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THE DISTRIBUTION OF A CRF-LIKE DIURETIC PEPTIDE IN THE BLOOD-FEEDING BUG *RHODNIUS PROLIXUS*

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Summary

The blood-feeding bug *Rhodnius prolixus* ingests a large blood meal, and this is followed by a rapid diuresis to eliminate excess water and salt. Previous studies have demonstrated that serotonin and an unidentified peptide act as diuretic factors. In other insects, members of the corticotropin-releasing factor (CRF)-related peptide family have been shown to play a role in post-feeding diuresis. Using fluorescence immunohistochemistry and immunogold labelling with antibodies to the *Locusta* CRF-like diuretic hormone (*Locusta*-DH) and serotonin, we have mapped the distribution of neurones displaying these phenotypes in *R. prolixus*. Strong *Locusta*-DH-like immunoreactivity was found in numerous neurones of the central nervous system (CNS) and, in particular, in medial neurosecretory cells of the brain and in posterior lateral neurosecretory cells of the mesothoracic ganglionic mass (MTGM). Positively stained neurohaemal areas were found associated with the corpus cardiacum (CC) and on abdominal nerves 1 and 2. In addition, *Locusta*-DH-like immunoreactive nerve processes were found over the posterior midgut and hindgut. Double-labelling studies for *Locusta*-DH-like and serotonin-like immunoreactivity demonstrated some co-localisation in the CNS; however, no co-localisation was found in the medial neurosecretory cells of the brain, the posterior lateral neurosecretory cells of the MTGM or neurohaemal areas. To confirm the presence of a diuretic factor in the CC and abdominal nerves, extracts were tested in Malpighian tubule secretion assays and cyclic AMP assays. Extracts of the CC and abdominal nerves caused an increase in the rate of secretion and an increase in the level of cyclic AMP in the Malpighian tubules of fifth-instar *R. prolixus*. The presence of the peptide in neurohaemal terminals of the CC and abdominal nerves that are distinct from serotonin-containing terminals indicates that the peptide is capable of being released into the haemolymph and that this release can be independent of the release of serotonin.

Key words: peptide, *Rhodnius prolixus*, diuresis, corticotropin releasing factor, serotonin, immunoreactivity.

Introduction

Larvae of the blood-feeding hemipteran *Rhodnius prolixus* ingest large blood meals of up to 10 times their initial body mass. In this engorged state, the bug is vulnerable to predation and must eliminate excess water and salt to reduce the volume of its meal. Rapid elimination of urine usually commences within 2–3 min of feeding and lasts for the next 3 h, during which the insect may lose 40 % of the mass of the meal. The rate of post-feeding diuresis is one of the fastest of all insects (Nicolson, 1993) and, *in vivo*, is 0.4–0.7 μl min^{-1} for the first 2–3 h (Maddrell, 1964a,b). This diuresis in *R. prolixus* is under the control of one or more diuretic hormones that cause a 1000-fold post-feeding increase in the rate of fluid transport by the Malpighian tubules (Maddrell, 1966). Haemolymph taken from a recently gorged insect has potent diuretic activity when tested on isolated Malpighian tubules (Maddrell, 1963). Maddrell (1963) also tested tissue homogenates on isolated Malpighian tubules and found diuretic activity in all parts of the central nervous system (CNS) of *R. prolixus* except for the corpora cardiaca (CC). The majority of the diuretic activity was in the mesothoracic ganglionic mass (MTGM), and 90 % of this activity resided in posterior lateral cell groups. The release of the diuretic factor into the haemolymph appears to be from neurohaemal sites on the abdominal nerves (Maddrell, 1966; Berlind and Maddrell, 1979). Aston and White (1974) determined that the diuretic factor present in homogenates was peptidergic in nature. In addition to this unidentified peptide, the amine serotonin has been reported to be a true diuretic hormone in *R. prolixus* (Maddrell et al., 1991). Serotonin is released from neurohaemal areas, and haemolymph serotonin levels are elevated to 10^{-7} mol l^{-1} within 5 min of feeding in fifth-instar *R. prolixus* (Lange et al., 1989). These levels drop over the next 20 min to less than 10^{-8} mol l^{-1}. Serotonin stimulates fluid secretion, with a threshold of 5×10^{-8} mol l^{-1} (Maddrell et al., 1969), and elevates cyclic AMP levels in

Malpighian tubules (Barrett and Orchard, 1990). Both serotonin (Barrett and Orchard, 1990; Montoreano et al., 1990) and at least one peptide diuretic hormone (Aston, 1975) are believed to act *via* a cyclic-AMP-dependent pathway. Barrett and Orchard (1990) suggested a possible synergistic role for serotonin and the diuretic peptide, and Maddrell et al. (1993) showed that serotonin, indeed, acts synergistically with forskolin and the peptide diuretic hormone(s) to increase rates of fluid secretion.

In other insects, two families of diuretic peptides (DPs), the corticotropin-releasing factor (CRF)-like and kinin peptides (Coast, 1996), have been identified and sequenced. In *Drosophila melanogaster*, cardioactive peptide 2b (CAP_{2b}) has also been identified as a stimulatory factor of Malpighian tubules (O'Donnell et al., 1996). In *R. prolixus*, however, CAP_{2b} has recently been shown to inhibit diuresis (Quinlan et al., 1997).

The CRF-like family of diuretic peptides (DPs) includes eight published sequences that have a high degree of sequence identity to a superfamily of vertebrate peptides that includes sauvagine, corticotropin-releasing factor (CRF= corticoliberin), urotensin I and urocortin. The first insect diuretic peptide was isolated from *Manduca sexta* (Kataoka et al., 1989) and termed a diuretic hormone (DH); it is 41 amino acid residues in length with an amidated carboxyl terminus. Subsequently, a second diuretic peptide/diuretic hormone was isolated from *M. sexta* and termed *M. sexta* DPII (Blackburn et al., 1991); it contains only 30 amino acid residues. Three diuretic peptides all containing 46 amino acid residues were isolated from *Locusta migratoria* (Kay et al., 1991b; Lehmberg et al., 1991), *Acheta domesticus* (Kay et al., 1991a) and *Periplaneta americana* (Kay et al., 1992). A 44-amino-acid diuretic peptide has been identified from both *Musca domestica* and *Stomoxys calcitrans* (Clottens et al., 1994). All these insect diuretic peptides/diuretic hormones have high biological activity on *M. sexta* Malpighian tubules (Audsley et al., 1995). More recently, two diuretic hormones were identified from *Tenebrio molitor*, containing 37 (Furuya et al., 1995) and 47 (Furuya et al., 1998) amino acid residues; unlike any other CRF-like peptides, *T. molitor* DH_{37} and DH_{47} both have non-amidated carboxyl termini, which are probably responsible for their lack of detectable biological activity on Malpighian tubules of *M. sexta* (Furuya et al., 1995). However, *T. molitor* DH_{37} in particular has high biological activity on *T. molitor* Malpighian tubules (Furuya et al., 1995). Ignoring the identical diuretic peptide isolated from the two species of fly, the identities of members of the CRF-like diuretic peptides/diuretic hormones show 20–76 % identity (this value depends on the alignment used; these values are from the most recently published alignment, that of Furuya et al. (1998). While a number of researchers have chosen to call these diuretic peptides rather than hormones, Patel et al. (1995) have now presented 'unequivocal evidence of a hormonal function for *Locusta*-DP in the control of primary urine production' in *L. migratoria*. Hence, the *Locusta* diuretic peptide is now referred to as *Locusta* diuretic hormone (*Locusta*-DH).

CRF-like peptides increase cyclic AMP content (Coast, 1996), transepithelial potential (O'Donnell et al., 1996; Nicolson, 1993) and rate of secretion in insect Malpighian tubules (Coast, 1996). Recently, Coast (1996) demonstrated that *Locusta*-DH stimulated fluid secretion in *R. prolixus* Malpighian tubules, indicating that *R. prolixus* may well contain member(s) of this family of peptides.

The purpose of our research is to characterize more fully the neurohormonal regulation of diuresis in *R. prolixus*. In this paper, we report the localisation (and co-localisation) of two identified diuretic factors, a *Locusta*-DH-like factor and serotonin. Since a CRF-like diuretic peptide has not been sequenced from *R. prolixus*, we have been unable to use a species-specific antibody for immunolocalisation studies. However, the sequence of the *Locusta*-DH is known, and an antiserum to the carboxyl terminus (residues 29–46) of this peptide has been raised (Patel et al., 1994). Using this antiserum, together with fluorescence immunohistochemistry and immunogold techniques, the distribution of *Locusta*-DH-like material in the central nervous system (CNS) and gut of fifth-instar *R. prolixus* has been studied. In addition, we have looked for co-localisation with the other known diuretic hormone in *R. prolixus*, serotonin. We have also tested CC and abdominal nerve tissue extracts in Malpighian tubule secretion and cyclic AMP assays.

Materials and methods

Insects

Fifth-instar larvae of *Rhodnius prolixus* Stål were taken from a long-standing colony maintained at 25 °C under high humidity. The insects were unfed, 6–8 weeks post-emergence and had been fed on rabbit blood as fourth instars.

Fixation and staining

The fifth-instar larvae were secured on a piece of dental wax in a dissecting dish with the dorsal cuticle uppermost. Under physiological saline (Lane, 1975), the dorsal cuticle was removed, exposing the CNS and visceral tissues, and the tissues were then fixed *in situ* using 2 % paraformaldehyde. In some preparations, the dorsal abdominal cuticle was also processed. The tissues were fixed and stained as described by Tsang and Orchard (1991), with some minor modifications. In brief, the tissues were fixed for approximately 2 h at room temperature (22–24 °C), washed in phosphate-buffered saline (PBS), then transferred into 4 % Triton X-100 with 2 % bovine serum albumin (BSA) and 10 % normal sheep serum (NSS) for 1 h. The preparations were transferred to the primary antiserum solution and placed on a flatbed shaker at 12 °C for 24–48 h. The polyclonal antisera were raised in rabbit against serotonin (Incstar, Stillwater, MN, USA), *Locusta*-DP residues 29–46 (Patel et al., 1994) and *Manduca*-DH residues 1–41 and 29–41. The *Manduca sexta* peptide and fragment 29–41 were conjugated to glutaraldehyde, and the rabbits were injected using keyhole limpet haemocyanin (KLH). The *Manduca sexta* antibodies were immunoaffinity-purified using hapten-

conjugated BSA. Anti-serotonin or anti-*Locusta*-DH antisera were used at concentrations of 1:1000 or 1:4000 in 0.4 % Triton X-100 with 2 % BSA and 10 % NSS or normal goat serum (NGS) (depending on the secondary antibody to be used). The anti-*Manduca*-DH antisera were used at concentrations of 1:250, 1:500 or 1:1000 in 0.4 % Triton X-100 with 2 % BSA and 10 % NSS. The preparations were then washed in PBS for 24 h at 12 °C. Three different procedures for processing with secondary antibody were used. (i) The preparations were placed in Cy3-labelled sheep anti-rabbit immunoglobulin solution (Sigma Chemicals, St Louis, MO, USA) at 1:200 in PBS with 10 % NSS for 12 h, and then washed for 18 h at 12 °C or 5 h at room temperature in PBS. (ii) The preparations were placed in biotin-labelled anti-rabbit immunoglobulin solution (Biocan Scientific, Mississauga, Ontario, Canada) at 1:200 with 10 % NGS for 18 h at 12 °C, washed for 18 h in PBS followed by Cy3-labelled streptavidin (Biocan Scientific). (iii) The preparations were placed in FITC-conjugated anti-rabbit immunoglobulin (IgG; Biocan Scientific) for 18 h at 12 °C and again washed for 18 h at 12 °C or 5 h at room temperature in PBS. Double-labelled preparations were stained serially, the primary antiserum followed by the secondary antiserum, for *Locusta*-DH (secondary FITC), then serotonin (secondary, Texas-Red-conjugated anti-rabbit IgG; Biocan Scientific). All preparations were mounted in a solution of 80 % glycerol containing 5 % *n*-propyl gallate, pH 7.3, and were then viewed under an epifluorescence microscope equipped with a drawing tube and/or a confocal microscope (Viewscan DVC-250, Biorad, Hercules, CA, USA).

Control experiments, where required, were run in which the primary antiserum was omitted or in which the primary antiserum (1:1000) was preincubated with 10 μmol l^{-1} *Locusta*-DH.

Immunogold electron microscopy

Electron microscopic examination and immunocytochemistry were performed as described previously (Miksys and Orchard (1994). The corpora cardiaca, aorta and MTGM with abdominal nerves attached were exposed under physiological saline and then fixed *in situ* at room temperature for 1 h in 3 % glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.0). Tissues were dissected and fixed for a further 30 min in fresh fixative, rinsed in buffer and embedded in 1.5 % aqueous agarose. They were then post-fixed in 0.5 % osmium tetroxide in the same buffer for 10 min, before dehydration and embedding. The agarose blocks were embedded in an Epon-Araldite (J.B. EM, Dorval, Quebec, Canada) mixture *via* propylene oxide. Silver/gold sections (100–110 nm) were collected on uncoated 200 mesh nickel grids, etched with fresh 4 % aqueous sodium metaperiodate for 10 min at room temperature, rinsed in distilled water and incubated in 0.05 mol l^{-1} Tris buffer (pH 7.2) with 0.5 % bovine albumin (fraction V, protease-free, Sigma) and 0.1 % NGS (TBS/BA/NGS) for 15–60 min at room temperature. Grids were then incubated in the rabbit anti-*Locusta*-DH (1:700 in TBS/BA/NGS) for 18 h at 4 °C, washed three times by rotation in TBS/BA/NGS for 20 min at room temperature and then incubated in goat anti-rabbit IgG conjugated to 10 nm colloidal gold particles (Sigma) 1:50 in TBS/BA/NGS for 1 h at room temperature. Grids were again washed three times by rotation in TBS/BA/NGS for 20 min. The sections were then stained for 20 min in aqueous uranyl acetate and washed three times by rotation in TBS/BA/NGS for 20 min. The grids were rinsed in distilled water for 10 min and viewed with a Hitachi H7000 electron microscope. Granules were measured, and the true diameters were calculated according to the method of Froesch (1973).

Controls were performed either by omitting the primary antiserum or by pre-absorbing the antiserum with *Locusta*-DH at 10 μmol l^{-1} for 3.5 h at room temperature.

Tissue extracts

Corpora cardiaca and abdominal nerves (1–5) were dissected from *R. prolixus* fifth instars and collected into ice-cold methanol:acetic acid:water (90:9:1). The tissues were frozen at −20 °C, then thawed, sonicated and centrifuged at 8800 ***g*** for 10 min. The supernatant was decanted and dried in a Speed-Vac (Savant, Farmingdale, NY, USA). These tissue extracts were then applied to a C_{18} Sep-Pak cartridge (Waters Associates, Mississauga, Ontario, Canada) previously equilibrated as described by Miggiani et al. (1999). The cartridge was then washed sequentially with 3.0 ml each of water, 30 %, 60 % and 100 % acetonitrile (Burdick and Jackson, Muskegon, MI, USA) with 0.1 % trifluoroacetic acid (BDH, Toronto, Ontario, Canada), and the elutant was collected. The collected extracts were dried in the Speed-Vac and frozen at −20 °C until use. Extracts were reconstituted in *Rhodnius* saline at a concentration of one tissue equivalent per 10 μl.

Malpighian tubule secretion assay

The fifth-instar larvae were secured and dissected open as described above. Under physiological saline, the Malpighian tubules were freed from trachea and fat with the aid of fine glass rods. The upper portions of the tubules were transferred to a 20 μl drop of physiological saline under water-saturated heavy mineral oil. The open end of the tubule was pulled out and wrapped around a minuten pin 2 mm away from the edge of the 20 μl drop. The tubules were allowed to equilibrate for 20 min. Droplets of urine from the cut end of the tubule were removed by sucking up the drop into an oil-filled fine polypropylene pipette. The drop was then transferred and gently blown out under the oil and allowed to settle on the Sylgard-coated bottom of the dish. The diameter of the sphere was measured using an eye-piece micrometer, and the volume was calculated. Saline containing the various tissue extracts was exchanged for the equilibrating saline. The tubules were allowed to secrete for 20–30 min. The maximum rate of secretion for each tubule was determined using 10^{-6} mol l^{-1} serotonin (Sigma). Rates are calculated as a percentage of the maximum rate of secretion.

Malpighian tubule cyclic AMP assay

Malpighian tubules from fifth-instar larvae were dissected under *Rhodnius* saline. The tubules were then transferred to a microfuge tube containing 5×10^{-4} mol l^{-1} 3-isobutyl-1-methylxanthine (IBMX; Sigma), a phosphodiesterase inhibitor, and tissue extract, saline only or 10^{-6} mol l^{-1} serotonin. Tubules were incubated for 10 min, and the reaction was then stopped with 500 µl of boiling 0.05 mol l^{-1} sodium acetate. Samples were placed in a boiling water bath for 5 min, then frozen at –20 °C until assayed. To assay the cyclic AMP content of the Malpighian tubules, the samples were thawed, sonicated and centrifuged at 8800 ***g*** for 10 min, and the supernatant was decanted. The cyclic AMP in the supernatant was measured using a radioimmunoassay kit (Mandel/NEN, Guelph, Ontario, Canada) with modifications as described by Lange and Orchard (1986).

Results

Distribution of Locusta*-DH-like immunoreactivity in unfed* R. prolixus

The antisera raised against *Manduca*-DP 1–41 and 29–41 at concentrations of 1:1000–1:250 produced no immunoreactive staining in the CNS of fifth-instar *R. prolixus*. In contrast, the anti-*Locusta*-DH antiserum generated against residues 29–46 showed immunoreactivity distributed throughout the CNS and gut. A composite *camera lucida* drawing of the cell bodies in the CNS that were immunoreactive to the anti-*Locusta*-DH antiserum is shown in Fig. 1. Although staining is found throughout the CNS, there are two areas of particular interest: (a) the medial neurosecretory cells and their projections to the CC; and (b) the posterior lateral neurosecretory cells in the MTGM, which send projections out to abdominal nerves 1 and 2 and form neurohaemal-like areas on these nerves.

Control experiments in which the primary antiserum was omitted or preabsorbed with *Locusta*-DH (10 µmol l^{-1}) resulted in the abolition of staining in the CNS of *R. prolixus.*

Brain and retrocerebral complex

Approximately 450 cells in the brain showed positive stianing, with 40–46 being very intensely stained (Fig. 2A). Most of these cell bodies were found in the protocerebrum of the brain. Large numbers of immunoreactive cells were found at the base of the optic lobes. In addition, numerous cells stained along the posterior margin of the protocerebral lobes, including a cluster of five brightly stained cells in each hemisphere. Processes from this group of cells could be followed for a short distance and appeared to exit *via* a small nerve (Chiang and Davey, 1988) on the posterior margin of the protocerebral lobes. These projections appeared to join up to the anterior of the CC. Twelve to fourteen medial neurosecretory cells in each lobe of the brain stained intensely (Fig. 2B). The processes of these cells projected to the midline of the brain, where they converged and then descended ventrally to above the oesophagus. At this point, the tracts again separated, with each branch passing posteriorly on the ventral side of the brain and exiting the procerebral lobes at the nervus corporis cardiaci (NCC). Some varicosities were found on the anterior surface of the brain. No extensive aborizations of the projections from the cell bodies were seen in the brain.

The retrocerebral complex in *R. prolixus* is composed of a fused CC, a single corpus allatum, the aorta and the NCC (Chiang and Davey, 1988). The aorta is attached to the CC and the posterior margin of the brain. The strong staining in the projections from the medial neurosecretory cells could be followed into the CC (Fig. 2C). The CC stained very intensely, revealing an extensive plexus of immunoreactive varicosities, with weak staining extending a short distance along the aorta (Fig. 2C,D).

Suboesophageal and prothoracic ganglion

In the suboesophageal ganglion (SOG), 122–130 cells

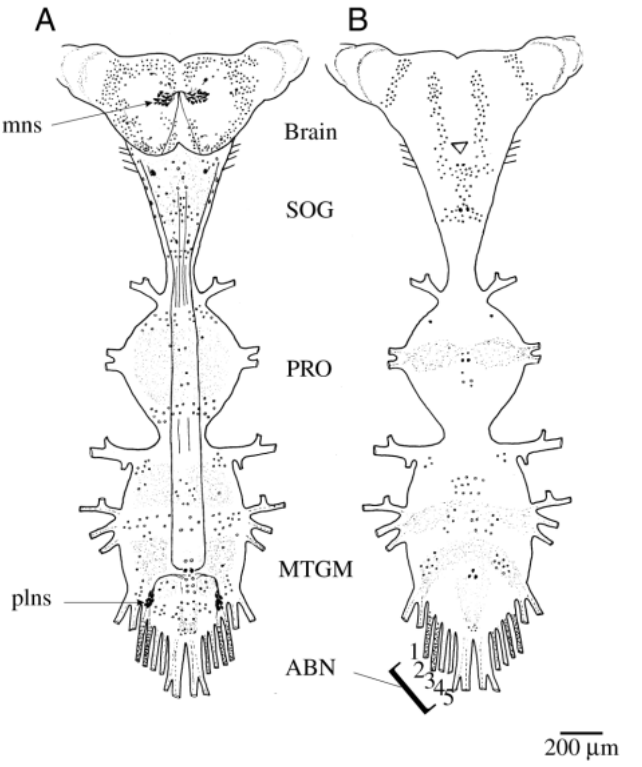

Fig. 1. Composite *camera lucida* drawing of dorsal (A) and ventral (B) aspects of the central nervous system of *Rhodnius prolixus.* Filled cells indicate strong immunoreactivity to *Locusta*-DH antiserum. Intensely stained medial neurosecretory cells (mns) in the brain send processes medially and ventrally. These processes travel through the brain and exit to the corpus cardiacum (not shown). The intensely stained posterior lateral neurosecretory cells (plns) in the mesothoracic ganglionic mass (MTGM) send processes centrally and out through abdominal nerves 1 and 2. Stippled areas in the suboesophageal ganglion (SOG), prothoracic ganglion (PRO) and MTGM indicate neuropile. Fine processes can be seen in all the abdominal nerves (ABN1–ABN5) in the MTGM. Abdominal nerves 1 and 2 have extensive neurohaemal areas along the length of the nerves. Scale bar, 200 µm.

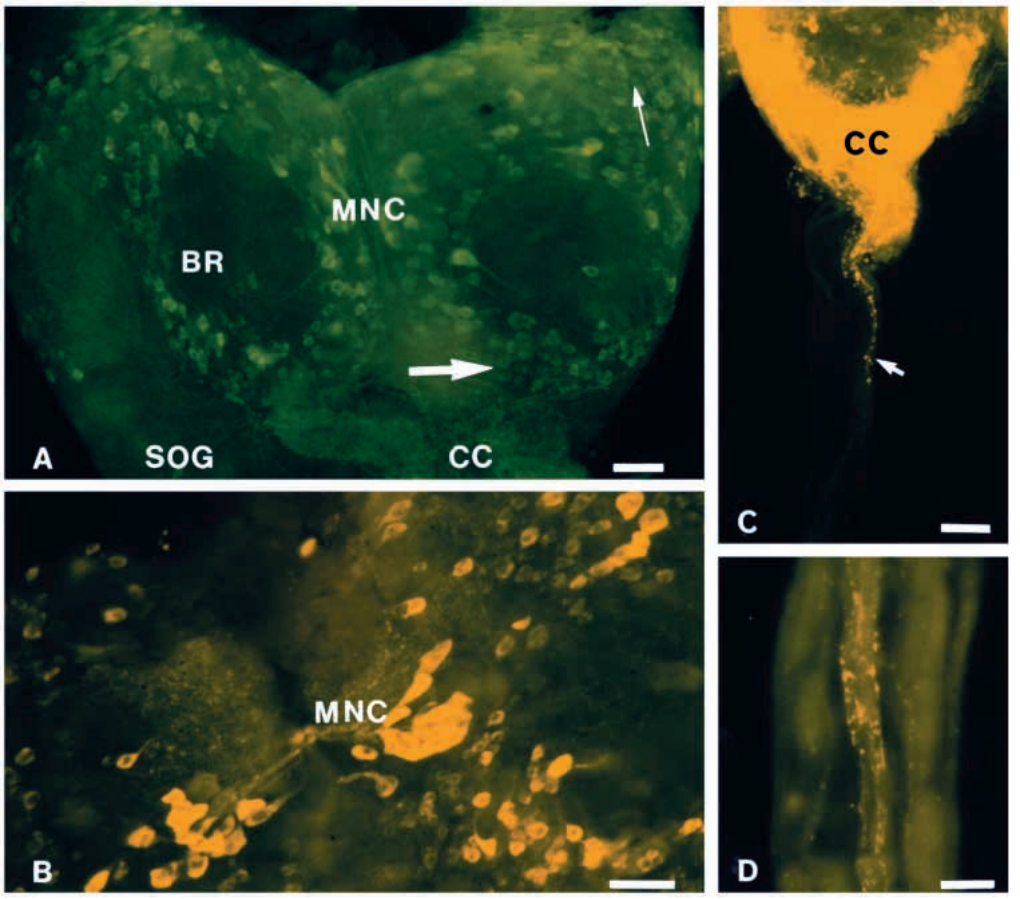

Fig. 2. (A) Whole-mount of a fifth-instar *Rhodnius prolixus* brain (BR), suboesophageal ganglion (SOG) and corporus cardiacum (CC) stained using the *Locusta*-DH antiserum. A large number of cells in the optic lobe/brain junction (thin arrow) and many cells along the posterior edge of the lobes of the brain (thick arrow) are immunoreactive. The medial neurosecretory cells (MNC) are partially obscured. Scale bar, 50 µm. (B) Medial neurosecretory cells of the brain (MNC). Scale bar, 50 µm. (C) Corporus cardiacum (CC) and aorta. Note the stain running a short distance along the aorta (arrow). Scale bar, 50 µm. (D) Higher magnification of the immunoreactive staining in the aorta near the CC. Scale bar, 25 µm.

stained positively. Most of these cell bodies were bilaterally paired. Some strongly staining cells were found on the lateral margin of the SOG. Two bilaterally paired cells stained strongly in the midline of the ventral anterior SOG, and there were also other more faintly stained central cells. In the prothoracic ganglion, 58–62 bilaterally paired cells stained at the anterior and posterior ends of the ganglion (Fig. 3A). Several pairs of axon processes, two of which could be traced from the base of the brain to the MTGM (their origin unknown), ran through the connectives into each of the ganglia, where they arborized extensively in the neuropile.

Mesothoracic ganglionic mass

The MTGM had 250–260 immunoreactive cells (Fig. 3B) that stained with anti-*Locusta*-DH antiserum. In the central midline of the fused ganglia, 6–8 paired cells were immunopositive, of which 2–4 stained very strongly. There was an extensive immunoreactive neuropile in the anterior, midlateral and central posterior portions of the MTGM. Axon tracts could be followed along the connectives from the prothoracic ganglion into the MTGM (Fig. 3A). With the exception of one pair of tracts, these projections were lost in the anterior neuropile. The one pair of tracts that did not enter into this anterior neuropile extended posteriorly along the lateral portions of the MTGM, then turned towards the mid-region, ending close to the pair of strongly stained cells in the central midline of the MTGM. Anterior, medial and posterior lateral groups of stained cells were also evident. The posterior lateral neurosecretory cell groups of the MTGM were very intensely stained (Fig. 3B). There were 10–12 cells in this position. The processes from these cells bifurcated at some point anterior to the cell body. One set of branches could be traced into the neuropile. The other set passed out through abdominal nerves 1 or 2 and resulted in positively stained neurohaemal areas lying on the surface of these nerves (Fig. 3C). The staining on nerves 1 and 2 could also be followed out to the body wall, where some staining was seen around the spiracles. Fine axon tracts could also be seen in abdominal nerves 3–5 and in the genital nerves.

Digestive system

Immunoreactive staining on the hindgut was consistent in all the preparations studied. The hindgut and the posterior midgut had a very extensive staining pattern of fine nerve processes over their entire surfaces (Fig. 3D). No nerve processes were seen over the crop (anterior midgut) or the foregut. A few immunoreactive endocrine-like cell bodies were seen in the crop and posterior midgut in only two preparations, from insects that had been starved for 10 weeks and using the sensitive Cy3-conjugated secondary antibody. These cells were triangular in shape, but were not strongly stained or clearly defined. Lateral extensions were not visible.

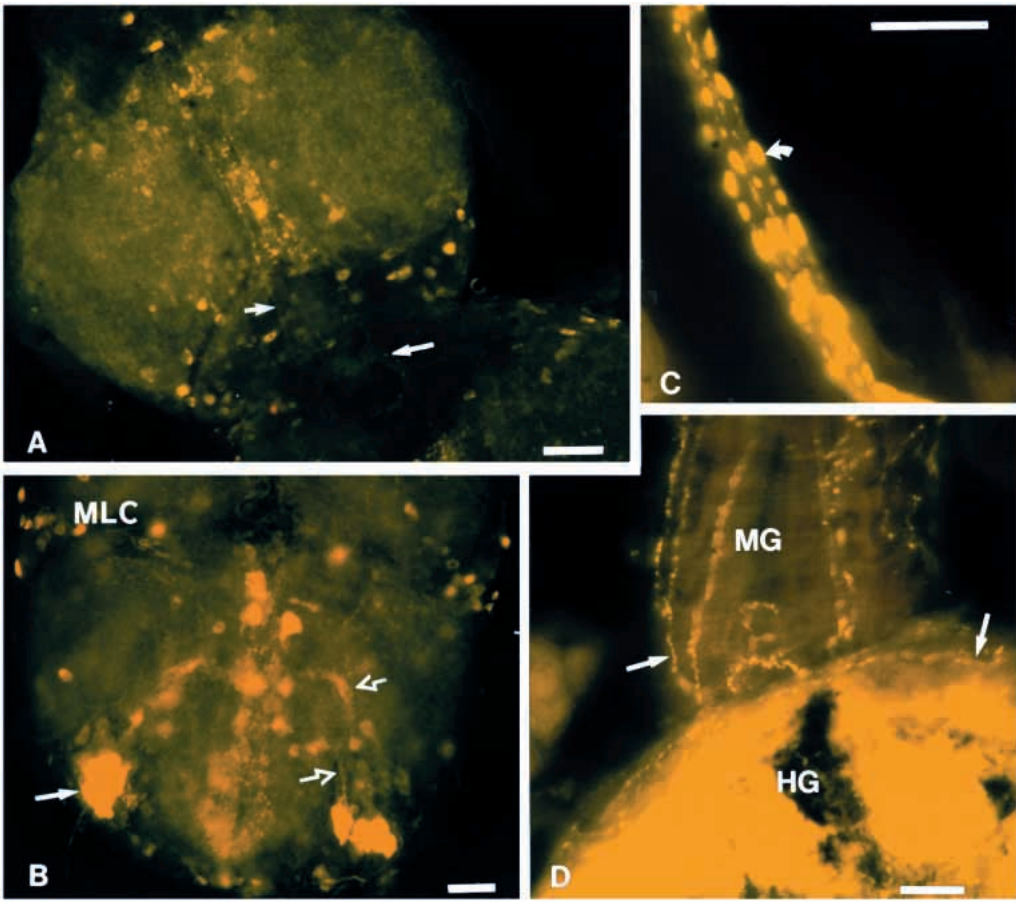

Fig. 3. (A) Prothoracic ganglion showing the strongly stained neuropile and cell bodies anteriorly and posteriorly in the ganglion. Note the axon tracts which project through the ganglion and connective to the mesothoracic ganglionic mass (MTGM) (arrows). Scale bar, 50 µm. (B) The MTGM showing the strongly stained posterior lateral neurosecretory cells (filled arrow) and processes (open arrows) which are seen to project towards the central MTGM. Groups of mid-lateral cells (MLC) are also seen in the MTGM. Scale bar, 50 µm. (C) Neurohaemal staining (curved arrow) on the abdominal nerve. Scale bar, 25 µm. (D) Hindgut (HG) and posterior midgut (MG) showing fine processes (arrows) on the posterior midgut and covering the entire hindgut. Scale bar, 50 µm.

Serotonin-like and Locusta*-DH-like double-label immunohistochemistry*

Using double-label immunohistochemistry, we compared the distribution of serotonin-like and *Locusta*-DH-like immunoreactivity. In the brain of fifth-instar *R. prolixus*, some cells were double-labelled for both serotonin and the peptide. These occurred at the margin of the optic lobes and the brain (five cells) and at the posterior margin of the brain (four cells), with one strongly double-labelled cell in the medial part of the brain (Fig. 4A). The medial neurosecretory cells, however, were not double-labelled and only revealed labelling for *Locusta*-DH-like immunoreactivity (Fig. 4A). The CC had neurohaemal-like staining for both serotonin-like and *Locusta*-DH-like immunoreactivity, but these terminals were not double-labelled. Dorsal unpaired medial (DUM) neurones located in the MTGM are the major source of serotonin-like neurohaemal staining on the five abdominal nerves (Orchard et al., 1989), whereas the posterior lateral neurosecretory cells of the MTGM appeared to be the major contributors to the *Locusta*-DH-like neurohaemal staining on abdominal nerves 1 and 2. In the MTGM, there was some co-localisation of serotonin and the peptide in cell groups flanking the posterior lateral neurosecretory cell groups (Fig. 4B), but not in the posterior lateral neurosecretory cell groups, the DUM neurones or the neurosecretory terminals on the abdominal nerves (Fig. 4C).

Immunogold electron microscopy

At the electron microscope level, the aorta and abdominal nerves were surrounded by a basal membrane, which is an acellular sheath above the perineural layer of cells. Below the perineural layer were axons of various diameters. The perineural layer provides a selective barrier between the haemolymph and the axons. Thin sections of the CC, the aorta and the abdominal nerves showed the presence of *Locusta*-DH-like immunoreactive material as shown by 10 nm gold particles lying over electron-dense granules in neurosecretory terminals (Fig. 5A–D). These neurosecretory terminals were located between the basal membrane and the perineural layer in the aorta and abdominal nerves (Fig. 5C,D). In the CC, two types of granules, found in different terminal types, were observed to be immunoreactive (Table 1), one type smaller and round and the second oval. The oval granules were found in terminals containing granules of irregular profile. Only granules in which a full profile was seen were measured. In the aorta, only oval granules were found to be immunoreactive. The electron micrographs of the abdominal nerves showed similar types of neurosecretory terminals to those found in the CC and aorta. However, in these terminals, only a single type of immunoreactive granule was found. These granules were round, but slightly larger in size than those of the CC (Table 1).

Both omission of primary antiserum and preabsorption of

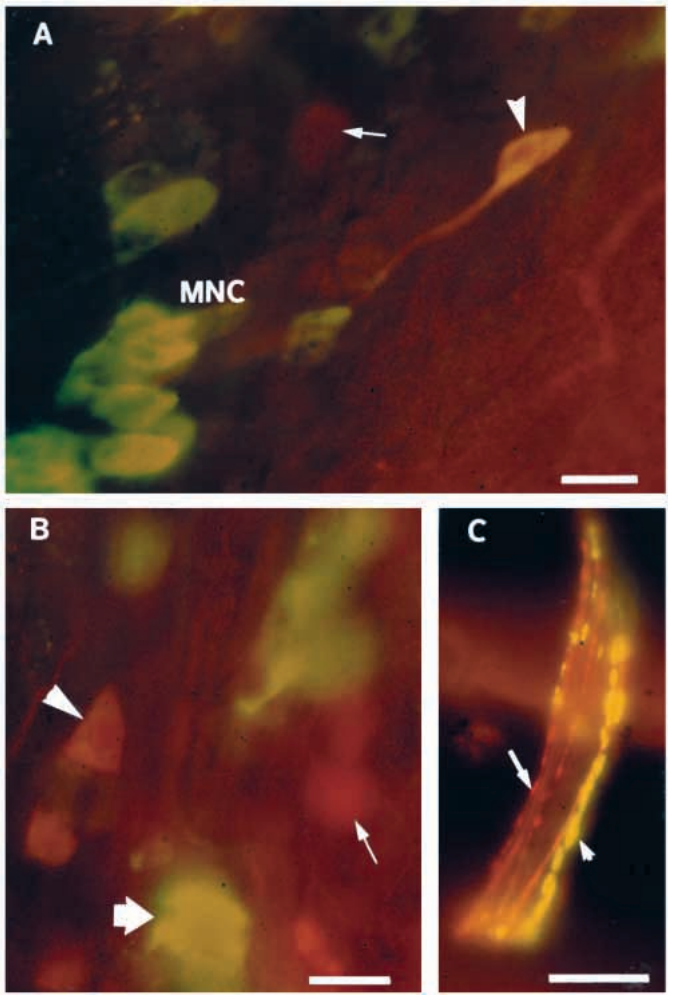

Fig. 4. (A) A brain processed for both *Locusta*-DH-like immunoreactivity (FITC) and serotonin-like immunoreactivity (Texas Red) showing single-labelled medial neurosecretory cells (MNC) and a single-labelled serotonin-like immunoreactive cell (arrow). Note the double-labelled cell (arrowhead). Scale bar, 25 µm. (B) The mesothoracic ganglionic mass showing posterior lateral cells single-labelled for *Locusta*-DH-like immunoreactivity (thick arrow), single-labelled for serotonin-like immunoreactivity (thin arrow) and double-labelled (arrowhead). Scale bar, 25 µm. (C) Abdominal nerve 2 showing the single-labelled *Locusta*-DH-like (arrowhead) and single-labelled serotonin-like (arrow) immunoreactive neurohaemal sites. Scale bar, 25 µm.

primary antiserum with *Locusta*-DH (10 µmol l^{-1}) abolished all immunogold staining.

Malpighian tubule secretion assay

To gain some experimental evidence for the presence of the CRF-like diuretic peptides in *R. prolixus* neurohaemal tissues, we processed these tissues through Sep-Pak, eluted with 30, 60 and 100 % acetonitrile in 0.1 % TFA, and assayed the individual fractions using the *R. prolixus* Malpighian tubule secretion assay. Material eluting with 60 % acetonitrile in 0.1 % TFA possessed diuretic activity. The 60 % acetonitrile cut of the CC had activity reaching 41.7±6.6 % (mean ± S.E.M., *N*=6) of maximum secretion rate tested at 2 tissue equivalents, while the 60 % cut of the abdominal nerves had activity reaching 27.01±5.8 (*N*=8) of maximum secretion rate tested at 2 tissue equivalents. Interestingly, while the 30 % and 100 % cuts from the CC extracts did not alter basal secretion rates, the 30 % cut from the abdominal nerves did possess activity reaching 18.45±6.6 % of maximum secretion rate, suggesting the probability that more than one diuretic factor is associated with the abdominal nerves.

Table 1. Locusta-*DH-like immunoreactive granule morphology for granules found in the corpus cardiacum, aorta and abdominal nerves*

Tissue	Granule type	Mean diameter (nm)	True diameter (nm)
CC	Round	73.2±2.5 (24)	77.6
CC	Oval	121.1±6.3×73.9±3.3 (18)	135.6×79.4
Aorta	Oval	114.9±3.6×76.7±2.5 (31)	129.12×83.5
Abdominal nerves	Round	112.5±3.4 (33)	124.5

The corpus cardiacum (CC) contains two different granule types differing in shape and size.
True diameter was calculated according to Froesch (1973).
Values are means ± S.E.M. (*N*).

Malpighian tubule cyclic AMP assay

Since the 60 % acetonitrile cut of both the CC and abdominal nerves possessed diuretic activity when tested on isolated Malpighian tubules, and the CRF-like insect diuretic peptides have previously been shown to elevate the cyclic AMP content of Malpighian tubules, we assayed these fractions for their ability to elevate cyclic AMP levels in *R. prolixus* Malpighian tubules. The fraction eluting with 60 % acetonitrile in 0.1 % TFA from Sep-Pak C_{18} from both CC and abdominal nerves was capable of increasing the cyclic AMP content of *R. prolixus* Malpighian tubules in the presence of IBMX. When tested at 4 tissue equivalents per 50 µl, the CC increased cyclic AMP content 3.8-fold (*N*=4), whereas the abdominal nerves, when tested at 4 tissue equivalents per 100 µl, increased cyclic AMP content 2.3-fold (*N*=4). In comparison, 10^{-6} mol l^{-1} serotonin increased cyclic AMP content by 3.5-fold over the saline control values.

Discussion

The results demonstrate that *R. prolixus* possesses at least one peptide related to the insect CRF-like diuretic peptide family. The neurones expressing this phenotype are extensively distributed in the CNS and project to neurohaemal sites in the CC and on the abdominal nerves and to the hindgut. In addition, endocrine-like cells of the midgut may also express these peptides, although not strongly. These data were obtained using an antiserum raised against *Locusta*-DH which has been

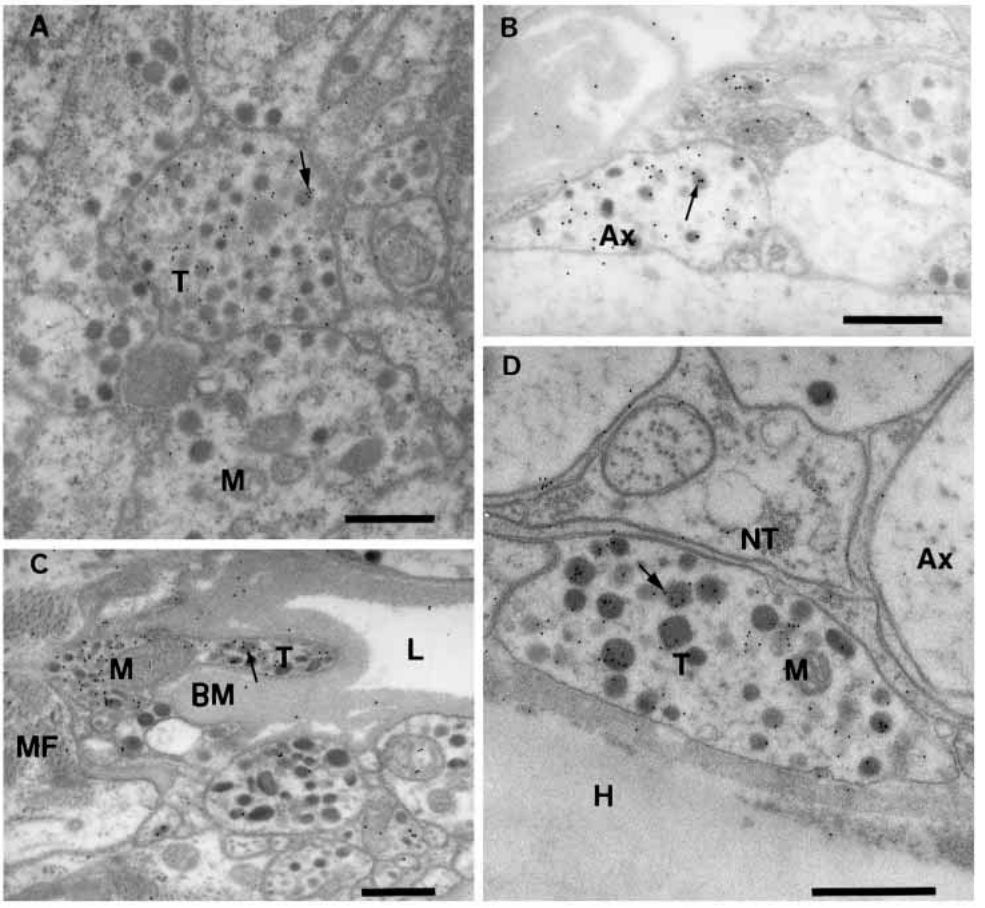

Fig. 5. (A) Electron micrograph of the corporus cardiacum showing a neurosecretory axon terminal (T) with colloidal gold particles concentrated on neurosecretory granules (arrow) showing *Locusta*-DH-like immunoreactivity. Note the mitochondria (M). Scale bar, 0.5 µm. (B) Section of the aorta, with a nerve containing axons (Ax) with immunogold labelling of *Locusta*-DH-like immunoreactive neurosecretory granules (arrow). Scale bar, 0.5 µm. (C) Section through the aorta showing the lumen (L) of the aorta and an axon terminal (T) containing immunogold labelling of *Locusta*-DH-like immunoreactive neurosecretory granules (arrow). The terminal lies against the basement membrane (BM). Note the mitochondria (M) and the muscle fibres (MF) of the aorta. Scale bar, 0.5 µm. (D) Section of abdominal nerve 2 showing an axon (Ax) and an axon terminal (T) containing *Locusta*-DH-like immunogold-labelled neurosecretory granules (arrow). The terminal lies against the basement membrane next to the haemolymph (H). Note the neurotubules (NT) and mitochondria (M). Scale bar, 0.5 µm.

shown to recognise the CRF-like *Locusta*-DH in *Locusta migratoria* (Patel et al., 1994). Patel et al. (1994) used a combination of high-performance liquid chromatography, mass spectrometry, bioassay and immunoassay to show that the antiserum recognised authentic *Locusta*-DH. Moreover, Audsley et al. (1997) used RIA to show that the antiserum recognised CRF-related peptides, but not unrelated peptides. Interestingly, the antisera raised against residues 29–41 and 1–41 of *Manduca*-DH did not result in any immunofluorescence in the CNS of *R. prolixus*. Preabsorption of the *Locusta*-DH antiserum with *Locusta*-DH abolished staining in the CNS, indicating a degree of specificity of the antiserum. Whilst the blocking of staining does not remove the possibility that the antiserum cross-reacts with another peptide(s) (see Nässel, 1996), the fact that this antiserum stains the posterior lateral neurosecretory cells of the MTGM, cells that have been shown previously to possess diuretic activity (Maddrell, 1966; Berlind and Maddrell, 1979), certainly suggests the antiserum is recognising a diuretic peptide in *R. prolixus*. The projections from these cells and the neurohaemal distribution on abdominal nerves 1 and 2 are consistent with those described by Maddrell (1966).

The wide distribution of *Locusta*-DH-like staining in the CNS of *R. prolixus* is similar to the distribution of CRF-like peptides in *Locusta migratoria* and *Manduca sexta* (Patel et al., 1994; Emery et al., 1994; Veenstra and Hagedorn, 1991; Chen et al., 1994). Medial neurosecretory cells have been found to stain positively for *Locusta*-DH in *L. migratoria* (Patel et al., 1994) and for both *Manduca*-DH and *Manduca*-DPII in *M. sexta* (Veenstra and Hagedorn, 1991; Emery et al., 1994). The neurosecretory cell groups of the brain of *R. prolixus* have previously been described by Steel and Harmsen (1971) using a variety of staining techniques. These groups include 17 medial neurosecretory cells, a group of five cells along the posterior margin close to where NCC1 exits the brain, two cells in the dorsolateral region of the protocerebrum adjacent to its junction with the optic lobes, and a single neurosecretory cell located on the ventral surface posterior to the medial neurosecretory cells on both sides of the brain. The *Locusta*-DH antiserum appears to recognise cells in each of these positions, as well as others. The CC is an important neurohaemal organ, and we have shown that there is intense staining over the entire CC of *R. prolixus*, extending a short distance along the aorta. The immunofluorescence in the CC is consistent with the results in *L. migratoria* (Patel et al., 1994), in which the CC was shown to be highly immunoreactive to the *Locusta*-DH antiserum.

There are strongly stained cells in the SOG of *R. prolixus* in more lateral positions. In *M. sexta*, Emery et al. (1994) demonstrated a population of cells staining in the SOG with

anti-*Manduca*-DHII antiserum. However, Veenstra and Hagedorn (1991), using the anti-*Manduca*-DH antiserum, found no staining in the SOG. In *L. migratoria*, interneurones project through all the ganglia in the CNS, suggesting a central role for *Locusta*-DH as a neurotransmitter/neuromodulator (Patel et al., 1994). Similar results were found in *R. prolixus*.

The staining of posterior lateral neurosecretory cell groups in the MTGM of *R. prolixus* is consistent with the staining pattern found in other insects. Posterior lateral neurosecretory cells, staining for CRF-like diuretic peptides, have been identified in the abdominal ganglia of *L. migratoria* (Patel et al., 1994; Thompson et al., 1995) and *M. sexta* (Chen et al.,1994). These cell bodies send processes out of the abdominal nerves to their respective neurohaemal organs. The posterior lateral neurosecretory cells of *R. prolixus* are in a position consistent with that of the cells described by Maddrell (1966) and Berlind and Maddrell (1979) and have been shown to possess diuretic activity. Maddrell (1966) demonstrated that the abdominal nerves of *R. prolixus* were a site of release of the 'diuretic hormone', with the greatest amount of diuretic activity being present in the proximal lengths of abdominal nerves 1, 2 and 3. The intense neurohaemal-like immunoreactive staining found in this study, on abdominal nerves 1 and 2, is again consistent with these findings.

The crop, or anterior midgut, is innervated by the frontal ganglion through the recurrent nerve to the hypocerebral and ingluvial ganglia (Tsang and Orchard, 1991). Endocrine-like cells have also been described in insect midgut (Žitňan et al., 1993). No staining was observed using the anti *Locusta*-DH antiserum in the frontal ganglion, and the staining of the midgut endocrine-like cells in *R. prolixus* was weak and inconsistent. However, midgut cells, as well as endocrine cells in the ampulla of the midgut of *L. migratoria*, have been shown to stain positively for CRF-like peptides in *Aedes aegypti* (Veenstra et al., 1995) and *L. migratoria* (Montuenga et al., 1996). These peptides may play a role in controlling enzyme secretion and salt and water transport, although little is known about the physiological role of midgut peptides. Blake et al. (1996) found that, in addition to stimulating secretion in Malpighian tubules, *Acheta*-DP increased the frequency and amplitude of myogenic contractions in isolated *Acheta domesticus* foregut. This stimulation of contraction rate may play a role in the movement and mixing of food in the gut, as well as in the mixing of the insect haemolymph.

With regard to the hindgut of *R. prolixus*, immunoreactive processes were found over the entire structure. The role of the hindgut has not been studied in detail in *R. prolixus*. During the fast phase of diuresis, the hindgut collects urine and expels it every 2–3 min through the anus. However, between feeds, the hindgut could play a role in water recycling. The *Locusta*-DH-like material in *R. prolixus* may play a role in hindgut contraction, in the mixing of the hindgut contents and in the expulsion of urine.

Serotonin and *Locusta*-DH have both been shown to have diuretic activity on isolated *R. prolixus* Malpighian tubules. The double-labelling experiments show clearly that there are some cells in the brain and MTGM that contain both serotonin-like and *Locusta*-DH-like material. However, this is not true of the medial neurosecretory cells or posterior lateral neurosecretory cells of the MTGM or of their respective neurohaemal areas. Thus, serotonin-like and *Locusta*-DH-like material can potentially be released into the haemolymph independently of one another. This has some significance in the context of the synergistic control of Malpighian tubules by these two diuretic factors and the possibility of their independent control over Malpighian tubules during certain stages of the insect life history.

The immunogold studies demonstrate the presence of electron-dense neurosecretory granules in the nerve terminals of the CC, aorta and abdominal nerves that are *Locusta*-DH-like immunoreactive. The terminals on the aorta and abdominal nerves are clearly neurohaemal in nature, the terminals being found directly under the basement membrane. While we have not yet demonstrated the presence of the *Locusta*-DP-like material in the haemolymph of *R. prolixus*, Audsley et al. (1997) demonstrated the presence of *Locusta*-DH in the haemolymph of *L. migratoria* and found that the titre increased after feeding, confirming the role of CRF-like peptide(s) as a diuretic hormone in *L. migratoria*.

The presence of morphologically different immunoreactive granule types (Table 1) suggests the presence of different CRF-like peptides in the CC and perhaps of a third type in the abdominal nerves. Certainly, two forms of CRF-like peptide exist in *M. sexta* (Kataoka et al., 1989; Blackburn et al., 1991). These different forms of the diuretic peptides are found in cells with projections to the CC (Veenstra and Hagedorn, 1991; Emery et al., 1994). Miksys and Orchard (1994), using immunogold techniques, have previously suggested the presence of at least four terminal types on the five abdominal nerves of *R. prolixus*. The present immunogold studies suggest that *Locusta*-DH-like terminals contain granules morphologically similar to those in serotonergic terminals, although the *Locusta*-DH-like granules are somewhat smaller. This now suggests there may, in fact, be five terminal types on certain nerves. Serotonin-like immunoreactive terminals are found on all five abdominal nerves, whereas *Locusta*-DH-like terminals are found on abdominal nerves 1 and 2. Although double-labelling of the terminals at the electron microscope level is not possible because of differences in fixation procedures for serotonin and peptides, the immunofluorescence double-labelling experiments clearly show that serotonin and the peptide are located in different neurohaemal terminals.

The Malpighian tubules secretion studies demonstrate the presence of a diuretic factor in the 60 % acetonitrile cut of the CC extracts and in the 30 % and 60 % cuts of the abdominal nerve extracts. The partial purification of the tissue through Sep-Pak would have removed serotonin, while the CRF-like peptides have been shown to elute from Sep-Pak C_{18} with a 40–60 % cut of acetonitrile (Kay et al., 1991a,b, 1992; Patel et al., 1994). A significant increase in the content of cyclic AMP in Malpighian tubules exposed to the 60 % acetonitrile cuts for

both the CC and abdominal nerves was observed following a 10 min incubation in the presence of the phosphodiesterase inhibitor IBMX. CRF-like peptides have previously been shown to act through cyclic AMP (Kay et al., 1991b), and Aston (1975) suggested that the *R. prolixus* diuretic peptide/diuretic hormone(s) act *via* a cyclic-AMP-dependent pathway. In addition, *Locusta*-DP also increases cyclic AMP levels in *R. prolixus* Malpighian tubules (V. A. Te Brugge, unpublished observations). Taken together, the immunohistochemistry, immunogold labelling, secretion and cyclic AMP assays suggest the presence of a CRF-like diuretic peptide in the CC and abdominal nerves of *R. prolixus*. Previous work using homogenates has shown diuretic activity in all parts of the CNS except the CC (Maddrell, 1963). Maddrell (1963) suggested that most of the activity was found in the MTGM and that the majority of this was in the posterior lateral neurosecretory cells and was released from neurohaemal areas of the abdominal nerves. Since then, much of the research on *R. prolixus* diuresis has concentrated on the MTGM and neurohaemal areas on the abdominal nerves (Maddrell, 1966; Berlind and Maddrell, 1979; Maddrell et al., 1991, 1993). Maddrell (1963, 1964b) found no reduction in the rate of diuresis upon decapition or constriction of the bug anterior to the MTGM, and no diuretic activity in homogenates of the CC. Interestingly, Nuñez (1962, 1963), Coles (1966) and Baehr and Baudry (1970) have reported that there was a reduction in diuresis in response to neck ligation or decapitation. Whether this represents a blocking of sensory information or the release of hormone or both is unclear, although Nuñez (1963) certainly suggested that a diuretic factor was present in the head of *R. prolixus*. These findings are difficult to reconcile, but it must be borne in mind that different methods of measuring diuresis were employed and that homogenisation in saline at room temperature is likely to result in liberation of proteases as well as diuretic hormone.

This study provides evidence for the presence of a CRF-like diuretic peptide in the CNS and digestive system of *R. prolixus*. This peptide resides in neurohaemal terminals of the CC, aorta and abdominal nerves and can potentially be released independently of serotonin. Although a synergistic role has been suggested for serotonin and the diuretic peptide (Barrett and Orchard, 1990; Maddrell et al., 1991, 1993), no experiments have been performed using purified *R. prolixus* diuretic peptide alone and/or in combination with serotonin. Interestingly, Coast (1996) found no synergism between *Locusta*-DH and serotonin in *R. prolixus* Malpighian tubules. Understanding the timing of release of the peptide(s) and serotonin and their interaction must await the purification and sequencing of the diuretic peptide(s) in *R. prolixus*. This will then provide a more complete understanding of the neurohormonal control of rapid diuresis and water cycling in *R. prolixus*.

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