Histochem Cell Biol (1995) 104:337–347

ORIGINAL PAPER

Jan A. Veenstra · Greg W. Lau
Hans-Jürgen Agricola · David H. Petzel

Immunohistological localization of regulatory peptides in the midgut of the female mosquito *Aedes aegypti*

Accepted: 11 May 1995

Abstract The midgut of the female mosquito *Aedes aegypti* was studied immunohistologically with antisera to various regulatory peptides. Endocrine cells immunoreactive with antisera to perisulfakinin, RFamide, bovine pancreatic polypeptide, urotensin 1, locustatachykinin 2 and allatostatins A1 and B2 were found in the midgut. Perisulfakinin, RFamide and bovine pancreatic polypeptide all react with the same, about 500 endocrine cells, which were evenly distributed throughout the posterior midgut, with the exception of its most frontal and caudal regions. In addition, these antisera recognized three to five neurons in each ingluvial ganglion and their axons, which ran longitudinally over the anterior midgut, as well as axons innervating the pyloric sphincter. The latter axons appear to be derived from neurons located in the abdominal ganglia. Antisera to two different allatostatins recognized about 70 endocrine cells in the most caudal area of the posterior midgut and axons in the anterior midgut whose cell bodies were probably located in either the brain or the frontal ganglion. Antiserum to locustatachykinin 2 recognized endocrine cells present in the anterior midgut and the most frontal part of the posterior midgut, as well as about 50 cells in the most caudal region of the posterior midgut. Urotensin 1 immunoreactivity was found in endocrine cells in the same region as the perisulfakinin-immunoreactive cells, but no urotensin-immunoreactive axons were found in the midgut. Double labeling experiments showed that the urotensin and perisulfakinin immunoreactivities were located in different cells. Such experiments also showed that the locustatachykinin and allatostatin immunoreactivities in the most caudal area of the posterior midgut were present in different cells. No immunoreactivity was found in the mosquito midgut when using antisera to corazonin, allatotropin or leucokinin IV. Since these peptides have either been isolated from, or can reasonably be expected to be present in mosquitoes, it was concluded that these peptides are not present in the mosquito midgut.

J.A. Veenstra (✉) · G.W. Lau
Department of Entomology and Center for Insect Science,
The University of Arizona, AZ 85721, Tucson, USA
Tel. 520 621 1731; Fax 520 621 4886

H.-J. Agricola
Institut für Allgemeine Zoologie und Tierphysiologie,
Friedrich-Schiller-Universität, Jena, Germany

D.H. Petzel
Department of Biomedical Sciences, School of Medicine,
Creighton University, Omaha, USA

Introduction

A considerable number of different types of endocrine cells can be distinguished in the insect midgut on the basis of their morphology, type of secretory granules and immunoreactivity to various regulatory peptides (Nishiitsutsuji-Uwo and Endo 1981; Endo and Nishiitsutsuji-Uwo 1981; Endo et al. 1982a, b; Duve and Thorpe 1982; Iwanaga et al. 1981, 1986; Andriès and Tramu 1984, 1985a, b; Brown et al. 1985, 1986, 1990; Schols et al. 1987; Glättli et al. 1987; Andriès and Beauvillain 1988; Montuenga et al. 1989; Jenkins et al. 1989; Verhaert et al. 1989; Zitnan et al. 1993; Lundquist et al. 1993; Reichwald et al. 1994; Agricola and Bräunig 1995). It seems a reasonable expectation that, as in vertebrates, these cells are involved in the regulation of digestion. Release of elementary granules from the cell bodies has been demonstrated at the ultrastructural level (Endo and Nishiitsutsuji-Uwo 1982; Brown et al. 1985), but the specific functions of these cells remain unknown.

Female *Aedes aegypti* mosquitoes take a single large blood meal which is then digested in a relatively short time span. It is known that the initiation of digestion induces massive changes in the midgut. Thus, transcription, synthesis and release of trypsin, the major protease secreted into the gut lumen of mosquitoes, are dramatically increased after a blood meal in the mosquito *A. aegypti* (Briegel and Lea 1975; Barillas-Mury et al. 1991). Such changes might very well be mediated by regulatory peptides secreted by the endocrine cells of the midgut and mosquitoes are therefore likely to be good experimental model systems for the elucidation of

the physiological role of the endocrine cells in the insect midgut.

Previous work on the endocrine cells in the midgut of *A. aegypti* has already shown the presence of various cell types using electron microscopy (Brown et al. 1985). One of these cell types reacts with antisera to the molluscan cardioexcitatory tetrapeptide, FMRFamide, and bovine pancreatic polypeptide (Brown et al. 1986). We are interested in elucidating the functions of the endocrine cells, which will need the isolation and structural identification of the peptides produced by them. Since antisera to insect neuropeptides may be useful for the isolation of immunoreactive materials, we have concentrated in the present study on the use of such antisera for the immunohistological identification of regulatory peptides in the mosquito midgut.

Materials and methods

Insects

A. aegypti was reared as described by Shapiro and Hagedorn (1982). Throughout this study only adult non-blood-fed females of 3- to 7-days old have been used.

Materials

The allatotropin, corazonin and leucokinin IV antisera and their use in immunohistology have been described before (Veenstra and Davis 1993; Veenstra et al. 1994; Chen et al. 1994). The antisera to bovine pancreatic polypeptide (615-R110-146-17), urotensin 1, RFamide (145-IV), and allatostatin B1 were kind gifts from Drs R.E. Chance, K. Lederis, C.J.P. Grimmelikhuijzen, and R. Feyereisen, respectively. The perisulfakinin and locustatachykinin II (50820091) antisera were raised by one of us (H.-J.A.) as follows. Five milligrams of peptide dissolved in 2.5 ml of phosphate-buffered saline pH 7.2 (PBS) was mixed with 2.5 ml of 2% aqueous (v/v) glutaraldehyde. After incubation for 3 min at room temperature, 4 ml PBS containing 20 mg bovine thyroglobulin was added, the mixture stirred continuously for 2 h at 4° C, and dialyzed against PBS. Male rabbits (29–30 weeks old) were injected subcutaneously with 400 µg (160 µl) of the conjugates, emulsified in 100 µl Freund's complete adjuvant. Three months later, booster injections (200 µg of conjugate in 2 ml distilled water) were given on 3 consecutive days. The antisera were collected 6 days after the last injection.

The rhodamine-labeled goat anti-rabbit IgG was from Boehringer Mannheim Biochemicals (Indianapolis, Ind., USA), fluorescein-labeled goat anti-rabbit IgG from Molecular Probes (Eugene, Ore. USA) and the fluorescein-labeled Fab fragment of goat anti-rabbit IgG from Jackson ImmunoReseach Laboratories (West Grove, Pa.). Fluorescein-labeled and rhodamine-labeled anti-allatostatin B2 IgG and anti-perisulfakinin IgG were prepared in the laboratory as follows. Immunoglobulin G was purified from the antisera with octanoic acid as described by Harlow and Lane (1988), dialyzed against HPLC-grade water, and lyophilized. The perisulfakinin IgG was then reacted with fluorescein isothiocyanate (Boehringer Mannheim) in 0.1 *M* sodium bicarbonate buffer pH 9.0 for 4 h at room temperature. The allatostatin IgG and perisulfakinin IgG were reacted with tetramethylrhodamine succinimidylate (Molecular Probes) for 2 h at room temperature in 0.1 *M* sodium bicarbonate buffer pH 8.3, after which 1.5 *M* hydroxylamine pH 8.0 was added to a final concentration of 0.15 *M* and the incubation was continued for another 30 min. The labeled IgG were separated from the unincorporated fluorescent labels by gel filtration. The fluorescein- and rhodamine-conjugated perisulfakinin IgGs had molar fluorescent label:protein ratios of 2.15 and 0.94, respectively. Perisulfakinin was from Bachem Feinchemikalien (Bubendorf, Switzerland), locustatachykinin 2 and RFamide from Peninsula Laboratories (Belmont, Calif., USA) and allatostatin B2 a gift from Dr. R. Feyereisen.

Immunohistology

The whole-mount immunohistological methods used were adapted from Davis et al. (1993) and Tibbetts and Nichols (1993). Midguts were dissected from mosquitoes and fixed in 4% paraformaldehyde in phosphate buffer overnight at 4° C. All washes were performed in 0.1 *M* phosphate buffer, pH 7.4, containing 0.5% Triton X-100 and 0.1% sodium azide. After fixation the tissue was washed 6 times for 1 h at room temperature on a shaker; occasionally the last wash was performed overnight at 4° C. After washing the tissues, they were incubated for 1.5 h in 10% normal goat serum in washing buffer, followed by the application of the primary antiserum diluted in washing buffer containing 2% normal goat serum, after which the tissue was left overnight at 4° C on a shaker. The tissue was washed as after fixation and the secondary antiserum was applied in the same fashion. Antisera were diluted 1:1000 (urotensin 1, RFamide), 1:2000 (perisulfakinin, allatostatins, bovine pancreatic polypeptide), 1:5000 (locustatachykinin 2), or 1:40000 (perisulfakinin); secondary antisera were diluted 1:1000. After the final washes the guts were mounted in 80% glycerol made up to 20 m*M* sodium bicarbonate buffer, pH 9.5.

Double labelings were performed on selected midguts which had been labeled and viewed previously. After removing the cover slip the tissue was returned to the washing buffer, washed twice for 1 h, and pretreated for 2 h with 2% normal goat serum and 2% preimmune rabbit serum in washing buffer. The tissue was then incubated overnight at 4° C with either the fluorescein-labeled perisulfakinin or the rhodamine-labeled allatostatin IgG. Tissues were washed 6 times, and mounted as described above. Results obtained with the fluorescein-labeled goat anti-rabbit IgG in the double labeling procedures were unsatisfactory, and for double labelings involving rhodamine-labeled anti-perisulfakinin or anti-allatostatin B1, the first unlabeled antiserum was therefore visualized using a fluorescein-labeled Fab fragment of goat anti-rabbit IgG.

Immunohistological controls

Immunoreactivity was suppressed after preabsorption for 24 h with 10 nmol/ml of the peptide used to generate each antiserum, or with 100 nmol of RFamide for the RFamide antiserum. Such controls were not performed for the antisera to urotensin 1 and bovine pancreatic polypeptide, since the immunohistological specificity of these antisera on the mosquito midgut has already been reported (Brown et al. 1986; 1990) and these antisera were included in the present study only for comparative purposes.

Special controls were performed for the perisulfakinin antiserum. Since perisulfakinin has a C-terminal RFamide, it was expected that the antiserum might react with other insect neuropeptides having a C-terminal RFamide. In a competive ELISA for perisulfakinin set up according to the protocol described by Kingan (1989) the antiserum did not recognize RFamide, FMRFamide, or leucomyosuppressin in amounts of up to 10 nmol per well, while 100 fmol of perisulfakinin was easily detectable. Since such ELISA experiments are only indicative of the specificity of the antiserum in immunocytology, we also tested it in immunocytology on the central nervous system of prepupae of *Drosophila melanogaster*, the only insect species for which the differential localization of the the FMRFamides, leucomyosuppressins and sulfakinins has been determined (Schneider et al. 1991; O'Brien et al. 1991; Nichols 1992; Tibbetts and Nichols 1993).

High performance liquid chromatography (HPLC)

Midguts were dissected under saline and stored frozen at –70° C until a total of 1000 had been collected. The tissue was then homogenized in 15 ml of Bennett's mixture (Bennett et al. 1981),

centrifuged and separated on a previously activated and equilibrated C-18 Sep-Pak column. The Sep-Pak was washed with 5 ml water containing 0.1% trifluoroacetic acid (TFA), and the peptide-containing fraction eluted with 5 ml of 65% acetonitrile in water, containing 0.1% TFA. The peptide-containing fraction was reduced to about 1 ml and then diluted with 0.1% TFA in water to 4 ml, which was injected onto a reversed phase Econosil C-18 column (4.6×250 mm, 10 µm particle size, pore size 60 Å) from Alltech Associates (Deerfield, Ill.). The column had been equilibrated with 6.5% acetonitrile and 0.1% TFA in water and the flow rate was 1 ml/min. After injection the column was eluted isocratically with 6.5% acetonitrile and 0.1% TFA in water for 10 min, followed by a linear gradient over 50 min to 39% acetonitrile and 0.1% TFA in water. Immediately after the first gradient a second gradient was run to 65% acetonitrile and 0.1% TFA in water over 10 min. The column was then washed with 65% acetonitrile and 0.1% TFA in water. Delay between the pumps and the detector was about 9.5 min. Absorbance was monitored at 214 nm with 0.5 absorbance units full scale. Eighty 1-min fractions were collected and, after the addition of 100 µg radioimmunoassay-grade bovine serum albumin (Sigma Chemical, St. Louis, Mo., USA), lyophilized. Samples were then shipped to Omaha for assay on the transepithelial voltage of the Malpighian tubule.

Electrophysiology

Malpighian tubules of female *A. aegypti* were perfused in vitro according to the method of Burg et al. (1966). The tubules were perfused with symmetrical saline (150 m*M* NaCl, 25 mM HEPES, 5 m*M* glucose, 7 m*M* $CaCl_2$, 1.8 m*M* $NaHCO_3$, and 0.6 m*M* $MgCl_2$, pH 7.0) at room temperature. The transepithelial voltage was measured via Ag-AgCl electrodes with respect to ground in the bath. The lyophilized HPLC fractions were dissolved in 600 µl saline and injected into the bath to test for effects on the transepithelial voltage. No distal holding pipette was used, but this does not significantly affect the transepithelial voltage, since the electrical length constant of these tubules (about 300 µm) is much smaller than the length of the tubules used (always more than 1500 µm).

Results

The insect gut can be divided into three parts, the foregut, the midgut and the hindgut. Both the foregut and the hindgut have a cuticular lining on the inside, and it is the midgut where digestion takes place. Not surprisingly, then, endocrine cells have only been described from the insect midgut. In the adult female mosquito, the midgut is divided into two parts, the anterior midgut and the posterior midgut. It is in the posterior midgut that the blood meal is stored and digested.

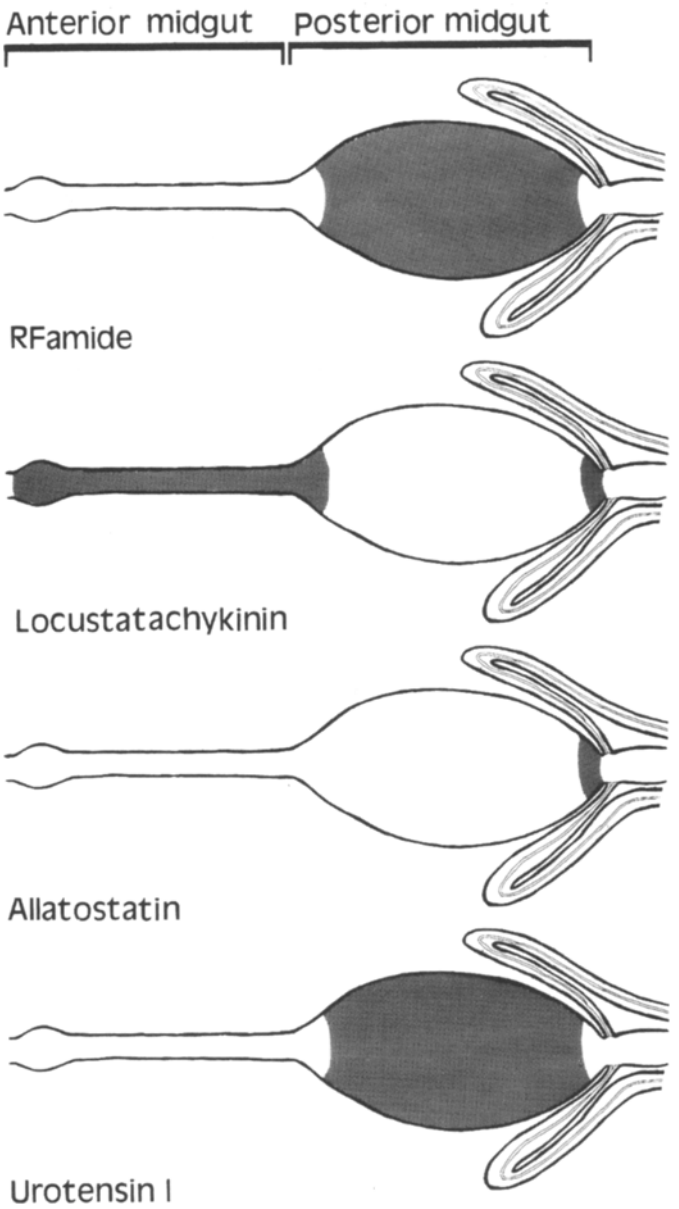
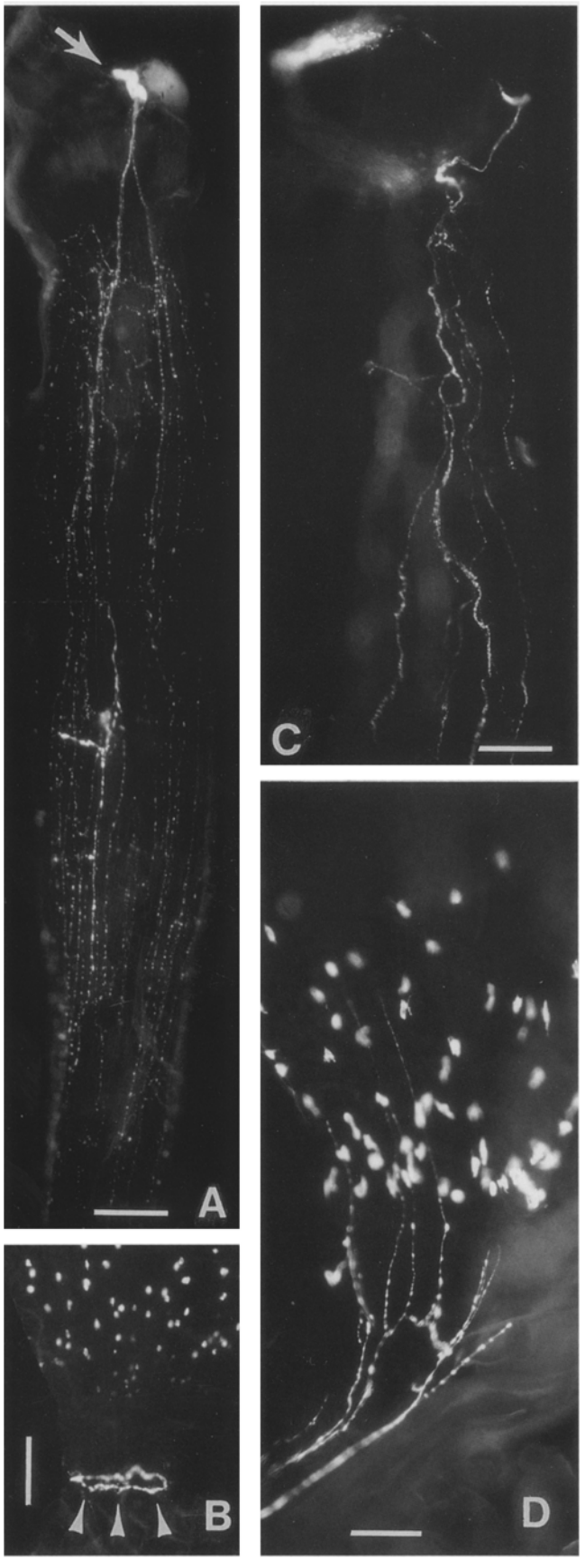


Fig. 1 Schematic localization of immunoreactive endocrine cells in the midgut of *Aedes aegypti* as revealed by antisera to RFamide, perisulfakinin and bovine pancreatic polypeptide *(top panel)*, locustatachykinin II *(second from top)*, allatostatins A1 and B2 *(third panel from top)* and urotensin 1 *(bottom panel)*

RFamide, perisulfakinin and bovine pancreatic polypeptide

The immunocytochemical results with the RFamide, bovine pancreatic polypeptide and high concentrations of perisulfakinin antisera were indistinguishable. Double labeling procedures in which bovine pancreatic polypeptide-or RFamide-immunoreactive cells and axons were first visualized with rhodamine using a two-step procedure followed by fluorescein-labeled perisulfakinin IgG confirmed that all immunoreactive cells contained both rhodamine and fluorescein labels. Similar experiments using urotensin 1 antiserum instead of bovine pancreatic polypeptide or RFamide antiserum yielded immunoreactive cells containing either rhodamine or fluorescein label (see below; Fig. 3E). It was therefore concluded that the double labeling of the same cells with these different antisera was not due to an artifact and that the three antisera all recognized the same cells in the mosquito midgut. Consequently, the positive RFamide-, bovine pancreatic polypeptide- and perisulfakinin-immunoreactive material will be described together.

Dispersed within the posterior midgut epithelium of the adult female there are about 500 immunoreactive endocrine cells, which are of the open columnar type (Figs.



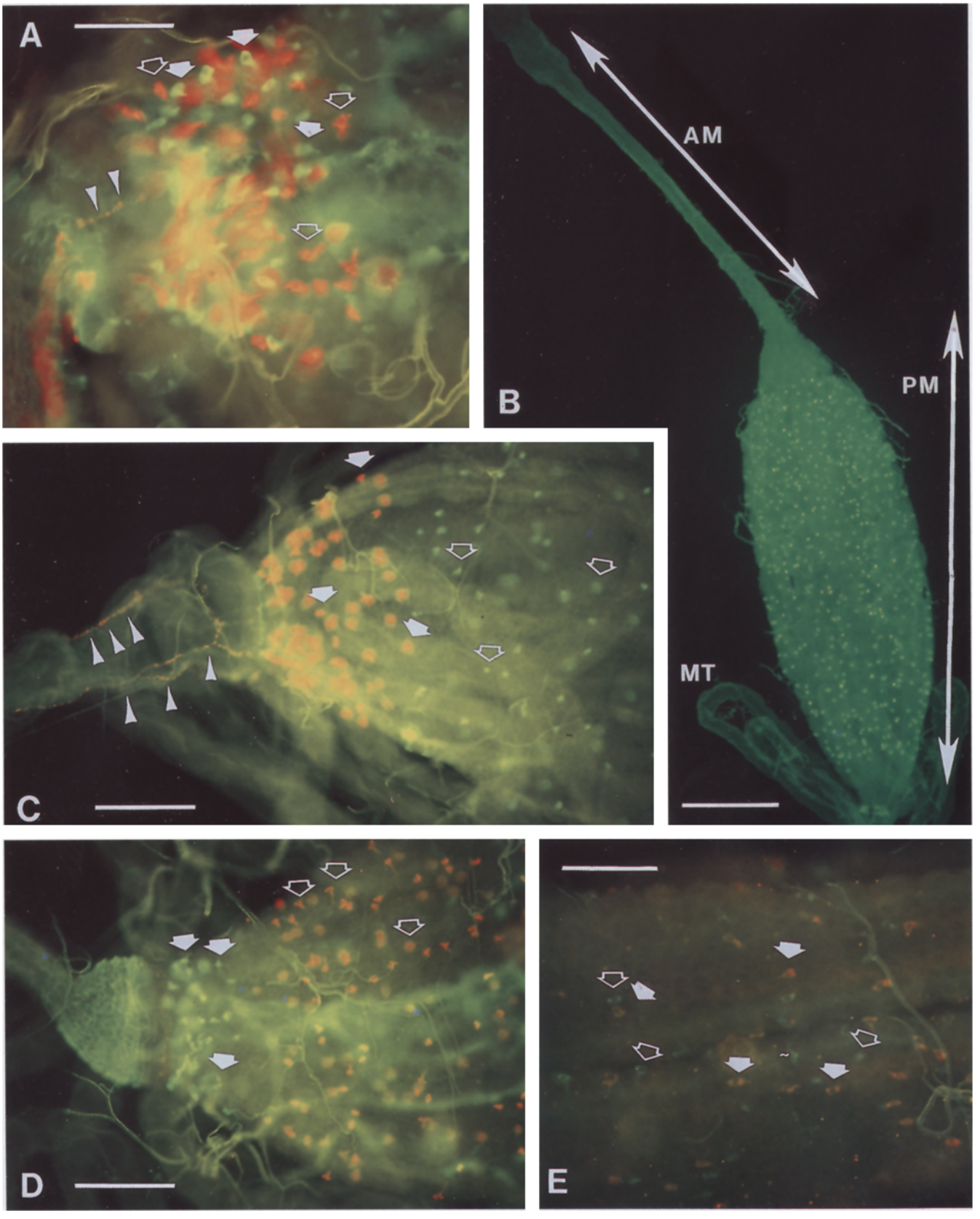
1, 3B). These cells measure about 10 μm, have basolateral extensions and stain equally intensely with all three antisera. In addition to these endocrine cells, there is a circular immunoreactive axonal ring around the pyloric sphincter (Fig. 2B). The source of the latter processes is from four axons which run in two symmetrical pairs in each proctodaeal nerve longitudinally over the hindgut. Their cell bodies are most likely located in the abdominal ganglia, but this could not be established beyond doubt. In the anterior part of the midgut there are several immunoreactive axons, which run longitudinally over the gut. They are derived from three to five immunoreactive cell bodies in each ingluvial ganglion (Fig. 2A).

Perisulfakinin

Experiments on *D. melanogaster* showed that the perisulfakinin antiserum was specific for sulfakinins when used at concentrations of 1:10 000 or lower. For the *Aedes* midgut,

Fig. 2 **A** RFamide-immunoreactive neurons in the gluvial ganglion and their axons running over the anterior midgut. Anterior top, posterior bottom. **B** RFamide-immunoreactive axon ring around the pyloric sphincter and some of the RFamide-immunoreactive endocrine cells in the posterior midgut. Note the absence of RFamide-immunoreactive endocrine cells in the most posterior region of the midgut. Anterior top, posterior bottom. **C** Allatostatin-immunoreactive axons on the anterior midgut. Anterior top, posterior bottom. **D** Allatostatin-immunoreactive axons and endocrine cells in the most posterior part of the midgut. Anterior top, posterior bottom. *Scale bars* indicate 100 μm (**A, C, D**) or 200 μm (**B**)

Fig. 3A–E Immunocytological localization of peptides in endocrine cells of the midgut of the female mosquito *A. aegypti.* **A** Double labeling of green locustatachykinin (*closed arrows;* locustatachykinin antiserum 1:5000, followed by fluorescein-labeled Fab fragment of affinity-purified anti-rabbit IgG) and red allatostatin (*open arrows;* rhodamin-labeled anti-allatostatin IgG) immunoreactivity in the caudal-most area of the posterior midgut. An allatostatin-immunoreactive axon is also in focus *(arrowheads).* Anterior to right, posterior to left. **B** Localization of perisulfakinin immunoreactivity. Note the presence of endocrine cells in the posterior midgut, with the exception of the frontal and caudal regions. Perisulfakinin primary antiserum, fluorescein-labeled secondary antiserum. Anterior up, posterior down. **C** Double labeling of orange/red allatostatin and green perisulfakinin immunoreactivity in the posterior part of the stomach. Rhodamine-labeled anti-allatostatin IgG and fluorescein-labeled anti-perisulfakinin IgG. Note allatostatin-immunoreactive endocrine cells in the most posterior part of the stomach, and the allatostatin-immunoreactive axons coming from the hindgut *(arrowheads).* Anterior to right, posterior to left. **D** Caudal area of the posterior midgut showing green locustatachykinin immunoreactivity (*closed arrows;* locustatachykinin antiserum 1:5000 and fluorescein-labeled Fab fragment of affinity-purified anti-rabbit IgG) and red perisulfakinin immunoreactivity (*open arrows;* rhodamine-labeled anti-perisulfakinin IgG). Anterior to right, posterior to left. **E** Double labeling of red urotensin 1 (*closed arrows;* primary antiserum 1:2000; rhodamine-labeled secondary antiserum) and green anti-perisulfakinin immunoreactivity (*open arrows;* fluorescein-labeled anti-perisulfakinin IgG) in the posterior midgut. Anterior to right, posterior to left. (*AM* anterior midgut, *PM* posterior midgut, *MT* Malpighian tubules). *Scale bars* indicate 125 μm (**A, E**), 500 μm (**B**) or 250 μm (**C, D**)


A
B
AM
PM
MT
C
D
E

the endocrine cells in the midgut were still immunoreactive when the antiserum was diluted at 1:40 000, but the immunoreactive cells in the ingluvial ganglion and the immunoreactive axon terminals in the hindgut were no longer recognized by the perisulfakinin antiserum at this dilution.

Locustatachykinin

The locustatachykinin 2 antiserum yielded a relatively high background, in particular it seemed to have a strong affinity for membranes. This background staining was inconsistent, sometimes it was relatively low, on other occasions it was very intense, but it was never reminiscent of the localization of a regulatory peptide. About 60–80 locustatachykinin-immunoreactive endocrine cells were located in the anterior midgut, about 20–40 in the most frontal region of the posterior midgut and another 30–40 in the most caudal region of the stomach (Figs. 1, 3A, D). The size of these cells was about 8–12 μm; they were of the open type and had basolateral extensions. No immunoreactive nerve fibres were detected in any part of the midgut or the hindgut.

Allatostatin

Allatostatin immunoreactivity was found in about 40–60 endocrine cells in the most caudal part of the posterior midgut, just in front of the pyloric sphincter (Fig. 1). These cells were also of the open type, had basolateral extensions and measured about 10–12 μm. In the same region of the posterior midgut there were allatostatin-immunoreactive fibres, intermingled with the allatostatin-immunoreactive neuroendocrine cells (Fig. 2D), which were derived from axons in the proctodaeal nerve, whose cell bodies were located in the terminal abdominal ganglion. In the anterior part of the midgut, allatostatin-immunoreactive fibres were also present (Fig. 2C).

Double labeling with perisulfakinin and allatostatin antisera established that the allatostatin-immunoreactive endocrine cells in the caudal part of the posterior midgut are different from both the perisulfakinin- and the locustatachykinin-immunoreactive endocrine cells in the midgut (Fig. 3A, C). The allatostatin-immunoreactive axons in the hindgut were present in the same nerves as the RFamide-immunoreactive axons and at first sight at low magnification they almost appeared identical. However, these were different axons, as could be most clearly seen in the location of their final destination (compare e.g. Fig. 2B with Fig. 2D). The allatostatin-immunoreactive axons in the anterior part of the midgut were similarly very closely associated with the RFamide-immunoreactive axons in the anterior midgut. However, the RFamide-immunoreactive neurons in the ingluvial ganglion were not recognized by the allatostatin antisera and the allatostatin-immunoreactive axons could be followed up to the cerebral neurohemal organ, which contained a considerable amount of allatostatin-immunoreactive axons and axon terminals. This made it impossible to establish where the cell bodies of the allatostatin-immunoreactive axons on the anterior midgut were located.

Urotensin 1

In general, immunoreactivity with this antiserum, although consistent, was weak and it had a relatively high background. A variable number of urotensin 1-immunoreactive cells was found in the epithelium of the posterior midgut. Double labeling experiments of urotensin 1 immunoreactivity labeled with the secondary rhodamine-labeled antibody and the fluorescein-labeled perisulfakinin IgG clearly established that these two types of immunoreactivity were located in different cells (Fig. 3E), even though the two cell types were present in the same general area. In the best preparation we could count up to 350 cells per midgut. Although a substantial portion of the urotensin 1-immunoreactive cells was of the open type, due to the less intensive staining with this antibody we were unable to ascertain whether this was true for all cells. The basolateral extensions were similarly much more difficult to distinguish.

Allatotropin, leucokinin, and corazonin

No immunoreactivity was observed in the midguts of adult females with antisera to allatotropin, leucokinin IV, or corazonin.

Electrophysiological evidence for a diuretic factor in the midgut

The presence of urotensin 1-like immunoreactive cells in the mosquito midgut suggested the possible presence of a related peptide in the mosquito midgut. Since urotensin 1 is related to corticotropin-releasing factor and insect diuretic hormone (e.g. Kataoka et al. 1989), the urotensin 1 immunoreactivity might be due to a diuretic peptide. We therefore extracted 1000 midguts, separated the extract by HPLC, and assayed the fractions on the transepithelial voltage of the mosquito. This assay has been shown to be very specific for diuretic factors and every factor known to stimulate fluid secretion by the Malpighian tubules also has clear effects on the transepithelial voltage (e.g. Petzel et al. 1985; Veenstra 1988). The results showed electrophysiological activity eluting in fractions 69–80, which is clearly different from the leucokinins recently isolated from *A. aegypti* (Veenstra 1994). The *Aedes* leucokinins also have very strong electrophysiological effects on the Malpighian tubules (Petzel, unpublished data), but elute much earlier on HPLC (Fig. 4). Since the electrophysiological activity was found in several fractions, it is possible that more than one peptide is involved.

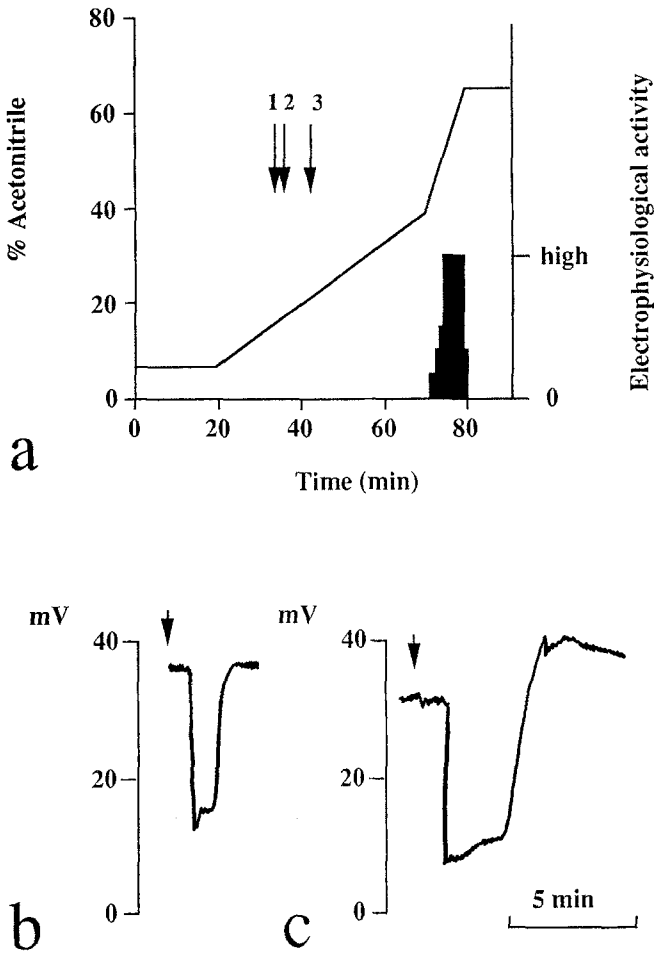


Fig. 4 **a** HPLC chromatogram of Sep-Pak prepurified extract of 1000 *A. aegypti* midguts. Fractions containing electrophysiological activity on the Malpighian tubules of the same species are indicated by histograms. Elution times of the *Aedes* leucokinins 1, 2 and 3 are indicated by *arrows 1, 2* and *3*. **b** Effects of material eluting between 72 and 73 min on the transepithelial voltage of a Malpighian tubule. **c** Effects of material eluting between 77 and 78 min on the transepithelial voltage of a Malpighian tubule

Discussion

Comparative aspects

Previous work on the endocrine cells in the mosquito *A. aegypti* had identified four different cell types using electron microscopy and two cells types using immunocytology with antisera to the molluscan cardioexcitatory tetrapeptide, FMRFamide, and the vertebrate peptides pancreatic polypeptide and urotensin 1 (Brown et al. 1985, 1986, 1990). We have extended this work by using antisera to various insect regulatory peptides and the results suggest the presence in these cells of regulatory peptides belonging to three well-characterized peptide families, the allatostatins, locustatachykinins, and the sulfakinins. It is interesting to note that the location of the four types of endocrine cells found here by immunocytology nicely coincides with the location of the four different endocrine cell types described by Brown et al. (1986) using electron microscopy. This suggests that there may be only four different types of endocrine cells in the midgut of the mosquito. However, in moths, cockroaches, and dragonfly larvae, a larger number of cell types was found (Endo and Nishiitsutsuji-Uwo 1981; Nishiitsutsuji-Uwo and Endo 1981; Andriès and Tramu 1985a, b), and it can therefore not be excluded that other cell types remain to be discovered in the mosquito midgut.

Identity of the immunoreactive materials

Five allatostatins were initially isolated from the cockroach *Diploptera punctata* by their ability to inhibit juvenile hormone biosynthesis (Woodhead et al. 1989; Pratt et al. 1989, 1991). These peptides are encoded by a single cDNA obtained from mRNA isolated from cockroach brain (Donly et al. 1993). Five peptides homologous to the cockroach allatostatins have been isolated and identified from the blowfly *Calliphora vomitoria* (Duve et al. 1993). Allatostatin immunoreactivity has been detected by immunohistology in brain as well as midgut of both cockroaches and flies (Duve et al. 1993; Reichwald et al. 1994), and it has been shown for the cockroach that the allatostatin gene expressed in the brain is also expressed in endocrine cells of the midgut (Reichwald et al. 1994). There is, thus, evidence for the existence of peptides homologous to allatostatins in Diptera, as well as for the expression of the allatostatin gene in the midgut. We therefore believe that the allatostatin-immunoreactive endocrine cells in the mosquito midgut produce mosquito homologs of this peptide family.

In an earlier study, the presence and distribution of FMRFamide and bovine pancreatic polypeptide-immunoreactive endocrine cells in the mosquito midgut has been reported (Brown et al. 1986). It has been shown elsewhere that these antisera cross-react on insect tissue (Veenstra 1984; Veenstra and Schooneveld 1984), and this appears also to be the case with the endocrine cells in the mosquito midgut (Brown et al. 1986). Evidence reported elsewhere suggests that the insect bovine pancreatic polypeptide immunoreactive material is more closely related to (FM)RFamide than the pancreatic polypeptides (Veenstra and Schooneveld 1984; Veenstra 1984; Myers and Evans 1985). Our double labeling experiments demonstrated that these cells are the same as the ones reacting with antisera to RFamide and perisulfakinin. The question then arises, which peptides are produced by these cells. Four (FM)RFamide-related peptide families have been isolated from insects, these include: (1) the "real" FMRFamides with a C-terminal sequence of Phe-Met-Arg-Phe-amide (Nambu et al. 1988; Schneider and Taghert 1988); (2) the myosuppressins with a C-terminal FLRFamide sequence (Holman et al. 1986; Robb et al. 1989; Kingan et al. 1990); (3) the sulfakinins with a C-terminal sequence of DDY(sulfate)GHMRFamide (Nachman et al. 1986a, b; Nichols et al. 1988; Veenstra 1989); and (4) the "head peptides" with their C-terminal sequence of SL(R/K)(L/T)RFamide, which were

initially isolated from head extracts from *A. aegypti* (Matsumoto et al. 1989) and more recently from the nervous system of the horseshoe crab (Gaus et al. 1993) and the cockroach midgut (Veenstra and Lambrou 1995). As with the FMRFamide-immunoreactive material in the nervous system, it is not immediately clear which of the identified FMRFamide-like peptides are responsible for the (FM)RFamide immunoreactivity.

In situ hybridization with probes for the *Drosophila* FMRFamide gene did not yield a signal in the FMRFamide-immunoreactive cells in the midgut, suggesting that these peptides are not present there (Schneider et al. 1991; O'Brien et al. 1991). It seems plausible, then, that the homologous gene in *A. aegypti,* if it exists, is similarly not expressed in the midgut. The presence of the so-called *Aedes* head peptides in the mosquito midgut has been suggested before (Brown et al. 1994), and our recent identification of a homologous peptide in the midgut of the American cockroach (Veenstra and Lambrou 1995) makes it very likely that the *Aedes* head peptides are present in the mosquito midgut. The RFamide-immunoreactive endocrine cells in the midgut are also recognized by the perisulfakinin antiserum, even when this is used in a sufficiently high dilution to be specific for sulfakinins in *Drosophila,* which suggests the presence of a sulfakinin in these cells. However, the presence of both the *Aedes* head peptides and the sulfakinins in the mosquito midgut needs to be confirmed and the presence of other RFamide-related peptides in the RFamide-immunoreactive endocrine cells of the mosquito midgut cannot be excluded.

The locustatachykinins were initially isolated from the locust *Locusta migratoria* (Schoofs et al. 1990a, b) and two related peptides have been found in the fly *C. vomitoria* (Lundquist et al. 1994). In blowflies, locustatachykinin-immunoreactive endocrine cells have also been found in the midgut (Lundquist et al. 1993), indicating the possibility that the locustatachykinins, like the allatostatins (Reichwald et al. 1994), are authentic insect brain-gut peptides. However, this will need to be confirmed by isolation and structural identification of the locustatachykinin-immunoreactive peptides.

The urotensin 1-immunoreactive cells described here from the midgut of *A. aegypti* have been described briefly before (Brown et al. 1990). Since insect diuretic hormones structurally related to corticotropin-releasing factor and urotensin have been isolated from several insect species (Kataoka et al. 1989; Lehmberg et al. 1991; Kay et al. 1991), and the gene encoding this hormone is expressed in the midgut of the sphinx moth *Manduca sexta* (Digan et al. 1992), it is tempting to speculate that these cells might produce a diuretic hormone. The presence in the midgut of *A. aegypti* of material with strong electrophysiological effects on the Malpighian tubules of the same species reinforces this hypothesis.

Antisera against leucokinin IV and allatotropin revealed neuroendocrine cells and neurons in the central nervous of *A. aegypti* (Chen et al. 1994; Veenstra, unpublished observations) and the identity of the leucokinin- and allatotropin-immunoreactive peptides of *A. aegypti* has been established (Veenstra 1994; Veenstra, unpublished data). Thus, while homologs of these peptides are present in *A. aegypti* and the antisera used here detect these peptides in the nervous system, no allatotropin- or leucokinin-immunoreactive endocrine cells or nerve fibers were found in the mosquito midgut. Hence, it appears that these peptides are not present in the midgut of *A. aegypti.* Corazonin has been well conserved during evolution in insects (Veenstra 1991) and the antiserum used here is useful for the immunohistological localization of this peptide in the nervous system of insects, including Diptera (Veenstra and Davis 1993; Cantera et al. 1994). Nevertheless, no corazonin immunoreactivity was found in the mosquito midgut, indicating that this peptide is not present in this tissue.

Possible functions

Endocrine cells in the digestive tract are generally believed to have a paracrine function, i.e. that the peptides they produce are locally released and effective on gut cells nearby; in addition they may also have effects on more distantly located target organs (e.g. Fujita et al. 1981). If their major effects are on cells nearby, then the location of the immunoreactive cells in the mosquito midgut may give important clues to their functions.

Locustatachykinins were isolated from locust brain by their ability to induce hindgut contractions. The posterior midgut needs to be firmly tightened at both ends in the mosquito until the blood meal is digested, after which the posterior end is loosened and the digested blood is moved into the hindgut. The presence of locustatachykinin-immunoreactive peptides in endocrine cells at both ends of the posterior midgut might, therefore, suggest the involvement of these peptides in keeping these ends contracted in order to keep the blood meal confined to the posterior midgut, while being digested.

In cockroaches, allatostatin is able to inhibit proctolin-induced contractions of the hindgut (Lange et al. 1993), while in flies, two allatostatin homologs have been shown to inhibit gut contractions in the fly ileum (Duve et al. 1993; Duve and Thorpe 1994). The presence of allatostatin-immunoreactive peptides in the most posterior end might, therefore, suggest the involvement of this peptide in relaxing the pyloric sphincter once the blood has been fully digested. It is interesting to note the presence of allatostatin-immunoreactive axons derived from neurons in the abdominal ganglia in the same area, suggesting the existence of both a paracrine and a nervous pathway to relax the pyloric sphincter. Similarly, the allatostatin-immunoreactive axons on the anterior midgut might release allatostatins during feeding in order to relax the anterior midgut and allow blood to flow into the midgut.

Apart from the induction of muscle contraction or its relaxation, peptides produced by the midgut endocrine cells are likely to be involved in the regulation of trypsin synthesis and release, which shows drastic changes im-

mediately after a blood meal (Briegel and Lea 1975; Barillas-Mury et al. 1991; 1995), as well as ion and water homeostasis. In blood-feeding insects, like the mosquito, ion and/or water homeostasis can be severely disrupted by a meal the size of the insect. Although it cannot be excluded that the locustatachykinin- and allatostatin-immunoreactive peptides are involved in the regulation of trypsin metabolism and ion and water homeostasis, it seems more likely that such a role would be played by the peptides produced in the RFamide- and/or urotensin-immunoreactive cells, since those cells are present in the same area of the posterior midgut epithelium where trypsin is produced (Graf et al. 1986) and where most of the water and ions from the blood meal are absorbed. Urotensin 1 is related to insect diuretic hormones (Kataoka et al. 1989) and in the moth, *M. sexta,* the diuretic hormone gene is expressed in the midgut (Digan et al. 1992), while sulfakinins are structurally related to cholecystokinin (Nachman et al. 1986a, b), which in vertebrates can stimulate trypsin biosynthesis (Rothman and Wells 1967; Rosewicz et al. 1989). It is therefore tempting to speculate that the RFamide-immunoreactive endocrine cells in the mosquito midgut contain sulfakinins and that these peptides are the regulators of trypsin synthesis and/or secretion, while the urotensin 1-immunoreactive cells are involved in the regulation of water and ion homeostasis. If the mosquito sulfakinins were indeed regulating trypsin synthesis and release, then not only would their structures be homologous to cholecystokinin, but also their function.

These hypotheses regarding the functions of the endocrine cells of the mosquito midgut can be tested once the peptides have been isolated and identified. We are therefore now directing our efforts to the isolation and identification of these peptides.

Acknowledgements We thank Dr. N.T. Davis for the use of the fluorescence microscope, Dr. R. Nichols for sharing unpublished information on the distribution of sulfakinins in *Drosophila,* Dr. J.B. Clark for several gifts of *Drosophila melanogaster* and Drs. R.E. Chance, R. Feyereisen, C.J.P. Grimmelikhuijzen and K. Lederis for their generous gifts of antisera to bovine pancreatic polypeptide, allatostatin B1, RFamide, and urotensin 1. This work was supported by grants from the National Institutes of Health (AI 33429) to J.A.V. and from the Bundesministerium für Forschung und Technologie (Project number 0316919A) to H.-J.A. G.W.L. was a participant in the University of Arizona Undergraduate Biology Research Program and supported in part by a National Science Foundation Research Experiences for Undergraduates Site grant to the University of Arizona (BIR 9322110).

References

Agricola H-J, Bräunig P (1995) Comparative aspects of peptidergic signaling pathways in the nervous systems of arthropods. In: Breidbach O, Kutsch W (eds) The nervous systems of invertebrates: an evolutionary and comparative approach. Birkhäuser, Basel, pp 303–327

Andriès J-C, Beauvillain JC (1988) Ultrastructural study of cholecystokinin-like immunoreactivity in endocrine cells of the insect midgut. Cell Tissue Res 254:75–81

Andriés J-C, Tramu G (1984) Détection immunohistochimique de substances apparentées à des hormones peptidiques de mammifères dans le mésentéron d'*Aesha cyanea* (Insecte, Odonate). C R Acad Sc Paris 299:181–184

Andriès J-C, Tramu G (1985a) Ultrastructural and immunohistochemical study of endocrine cells in the midgut of the cockroach *Blaberus craniifer* (Insecta, Dictyoptera). Cell Tissue Res 240:323–332

Andriès J-C, Tramu G (1985b) Distribution patterns of mammalian-like peptide immunoreactive cells in the midgut of *Aeshna cyanaea* (Insecta, Odonata). Experientia 41:500–503

Barillas-Mury C, Graf R, Hagedorn HH, Wells MA (1991) cDNA and deduced amino acid sequence of a blood meal-induced trypsin from the mosquito, *Aedes aegypti.* Insect Biochem 21:825–831

Barillas-Mury C, Noriega FG, Wells MA (1995) Early trypsin activity is part of the signal transduction system that activates transcription of the late trypsin gene in the midgut of the mosquito, *Aedes aegypti.* Insect Mol Biol 25:241–246

Bennett HPJ, Browne CA, Solomon S (1981) Purification of two major forms of rat pituitary corticotropin using only reversed-phase liquid chromatography. Biochemistry 20:4530–4538

Briegel H, Lea AO (1975) Relationship between protein and proteolytic activity in the midgut of mosquitoes. J Insect Physiol 21:1597–1609

Brown MR, Raikhel AS, Lea AO (1985) Ultrastructure of midgut endocrine cells in the adult mosquito, *Aedes aegypti.* Tissue Cell 17:709–721

Brown MR, Crim JW, Lea AO (1986) FMRFamide- and pancreatic polypeptide-like immunoreactivity of endocrine cells in the midgut of a mosquito. Tissue Cell 18:419–428

Brown MR, Crim JW, Lea AO (1990) Localization of Aea-HP-1 and vertebrate-like peptides in the neuroendocrine and midgut endocrine systems of *Aedes aegypti.* In: Hagedorn HH, Hildebrand JG, Law JH (eds) Molecular insect science. Plenum Press, New York London, p 284

Brown MR, Klowden MJ, Crim JW, Young L, Shrouder LA, Lea AO (1994) Endogenous regulation of mosquito host-seeking behavior by a neuropeptide. J Insect Physiol 40:399–406

Burg M, Grantham M, Abramow M, Orloff J (1966) Preparation and study of fragments of single rabbit nephrons. Am J Physiol 210:1293–1298

Cantera R, Veenstra JA, Nässel DR (1994) Postembryonic development of corazonin-containing neurons in the blowfly, *Phormia terranovae.* J Comp Neurol 350:559–572

Chen Y, Veenstra JA, Davis NT, Hagedorn HH (1994) A comparative study of leucokinin-immunoreactive neurons in insects. Cell Tissue Res 276:69–83

Davis NT, Homberg U, Dircksen H, Levine RB, Hildebrand JG (1993) Crustacean cardioactive peptide-immunoreactive neurons in the hawkmoth *Manduca sexta* and changes in their immunoreactivity during postembryonic development. J Comp Neurol 338:612–627

Digan ME, Roberts DN, Enderlin FE, Woodworth AR, Kramer SJ (1992) Characterization of the precursor for *Manduca sexta* diuretic hormone Mas-DH. Proc Natl Acad Sci USA 89:11074–11078

Donly BC, Ding Q, Tobe SS, Bendena WG (1993) Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach *Diploptera punctata.* Proc Natl Acad Sci USA 90:8807–8811

Duve H, Thorpe A (1982) The distribution of pancreatic polypeptide in the nervous system and gut of the blowfly, *Calliphora vomitoria* (Diptera). Cell Tissue Res 227:67–77

Duve H, Thorpe A (1994) Distribution and functional significance of Leu-callatostatins in the blowfly *Calliphora vomitoria.* Cell Tissue Res 267:367–379

Duve H, Johnsen AH, Scott AG, Yu CG, Yagi KJ, Tobe SS, Thorpe A (1993) Callatostatins: neuropeptides from the blowfly *Calliphora vomitoria* with sequence homology to cockroach allatostatins. Proc Natl Acad Sci USA 90:2456–2460

Endo Y, Nishiitsutsuji-Uwo J (1981) Gut endocrine cells in insects: the ultrastructure of the gut endocrine cells of the lepidopterous species. Biomed Res 2:270–280

Endo Y, Nishiitsutsuji-Uwo J (1982) Exocytotic release of secretory granules from endocrine cells in the midgut of insects. Cell Tissue Res 222:515–522

Endo Y, Iwanaga T, Fujita T, Nishiitsutsuji-Uwo J (1982a) Localization of pancreatic polypeptide (PP)-like immunoreactivity in the central and visceral nervous systems of the cockroach *Periplaneta.* Cell Tissue Res 227:1–9

Endo Y, Nishiitsutsuji-Uwo J, Iwanaga T, Fujita T (1982b) Ultrastructural and immunohistochemical identification of pancreatic polypeptide-immunoreactive endocrine cells in the cockroach midgut. Biomed Res 3:454–456

Fujita T, Iwanaga T, Kusumoto Y, Yoshie S (1981) Paraneurons and neurosecretion. In: Farner DS, Lederis (eds) Neurosecretion. Molecules, cells, systems. Plenum Press, New York London, pp 3–13

Gaus G, Doble KE, Price DA, Greenberg MJ, Lee TD, Batelle B-A (1993) The sequences of five neuropeptides isolated from *Limulus* using antisera to FMRFamide. Biol Bull 184:322–329

Glättli E, Rudin W, Hecker H (1987) Immunoelectron microscopic demonstration of pancreatic polypeptide in midgut epithelium of hematophagous Dipterans. J Histochem Cytochem 35:891–896

Graf R, Raikhel AS, Brown MR, Lea AO, Briegel H (1986) Mosquito trypsin: immunocytochemical localization in the midgut of blood-fed *Aedes aegypti.* Cell Tissue Res 245:19–27

Harlow E, Lane D (1988) Antibodies. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Holman GM, Cook BJ, Nachman RJ (1986) Isolation, primary structure and synthesis of leucomyosuppressin, an insect neuropeptide that inhibits spontaneous contractions of the cockroach hindgut. Comp Biochem Physiol 85C:329–333

Iwanaga T, Fujita T, Nishiitsutsujii-Uwo J, Endo Y (1981) Immunohistochemical demonstration of PP-, somatostatin-, enteroglucagon- and VIP-like immunoreactivities in the cockroach midgut. Biomed Res 2:202–207

Iwanaga T, Fujita T, Takeda N, Endo Y, Lederis K (1986) Urotensin I-like immunoreactivity in the midgut endocrine cells of the insects *Gryllus bimaculatus* and *Periplaneta americana.* Cell Tissue Res 244:565–568

Jenkins AC, Brown MR, Crim JW (1989) FMRF-amide immunoreactivity and the midgut of the corn earworm *(Heliothis zea).* J Exp Zool 252:71–78

Kataoka H, Troetschler RG, Li JP, Kramer SJ, Carney RL, Schooley DA (1989) Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta.* Proc Natl Acad Sci USA 86:2976–2980

Kay I, Coast GM, Cusinato O, Wheeler CH, Totty NF, Goldsworthy GJ (1991) Isolation and characterization of a diuretic peptide from *Acheta domesticus* – evidence for a family of insect diuretic peptides. Biol Chem Hoppe Seyler 372:505–512

Kingan TG (1989) A competitive enzyme-linked immunosorbent assay: applications in the assay of peptides, steroids, and cyclic nucleotides. Anal Biochem 183:283–289

Kingan TG, Teplow DB, Philips JM, Riehm JP, Rao KR, Hildebrand JG, Homberg U, Kammer AE, Jardine I, Griffin PR, Hunt DF (1990) A new peptide in the FMRFamide family isolated from the CNS of the hawkmoth, *Manduca sexta.* Peptides 11:849–856

Lange AB, Chan KK, Stay B (1993) Effects of allatostatin and proctolin on antennal pulsatile organ and hindgut muscle in the cockroach, *Diploptera puncata.* Archiv Insect Biochem Physiol 24:79–92

Lehmberg E, Ota RB, Furuya K, King DS, Applebaum SW, Ferenz H-J, Schooley DA (1991) Identification of a diuretic hormone of *Locusta migratoria.* Biochem Biophys Res Commun 179:1036–1041

Lundquist CT, Clottens F, Holman GM, Riehm JP, Bonkale W, Nässel DR (1993) Locustatachykinin immunoreactivity in the blowfly central nervous system and intestine. J Comp Neurol 341:225–240

Lundquist CT, Clottens FL, Holman GM, Nichols R, Nachman RJ, Nässel DR (1994) Callitachykinins I and II, two novel myotropic peptides isolated from the blowfly, *Calliphora vomitoria,* that have resemblances to tachykinins. Peptides 15:761–768

Matsumoto S, Brown MR, Crim JW, Vigna SR, Lea AO (1989) Isolation and primary structure of neuropeptides from the mosquito, *Aedes aegypti,* immunoreactive to FMRFamide antiserum. Insect Biochem 19:277–283

Montuenga LM, Barrenechea MA, Sesma P, López, Vázquez JJ (1989) Ultrastructure and immunocytochemistry of endocrine cells in the midgut of the desert locust, *Schistocerca gregaria* (Forskal). Cell Tissue Res 258:577–583

Myers CM, Evans PD (1985) The distribution of bovine pancreatic polypeptide/FMRFamide-like immunoreactivity in the ventral nervous system of the locust. J Comp Neurol 234:1–16

Nachman RJ, Holman GM, Haddon WF, Ling N (1986a) Leucosulfakinin, a sulfated insect neuropeptide with homology to gastrin and cholecystokinin. Science 234:71–73

Nachman RJ, Holman GM, Cook BJ, Haddon WF, Ling N (1986b) Leucosulfakinin II, a blocked sulfated insect neuropeptide with homology to cholecystokinin and gastrin. Biochem Biophys Res Commun 140:357–364

Nambu JR, Murphy-Erdosh C, Andrews PC, Gottfried J, Feistner GJ, Scheller RH (1988) Isolation and characterization of a *Drosophila* neuropeptide gene. Neuron 1:55–61

Nichols R (1992) Isolation and expression of the *Drosophila* drosulfakinin neural peptide gene product, DSK-I. Mol Cell Neurosci 3:342–347

Nichols R, Schneuwly SA, Dixon JE (1988) Identification and characterization of a *Drosophila* homologue to the vertebrate neuropeptide cholecystokinin. J Biol Chem 263:12167–12170

Nishiitsutsuji-Uwo J, Endo Y (1981) Gut endocrine cells in insects: the ultrastructure of the endocrine cells in the cockroach midgut. Biomed Res 2:30–44

O'Brien MA, Schneider LE, Taghert PH (1991) In situ hybridization analysis of the FMRFamide neuropeptide gene in *Drosophila.* II. Constancy in the cellular pattern of expression during metamorphosis. J Comp Neurol 304:623–638

Petzel DH, Hagedorn HH, Beyenbach KW (1985) Preliminary isolation of mosquito natriuretic factor. Am J Physiol 249:R701–R711

Pratt GE, Farnsworth DE, Siegel NR, Fok KF, Feyereisen RE (1989) Identification of an allatostatin from adult *Diploptera punctata.* Biochem Biophys Res Commun 163:1243–1247

Pratt GE, Farnsworth DE, Fok KF, Siegel NR, McCormack AL, Shabanowitz J, Hunt DF, Feyereisen R (1991) Identity of a second type of allatostatin from cockroach brains: an octadecapeptide amide with a tyrosine-rich address sequence. Proc Natl Acad Sci USA 88:2412–2416

Reichwald K, Unnithan GC, Davis NT, Agricola H, Feyereisen R (1994) Expression of the allatostatin gene in endocrine cells of the cockroach midgut. Proc Natl Acad Sci USA 91:11894–11898

Robb S, Packman LC, Evans PD (1989) Isolation, primary structure and bioactivity of SchistoFLRF-amide, a FMRF-amide-like neuropeptide from the locust, *Schistocerca gregaria.* Biochem Biophys Res Commun 160:850–856

Rosewicz S, Dubar Lewis L, Wang X-Y, Liddle RA, Logsdon CD (1989) Pancreatic digestive enzyme gene expression: effects of CCK and soybean trypsin inhibitor. Am J Physiol 256:G733–G738

Rothman SS, Wells H (1967) Enhancement of pancreatic enzyme synthesis by pancreozymin. Am J Physiol 213:215–218

Schneider LE, Taghert PH (1988) Isolation and characterization of a *Drosophila* gene that encodes multiple neuropeptides related to Phe-Met-Arg-Phe-NH_2 (FMRFamide). Proc Natl Acad Sci USA 85:1993–1997

Schneider LE, O'Brien MA, Taghert PH (1991) In situ hybridization analysis of the FMRFamide neuropeptide gene in *Drosophila.* I. Restricted expression in embryonic and larval stages. J Comp Neurol 304:608–622

Schols D, Verhaert P, Huybrechts R, Vaudry H, Jégou S, de Loof A (1987) Immunocytochemical demonstration of proopiomelanocortin- and other opioid-related substances and a CRF-like peptide in the gut of the American cockroach, *Periplaneta americana* L. Histochemistry 86:345–351

Schoofs L, Holman GM, Hayes TK, Nachman RJ, Loof A de (1990a) Locustatachykinin I and II, two novel insect neuropeptides with homology to peptides of the vertebrate tachykinin family. FEBS Lett 261:397–401

Schoofs L, Holman GM, Hayes TK, Kochansky JP, Nachman RJ, Loof A de (1990b) Locustatachykinin III and IV. Two additional insect neuropeptides with homology to peptides of the vertebrate tachykinin family. Regul Pept 31:199–212

Shapiro JP, Hagedorn HH (1982) Juvenile hormone and the development of ovarian responsiveness to a brain hormone in the mosquito, *Aedes aegypti.* Gen Comp Endocrinol 46:176–183

Tibbetts MF, Nichols R (1993) Immunocytochemistry of sequence-related neuropeptides in *Drosophila.* Neuropeptides 24:321–325

Veenstra JA (1984) Immunocytochemical demonstration of a homology in peptidergic neurosecretory cells in the suboesophageal ganglion of a beetle and a locust with antisera to bovine pancreatic polypeptide, FMRFamide, vasopressin and α-MSH. Neurosci Lett 48:185–190

Veenstra JA (1988) Effects of 5-hydroxytryptamine on the Malpighian tubules of *Aedes aegypti.* J Insect Physiol 34:299–304

Veenstra JA (1989) Isolation and structure of two gastrin/CCK-like neuropeptides from the American cockroach homologous to the leucosulfakinins. Neuropeptides 14:145–149

Veenstra JA (1991) Presence of corazonin in three insect species, and isolation and identification of [His^7]corazonin from *Schistocerca americana.* Peptides 12:1285–1289

Veenstra JA (1994) Isolation and identification of three leucokinins from the mosquito *Aedes aegypti.* Biochem Biophys Res Commun 202:715–719

Veenstra JA, Davis NT (1993) Localization of corazonin in the nervous system of the cockroach *Periplaneta americana.* Cell Tissue Res 274:57–64

Veenstra JA, Lambrou G (1995) Isolation of a novel RFamide peptide from the midgut of the American cockroach, *Periplaneta americana.* Biochem Biophys Res Commun (in press)

Veenstra JA, Schooneveld H (1984) Immunocytochemical localization of neurons in the nervous system of the Colorado potato beetle with antisera against FMRFamide and bovine pancreatic polypeptide. Cell Tissue Res 235:303–308

Veenstra JA, Lehman HK, Davis NT (1994) Allatotropin is a cardioacceleratory peptide in *Manduca sexta.* J Exp Biol 188:347–354

Verhaert P, Downer RGH, Huybrechts R, Loof A de (1989) A substance resembling somatomedin C in the American cockroach. Regul Pept 25:99–110

Woodhead AP, Stay B, Seidel SL, Khan MA, Tobe SS (1989) Primary structure of four allatostatins: neuropeptide inhibitors of juvenile hormone synthesis. Proc Natl Acad Sci USA 86:5997–6001

Zitnan D, Sauman I, Sehnal F (1993) Peptidergic innervation and endocrine cells of insect midgut. Archiv Insect Biochem Physiol 22:113–132