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Endothelin-like Immunoreactivity in Midgut Endocrine Cells of the Desert Locust, *Locusta migratoria*

LUIS M. MONTUENGA,* M. A. PRADO,* D. R. SPRINGALL,† J. M. POLAK,† AND P. SESMA*

**Department of Cytology and Histology, University of Navarra, Pamplona, Spain; and †Department of Histochemistry, Royal Postgraduate Medical School, London, United Kingdom*

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Endothelin-1-like immunoreactivity has been found in endocrine cells of the midgut of the desert locust *Locusta migratoria*. Several antisera have been directed against the whole molecule and its C-terminal sequence. Endothelin-1-immunoreactive cells are present in the main region of the midgut (ventriculus) and in the midgut caeca but not in the ampullae through which the malpighian tubules drain. Endothelin-1-like immunoreactivity colocalizes with FMRFa immunoreactivity in the cells of the main region of the midgut but not in those in the midgut caeca. Endothelin-1-immunoreactive cells are present not only in adults but also throughout the five instars of posthatching development. © 1994 Academic Press, Inc.

Endothelin (ET) is a 21-amino-acid peptide isolated and characterized from the culture supernatant of porcine aortic endothelial cells (Yanagisawa *et al.*, 1988). The reported biological actions of ET are widespread (for review see Yanagisawa and Masaki, 1989; Simonson and Dunn, 1990; Rubanyi, 1992) but its predominant role in mammals seems to be vasoconstriction. ET is one of the most potent long-lasting vasoconstrictor/pressor substances so far reported. The vasocontractile properties of ET have been shown on isolated mammalian blood vessel preparations (De Nucci *et al.*, 1988) and *in vivo* at the regional and systemic levels (Miller *et al.*, 1989). ET also induces contraction of nonvascular smooth musculature such as that of the gut wall (De Nucci *et al.*, 1988; Wollberg *et al.*, 1991; Bolger *et al.*, 1992)., Three different mammalian endothelin-related genes have been cloned that correspond to three different endothelins (ET-1, ET-2, and ET-3) that possess similar but not identical 21-residue peptides (Inoue *et al.*, 1989), each produced as a precursor molecule. ET-1 (the originally isolated endothelin), ET-2, and ET-3 were subsequently found in a variety of mammalian tissues (Matsumoto *et al.*, 1989).

In endothelial cells preproET is processed like many other peptide hormones and neuropeptides (Yanagisawa *et al.*, 1988, Itoh *et al.*, 1988). The mature form of ET is generated through the cleavage of a putative intermediate 39-amino-acid precursor called "big-endothelin" (bigET), which is about 140 times less potent than the mature 21-amino-acid peptide in its vasoconstrictor activity (Yanagisawa and Masaki, 1989).

Immunohistochemical evidence of the occurrence of ETs in the endothelial cells was shown initially by Hiroe *et al.*, (1989). Since then, further reports have demonstrated that ET is also expressed in a variety of mammalian cells, including brain and spinal cord neurons (Giaid *et al.*, 1989), bronchial epithelial cells (MacCumber *et al.*, 1989; Rozengurt *et al.*, 1990; Springall *et al.*, 1991), and macrophages (Ehrenreich *et al.*, 1990). There have also been reports of endothelin-like immunoreactivity in endocrine cells, for example, in the human

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pituitary (Naruse *et al.*, 1992), in rat and bovine parathyroid cells (Fujii *et al.*, 1991), and in the cells of the diffuse endocrine system in the human developing lung and gut (Giaid *et al.*, 1990; Gibson *et al.*, 1992; Escrig *et al.*, 1992).

The diffuse endocrine system of the gut of insects is a local regulatory system consisting of several types of endocrine cells spread among the enterocytes. The study of the gut endocrine cells is of particular importance for understanding the regulation of the digestive processes. These endocrine cells, being of a receptosecretory nature, might be involved in the response to the variations of diet composition in terms of control of gut motility and secretion (Simpson and Simpson, 1990), and some are known to react with antibodies directed against mammalian regulatory peptides (Sehnal and Zitnan, 1990; Zitnan *et al.*, 1993). Many of the peptides found in these gut endocrine cells are also present in neurons of the brain or of the segmental ganglia. One of the most investigated peptides in insects is the neuropeptide FMRFamide (FMRFa) which, together with related peptides, is present in both endocrine cells and neurons (Brown and Lea, 1988; Jenkins *et al.*, 1989). In the locust, FMRFa-like peptides have been partially characterized and some of their possible biological roles have already been assessed (Robb and Evans, 1990). The aim of the present investigation was to determine immunohistochemically whether ET-related molecules are present in the endocrine cells of the midgut of the adult and developing desert locust. The possible colocalization of the ET with FMRFa in these endocrine cells has also been studied.

MATERIAL AND METHODS

In the present study 3 specimens of each posthatching instar and 12 adult locusts of both sexes were used. The locusts were reared according to Barras (1964) at 28° with a 18:6 hr photoperiod and fed with grass and bran. To prevent amoebic infections trimetoprime was added to the food. The posthatching development in *Locusta migratoria* occurs in five instars, previous to the adult stage. Specimens belonging to the different developmental instars can be readily distinguished by observing the anatomy of the alar rudiments, the terminal abdominal segments, and the external genitalia (Uvarov, 1966).

Animals were killed by decapitation and dissected under a Ringer solution isotonic to locust (*Schistocerca gregaria*) hemolymph (Mordue, 1969). The whole gut was extracted, with the attached Malpighian tubules. The specimens were fixed in Bouin's fluid for 24 hr and embedded in paraffin.

Immunocytochemistry. Paraffin sections (4–6 μm) were treated with the avidin–biotin complexes (ABC) technique according to Hsu *et al.* (1981). After deparaffination with xylol, endogenous peroxidase was blocked by a treatment with 3% H_2O_2 in methanol. Sections were hydrated through alcohols and then placed in Tris–HCl buffer saline (TBS; 0.05 *M* Tris Buffer, pH 7.4, 0.5 *M* NaCl). Nonspecific binding sites were blocked with 5% swine immunoglobulins in TBS. Sections were incubated overnight at 4° with primary rabbit antiserum (see Table 1). After rinsing in TBS (5 min) the sections were incubated for 30 min at room temperature with biotinylated swine serum directed against rabbit immunoglobulins (Dakopatts, Glostrup, Denmark) diluted 1:200 in TBS. Following a second rinse in TBS, the sections were treated for 30 min at room temperature with avidin–biotin peroxidase complexes (Dakopatts) diluted 1:100 in TBS and prepared 30 min in advance. After additional washes, peroxidase was demonstrated by the diaminobenzidine/H_2O_2 method (Sigma Chemical Co., St Louis, MO). Sections were washed with distilled water, lightly counterstained with haematoxylin, dehydrated, and mounted in DPX.

Antisera. Table 1 summarizes the main data concerning the antisera used. The polyclonal antisera

TABLE 1
SURVEY OF THE ANTISERA USED

Antiserum	Dilution	Source and ref no.
FMRFa	1:3000	Peninsula 61009
ET-1 (human/porcine)	1:3000	RPMS[a] 1946 (CRB 58)
ET-1 (human/porcine)	1:1000	RPMS 1888 (CRB 45)
ET-1 (human/porcine)	1:1000	RPMS 1914 (CRB 42)
ET-1 (15–21)	1:2000	RPMS 2016 (Kimura anti-ETc)
ET-1 (15–21)	1:2000	RPMS 2085 (Kimura anti-ETc)

[a] Royal Postgraduate Medical School.

were raised in New Zealand white rabbits against synthetic whole ET-1 and ET-1 (15–21) (Giaid *et al.*, 1989 Yoshizawa *et al.*, 1990, respectively). Specificity of the immunostaining obtained with these antisera was tested by liquid-phase absorption test (10 nmol antigen per milliliter of optimally diluted antiserum, 16 hr) with synthetic whole ET-1 or bigET-1 (Peninsula Labs, Merseyside, UK). The immunostaining with the several antisera against ET-1 was always quenched by the ET-1 molecule. Cross-reactivity of the FMRFa antiserum with ET-1-related molecules and of the ET-1-related antisera with FMRFa (Peninsula Labs) was ruled out by means of crossed absorption test. Neither ET-1-like nor FMRFa immunoreactivity was absorbed when bigET-1 peptide was used. As controls for method specificity some sections were incubated with nonimmune rabbit serum instead of the primary antisera. Furthermore, when applicable, one of the stages of the avidin–biotin peroxidase complex procedure was omitted.

Quantification. The quantification studies were performed using the anti-ET-1 antiserum 2085. Four individuals of each of the posthatching stages were used for quantification. Four nonserial sections of the gut of each specimen were counted. To avoid cells appearing in multiple sections, nonserial sections (1 every 10 serial, each one being about 5 μm thick) were used for this part of the study. All the immunoreactive cells present in a totally longitudinal section of the midgut were counted. Only the cells with a visible nucleus were considered. The number of cells counted was related either to the total length (in mm) of the midgut, from the crop/midgut transition to the pyloric valve, or to the total length of the section of the caecum. Differences in cell density between gut areas and between instar groups were assessed by ANOVA and Scheffe F tests.

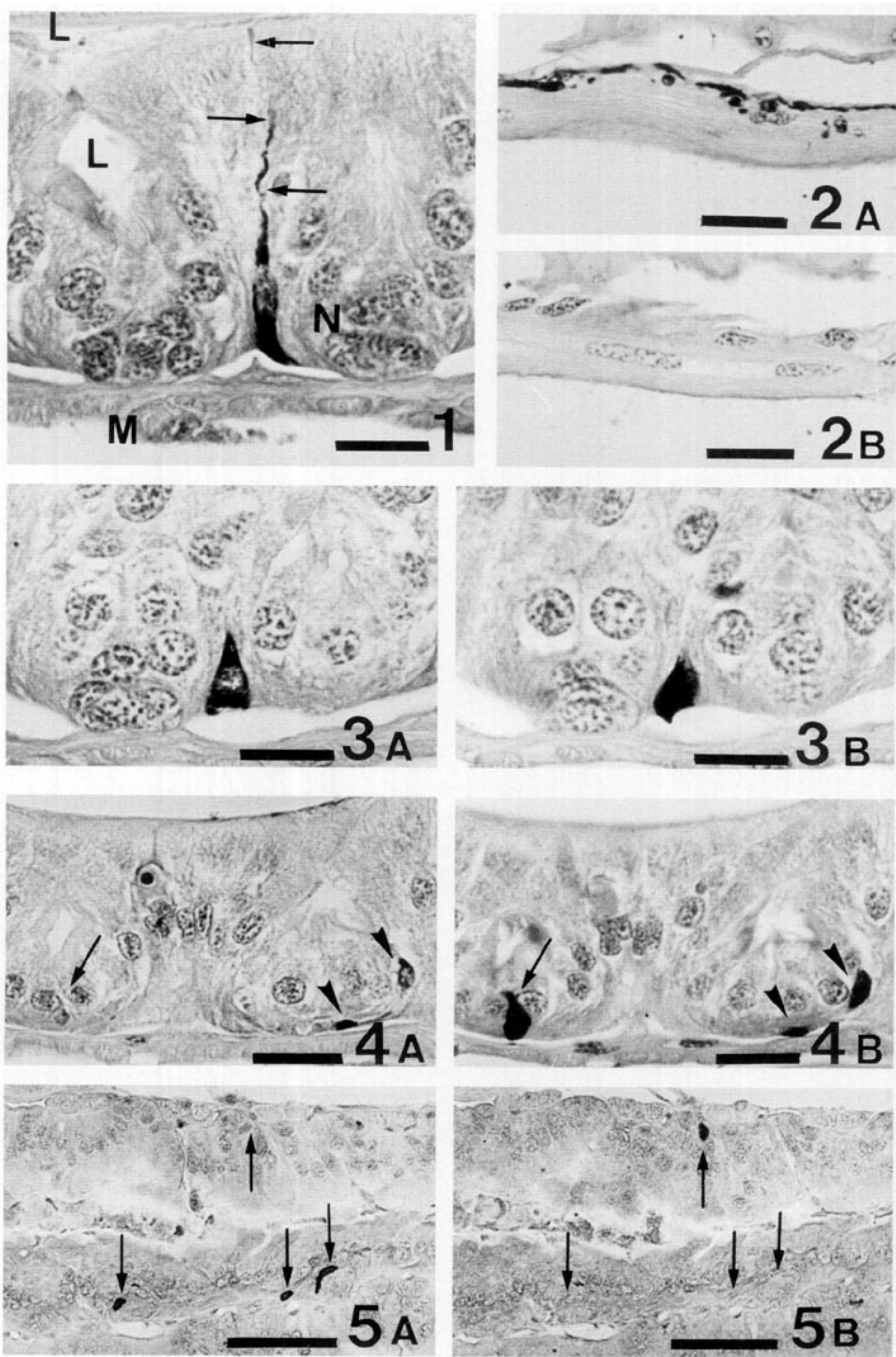
RESULTS

Immunoreactive endocrine cells were present in the midgut of all the specimens studied and were positive for the antibodies raised against FMRFa, ET-1 (1–21), and ET-1 (16–21) (see Table 1)(Figs. 1–9). ET-1-like immunoreactivity was clearly evident in cells throughout the whole midgut and caeca. In the ampullae through which the malpighian tubules drain many FMRFa-immunoreactive cells were present, but no ET-1-like immunoreactivity was observed. The ET-1-like immunoreactive cells were frequently found in the vicinity of the nidi of regenerative cells that are spread in the epithelial lining (Figs. 1, 3, and 4) and were also found in between the more differentiated cells of the epithelium. With the antisera used, no ET-1-like immunoreactivity was found in the nerve fibers that innervate the muscle layer of the midgut, while FMRFa immunoreactivity was clearly detectable in many of those fibers (Fig. 2).

ET-1-like immunoreactive cells were elongated or bottle-shaped and usually spanned the whole width of the midgut epithelium from the basal lamina to the lumen (Fig. 1). The cells reached the lumen by means of a thin cytoplasmic process. The total cell size and the size of the nucleus were considerably smaller than those of the neighboring enterocytes. The immunoreactive material was preferentially located in the basal regions of the cell below the nucleus, but was also usually present in the slender apical process that runs toward the lumen.

Colocalization studies were carried out with antibodies 2016 and 1946, respectively directed against ET-1(16–21) and the whole ET-1 molecule, and the antibody against FMRFa. Immunoreactivity with all the ET-1 antisera was detected in the same cells. Using serial reversed-face sections, ET-like immunoreactivity was often found to be colocalized with FMRFa-like immunoreactivity (Fig. 3). In the main segment of the midgut (ventriculus) all the ET-immunoreactive cells were also FMRFa-immunoreactive, although not all the FMRFa-immunoreactive cells present in this region seemed to store ET-like material (Fig. 4). In the midgut caeca there was no apparent correlation between ET-like and FMRFa-immunoreactive cells (Fig. 5). None of the FMRFa-immunoreactive cells that accumulate in the ampullae through which the malpighian tubules drain was labeled by any of the anti-ET-1 antibodies used.

ET-like immunoreactive cells were found in the main region and the caeca of the midgut throughout all the five instars of locust posthatching development (Fig. 10). In the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first four instars, the number of ET-like immunoreactive cells was small, frequently not more than 1–2 cells per longitudinal section of the whole gut. The numbers of ET-like immunoreactive cells per unit length of the main region of the midgut increased significantly from the 5th instar locust (0.30 ± 0.06; mean ± SEM; $n = 16$) to the adult (1.29 ± 0.05) ($P < 0.01$). In the same stages the density of FMRFa-immunoreactive cells also decreased significantly from 4.16 ± 0.22 cells/mm of midgut to 1.58 ± 0.07 cells/mm ($P < 0.01$). Similar results were found in the midgut caeca.

Cross-absorption tests (Table 2) did not reveal any cross-reactivity between the antisera against ET-1 and FMRFa and the alternative peptide. In both the main region of the midgut and the midgut caeca, immunoreactivity was not abolished when the anti-ET-1 antibodies were preabsorbed with the tetrapeptide FMRFa (Figs. 6 and 7) or when the anti-FMRFa antiserum was absorbed with ET-1 (Fig. 8). In the same tissue, staining was abolished by preabsorption with ET-1 of antisera 2016 and 2085 against ET-1 (16–21), and 1888, 1946, and 1914 against ET-1 (Figs. 9a and 9b).

DISCUSSION

In the present work ET-1-like immunoreactivity is shown in endocrine cells of the midgut of the desert locust. The colocalization of ET-1 in a subpopulation of FMRFa-immunoreactive endocrine cells is also demonstrated.

The use of antisera against vertebrate regulatory peptides has yielded a large number of findings both in the nervous system and in the endocrine cells of invertebrates. The interpretative problems associated with comparative immunohistochemical studies when antibodies directed against molecules from a different species are used have often been stressed and have led to the practice of reporting the positive findings such as peptide-like immunoreactivity. Nevertheless, the structural homology between the amino acid sequences of many vertebrate peptide hormones and the peptides isolated from invertebrate tissues (such as the "peptide families" of insulin, FMRFa, the adipokinetic hormone, proopiomelanocortin, the tachykinins, or gastrin/CCK; reviewed by De Loof *et al.*, 1990) justifies interest in this kind of immunocytochemical study. The number of vertebrate-related peptides which might have regulatory functions in insects seems to be very high; according to our findings, a molecule structurally related to the newly characterized vertebrate peptide endothelin should be added to this long list.

The antibodies used in the present study have been raised against either the whole mammalian ET-1 molecule or its C-terminal fragment. Our immunocytochemical find-

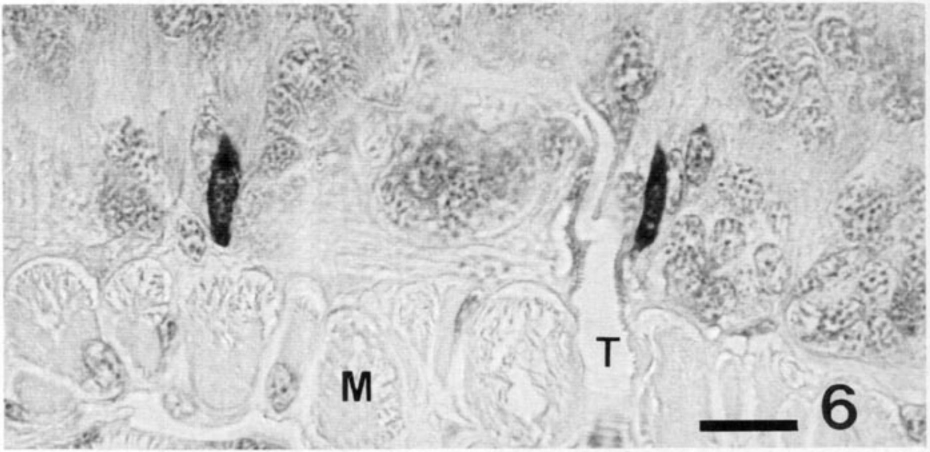
FIG. 1. ET-immunoreactive endocrine cell located in the basal region of the midgut epithelium. A fine cytoplasmic process (arrows) makes contact with the lumen (L). N, nidus of regenerative cells. M, Muscle cells. Paraffin section. ABC method. Bar; 20 μm. ×650.

FIG. 2. Serial reversed-face sections of the muscle layer of the midgut, immunostained for FMRFa (a) and ET (b). The nerve fibers that innervate the muscle layer are stained only when antibodies against FMRFa are used. Bars, 30 —m. ×400.

FIG. 3. Serial reversed-face sections of the same endocrine cell, immunoreactive for ET (a) and FMRFa (b). Bars, 20 μm. ×650.

FIG. 4. Serial reversed-face sections of the main region of the midgut immunostained for ET (a) and FMRFa (b), showing colocalization of both immunoreactivities in two cells (arrowheads). This colocalization does not occur in the FMRFa-immunoreactive cell pointed by the arrow. Bars, 30 μm. ×400.

FIG. 5. Serial reversed-face sections of the midgut caeca, immunostained for ET (a) and FMRFa (b). No colocalization of these immunoreactivities has been observed in endocrine cells of the midgut caeca (arrows). Bars, 100 μm. ×150.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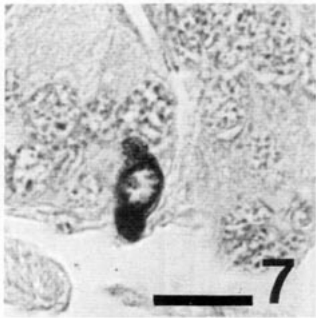
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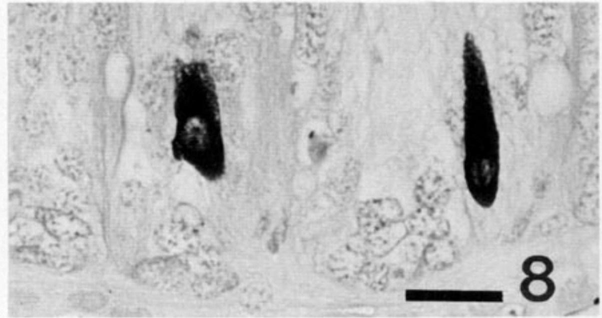
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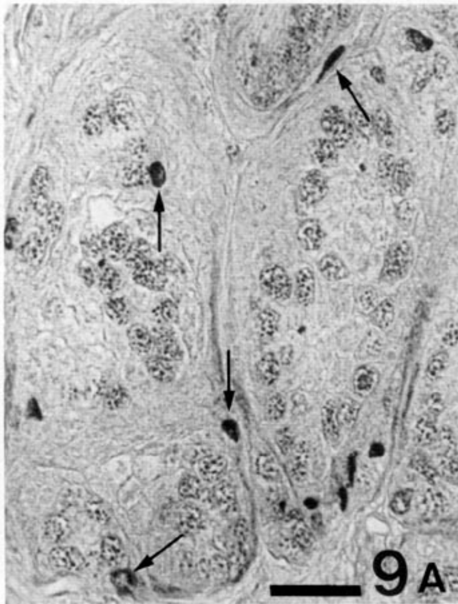
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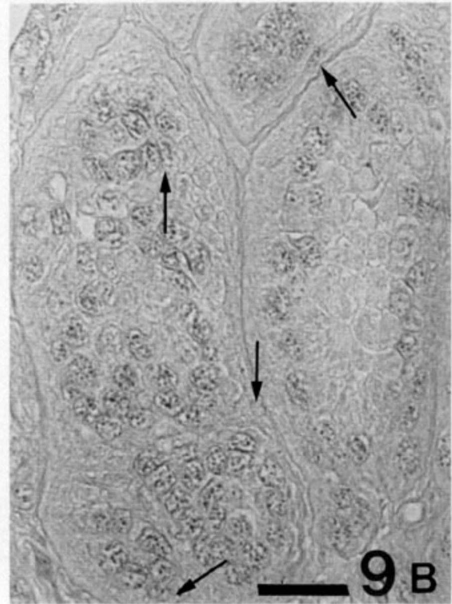
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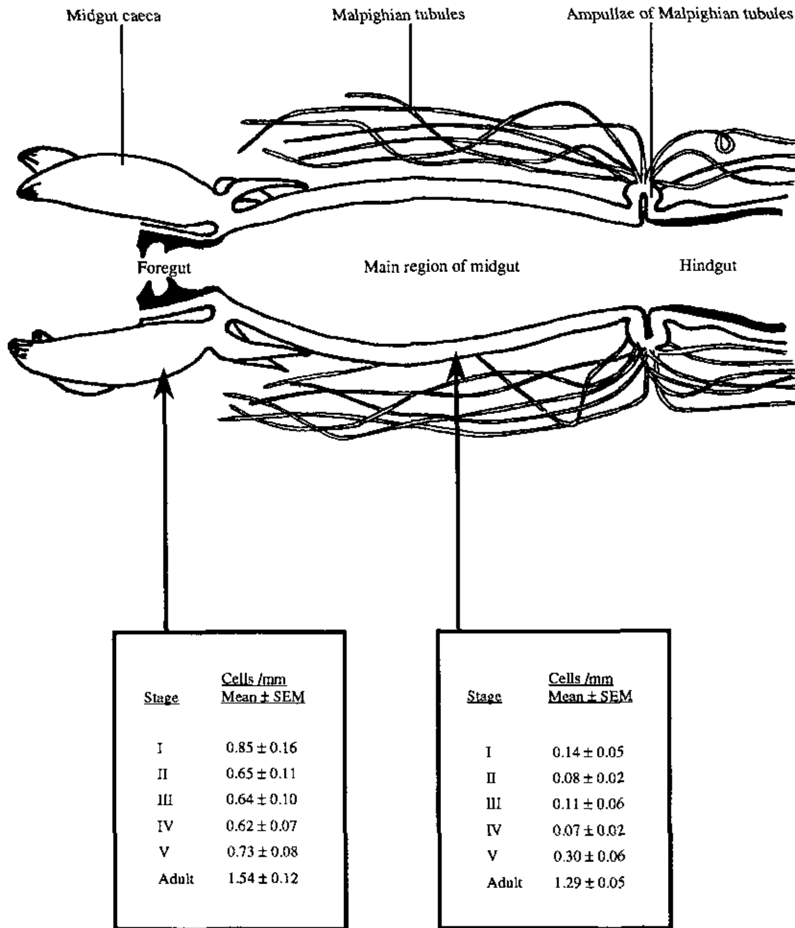

FIG. 10. Distribution of ET immunoreactive cells in the midgut of *Locusta migratoria* throughout the posthatching developmental stages and in adults. SEM values for the first stages are relatively high due to the numerous zero values (no ET-immunoreactive cell was found in the section).

ings suggest the presence of either ET-1 or at least an antigenically related molecule in a group of endocrine cells of the insect midgut. The positive results obtained in this study using several antibodies against the whole molecule and its C-terminal fragment support the hypothesis of the immunoreactive substance present in these endocrine cells being a truly ET-related molecule or at least a peptide including a sequence very similar to the C-terminal (15–21) amino acids of ET-1.

FIG. 6. Epithelium of the wall of the main region of the midgut immunostained with antibodies against ET absorbed with FMRFa. T, trachea. M, muscle cells. Bar, 20 μm. ×650.

FIG. 7. Epithelium of the wall of a midgut caecum immunostained with antibodies against ET absorbed with FMRFa. Bar, 20 μm. ×650.

FIG. 8. Epithelial layer of the wall of the main region of the midgut, immunostained with antibodies against FMRFa absorbed with ET. Bar, 20 μm. ×650.

FIG. 9. Serial sections of the wall of the main region of the midgut, immunostained with antibodies against ET (a) and with the same antiserum absorbed with ET (b). The immunoreactivity in endocrine cells was abolished (arrows). Bars, 30 μm. ×400.

TABLE 2
CROSS-ABSORPTION CONTROLS

Peptide	Antisera	
	ET-1	FMRFa
ET-1	–	+
bigET-1	+	+
FMRFa	+	–

Note. (+) Positive immunoreactivity; (–) negative immunoreactivity.

At present, little is known concerning the evolutionary origins of the endothelins (Landan *et al.*, 1991). In 1989, Kloog *et al.* reported striking similarities in sequence and bioactivity between ET and a small toxic peptide (sarafotoxin) isolated from the venom from the burrowing asp, *Atractaspis engaddensis,* and concluded that endothelins and sarafotoxins are members of the same family of peptides. In nonmammalian vertebrates, immunoreactive ET has been detected in the plasma of several species using radioimmunoassay for ET-1 (Udemura *et al.*, 1991). Concerning the invertebrates, there are also some isolated immunocytochemical studies that report immunoreactive endothelin-like substances being found in some central or peripheral neurons of two species of annelids, four species of mollusc, and one species of insect (Kasuya *et al.*, 1990, 1991; Hasegawa and Kobayashi, 1991; Giaid *et al.*, 1991). In the annelid worm *Neanthes diversicolor* ET-like immunoreactivity was found in several nervous elements, including those innervating the digestive tract (Kasuya *et al.*, 1990). In the species of molluscs studied and in the cricket, the ganglia involved in the visceral innervation contained ET-immunoreactive neurons (Kasuya *et al.*, 1990, 1991; Hasegawa and Kobayashi, 1991; Giaid *et al.*, 1991). In our study, only endocrine cells, and not nerve fibers, were immunostained with the anti ET-1 antibodies, while FMRFa-immunoreactive fibers could be detected. No report has been published so far of endothelin-like immunoreactivity in endocrine cells either in nonmammalian vertebrates or in invertebrates.

The presence of an ET-1-like peptide in the diffuse neuroendocrine system of the gut of invertebrates can be related to the already known functional involvement of endothelin in the mammalian gut neuroendocrine system. The immunocytochemical demonstration of ET-1 in the mammalian gut has already been reported (Inagaki *et al.*, 1991; Escrig *et al.*, 1992). ET-1 is the major isoform of endothelin in the adult and fetal human gut along with its precursor form bigET-1. ET-1-like immunoreactivity was found in the gastrointestinal tracts of both adult and fetal gut. Immunocytochemically it was localized to ganglion cells of the gut nervous plexi. Endocrine cells were immunostained for bigET-1 only in some phases of development (Escrig *et al.*, 1992). The possible functional role of endothelin and its precursor in the mammalian gut has been a matter of discussion. Specific binding sites for ET-1 have been demonstrated using *in vitro* autoradiography in the neural plexi and the mucosa of the human intestine, suggesting a role in the modulation of intestinal motility and secretion (Inagaki *et al.*, 1991). The involvement of endothelin in the regulation of the contraction of gastrointestinal smooth muscle has been shown by several groups (De Nucci *et al.*, 1988; Takahashi *et al.*, 1990; Wollberg *et al.*, 1991; Bolger *et al.*, 1992). Endothelin seems to stimulate ion secretion in stripped intestinal mucosa in a concentration-dependent manner (Brown and Smith, 1991; Roden *et al.*, 1992).

The endocrine cells of the gut of many insects are immunoreactive for FMRFa. FMRFa-like peptides belong to a growing interphyletic family of peptides originally isolated from molluscs (Price *et al.*, 1987). FMRFa and related peptides have potent modulatory actions on locust skeletal and visceral muscle and seem to function both as circulating neurohormones and as locally released neuromodulators and neurotrans-

mitters (Robb and Evans, 1990). The colocalization of ET and FMRFa supports a possible modulatory interaction between both peptides.

The results of our study lead to the conclusion that there are at least three different endocrine cell types in relation to their immunoreactivity to the antisera used against ET-1 and FMRFa. In the main region of the midgut, ET-1 immunoreactive cells are also immunoreactive with anti-FMRFa. Conversely, in the midgut caeca, the ET-1 immunoreactive cells are not FMRFa-immunoreactive. Finally, in all the three regions of the midgut (caeca, main region, and ampullae of the malpighian tubules) there are FMRFa-immunoreactive cells that are not stained by ET-1 antisera. The interpretation of the different response of FMRFa-immunoreactive cells to ET-1 antisera has to take into account that the antibodies directed against FMRFa most probably recognize a heterogeneous population of endocrine cells. It is known that the anti-FMRFa antisera are able to react with a variety of different peptides belonging to the same family containing an identical or similar C-terminal sequence. In insects, several neuropeptides belonging to this family have already been isolated (Holman *et al.*, 1986; Nambu *et al.*, 1988; Schneider and Taghert, 1988) mainly from the brain but also from the abdominal ganglia (Duve *et al.*, 1992).

Escrig *et al.* (1992) suggested that the presence of endothelin-related immunoreactivity in the endocrine cells of the mammalian gut mucosa during development may be related to the reported role of endothelins on the stimulation of cell division and on growth (Brown and Littlewood, 1989; Bobik *et al.*, 1990). Our quantitative study of the distribution of cells throughout the development of the posthatching locust shows that in the adults there is an increased number of ET-1-like immunoreactive cells per area of midgut tissue sectioned compared to the rest of the developing instars. Thus, in the case of the locust, the immunoreactivity for ET-1 is sparse in the phases of development in which the growth in size of the gut is taking place. There are several possibilities to explain the scarcity of ET-1-like immunoreactivity in the first instars. It could be due either to a low number of ET-1-like producing cells or to a nondetectable amount of ET-1 being produced, or to an increase in the release of the peptide. In the same way, the higher proportion of cells in the adults could be due either to an increased rate of differentiation of the ET-1-like cells from the regenerative nidi from where they originate (Endo *et al.*, 1983; Montuenga *et al.*, 1989), or alternatively to an increased storage of the peptide that renders more cells detectable with our immunocytochemical methods. The comparison of the quantitative data of the ET-like immunoreactive cell population with those of the FMRFa population gives some help in the clarification of the questions posed above. Our data show that, while the population of detectable FMRFa cells seems to be decreased when entering the adult stage, there is an apparent increase in the number of ET-immunoreactive cells, making the hypothesis of increased storage of the ET-1-like material more likely than that of an increased rate of endocrine cell differentiation. Moreover, the very few data available on the kinetics of the adult insect midgut point toward a slow mechanism of differentiation from the nidi as the way of appearance of new endocrine cells. In the gut of the adult cockroach, it takes between 7 and 14 days from the end of cell division for a precursor cell to acquire PP-like immunoreactivity (Endo *et al.*, 1983). If the rate of differentiation of ET-1-like immunoreactive cells from the nidi in the developing locust is similar to that measured for the PP-like cells of adult cockroaches, the sharp rise of endothelin-1-like immunoreactivity shown in our study could very unlikely be due to a process of differentiation of new cells. Fur-

ther experiments are required to correlate cell proliferation with the pattern of immunoreactivity and, eventually, to define whether the rise in ET-1 storage suggested for the adult stage is produced either by a lack of release or by an increased synthesis of the peptide.

Until the immunoreactive materials present in the midgut endocrine cells are fully characterized, it is too speculative to assess the physiological role of the endogenous endothelin-like peptides in *L. migratoria* and in other insect species. The precise functions of these peptides in the endocrine cells of the insect midgut remain to be elucidated.

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