Allatostatin-Like-Immunoreactive Neurons of the Tobacco Hornworm, Manduca sexta, and Isolation and Identification of a New Neuropeptide Related to Cockroach Allatostatins

N.T. DAVIS, J.A. VEENSTRA, R. FEYEREISEN, AND J.G. HILDEBRAND

1Arizona Research Laboratories, Division of Neurobiology, University of Arizona, Tucson, Arizona 85721
2Department of Entomology, University of Arizona, Tucson, Arizona 85721

Abstract

The YXFGLamide C-terminus serves to define most members of a family of structurally related neuropeptides, the YXFGLamides. These peptides have been identified from the nervous system of various insects and include the allatostatins of cockroaches and crickets, the schistostatins of locusts, and the callatostatins of blowflies. The YXFGLamides have been shown to have various functions, including inhibition of juvenile hormone biosynthesis in cockroaches and crickets and inhibition of contraction of certain insect visceral muscles. We wanted to know if these peptides occur in M. sexta and what functions they might have. A new peptide, AKSYNFGLamide, was isolated and identified from M. sexta and has been named “lepidostatin-1”; this is the first YXFGLamide to be found in a lepidopteran, and there are indications that additional YXFGLamides occur in M. sexta. An antiserum to cockroach allatostatins (YXFGLamides) was shown to recognize lepidostatin-1 of M. sexta and was used to map YXFGLamide-immunoreactive neurons in larvae. Because immunoreactive interneurons were found to form an extensive neuropil, YXFGLamides probably function as neuromodulators in M. sexta. Neuroendocrine cells in the brain, abdominal ganglia, and their respective neurohemal organs were YXFGLamide immunoreactive and appear to release YXFGLamides as neurohormones. Immunoreactivity to YXFGLamides and M. sexta diuretic hormone were found to be colocalized and appear to be coreleased in these neuroendocrine cells, indicating that YXFGLamides may be involved in regulation of fluid transport. Innervation of the corpora allata by YXFGLamide-immunoreactive processes was very sparse, suggesting that this innervation does not play an important role in allatostasis. Many thoracic motor neurons were YXFGLamide immunoreactive, suggesting that YXFGLamides may have a myomodulatory or myotrophic function in larvae. However, this immunoreactivity disappeared early in metamorphosis and did not reappear in the adult. The YXFGLamide-immunoreactive neurons in the terminal abdominal ganglion were found to innervate the hindgut, indicating that YXFGLamides may be involved in the control of the rate of myogenic contractions of the larval hindgut. J. Comp. Neurol. 385:265–284, 1997.

Indexing terms: insects; immunocytochemistry; SCPs; FMRFamide; diuretic hormone

The YXFGLamide peptide family is usually characterized by a Tyr-Xaa-Phe-Gly-Leu-amide C-terminus, in which Xaa may be Gly, Ala, Ser, or Asn. The first YXFGLamides were identified from brain extracts of the cockroach, Diploptera punctata, and because these peptides were shown to inhibit biosynthesis of juvenile hormone (JH) by corpora allata (CA) in vitro, they were named allatostatins (Pratt et al., 1989, 1991; Woodhead et al., 1989, 1994). Immunocytochemical studies of D. punctata showed that allatostatin-immunoreactive neuroendocrine cells in the brain innervate the corpora allata, and these cells apparently are responsible for inhibition of JH biosynthesis (Stay et al., 1992).
Allatostatins similar to those of D. punctata occur in, and inhibit JH biosynthesis by the American cockroach, Periplaneta americana, the German cockroach, Blattella germanica, and the cricket, G. bimaculatus (Belés et al., 1994; Weaver et al., 1994; Lorenz et al., 1995). Although YXFGLamides, known as schistostatins, have been identified from the locust Schistocerca gregaria, their inhibition of JH synthesis has not yet been shown in this species (Vedaert et al., 1995, 1996, 1997).

YXFGLamides, known as callistatins, have been identified in the blowfly, Calliphora vomitoria, but do not inhibit JH biosynthesis in this insect (Duve et al., 1993, 1994, 1995a). A YXFGLamide has been identified in the honeybee, Apis mellifera, and, as in the blowfly, this peptide has not been found to be allatostatic (H. Kaatz, unpublished observation). Similarly, in the present study, we have identified a YXFGLamide from M. sexta, but tests indicate that this peptide is not allatostatic in M. sexta (P.E.A. Teal, unpublished data). The evidence, therefore, suggests that YXFGLamides do not have an allatostatic function in holometabolous insects. Moreover, in M. sexta this function has been shown to be under the control of an allatostatic peptide unrelated to peptides of the YXFGLamide family (Kramer et al., 1991).

In addition to the role of YXFGLamides in the inhibition of JH biosynthesis in some insects, these peptides may have several other functions. In the German cockroach, an allatostatin has been shown to inhibit vitellogenesis (Martin et al., 1996). Studies show that in cockroaches, crickets, locusts, blowflies, and fruitflies many interneurons are YXFGLamide immunoreactive (Agricola et al. 1992; Stay et al., 1992; Duve et al., 1993, 1994; Duve and Thorpe, 1994; Neuhäuser et al., 1994; East et al., 1995; Vedaert et al., 1995; Yoon and Stay, 1995; Vitzthum et al., 1996), and this immunoreactivity of interneurons is an indication that YXFGLamides act as neuromodulators. Also, the innervation of the hindgut of cockroaches, locusts, and blowflies is YXFGLamide immunoreactive, and YXFGLamides modulate the rate of contraction of the hindgut and antennal heart muscles (Hertel and Penzlin, 1992; Lange et al., 1993, 1995; Duve and Thorpe, 1994; Duve et al., 1994, 1999; Knuth et al., 1995; Vitzthum et al., 1995, 1996). Endocrine cells that are YXFGLamide immunoreactive have been found in the posterior midgut of cockroaches, blowflies, and fruitflies (Duve et al., 1994; Duve and Thorpe, 1994; Reichwald et al., 1994; Yu et al., 1995; Yoon and Stay, 1995), indicating that YXFGLamides of these cells have an endocrine or paracrine function.

It is clear that the YXFGLamides are multifunctional, but much is to be learned of these functions. Our study was undertaken because M. sexta appeared to be ideally suited for investigating the YXFGLamides. Because the neuroanatomy of M. sexta has been studied in depth, the mapping of neurons with allatostatin-like (ASL) immunoreactivity in this species offered considerable promise in showing this immunoreactivity in previously identified neurons. Moreover, it seemed likely that identification of ASL-immunoreactive (ASL-ir) neurons that project to peripheral targets or neurohemal organs would be useful in indicating possible functions of YXFGLamides. To help validate these immunocytochemical studies, we also undertook the isolation and identification of YXFGLamides in M. sexta. For practical reasons, our initial study has been limited to the identification and synthesis of one YXFGLamide-type peptide from M. sexta and to the mapping of ASL-ir neurons in larvae. In the future we hope to identify additional YXFGLamides, and we have undertaken studies of ASL-ir cells in the adult central and stomatogastric nervous systems and in the enteric endocrine system. Because a synthetic YXFGLamide of M. sexta is now available, we will perform bioassays on the functions of this peptide.

MATERIALS AND METHODS

Animals

Manduca sexta (Lepidoptera: Sphingidae) were reared at 25°C and 50–60% relative humidity under a long-day photoperiod regimen (17 hours light-7 hours dark). Larvae were fed on an artificial diet adapted from that of Bell and Joachim (1976).

Immunocytochemistry

Primary antiseras. Allatostatin-like (ASL) immunoreactivity was shown by using an antiserum raised to the octadecapeptide allatostatin, ASB2, (AYSYVEYKALPVYNFGL-NH2) of D. punctata (Pratt et al., 1991). Production and testing of this antisera (No. 368T) was reported by Reichwald et al. (1994). Because the antisera immunostains the same cells that are recognized by several other antiseras raised to allatostatins (N.T. Davis, unpublished data), it probably recognizes the common C-terminus (YXFGLamide) of the YXFGLamides. The capacity of this antisera to recognize AKSYNFGLamide, the YXFGLamide identified from M. sexta, was tested by liquid-phase preadsorption (24 hours at 4°C) of the working dilution (1:2,000) of the antisera with 100 nM synthetic AKSYNFGLamide. All specific immunostaining was eliminated by this test, indicating that the antisera recognizes AKSYNFGLamide of M. sexta. In addition, the antisera probably recognizes other allatostatin-like peptides in this insect, and so, neurons immunoreactive to the anti-allatostatin antisera will be referred to as ASL-ir neurons.

Antiseras to leucokinin-IV and to M. sexta diuretic hormone were used to show colocalization of their immunoreactivities with that of ASL immunoreactivity. Production and testing of the DH and LK antisera are described by Chen et al. (1994a) and Veenstra and Hagedorn (1991),

**Abbreviations**

I1a, I1b types of median neuroendocrine cells of the brain
AG1-6 abdominal ganglia of segments one through six
ASL-ir allatostatin-like-immunoreactive
CA corpus allatum
CAP2b cardioaccelerator peptide 2b
DH diuretic hormone of M. sexta
FMRFamide molluscan cardioactive tetrapeptide
JH juvenile hormone
L1-4 lateral neurosecretory cells of abdominal ganglia one through six
LK leucokinin
M5 median neurosecretory cell five of abdominal ganglia
M9 median neurosecretory cell of the ninth neurone of the terminal abdominal ganglion
NCC1-2 fused nerves one and two of the corpus cardiacum
PM1-3 proctodal median neurons one through three
PvO perivisceral organ
SCP molluscan small cardioactive peptide
SeG subesophageal ganglion
TG1-3 thoracic ganglia one through three
TAG terminal abdominal ganglion
respectively, and cells immunoreactive to these antisera are referred to here as DH-ir and LK-ir.

Cross-reactivity between the anti-allatostatin and anti-diuretic hormone sera was tested by preadsorption of each antiserum (washing dilution) with 100 nM of the heterologous antigen. These tests showed that the antisera are not cross-reactive at the dilutions used in this study.

An antiserum (No. 232) to the molluscan cardioactive peptide, FMRFamide (a gift from W.H. Watson III, University of New Hampshire, Durham, NH) and a monodonal antibody to SCPB (Monodonal Laboratory, University of Washington, Seattle, WA) were used to help establish the identity of certain ASL-ir neurons. The anti-FMRFamide serum has been characterized by O'Donahue et al. (1984). The SCPB antibody was characterized by Masinovsky et al. (1988) and Arbiser and Beltz (1991). Cells immunostained by the anti-FMRFamide serum will be referred to as FMRFamide-ir, and those immunostained by the SCPB antibody are referred to as SCP-ir.

Cardioacceleratory peptide 2b (CAP2b), which was identified from M. sexta by Huesmann et al. (1995), has a C-terminus (YLAFPRMamide) similar to that of SCPB (YLAPFRVamide), and, therefore, this peptide was used in a preadsorption control of the SCPB antibody. Preadsorption of a 1:1,000 dilution of the antibody supernatant with 100 nM synthetic CAP2b eliminated all specific immunostaining by the antibody, indicating that this monodonal antibody can recognize CAP2b.

**Tissue preparation.** Insects were anesthetized by chilling on ice, and dissections were performed in a physiological saline solution formulated for M. sexta (Christensen and Hildebrand, 1987). Brains and ventral nerve cords of larvae of various stages were removed and fixed for approximately 18 hours at 4°C in 4% (w/v) paraformaldehyde in sodium phosphate buffer (0.1 M, pH 7.4).

**Immunostaining protocols.** Whole-mounted preparations were studied by an immunofluorescence method adapted from that of Davis (1987). Briefly, phosphate-buffered (0.01 M, pH 7.4) normal saline containing 0.5% (v/v) Triton X-100 was used for washing tissues, and this solution, containing 10% (v/v) normal goat serum, was used as a blocking solution and as a diluent for the primary and secondary antisera. At room temperature and with gentle agitation, the tissues were treated for approximately 18 hours with the primary antiserum diluted 1:2,000 (anti-allatostatin, anti-leucokinin, anti-diuretic hormone, and anti-FMRFamide) or 1:1,000 (anti-SCP). For labeling by the secondary antiserum, the tissues, after thorough washing, were treated with a 1:200 dilution of goat anti-rabbit IgG conjugated to rhodamine (Jackson ImmunoResearch Laboratories) for approximately 18 hours at 4°C. In addition, 2% (w/v) rhodamine-dextran (MW 3,000; Molecular Probes, Eugene, OR) was used for some backfills, and backfilling and fixation were performed overnight as described above. By either method, successfully stained backfills were then double-stained for ASL immunoreactivity, by using fluorescein- or Cy5-conjugated goat anti-IgG for immunostaining.

**Confocal microscopy** Images of the immunostained and backfilled preparations were obtained with a Bio-Rad MRC 600 laser-scanning confocal microscope with a BHS, YHS, or RHS filter block with the argon-krypton laser or a GHS filter block with the argon laser. Images were processed on Adobe Photoshop and PowerPoint programs, and the pictures of the digitized images were made with a Lasergraphics slide maker. The color prints were made from Ektachrome Lumiere 100 transparencies. Where needed, the digitized images were modified only to enhance contrast, to merge images of double-stained tissue, and to provide pseudocolor.

**Peptide isolation, identification, and synthesis** Isolation of M. sexta YXFGLamides was performed according to the same general purification schedule successfully applied for the isolation of several mosquito peptides (Veenstra, 1994; Veenstra et al., 1997; Veenstra, unpublished data) and a cockroach midgut peptide (Veenstra and Lambrou, 1995). Five hundred ventral nerve cords of late pharate-adult M. sexta were dissected and stored frozen at −80°C. Then they were extracted in Bennett's mixture (1% NaCl, 5% formic acid, 1% trifluoroacetic acid [TFA], and 1 M HCl in water; Bennett et al., 1981) and prepurified on a C18 reversed-phase cartridge (Waters Corporation, Milford, MA). The HPLC separations were performed on an Econosil C-18 column (10 × 250 mm, 10 µm) from Alltech Associates, Inc. (Deerfield, IL) and a Microsorb phenyl column (4.6 × 250 mm, 5 µm) from Rainin Instrument Company, Inc. (Woburn, MA). Aliquots of fractions were
analyzed by using a competitive ELISA for allatostatin ASB2 of Diploptera punctata (AYSYVSEYKRLPYNFGLamide), as described by Reichwald et al. (1994). Sequence analysis was performed by the Division of Biotechnology of the University of Arizona, by using an Applied Biosystems model 477A pulsed-laser nitrogen protein sequencer with an online Applied Biosystems model 120A phenylthiohydantoin amino acid analyzer.

AKSYNFGLamide, the YXFGLamide-type peptide identified from M. sexta and CAP2B (pELYAFPRVamide) were synthesized by the Division of Biotechnology of the University of Arizona, by using a Gilson model 422 multiple peptide synthesizer and 9-fluorenylmethoxy-carbonyl chemistry. The crude peptides were purified by HPLC and their sequences were confirmed by tandem mass spectral analysis on a Finnigan TSQ7000 (Palo Alto, CA) instrument at the Southwest Environmental Health Sciences Center of the University of Arizona.

RESULTS

Background: Anatomy of the larval CNS

A brief summary of the anatomy of the larval CNS of M. sexta is necessary to establish the locations and projections of various ASL-ir neurons. The CNS consists of a dorsal brain (Br) and a ventral nerve cord formed by a chain of eleven segmental ganglia, namely, the subesophageal ganglion (SeG), three thoracic ganglia (TG1-3), six unfused abdominal ganglia (AG1-6), and the terminal abdominal ganglion (TAG; Fig. 1A–C). The SeG is a composite ganglion consisting of the neuromeres of the labial, maxillary, and mandibular segments. The TAG is formed by the fusion of the neuromeres of the seventh through ninth abdominal segments, plus almost indistinguishable remnants of the neuromere of the greatly reduced tenth segment.

Most efferent and afferent processes of each unfused segmental ganglion travel via two pairs of lateral nerve trunks, the dorsal and ventral nerves (Figs. 1A,B, DN, VN). In addition, a third pair, the intersegmental nerves, arises from the TG1-2 and TG2-3 connectives (Fig. 1A, IsN). These nerves are so named because they contain the motor axons that innervate most of the longitudinal and oblique trunk muscles of the next posterior segment. Thus, most motor neurons of trunk muscles are located in the ganglion of the segment anterior to that of their muscles. The intersegmental nerve of most segments is incorporated completely into the adjacent connective and is confluent with the dorsal nerve of the next posterior segment (Fig. 1A, DN of TG1; Fig. 1B, DN of AGs); only in the mesothoracic and metathoracic segments can portions of intersegmental nerves be distinguished. However, studies of motor neurons of the trunk muscles have shown that the axons of these neurons extend posteriorly through their ipsilateral connective and into the dorsal nerve of the next segment (Taylor and Truman, 1974; Casaday and Camhi, 1976). This pathway is the remnant of the intersegmental nerve and is designated here as the intersegmental motor tract.

In addition to the pairs of lateral nerve trunks, a median nerve extends posteriorly from each unfused ganglion and then divides to form the transverse nerves (Fig. 1A,B, MRN, TN). The median and transverse nerves contain motor axons of the spiracular muscles of the next posterior segment (Taghert and Truman, 1982). Each transverse nerve also contains the terminal processes of several neuroendocrine cells, and much of the surface of this nerve appears to be neurohemal. In the abdomen, the proximal portion of each transverse nerve is a slightly thickened structure called the perivisceral organ (PVo; Truman, 1973; Fig. 1B,C). Neuroendocrine cells project to the PVos via three pathways (a, b, c in Fig. 2), and their somata are median or lateral in the ganglia (Taghert and Truman, 1982; L1-4, M4-5 in Fig. 2).

Immunocytochemistry

A very large number of ASL-ir neurons and processes were immunostained throughout the larval CNS. Most of these cells appeared to be interneurons, and because numerous processes in the connectives were stained (see Fig. 5A, arrow), many of these ASL-ir cells must be ascending or descending interneurons. An extensive ASL-ir neuropil was stained in the brain and ganglia, and the processes were mostly very fine and uniform, rather than thick and varicose. Relatively few ASL-ir processes were stained in peripheral nerves, and these processes project to neurohemal organs, to the hindgut, and to thoracic muscles. The abundance of ASL immunostaining made identification of individual neurons and their processes very difficult, and therefore, the scope of this study had to be limited to ASL-ir neuroendocrine cells and certain distinctive interneurons and motor neurons.

ASL-ir interneurons of the larval brain. Staining for ASL immunoreactivity resulted in labeling of extensive areas of neuropil and numerous somata in the brain (Fig. 3A,B). Several ASL-ir interneurons are conspicuous or identifiable by their respective positions. The first is a group of approximately seven somata (Fig. 3A,E, straight arrow), of which one is especially prominent (Fig. 3C), in the anterior cortex of each lobe of the tritocerebral; these cells appear to contribute ASL-ir fibers to the tritocerebral neuropil, and some of these cells project through the tritocerebral commissure to bearborize in the contralateral tritocerebral neuropil. Nearby, there are rather diffuse ASL-ir processes in the neuropil of the larval antennal center and a few, weakly staining ASL-ir cells that may be the origin of these processes (Fig. 3D).

Densely arrayed ASL-ir processes are found throughout most of the protocerebrum (Fig. 3F,G,H), but, with the exception of the larval optic center (Fig. 3H, open arrow), specific areas of organized neuropil are difficult to distinguish. Several intensely stained ASL-ir interneurons apparently contribute to much of the ASL-ir neuropil of the protocerebrum. One pair of these neurons is located in the posterior lateral cortex of each protocerebral hemisphere (Fig. 3B,H, arrowhead), and as shown in Figure 3B, the initial projections of these interneurons can be distinguished from the rest of the neuropil. A strongly stained, ASL-ir interneuron that contributes many processes to the anterior and dorsal regions of the protocerebral neuropil is found in the dorsal cortex of each protocerebral hemisphere (Fig. 3A,F, arrowhead). Often this cell is accompanied by one or two weakly stained somata. Finally, a pair of ASL-ir interneurons lies in a paramedian position in the anterior ventral cortex, and these cells send processes to the ventral median region of the neuropil (Fig. 3A,E, curved arrow).

ASL-ir neuroendocrine cells in the brain and colocalization of diuretic hormone immunoreactivity in these cells. There are two pairs of ASL-ir cells in the pars intercerebralis (Fig. 3A, open arrow), each of which has projections that can be followed ventrally through a me-
Fig. 1. Depiction of the central nervous system and nerve trunks of an M. sexta larva (dorsal view; anterior is up). **A:** Brain, subesophageal ganglion, and thoracic ganglia (arrow, motor nerve of muscle VO1; Br, brain; CC, corpus cardiacum; CeC, circumesophageal connective; DN, dorsal nerve; FG, frontal ganglion; IsN, intersegmental nerve; MRN, median recurrent nerve; NCC3, third nerve of the CC; TcC, tritocerebral commissure; TG1-3, thoracic ganglia; TN, transverse nerve; VN, ventral nerve). **B:** Unfused abdominal ganglia representing ganglia of the first through sixth segments (AG, abdominal ganglion; DN, dorsal nerve; MRN, median recurrent nerve; TN, transverse nerve; VN, ventral nerve). **C:** Terminal abdominal ganglion (DN7&8, dorsal nerves of the seventh and eighth abdominal segments; NM7, neuromere of the seventh abdominal segment; PN, proctodeal nerve; TmN, terminal nerve; VN7&8, ventral nerves of seventh and eighth abdominal segments). Also see Abbreviations list.
Two of these five subtypes, IIa4 and IIa5, can be distinguished by the fact that each is represented by only two pairs of neuroendocrine cells, and so the two pairs of ASL-ir neuroendocrine cells must be one of these two subtypes. The IIa4 cells are labeled by the anti-diuretic hormone serum (Veenstra and Hagedorn, 1991; Chen et al., 1994b; Zitnán et al., 1995), and the IIa5 cells are immunostained by the SCPB antibody (Homberg et al., 1991). Because ASL immunoreactivity was colocalized with that of diuretic hormone in the IIa4 cells (Fig. 4A, arrows) but not with that of SCP in the IIa5 cells (Fig. 4A, arrowheads), the results indicated that the ASL-ir cells are the same as the IIa4 cells.

Double immunostaining showed colocalization of diuretic hormone and ASL immunoreactivities in processes of the IIa4 cells only in the corpora cardiaca allata (Fig. 4C, CC), and the aforementioned ASL-ir processes that extend into the corpora allata did not show DH-ir (Fig. 4C, CA). Therefore, these ASL-ir processes of the CA probably do not originate from the IIa4 neuroendocrine cells. Although we were unable to identify origin of these processes, it seems likely that they are from small neuroendocrine cells in the pars intermedialis.

**ASL-ir interneurons of AG1 through AG6.** In the unfused abdominal ganglia (AG1-6), there are four distinct types of ASL-ir interneurons, identifiable as such because they do not have immunoreactive processes projecting into peripheral nerves. Each type is recognized on the basis of its respective location, projection, and immunostaining characteristic. These four types serve as a basis for recognizing comparable ASL-ir interneurons of other ganglia. In addition to these interneurons, a few other interneurons were immunostained in AG1-6, but their staining was weak or inconsistent.

The type-1 ASL-ir interneurons are a pair of relatively large, weakly stained cells in the anterior ventral cortex; their projections are difficult to distinguish (Fig. 5A, T1). Representing the second type of ASL-ir interneurons is a group of three somata in each anterior, lateral cortex (Fig. 5A, T2). One of these somata is more dorsal and always stains more intensely than the others. The type-3 ASL-ir interneurons are a triplet of cells found in the posterior dorsolateral cortex, one of which is always less intensely immunostained than the others (Fig. 5A, T3). The neurites of these interneurons extend in a transverse tract leading to the contralateral neuropil, where they arborize. The fourth type of ASL-ir interneuron has a median, unpaired soma located in the posterior ventral cortex (Fig. 5A, T4). A bifurcated neurite extends anteriorly from this cell, but its finer arborizations could not be distinguished (Fig. 5B).

**ASL-ir neuroendocrine cells in AG2-6.** In AG2-6 (but not in AG1), ASL-ir staining revealed a lateral neuron that projects ipsilaterally into each ventral nerve and from there into the PVO of the next posterior segment (Fig. 5A, L3, curved arrow). The terminal processes of these ASL-ir cells have many prominent varicosities located near the surface of the PVO (Fig. 5A, PVO), a characteristic of neurohemal release sites and an indication that these ASL-ir cells have a neuroendocrine function.

The pattern of projection of the ASL-ir neuroendocrine cell is comparable with that of the L2-4 neuroendocrine cells of abdominal ganglia and previously described by Taghert and Truman (1982), Davis et al. (1993), and Chen et al. (1994b). Unlike neuroendocrine cell L1, cells L2-4 (and the ASL-ir neuroendocrine cell) project to the PVO via the ipsilateral ventral segmental nerve (Fig. 2, L2-4, a). To confirm that the ASL-ir neuroendocrine cell is one of the L2-4 cells, these cells were backfilled with Neurobiotin and then stained with streptavidin-fluorescein. Next, the ganglion was stained for ASL immunoreactivity and rhodamine-conjugated goat anti-rabbit IgG used for visualization. One of the L2-4 cells was doubly stained by this procedure, indicating that the ASL-ir neuroendocrine cell belongs to the L2-4 group (Fig. 4B, arrow). In addition, one pair of median neuroendocrine cells, the M5 cells (Fig. 4B,
Fig. 3. ASL immunostaining of cells and neuropil of the larval brain (frontal views; dorsal is up). Photographs in this and other figures are digitized, confocal microscopic images of merged optical sections. **A:** Anterior half of the third instar brain, showing ASL-ir cells and neuropil of the tritocerebrum (straight arrow), protocerebral interneurons characterized by strong immunostaining (curved arrow and arrowhead), and type IIa neurosecretory cells of the pars intermedialis (open arrow). **B:** Posterior half of the third instar brain, showing ASL-ir cells and neuropil (arrowhead), one of several protocerebral interneurons characterized by strong immunostaining; arrow, ASL-ir processes in nerve NCC 1+2. **C:** ASL-ir cells and neuropil of the tritocerebrum. **D:** ASL-ir cells and neuropil of the olfactory center. **E-H:** ASL-ir cells and neuropil in serial Vibratome sections (50 µm) of brain of fifth instar larva (straight arrow, ASL-ir cells and neuropil of the tritocerebrum; curved arrow and arrowheads, interneurons of the protocerebrum characterized by strong immunostaining; open arrow, ASL-ir immunostaining of neuropil of the optic center). Scale bars = 100 µm for A, B, 50 µm for C, D, 200 µm for E–H.
M), also was stained by this backfilling procedure but did not exhibit ASL immunoreactivity.

Colocalization of ASL, leucokinin, and diuretic hormone-immunoreactivities in the L3 neuroendocrine cells. Next, we were interested in determining which of the three lateral neuroendocrine cells is ASL-ir. A previous study had shown that L3 and L4, but not L2, can be immunostained by an anti-leucokinin serum (Chen et al., 1994b). Therefore, abdominal ganglia were doubly stained for leucokinin and ASL immunoreactivity. Leucokinin and ASL immunoreactivities were colocalized in one of the two LK-ir neuroendocrine cells (Figs. 5C,D, arrow), indicating that the ASL-ir neuroendocrine cell is not L2 but could be L3 or L4.

Because the study by Chen et al. (1994b) also had shown that the L3 cells can be immunostained by an antiserum against M. sexta diuretic hormone, abdominal ganglia were doubly stained for diuretic hormone and ASL immunoreactivity. The results showed that diuretic hormone and ASL immunoreactivities are colocalized in the L3 cells and in the varicosities in the PVo (Figs. 5E,F, arrow, PVo). Therefore, the ASL-ir neuroendocrine cells of the abdominal ganglia are the L3 cells.

ASL-ir interneurons in the terminal abdominal ganglion. A constriction in the terminal abdominal ganglion (TAG) serves to distinguish the seventh neuromere from the remainder of the ganglion (Fig. 1C, Nm7), and ASL-ir interneurons found in the seventh neuromere are mostly comparable with those of AG1-6 (Fig. 6A, T3; Fig. 6B, T2, T4).

The terminal region of the TAG contains a number of ASL-ir interneurons, but it was not possible to relate these neurons to the types of ASL-ir interneurons found in AG1-6 (Figs. 6A,B; note unlabeled ASL-ir somata). Double immunostaining, initially done in a study of ASL-ir neuroendocrine cells of the TAG (see below), revealed colocalization of diuretic hormone and ASL immunoreactivities in a prominent pair of paramedian interneurons at the posterior end of the TAG (Figs. 4D,E; 6A, IN). These are local interneurons, and they have extensive arborizations in the neuropil of the ninth neuromere (see Fig. 8A). The diuretic-hormone immunoreactivity of these interneurons was described by Chen et al. (1994b).

ASL-ir neuroendocrine cells of the TAG. The L3 neuroendocrine cells of neuromere seven of the TAG are ASL-ir (Fig. 6A, L3), and, as in the unfused abdominal ganglia, double staining showed that ASL, diuretic hormone, and leucokinin immunoreactivities are colocalized in these cells (Fig. 4E, L3a).

The presence of ASL-ir processes in the terminal nerves and in the ventral segmental nerves of the eighth segment (Fig. 6A, arrows) indicated that there are efferent ASL-ir neurons in the TAG or, possibly, in ganglia more anterior to it. A single ASL-ir process in the ventral nerve of the eighth segment was traced proximally to an intensely immunostained, ipsilateral cell in the eighth neuromere of the TAG (Fig. 6A, curved arrow, L3a). Because this neuron appears to be a segmental homolog of the L3 neuroendocrine cells of other abdominal ganglia, it is designated L3a.

The ASL-ir process of this L3 cell was traced distally through the eighth ventral nerve and into a nerve formed by the confluence of a branch from the eighth ventral nerve and the terminal nerve (Fig. 7A, VN8, TMn, arrow). By way of this nerve, the ASL-ir process enters the cryptonephridial chamber of the rectum and extends varicose branches that, along with other ASL-ir processes (see below), form a diffuse neurohemal-like system in the chamber.

The location and projection pattern of the L3 neurons of the eighth neuromere are very similar to those of diuretic hormone-immunoreactive neuroendocrine cells previously described by Chen et al. (1994b). The results of double-immunostaining experiments indicated that diuretic hormone and ASL immunoreactivities are, indeed, colocalized in these cells (Figs. 4D,E, L3a).

Staining for ASL immunoreactivity also showed a pair of cells in the posterior, midventral cortex of the TAG, just anterior to the DH/ASL-ir interneurons noted above (Figs. 4E, 6A, M5). These cells, which are median and project bilaterally, appear to be comparable with the median neuroendocrine cells of the unfused abdominal ganglia, but because they are not clearly related to any one type of the median abdominal neuroendocrine cells, they are...
designated simply as median neuroendocrine cells of the ninth abdominal neuromere, or MA9. The processes of the MA9 cells project bilaterally into each terminal nerve (Fig. 8B) and then into a branch that, as noted above, enters the cryptonephridial chamber (Fig. 7A, arrow). There the ASL-ir processes of the MA9 cells extend with ASL-ir branches from the L3A8 neuroendocrine cell to form the neurohemal-like structure noted above.

The earlier demonstration (Chen et al., 1994b) of a similar pair of leucokinin-ir neuroendocrine cells in the
TAG prompted our use of double staining for ASL and leucokinin immunoreactivities. These experiments showed that ASL and leucokinin immunoreactivities are colocalized in the M$_{Ag}$s cells (Fig. 4L, M$_{Ag}$) and in the L3 cells of the seventh neuromere, but not in the L3 cells of the eighth neuromere (Fig. 4L, L3$_{Ag}$, L3$_{AB}$).

**ASL-ir visceromotor neurons of the TAG.** As depicted in Figure 7A, the proctodeal nerve innervates the anterior rectum, ileum, ileal bulb, pylorus, and ampullae of the Malpighian tubules (terminology of Reinecke et al., 1973; Fig. 7A, PN, Im, Py, MA). We observed immunostaining of two or three ASL-ir processes that extend into the proctodeal nerve via the terminal nerve (Fig. 6E) and branching processes on the visceral muscles of the pyloric and ileal regions of the hindgut (Fig. 6F). We were unable to follow these ASL-ir processes back to their somata. Therefore, as a first step in identifying the ASL-ir neurons that innervate the hindgut, the proctodeal nerve was backfilled with dextran-rhodamine or dextran-fluorescein. Among the cells stained by bilateral backfilling were two or three ASL-ir processes that extend into the proctodeal nerve and ileal region of the hindgut (Fig. 6E). We were unable to follow these ASL-ir processes back to their somata.

SCP immunostaining of the TAG backfilled through the proctodeal nerve resulted in the double staining of the PL1-2 neurons and sometimes PL3 as well (Fig. 4F, PL1-3). The PL1 soma is located just anterior, and PL2 just posterior, to L3$_{AB}$ (Fig. 4D, PL1, PL2, L3$_{AB}$). A weakly stained SCP-ir cell, which appears to be an interneuron, is also located between PL1 and PL2 (Fig. 4F, IN). Two or three SCP-ir processes were observed in the proctodeal nerve, and scattered SCP-ir endings were observed on the pyloric region of the proctodeum. Double immunostaining showed that SCP and FMRFamide immunoreactivities are colocalized in neurons PL1-2 and that PL3 is FMRFamide-ir but usually not SCP-ir (Fig. 6G, PL1-2; Fig. 6H, PL1-3). In addition, neuroendocrine cell L3$_{AB}$ and other neurons of the TAG are FMRFamide-ir but not SCP-ir (compare Fig. 6G with 6H). Unlike the immunoreactivity of the ASL-ir median cells innervating the hindgut (PM2$_{A7}$, PM2$_{A8}$, PM3), the PL1-3 cells did not lose their SCP/FMRFamide immunoreactivity at the onset of metamorphosis.

**ASL-ir interneurons of the thoracic ganglia.** ASL immunostaining of the thoracic ganglia (TGs) resulted in the labeling of many more somata than were found in the unfused abdominal ganglia (compare Fig. 5A with Figs. 8C, D, and some of these additional somata appeared to be motor neurons (see below). The type-1 abdominal neurons (paramedian in the anterior, ventral cortex) are present, and occasionally a cell comparable with the type-4 neuron (unpaired, median in the posterior ventral cortex) can be distinguished (Fig. 8D, T1, T4). Somata comparable with the type-2 neurons (in the anterior dorsolateral cortex) are present, as are the somata of the type-3 neurons (triplet in the posterior dorsolateral cortex; Fig. 8C, T2, T3).

**Absence of ASL-ir neuroendocrine cells in the TGs.** Lateral neuroendocrine cells of each of the thoracic ganglia project via the median recurrent nerve to neuromere-8 of the thoracic and first abdominal segment (Wall and Taghert, 1991). These lateral neuroendocrine cells are labeled by the SCP antibody (Mesce et al., 1993), but double immunostaining for SCP and ASL immunoreactivity showed that these cells are not ASL-ir. Moreover, there are no ASL-ir processes in the median and transverse nerves of the thoracic and first abdominal segment (Fig. 8F, TN). Thus, we conclude that the ASL-ir cells of the thoracic ganglia do not have a neuroendocrine function.

**ASL-ir motor neurons of the TGs.** Several ASL-ir processes extend into the ventral nerve trunks of TG1, 2, and 3 and enter nerve branches innervating the leg muscles (Fig. 8E, arrows). The ASL-ir processes also extend from TG1 and 2 into the intersegmental nerve (Fig. 8F, ISN, double arrow) and could be traced into nerve branches innervating various ventral longitudinal and oblique muscles (Fig. 8H). In TG2 and 3, ASL-ir processes in the intersegmental nerve extend into the dorsal nerve via the connection between the intersegmental and dorsal nerve (Fig. 8F, DN, arrowhead), but no ASL-ir processes enter the dorsal nerve via the trunk of this nerve (Fig. 8F, DN).

Most of the motor neurons in the intersegmental nerve of TG2 have not been identified, and that identification was beyond the scope of this study. Nevertheless, we wished to identify at least one ASL-ir motor neuron for this study. Fortunately, we were able to locate a single ASL-ir process that extends from the metathoracic intersegmen-
The dorsal nerve of TG1 contains ASL-ir processes of the intersegmental motor tract, which descends from the labial neuromere of the SeG, and so some of the ASL-ir neurons of this neuromere are probably motor neurons of muscles of the prothoracic segment.

**Peptide isolation and identification**

Five hundred abdominal ventral nerve cords of late pharate adults were homogenized in Bennett’s mixture, prepurified on a C-18 Seppak, and the peptide fraction injected onto a C-18 column. Analysis of allatostatin immunoreactivity by ELISA indicated two major immunoreactive peaks and three or four minor ones (Fig. 9). The two major immunoreactive peaks were injected on the same C-1 column used previously to purify various insect neuropeptides (Veenstra, 1994; Veenstra and Lambrou, 1995; Veenstra et al., 1997; Veenstra, unpublished data), but no immunoreactivity was recovered. The major difference between this and previous purifications was the tissue used for isolation. In previous efforts rather large amounts of complex material were used for purification, whereas here we used cleanly dissected nerve cords. Our results suggested that recovery of peptides from this column is severely compromised if the injected material is a limited quantity of an already relatively pure peptide. Therefore, to purify the immunoreactive peak eluting between 46 and 48 minutes on the first HPLC column, we used a Microsorb column and 0.1% heptfluorobutyric acid as a pairing ion. A single immunoreactive peak was recovered and submitted to a final purification on the same column, by using 0.1% TFA as the pairing ion. The final chromatogram showed a single UV-absorbing peak that was immunoreactive (Fig. 10). We estimate that less than 100 pmol of the peptide were recovered, and virtually all of this material had to be used for sequence analysis. Therefore, an insufficient amount of the peptide was available for later comparison with the synthetic peptide.

Analysis yielded the following unambiguous sequence: Ala-Lys-Ser-Tyr-Asn-Phe-Gly-Leu. The largest amount of any other amino acid in the first cycle was 3.6 pmol of Gly; carryover was similarly very limited, the largest amount being 4.9 pmol of Asn in the sixth cycle (Table 1).

All insect peptides related to this sequence of amino acids have an amided C-terminus, and the anti-allatostatin serum used in the ELISA requires an amided C-terminus for recognition. The peptide, therefore, was synthesized with a C-terminal amide. The synthetic peptide had the same retention time on reverse-phase HPLC as the natural peptide, whereas the retention times of C-terminally amided oligopeptides and their analogs with a C-terminal acid differ profoundly under these conditions (Veenstra, 1994). It was, therefore, concluded that the structure of the peptide is AKSYNFGLamide. The YNFGLamide C-terminus of this peptide indicates that it belongs to the YXFGlamide family of peptides. This peptide is the first one in the YXFGlamide family to be identified from the Lepidoptera.

**DISCUSSION**

**ASL-ir interneurons**

We have shown that there are many ASL-ir interneurons in the brain and ventral ganglia of M. sexta larvae and that some of these cells are ascending or descending interneurons. Because synaptic neurotransmitters are
small, fast-acting molecules, it is likely that YXFGLamides of the ASL-ir interneurons function as neuromodulators rather than as synaptic neurotransmitters. In theory, such functions could be manifested through a general paracrine release, i.e., via nonsynaptic exocytosis and broad diffusion to relatively distant receptors in the neural tissue. Alternatively, the release could be parasynaptic, i.e., involving focal diffusion over relatively short distances to pre- or postsynaptic receptors. These two types of release probably represent the extremes of a continuum.
Fig. 8. **A:** Diuretic hormone immunoreactivity of the soma and neuropil of a local interneuron of neuromere-9 of the TAG. These cells are also ASL-ir. **B:** Leucokinin immunoreactivity of paired median neurosecretory cells of neuromere-9 and their bilateral projection into the terminal nerve. These cells are also ASL-ir. **C,D:** Frontal sections of the upper third (C) and lower two-thirds of the metathoracic ganglion of a third instar larva, showing four types of ASL-ir interneurons (T1-4). Some of the unlabeled ASL-ir somata in (D) are believed to be motor neurons. **E:** ASL-ir processes (arrows) in branches of the ventral nerve of the mesothoracic ganglion of a fourth instar larva. **F:** ASL-ir processes (double arrows) in the intersegmental nerve (IsN) of the mesothoracic ganglion of a fourth instar larva. ASL-ir processes also extend from this nerve into the dorsal nerve (arrowhead) and into a branch (arrow) that innervates ventral oblique muscle 1. No ASL-ir processes are found in the neurohemal transverse nerve (TN) and in the base of the dorsal nerve (DN). **G:** Motor neuron of ventral oblique muscle 1, shown by backfilling the motor nerve with dextran-rhodamine of a fourth instar larva. **H:** ASL-ir processes on ventral oblique muscle 1 of a fourth instar larva. **I,J:** ASL-ir processes and somata in frontal sections of the dorsal two-thirds (I) and ventral one-third of the subesophageal ganglion of a third instar larva. Some of the ASL-ir processes extend into the labial nerve (arrow). Scale bars = 50 µm in A–D, G, I, J, 200 µm in E, F, H.
and there may be instances in which parasympathetic release can affect synaptic domains of various sizes. Examples of both nonsynaptic and parasympathetic release have been found in nervous systems (see reviews by Hökfelt, 1991; Golding, 1994), but these phenomena have received little attention in insects.

Some peptidergic interneurons of insects are characterized by arborizations that are thicker, more irregular, and more varicose than most processes in the neuropil. The appearance of these branches is very similar to that of the neuroendocrine processes found in neurohemal organs, suggesting that peptidergic interneurons that have neurosecretory-like processes provide a general paracrine (nonsynaptic) release within the nervous system. Examples of this type of peptidergic interneuron include in the Arg-vasopressin-immunoreactive, descending interneurons of the SeG of cockroaches and grasshoppers (Davis and Hildebrand, 1992; Tyrer et al., 1993) and the PBAN-ir descending interneurons and CCAP-ir interneuron 704 of *M. sexta* (Davis et al., 1993, 1996). Moreover, nonsynaptic exocytosis of dense-core vesicles has been shown in the neuropil of cockroaches (Buma and Roubos, 1986).

Our study has shown that the ASL-ir neuropil of the brain and ganglia is very extensive, regular, and usually fine textured. Thus, unlike the examples cited above, most ASL-ir interneurons appear not to be general nonsynaptic paracrine cells but probably provide parasympathetic release of their neuropeptide. The coexistence of small-molecule, fast-acting synaptic transmitters with one or more peptides has been reported in many neurons and may be characteristic of most neurons; in addition, there is evidence that parasympathetic release of neuropeptides is activity dependent (see reviews by Hökfelt, 1991; Golding, 1994). The demonstration of colocalization of a classic synaptic transmitter and of a neuropeptide, therefore, may be an identifying feature of interneurons capable of both synaptic modulation and synaptic transmission. It may be that there is a class of peptidergic paracrine neurons that lack classic synaptic transmitters, but we suspect that most, if not all, of the ASL-ir interneurons also contain a classical synaptic transmitter.

It may be argued, moreover, that the extent of modulatory function of an interneuron is reflected in the level of production of its neuropeptide(s) and that interneurons that have a high peptide content may be expected to have major modulatory functions. Accordingly, many of the ASL-ir interneurons appear to serve major modulatory functions.

### ASL-ir neuroendocrine cells

We have shown allatostatin-like immunoreactivity in the IIa4 neuroendocrine cells of the brain and the L3 neuroendocrine cells of the abdominal ganglia. All of these cells also have colocalized diuretic hormone and leucokinin immunoreactivities (Chen et al., 1994a), and, therefore,
the L1a8 and L3 neuroendocrine cells appear to function in the regulation of fluid transport. Because allatostatin-like immunoreactivity is found in these neuroendocrine cells and in neuroendocrine cells projecting into the cryptonephridial chamber (L3aB, M A9), YXFGLamides also may have functions directly or indirectly related to the regulation of fluid transport. Bioassays now are needed to determine if these peptides do, in fact, have such functions.

A peptide unrelated to peptides of the YXFGLamide family has been shown to have an allatostatic function in M. sexta (Kramer et al., 1991). Zitrik et al. (1995) have shown that several neuroendocrine cells of the pars lateralis of the protocerebrum are immunoreactive to this allatostatic peptide and that these cells terminate in extensive arborizations in the corpora allata. In contrast we have found that there are very few ASL-ir processes in the corpora allata; the paucity of ASL-ir innervation of the CA appears to indicate that this innervation has no major role in the regulation of juvenile hormone biosynthesis in M. sexta larvae. There is a possibility that inhibition of JH biosynthesis in the larva could be humoral rather than neural, but it has been shown that the YXFGLamide identified in this study (AKSYNFGLamide) does not inhibit JH biosynthesis by the CA of M. sexta in vitro (P.E.A. Teal, unpublished data).

**ASL-ir skeletomotor neurons**

Many of the motor neurons innervating the thoracic and maxillary muscles are ASL-ir, and, except for a few motor neurons innervating the first abdominal segment, none of the motor neurons of the abdominal muscles are ASL-ir. This ASL-ir innervation can be seen in all larval stages, and therefore, it may be that YXFGLamides modulate the contraction of larval thoracic muscles. In cockroaches and locusts, such a function has been shown for the peptide, proctolin (Adams and O'Shea, 1983; Baines et al., 1990). However, most thoracic and abdominal trunk muscles of M. sexta larvae have essentially the same locomotor functions, and, if YXFGLamides do serve as myomodulators, it seems remarkable that only the thoracic muscles have ASL-ir innervation. It remains to be determined if this difference is related to the fact that there is putative release of YXFGLamide-like neurohormones in the abdomen but not in the thorax, and that YXFGLamides, therefore, might not be a suitable agents for modulation of abdominal muscles.

The ASL-ir of the thoracic motor neurons disappears early in pupal development and does not reappear in the motor neurons of adults. If YXFGLamides serve as myomodulators, this change could reflect a difference between larval and adult control of the thoracic muscles. It is known that neurons, which provide slow-type motor innervation of larval muscles, change during metamorphosis to provide fast innervation of the flight muscles of adults (Rheuben and Kammer, 1980), and the loss of YXFGLamide immunoreactivity in thoracic motor neurons at metamorphosis might possibly be related to such a change in function.

Another possibility is that a YXFGLamide serves as a trophic factor for the thoracic muscles. Myotrophic functions have been shown for a number of neuropeptides in vertebrates (Hökfelt, 1991), and trophic functions have been proposed for the neurosecretory axons associated with certain insect muscles (reviewed by Rheuben, 1995).

At the start of metamorphosis the thoracic muscles of Lepidoptera regress and are later replaced by a set of adult muscles (reviewed by Nüesch, 1985), but many or all of the motor neurons of the adult thoracic muscles persist and are remodeled from those of the larval stage (reviewed by Kent et al., 1995). If the thoracic muscles are denervated just before metamorphosis, the adult thoracic muscles fail to develop normally, and this effect of denervation is taken to indicate that the normal development of the adult thoracic muscles depends on a trophic effect provided by their motor neurons (Nüesch, 1985; Kent et al., 1995). Therefore, YXFGLamides possibly have a trophic influence on the development of the adult thoracic muscles. Immunostaining indicates that a YXFGLamide is present in thoracic motor neurons late into the prepupal stage, but that this peptide disappears from the motor neurons by a day after pupal ecysis and does not return in the adult. Therefore, if YXFGLamides do exert a trophic effect on the development of the adult muscles, that effect would have to be at a very early stage (prepupal) in the development of the adult muscles.

It is noteworthy that the larval motor neurons of M. sexta are FMRFamide-ir, that the number of these immunoreactive motor neurons increases with larval development, that this immunoreactivity declines during metamorphosis, and that the motor neurons of adults are not FMRFamide-ir (Witten and Truman, 1996). Thus, the pattern of developmental change in ASL-ir immunoreactivity of motor neurons is very similar to that of FMRFamide-ir motor neurons, but, as we have shown, ASL-ir motor neurons are limited to the thoracic ganglia, whereas FMRFamide-ir motor neurons are found in the thoracic and abdominal ganglia (Witten and Truman, 1996).

**ASL-ir visceromotor neurons of the hindgut**

As in other insects, the rhythmic contractions of the hindgut of M. sexta are myogenic (Tublitz et al., 1992). The demonstration that the PM2A7, PM2A8, and PM3 neurons are ASL-ir and that they terminate on the visceral muscles of the hindgut of M. sexta suggests that YXFGLamides might modulate contractions of the hindgut of this species. Inhibition of the rate of hindgut contractions by YXFGLamides has been shown in cockroaches and blowflies (Lange et al., 1993, 1995; Duve et al., 1994, 1995a). In a continuing part of our study of M. sexta, we also have found that application of a physiological concentration (10^{-7} M) of lepidostatin-1 resulted in inhibition of the rate and amplitude of contractions of larval hindgut in vitro (N.T. Davis, unpublished data).

Soon after the commencement of the active movements of gut emptying in the last instar larva, staining of the ASL-ir hindgut motor neurons can no longer be detected. Thus, this loss of ASL-ir comes at a time when there may be no need for inhibition of hindgut movements.

**SCP/FMRFamide-ir visceromotor neurons of the hindgut**

The array of neurons innervating the proctodeum that we have shown by backfilling through the proctodeal nerve is comparable with, but somewhat more extensive than, that described previously by Thorn and Truman (1989) and Tublitz et al. (1992). We have shown that the hindgut is innervated by the PL1 and PL2 neurons of the TAG and that these cells are SCP- and FMRFamide-ir. The immunoreactivity of these...
cells suggests that myogenic hindgut contractions also may be modulated by SCP- and/or FMRFamide-like peptides. Because SCP and FMRFamide immunoreactivities are colocalized in the PL1 and PL2 cells, the question arises as to whether this immunoreactivity is due to the colocalization of distinct peptides or the colabeling of the same peptide(s). Colocalization of SCP and FMRFamide immunoreactivities often have been observed in arthropods, but Arbiser and Beltz (1991) have shown that, in lobsters, immunostaining by the SCP antibody can be abolished by preadsorption with a heptameric FLRFamide. Therefore, the colocalized SCP and FMRFamide immunostaining cannot be due to colabeling of the same FLRFamide. Similar heptameric FLRFamides have been identified from ventral nerve cords of larval M. sexta (Kingan et al. 1996), and so these peptides could be responsible for the colocalized SCP and FMRFamide immunostaining observed in the PL1 and PL2 cells. Colabeling of FLRFamides in these cells, however, does not eliminate the possibility that the SCP immunostaining could be due in part to an SCP-like peptide. This possibility is more than theoretical because an SCP-like peptide, CAP2b, has been identified in M. sexta (Huesmann et al., 1995). The sequence of the last five amino acids of the C-terminus (AFPRVamide) of CAP2b is identical to that of SCPa, except that the terminal amino acid of SCPa is methionine rather than valine. Masinovsky et al. (1988) concluded that the epitope of the SCP antibody includes all or part of a six amino acid sequence located at the C-terminus of SCPS. We have shown that the SCP antibody can be preadsorbed with synthetic CAP2b; these results probably are due to the similarity of the C-terminus of CAP2b to that of the SCPa. Consequently, the SCP immunostaining of the PL1 and PL2 cells could be due to CAP2b.

Indirect evidence suggests that the function of the PL1 and PL2 cells is to accelerate the rate of hindgut contractions and that this effect may be mediated by CAP2b and/or FLRFamides. The supporting evidence with regard to CAP2b is as follows. Tublitz and Truman (1985) isolated a FLRFamide. The supporting evidence with regard to the possibility that the SCP immunostaining could be due to CAP2b is as follows. Kingan et al. (1990, 1996) have identified three extended FLRFamides (Mas-FLRFamide I, II, and III) from M. sexta, and Mas-FLRFamide II and III were found to be the most abundant FLRFamides of the ventral nerve cord of larvae. In M. sexta hindgut bioassays, these two peptides were very effective in accelerating hindgut contractions (Kingan et al., 1996). Because the FMRFamide-ir of the PL1 and PL2 cells could be due to Mas-FLRFamide II and/or III, acceleration of the rate of hindgut contractions might be mediated by the release of these FLRFamides from the PL1 and PL2 cells.

In any case, the ASL-ir (PM2 and PM3) and SCP/FMRFamide-ir (PL2 and PL3) visceromotor neurons of the hindgut appear to be antagonistic systems regulating hindgut contractions. Some of the somata shown by back-filling the proctodeal nerve were neither ASL-ir or SCP/FLRFamide-ir, and therefore, the control of hindgut movements may also involve release of neuroactive substances other than those that are SCP/FMRFamide- and ASL-ir.

Identification of AKSYNFGLamide (lepidostatin-1)

The peptide that we have identified from M. sexta is yet another novel member of the YXFGLamide family of peptides. It differs from all other YXFGLamides that have been isolated from cockroaches, crickets, locusts, blowflies, and mosquitoes (Pratt et al., 1989, 1991; Woodhead et al., 1989, 1994; Duve et al., 1993, 1994, 1995a; Bellés et al., 1994; Neuhäuser et al., 1994; Weaver et al., 1994; Veeelaert et al., 1996; J. A. Veenstra et al., 1997), and it differs from YXFGLamides predicted from the sequences of their cDNA’s (Donly et al., 1993; Ding et al., 1995; J. A. Veenstra et al., 1997).

Although only one peptide was identified, there are at least two, and probably four, more YXFGLamides present in the ventral nerve cord of M. sexta. It seems very likely that the same or very similar peptides will be found in other lepidopterans. Therefore, “lepidostatin-1” is proposed as the trivial name of this peptide, and it is hoped that as other lepidopteran YXFGLamides are identified, they can be so named in numerical sequence.

The identification of lepidostatin-1 will be helpful in the isolation of the cDNA encoding it and all other YXFGLamides of M. sexta. In addition, the availability of the synthetic peptide will enable the use of various bioassays in the study of its functions.

The structure of this peptide is very similar to that of cockroach allatostatins, but designation of it as an allatostatin could cause confusion because an allatostatic peptide unrelated to the YXFGLamides has already been identified from M. sexta and has been named “allatostatin” (Kramer et al., 1991). Moreover, the YXFGLamide of M. sexta is not allatostatic in M. sexta (P.E.A. Teal, unpublished data). In response to a similar indication that YXFGLamides of the blowfly Calliphora vomitoria are not allatostatic, these peptides have been named “callatostatins” (Duve et al., 1993), and this precedence has been followed by (Veeelaert et al., 1997a, 1997b) in the use of “schistostatin” for naming the YXFGLamides of Schistocerca gregaria. This generic-based method of naming YXFGLamides avoids the implication that these peptides are allatostatic, but given the very large number of insect genera and the probability that identical YXFGLamides will be found even in unrelated genera, the list of names named by this method may eventually prove to be very extensive and full of synonyms. This probability is illustrated by what is known of the peptides of adipokinetic hormone family: so far, the primary structures of approximately 31 different peptides have been reported, these occur in one or more of approximately 60 genera (Gåde, 1996), and the numbers are still growing.

Perhaps a suitable alternative to the genera-based naming of the YXFGLamides would be to use a prefix referring to the insect order in which the respective "statin" (YXFGLamide) has been reported and to follow the ordinal base name with the numerical order of the identification of the peptides in each order. Such a system would limit the list of names applied to the YXFGLamides to a comprehensible size and minimize the synonymy.
ACKNOWLEDGMENTS
We thank Wallace Clark for the sequence analysis and Thomas McClure for the mass spectrometry.

LITERATURE CITED
Adams, M.E., and M.O'Shea (1983) Peptide cotransmitter at a neuromuscular
Agricola, H., K. Schildberger, A. Schmidt, W. Naumann, S. Reissmann, F.
Huber, and H. Penzlin (1992) Immunochemical distribution of
allatostatin in the nervous system of the cockroach Periplaneta americana.
In N. Elsner and D.W. Richter (eds): Rythmogenesis in Neurons and
Arbiser, Z.K., and B.S. Beltz (1991) SCP B- and FMRFamide-immunoreactivi-
ties in lobster motor neurons: Colocalization of distinct peptides or
c labeling of the same peptide(s)? J. Comp. Neurol. 306:417–424.
innervation of locust mandibular and course muscle modulates contractions
through the elevation of inositol triphosphate. J. Comp. Neurol. 297:479–
494.
Am. 69:365–373.
Bellé, X., J.L. Maestro, M.D. Piulachs, A.H. Johnson, H. Duve, and A.
Thorpe (1994) Allatostatic neuropeptides from the cockroach Blattella
germanica (Dictyoptera, Blattellidae). Identification, immunolocal-
major forms of rat pituitary corticotropin using only reversed-phase
Buna, P., and E.W. Rousbas (1986) Ultrastructural demonstration of
non-synaptic release sites in the central nervous system of the snail
Lymnaea stagnalis, the insect Periplaneta americana and the rat.
Neuroscience 17:867–878.
Buys, C.M., and D. Gibbs (1981) The anatomy of neurons projecting to the
Casaday, G.B., and J.M. Camhi (1976) Metamorphosis of flight motor
neurons in the moth Manduca sexta. J. Comp. Physiol. 112:143–158.
Chen, Y., J.A. Veenstra, N.T. Davis, and H.H. Hagedorn (1994a) A compara-
Chen, Y., J.A. Veenstra, H. Hagedorn, and N.T. Davis (1994b) Leucokinin
and diuretic hormone immunoreactive neurons in the tobacco
Chen, Y., H. Hagedorn, J.D. Davis, and H.H. Hagedorn (1994a) A compara-
Chen, Y., J.A. Veenstra, C. A. Miller, H.Kataoka, G.B. Quistad, J.P. Li, R.L.
allatostatins from the cricket, Gryllus bimacularus de Geer (Ensifera,
Gryllidae); Additional members of a family of neuropeptides inhibiting


