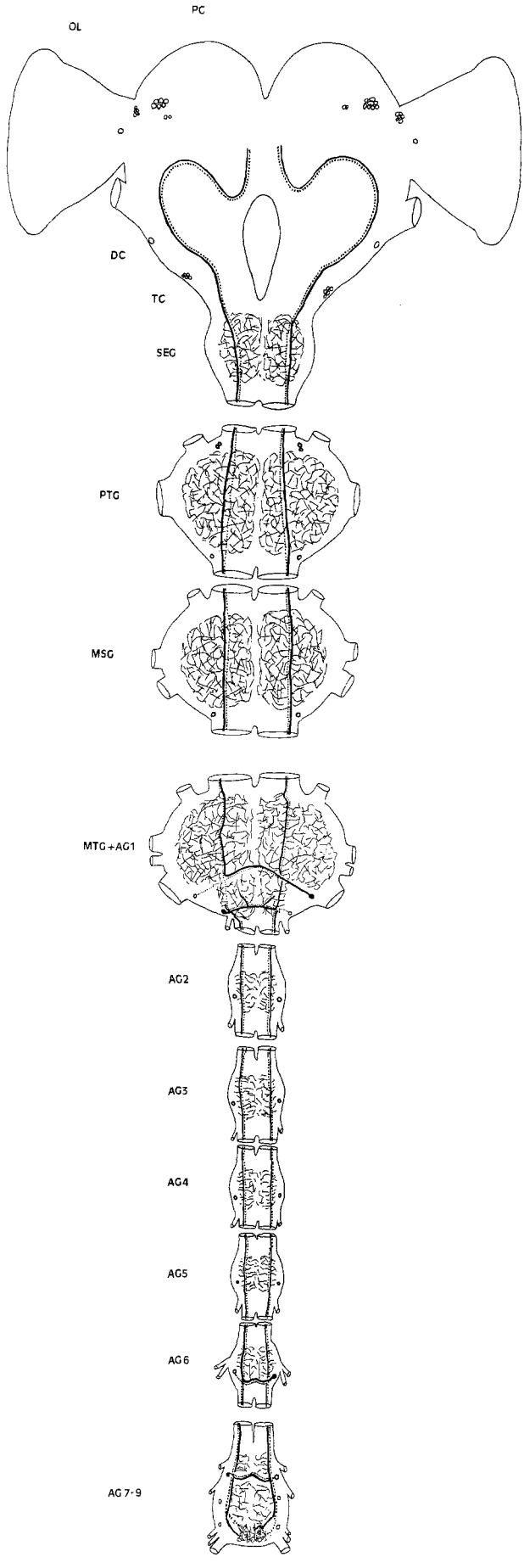

Fig. 4. Schematic representation of corazonin immunoreactivity as determined by corazonin antisera obtained after the seventh booster. Localization of cell bodies is indicated by *circles*, and the major processes of the corazonin-immunoreactive neurons in the metathoracic ganglion and abdominal ganglia 1, 6 and 7 have been drawn by thick *stippled lines*. The extent of their neuropil branches within the ventral ganglia has been indicated. *AG1-9* Abdominal ganglia 1-9; *DC* deutocerebrum; *OL* optic lobe; *PC* protocerebrum; *PTG* prothoracic ganglion; *MSG* mesothoracic ganglion; *MTG* metathoracic ganglion; *SEG* suboesophageal ganglion; *TC* tritocerebrum

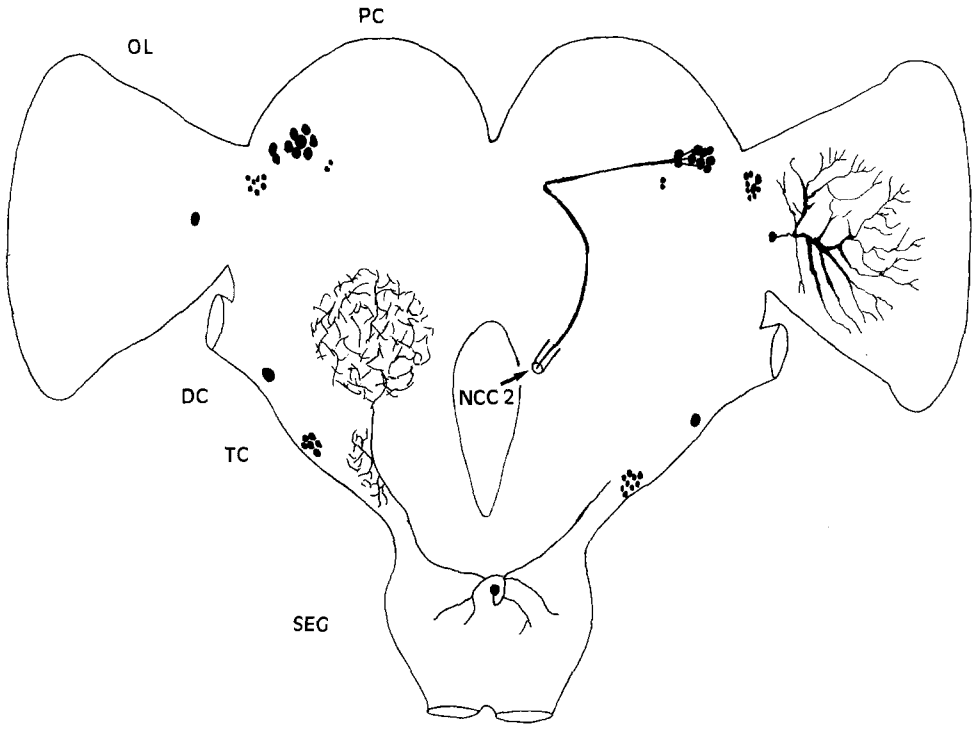

Fig. 5. Schematic localization of the corazonin-immunoreactive neurons in the brain and suboesophageal ganglion. The major axon processes and arborizations of the dorsal unpaired median neuron in the suboesophageal ganglion, the interneuron in the optic lobe and the lateral neurosecretory cells have been indicated. The axon of the dorsal unpaired median neuron has been traced only on one side. *NCC2* Nervus corporis cardiaci 2; other abbreviations as in Fig. 4

projections of the lateral neurosecretory cells were found to follow the classical pathway to the nervus corporis cardiaci II, and they branched and terminated in the corpora cardiaca (Figs. 3, 6). In the ventral nerve cord the antisera reacted with a posterior bilateral neuron in each neuromere of the thoracic and abdominal ganglia, but not in the suboesophageal ganglion.

To reveal the morphology of the immunoreactive neurons, whole-mount immunofluorescence preparations were prepared. The corazonin-immunoreactive arborizations in the neuropil of the ventral ganglia appear to be derived exclusively from paired interneurons in the methathoracic ganglion and in abdominal ganglia 1, 6 and 7 (Fig. 4). The neurite of each interneuron crosses over to the contralateral site of the ganglion, and its axon runs anteriorly through the connectives. The axons of the neurons in the abdominal ganglia 6 and 7 appear to terminate in the metathoracic ganglion. The axons of the neurons in the metathoracic and first abdominal ganglia extend to the protocerebrum, but their branching pattern could not be established clearly. In all ganglia a number of small dendrites arborize through the neuropil (Figs. 4, 9, 10). The projections of the other corazonin-immunoreactive interneurons in the thoracic and abdominal ganglia were not discernible. It could, therefore, not be established, whether these cells are local interneurons or ascending interneurons.

In the fluorescence whole-mount preparations some immunoreactive neurons were found that had not been seen in sectioned material (Figs. 4, 5). Small neurons close to the base of the optic lobe occured consistently in all preparations, but their axonal branching pattern could not be established. A single interneuron in the optic lobe also was found consistently (Fig. 7). Its axons penetrate the lamina, medulla and lobula. Close to the lateral neurosecretory cells, a pair of small immunoreactive interneurons was found; although their initial processes were clearly visible, it was not possible to follow them to their destination. Faintly immunoreactive neurons were found in the deutocerebrum and tritocerebrum, but their projections were not discernible (Fig. 9). A dorsal unpaired median (DUM) neuron was found in the suboesophageal ganglion (Fig. 8); occasionally its soma was displaced laterally. The branching pattern of this cell could not be established in its entirety, but in a few preparations it was possible to follow its axon into the circumoesophageal connectives and to arborizations in the tritocerebrum and the antennal lobe of the deutocerebrum (Fig. 9). Other cells, which had not been found in the sectioned material, include two to three very faintly reacting, small interneurons in the anterior region of the prothoracic ganglion. When these cells could be distinguished, the posterior pair of corazonin-immunoreactive interneurons in the same ganglion sometimes appeared to be absent.

ELISAs

Extracts of brains, corpora cardiaca and ventral nerve cords were separated by HPLC and individual fractions assayed for corazoninlike immunoreactivity (Fig. 11). In all tissues the major peak of corazoninlike immunoreactivity eluted at the same time as authentic corazonin. In an earlier experiment using HFBA as the pairing ion in HPLC, the corazonin-immunoreactive material from the abdominal nerve cord, the brain and the retrocerebral complex also eluted at the same time as synthetic corazonin (results not shown). The relative amount of corazonin in the different tissues was different. Thus,

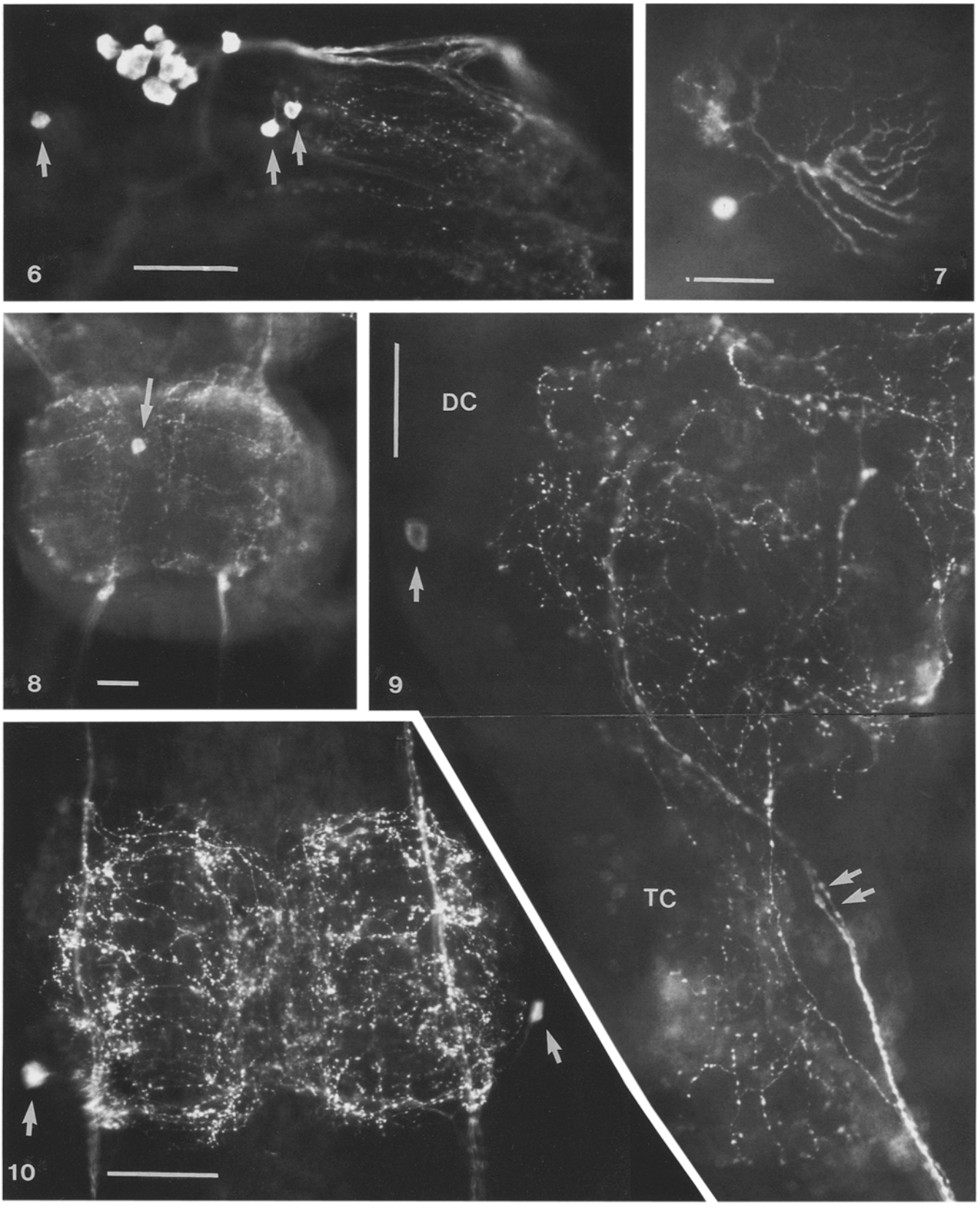

Fig. 6. Posterior view of the protocerebrum, showing the lateral neurosecretory cells and small neurons (*arrows*) that are immunoreactive with the corazonin antisera. *Scale bar:* 100 μm

Fig. 7. Posterior view of the optic lobe showing the corazonin-immunoreactive interneuron. *Scale bar:* 100 μm

Fig. 8. Dorsal view of the suboesophageal ganglion, showing the corazonin-immunoreactive DUM neuron (*arrow*). *Scale bar:* 100 μm

Fig. 9. Deutocerebrum (*DC*) and tritocerebrum (*TC*) with corazonin-immunoreactive processes derived from suboesophageal DUM neuron; also visible are an immunoreactive neuron in the deutocerebrum (*arrow*) and the two axons from the corazonin-immunoreactive interneurons in the metathoracic ganglion (*double arrows*). *Scale bar:* 100 μm

Fig. 10. Sixth abdominal ganglion, showing corazonin-immunoreactive interneurons (outside the plane of focus, *arrows*), and some of their arborizations in the neuropil. *Scale bar:* 100 μm

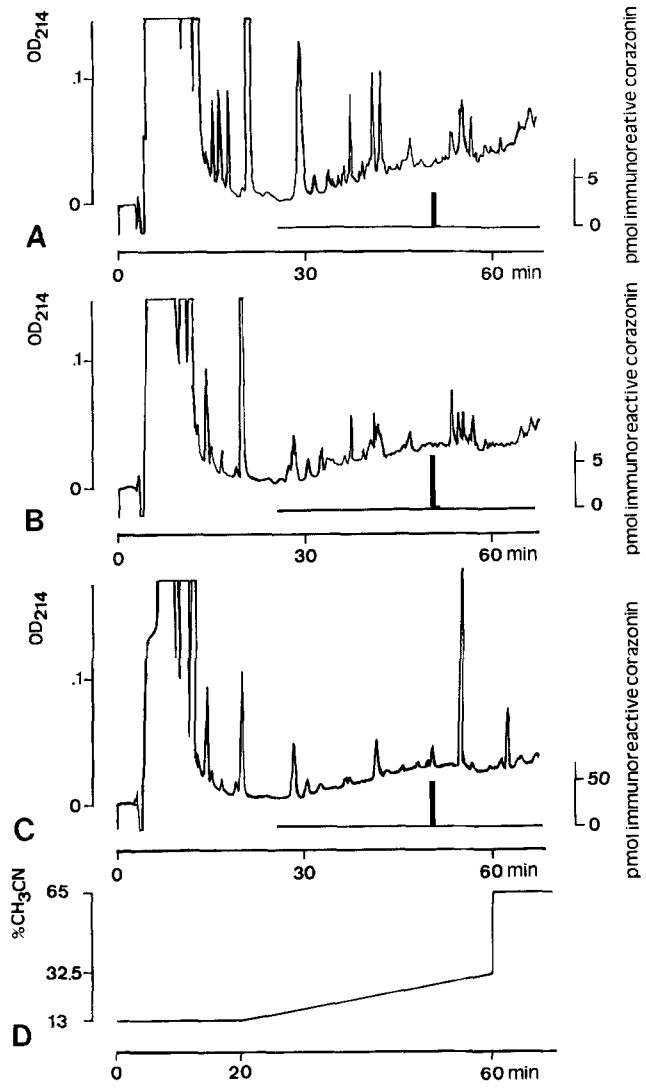

Fig. 11. HPLC chromatograms of Sep-Pak prepurified extracts from 10 abdominal nerve cords (**A**), 10 brains (**B**), or 10 retrocerebral complexes (**C**) from *Periplaneta americana*. Separations were performed on a C18 reversed phase column using TFA as a pairing ion with a gradient between water and acetonitrile as indicated (**D**). Optical density was measured at 214 nm and 1-ml fractions were collected aliquots of which were assayed by ELISA for corazonin and the total amount in each fraction calculated. Note that in all 3 tissues there is a single corazonin-immunoreactive peak with a retention time identical to that of synthetic corazonin, and that the amount of corazonin in the retrocerebral complex is ten times higher than in the brain or the abdominal nerve cord

while the corpora cardiaca contained about 5 pmol per animal, only one-tenth that amount was present in the abdominal nerve cord and brain (Fig. 11).

Discussion

Identity of the immunoreactive material

In this paper we describe the localization of corazonin-like immunoreactive material in the American cockroach. It is well known, that antisera raised to a peptide may cross-react with other peptides, even though the immunoreactivity is abolished by preincubation of the antiserum with the peptide (e.g., Swaab et al. 1977; Veenstra 1988). Since the antisera were raised against a peptide that is relatively abundant in the corpus cardiacum of the American cockroach (Veenstra 1989), it seemed possible initially that all the immunoreactive processes found in the corpus cardiacum, as well as the cells from which these processes originated, contained corazonin. However, staining alternating sections through the brain revealed that antisera from the early bleeds of the two rabbits recognized different cell types, with few cells being recognized by antisera from both rabbits.

To determine which immunoreactive cells produced authentic corazonin, a sensitive ELISA for this peptide was developed (Veenstra 1991). Since the initial antisera were not specific enough, booster inoculations were continued. As measured by ELISA, the titers of these antisera continued to improve. However, when the seventh bleeds of the two rabbits were tested in immunocytology, it appeared that not only their titers, but also their specificities had improved significantly. Antisera from both rabbits now labelled the same cells, most prominently a subgroup of neurosecretory cells in the pars lateralis and a bilateral neuron in each ganglion of the ventral nerve cord. Evidence from a combination of HPLC and ELISA confirmed the presence of authentic corazonin in both the neurosecretory cells that project to the corpus cardiacum and the bilateral neurons in the abdominal ganglia. Thus in two different HPLC systems only a single corazonin-immunoreactive peak was found in acid extracts of the corpora cardiaca and abdominal ganglia; this material has the same retention time as synthetic corazonin. Since in both the corpora cardiaca and the abdominal ganglia there is only a single type of immunoreactive cell, the processes of neurosecretory cells in the pars lateralis and the abdominal interneurons, respectively, these two cell types must contain authentic corazonin. Such evidence is lacking for the other immunoreactive cells, such as the dorsal unpaired median (DUM) neuron in the suboesophageal ganglion and the small neurons located near the base of the optic lobe. It is, therefore, possible that these cells produce a peptide other than corazonin. However, it is possible that the cells that were immunoreactive in the whole-mounts, but were not found in sectioned material, contain corazonin, but in very small quantities – quantities that are perhaps too small to be detectable after GPA fixation. This suggestion is supported by the findings that the processes of the suboesophageal DUM neuron were immunoreactive in the deutocerebrum and tritocerebrum after GPA fixation as well as in whole-mounts, while the soma was only found in whole-mounts. The immunoreactive neurosecretory cells in the pars lateralis also were only lightly labelled in the sectioned material after GPA fixation (Fig. 2), while staining intensively in the whole-mounts (Fig. 6).

There is obviously a risk in relying only on immunocytological localizations to ascertain the presence of a particular neuropeptide in a cell type. If we had not found such large differences between the antisera from

the two rabbits, we might easily have stopped injecting the rabbits after three boosters and, using these antisera, incorrectly concluded that corazonin, like many other neuropeptides, is widely distributed within the central nervous system. Our findings suggest that it may be unwise to use antisera to neuropeptides obtained after only two or three booster injections, especially when the resulting sera recognize more than one cell group. Our results also demonstrate the importance of injecting two or more rabbits and comparing their antisera in immunocytology.

Comparative aspects of corazonin-immunoreactive cells

The neurosecretory cells in the pars lateralis of the American cockroach have been described before by Pipa (1978) and Koontz and Edwards (1980) who used cobalt backfilling through the nervi corporis cardiaci to localize their cell bodies. In each hemisphere about 30 cell bodies were found, and these project to the ipsilateral corpus cardiacum via the nervi corporis cardiaci II. Thus, quantitatively, the corazonin-immunoreactive cells are an important component of the lateral neurosecretory cell group. In the cockroach *Diploptera punctata*, which has about 25 lateral neurosecretory cells (Lococo and Tobe 1984), the processes of some of the lateral neurosecretory cells terminate and branch in the corpus allatum (Thompson et al. 1987), but do not, like the corazonin-producing neurosecretory cells, arborize in the corpus cardiacum. At least some of the cells projecting to the corpora allata are immunoreactive with a monoclonal antibody to allatostatin I (Stay et al. 1992), one of a group of recently isolated allatostatins (Woodhead et al. 1989; Pratt et al. 1989, 1991).

The presence of FMRFamide immunoreactivity has previously been reported in locust DUM neurons (Ferber and Pflüger 1992), and the presence of corazonin-like immunoreactivity in a suboesophageal DUM neuron is another example of the presence of a peptide in this class of neurons, which usually contain octopamine (Evans 1980; Eckert et al. 1992). However, a detailed analysis of the DUM neurons in the suboesophageal ganglion of the locust *Locusta migratoria* found more DUM neurons than octopamine-immunoreactive neurons, indicating the possibility that some DUM neurons do not use octopamine (Bräunig 1991); alternatively, this neuron may produce both octopamine and corazonin.

Very little is known about the function of corazonin; the only effect described so far is its stimulation of heart contraction in the American cockroach (Veenstra 1989), and it is possible that this is not its only effect or even its major function. A striking aspect of the morphology of the corazonin interneurons in the ventral nerve cord is that their dendritic fields appear to be complementary but do not significantly overlap, suggesting that they form a single functional unit. The integration of the immunoreactive DUM neuron in the suboesophageal ganglion or the immunoreactive neurons and neurosecretory cells in the protocerebrum into this putative functional unit must remain speculation for the time being, but is an attractive hypothesis.

The corazonin-immunoreactive neurons in the ventral nerve cord are present in similar, segmentally repeated, locations in the ganglia and are, therefore, considered segmental homologues. Since the peptide they produce is indeed corazonin, it is tempting to speculate, that the lateral neurosecretory cells in the brain that produce corazonin are ancient segmental homologues of the abdominal interneurons. If this were correct, it would be interesting to know whether the lateral neurosecretory cells have gained a neurosecretory function during evolution, or whether the abdominal interneurons have lost their neurosecretory function. An answer to this question may well be very difficult, if not impossible, to obtain but could be an important clue to the understanding of the evolution of the insect nervous system in particular, and nervous systems in general.

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