Cells immunoreactive to an antisem specifically directed against vertebrate α-neo-endorphin (α-NE) were detected in the internal wall of anterior and posterior suckers of the rhynchobdellid leech Theromyzon tessulatum. These cells have morphological and ultrastructural characteristics close to the “releasing gland cells” of adhesive organs. The epitope recognized by anti-α-NE was contained in granules having a diameter of 0.2-0.3 µm. Previous works involving the brain of this leech demonstrate the existence of ~14 neurons immunoreactive to the anti-α-NE. Following an extensive purification including high pressure gel permeation and reversed-phase high performance liquid chromatography, epitopes contained in both suckers and central nervous system were isolated. Purity of the isolated peptides was controlled by capillary electrophoresis. Their sequences were determined by a combination of automated Edman degradation, electrospray mass spectrometry measurement, and coelution experiments in reversed-phase high performance liquid chromatography with synthetic α-NE. The results demonstrate that epitopes recognized by the anti-α-NE in the suckers and the central nervous system are identical to vertebrate α-NE (YGGFLRKYPK). This finding constitutes the first biochemical characterization of a prodynorphin-derived peptide in invertebrates. Moreover the isolation of this peptide in the annelida establishes the very ancient phylogenetic origin of α-NE as well as its conservation in evolution.

In vertebrates, all known opioids are cleavage products of three different precursors, i.e. proopiomelanocortin (POMC),1 proenkephalin, and prodynorphin (1). Processing of the prodynorphin yields a number of bioactive peptides including leucine-enkephalin, neo-endorphins (A and B) (Patey and Rossier, 1986). Among these peptides, α-neo-endorphin (α-NE) has been isolated from all vertebrates phyla (Sei et al., 1989; Goldsmith, 1992). The α-NE isolated from different tetrapods revealed by reversed-phase HPLC similar biochemical properties, reflecting the great conservation of this peptide in tetrapods (Sei et al., 1989, Goldsmith, 1992). NE immunocytochemical probes in cyclostomes, holostean and teleostean fish, proved negative (Dores and Gorbman, 1989; Dores et al., 1993b; Dores and McDonald, 1992). This result may be due to the following: 1) α-NE sequence changes that render this opioid undetectable to heterologous mammalian antisera, 2) a unique set of posttranslational processing reactions in which α-NE is not liberated from fragments of the prodynorphin precursor, or 3) the possibility that during evolution the prodynorphin precursor may be absent in fishes (see Dores et al. (1993b)).

If these three genes encoding opioid peptides are related (Gubler, 1987), one of the central questions is in what order did these genes evolve from a hypothetical ancestral gene? In this context research on different opioid peptides in invertebrates is essential (Stefano, 1991).

For example, enkephalin peptides have been isolated in crustacean (Luschen et al., 1991), in mollusks (Leung and Stefano, 1984), and in annelids (Salzet et al., 1995a). Moreover, a peptide derived from POMC maturation in vertebrates, γ-MSH, has also been characterized in the leech Theromyzon tessulatum (Salzet et al., 1994).

Given this amount of information in invertebrates concerning opioid peptides, little is known about α-NE in these animals. This peptide was not detected by immunocytochemistry in the mollusk Lymnaea stagnalis (Boer and van Minnen, 1985). By contrast, immunoactive material was present in annelids i.e. polychaeta (Dhainaut-Courtois et al., 1986) and achaeta (Verger-Bocquet et al., 1987a, 1987b, 1988). In the leech T. tessulatum, a great number of cells immunoreactive to the antisem raised against α-NE were found in the proboscis (Verger-Bocquet et al., 1987a, 1988) and in the brain (6-14 neurons) (Verger-Bocquet et al., 1987a, 1987b).

The aim of the present study is to fully characterize the α-NE-like peptide(s) in the leech T. tessulatum. Here we report for the first time that such material is present in the central nervous system and in a novel type of cell in the suckers.

MATERIALS AND METHODS

Animals

Mature specimens of the rhynchobdellid leech T. tessulatum were reared under controlled laboratory conditions as described in detail by Malecha et al. (1989).

Dissections and Surgical Procedures

After anesthesia with chloretone, animals were pinned flat, ventral side up in leech saline solution (Mueller et al., 1981). Central nervous...
system (CNS: brain and nerve cord) and anterior and posterior suckers were excised. Except for the purpose of immunocytochemistry, in which case they were fixed, suckers and CNS were immediately frozen in liquid nitrogen and stored at −70 °C for storage.

Antiserum

Polyclonal antiserum anti-α-Neo-endorphin (anti-α-NE) was raised in rabbits and provided by Dr. G. Tramu (Laboratoire de Neurocystochimie fonctionnelle, Université de Bordeaux I, Talence, France). Anti-α-NE specificity was determined by radioimmunoassay as noted elsewhere (Fallon et al., 1990). No significant cross-reactivity was found with methionine-enkephalin, leucine-enkephalin, dynorphin 1–17 and dynorphin 1–8, or α or β endorphin.

Immunocytochemical Procedures

Light Microscopy

Anterior and posterior parts of T. tessulatum (including suckers) were fixed overnight at 4 °C in Bouin-Hollandé fixative (+10% HgCl₂ saturated solution). They were then embedded in paraffin and serially sectioned at 7 μm. After removal of paraffin with toluene, the sections were successively treated with the anti-α-NE diluted 1:200 and with goat anti-rabbit IgG conjugated to horseradish peroxidase as described elsewhere (Verger-Bocquet et al., 1992). The specificity of the antiserum was tested on consecutive sections mounted on different slides by preadsorbing the antiserum overnight at 4 °C with the homologous antigen (synthetic α-NE, Sigma) at a concentration of 500 μg/ml pure antiserum.

Electron Microscopy

Anterior and posterior suckers were fixed for 2 h at 4 °C in a mixture of 4% paraformaldehyde, 0.2% picric acid, and 0.1% glutaraldehyde in 0.1 M phosphate buffer. The tissue were post-fixed in 1% OsO₄ 2 h and acidified water (0.1% trifluoroacetic acid). Elution was performed with a discontinuous linear gradient of acetonitrile in acidified water over 0–15% for 10 min and over 15–45% for 30 min at a flow rate of 1 ml/min. The column effluent was monitored by absorbance at 226 nm and the presence of α-NE-like material detected as above.

Suckers

Suckers underwent same purification procedure, except an additional preparative step was added. The supernatant obtained after acidic extraction contained green and yellow pigments as well as mucus. They were removed early on the procedure by precipitation with water/acetone (20/80, v/v). The acetic fraction was reduced 20-fold by freeze-drying, fraction aliquots of 0.5 μl were tested by DIA.

TABLE I

| Step | Column material | Gradient of acetonitrile | Yield in brain |
|------|-----------------|--------------------------|----------------|
| 1. Prepurification | Waters Sep-Pak C₁₈ | 50 | 100 |
| 2. HPGPC | Sec2000, 7.5 × 300 mm | 30 | 78 |
| 3. Reversed-Phase HPLC | Vydac C₁₈, 4.8 × 250 mm | 0–15 (10 min) | 70 |
| 4. Reversed-Phase HPLC | Vydac C₁₈, 4.8 × 250 mm | 15–45 (30 min) | 69 |
| 5. Reversed-Phase HPLC | Vydac C₁₈, 4.8 × 250 mm | 15–45 (40 min) | 65 |
| 6. Final purification | ODS C₁₈, 2 × 250 mm | 0–60 (60 min) | 65 |

Immunosassays

Enzyme-linked immunosorbent assays (ELISA) and dot immunobinding assay (DIA) were conducted according to Salzet et al. (1993a) with anti-α-NE employed at a dilution of 1:1000. As a control, preadsorption of α-NE was carried out using homologous peptide. Prior to ELISA and DIA, anti-α-NE, at its working dilution was incubated overnight at 4 °C with synthetic α-NE (Sigma) (300 μg/ml undiluted anti-α-NE).

Purification

A four-step procedure was employed (Table I).
Fig. 1. Light microscopy of the suckers of T. tesselatum showing immunoreactive cells with anti-α-NE antibody (panels 1-5) and electron micrographs of α-NE-like cells (panels 6-8). Panel 1, general view of the anterior sucker showing the distribution of the α-NE-like cells. Scale bar, 200 μm. Panel 2, numerous α-NE-like cells detected in the posterior sucker. Scale bar, 33 μm. Panel 3, α-NE-like cells with long processes reaching the cuticle. Scale bar, 20 μm. Panels 4 and 5, immunoreactive cells with anti-α-NE antibody (4) in anterior sucker. The same destained cells are stained with hemalun picroindigo carmin (5). Each arrow shows the same cell in 4 and 5. Scale bar, 10 μm. Panels 6 and 8, immunogold technique. Note the presence of colloidal gold particles on granules in the cellular body (6) and its process (8). Scale bar, 0.5 μm. Panel 7, ending of the process with numerous electron dense granules. Scale bar, 0.5 μm; a.j., adherens junction; s.j., septate junctions.

Electrospray Mass Spectrometry

The purified peptide was dissolved in water/methanol (50/50, v/v) containing 1% acetic acid and analyzed on a VG Biotech BioQ mass spectrometer (Manchester, UK). Details of the method have been described elsewhere (Salzet et al., 1993a).

RESULTS

Immunocytochemical Investigations

In addition to the cells previously described in the proboscis (Verger-Bocquet et al., 1987a) and in the brain (Verger-Bocquet et al., 1987b, 1988) of T. tesselatum, numerous α-NE immunoreactive cells are observed in the epithelium of anterior and posterior suckers (Figs. 1, panels 1 and 2). In T. tesselatum, the α-NE-like material exhibited subepidermal bodies located among muscular and mucous gland cells. These small cells, 6–10 μm in diameter, possess a large nucleus (3–5 μm) for their size (Fig. 1, panels 4 and 5). They bear elongated processes that terminate immediately beneath the cuticle (Fig. 1, panel 3) and are abundant (>12,000/mm²). None of the processes had pores through the cuticle (Fig. 1, panel 7). At the ultrastructural level, numerous granules can be noted in the cell body and in the processes. These ovoid or somewhat crescent-shaped granules (0.2–0.3 μm) are characterized by a homogenous electron-dense material (Fig. 1, panel 7). After immunogold labeling, numerous colloidal gold particles are observed in these granules (Fig. 1, panels 6 and 8).

Biochemical Investigations

Isolation of the α-NE Substance—Central nervous systems (1000) or suckers (400) were subjected to a peptide extraction in 1 M acetic acid, pH 2. ELISA revealed crude extract 6.32 ± 1.8 pmol of α-NE-like material/CNS and 15.45 ± 4.6 pmol of α-NE-like material/sucker (after acetonitrile precipitation). Crude extracts were prepurified using Sep-Pak C₁₈ cartridges. The fraction eluted by 50% of acetonitrile was reduced 20-fold by freeze-drying and applied to a HPGPC column. Eluted fractions tested in DIA revealed a single immunoreactive zone in the two cases (CNS or suckers) corresponding to peptides with molecular mass of 1–4.5 kDa (data not shown). An amount of 5.45 ± 0.75 pmol of α-NE-like material/CNS (recovery of 81%) and of 13.44 ± 2.25 pmol of α-NE-like material/sucker (recovery of 82%) was obtained. Results obtained after preadsorption of the antiserum by synthetic α-NE established the specificity of the immunodetection. Each immunoreactive fraction was then concentrated 20-fold and applied to a reversed-phase HPLC.

In a first step of reversed-phase HPLC on a Vydac C₁₈ column, α-NE-like substances (suckers or CNS) eluted at a same retention time (RT) comprised between 21–22 (corresponding to 26–27% of acetonitrile) (Fig. 2). In these conditions the vertebrate α-NE eluted at a RT of 21.85 min. Total amount of α-NE-like material determined by ELISA at this step of purification was 4.85 ± 0.92 pmol of α-NE-like material/CNS (recovery of 80%) and of 12.63 ± 4.25 pmol of α-NE-like material/sucker (recovery of 81%). The immunoreactive zone containing this material (suckers or CNS) was analyzed on the
same column with a shallower gradient. A peak immunoreactive to anti-\(\alpha\)-NE, at a RT comprised between 25 and 25.3 min (corresponding to 26.25–26.48% of acetonitrile), was resolved in both cases. At this step of purification, quantification by ELISA indicated an amount of 4.15 ± 0.75 pmol of \(\alpha\)-NE-like material/CNS (recovery of ~66%) and of 10.25 ± 3.75 pmol of \(\alpha\)-NE-like material/sucker (recovery of ~66%). In both cases (suckers or CNS), a peak was then purified to homogeneity on an ODS C\(_{18}\) reversed-phase column and gave in each case a single peak at a RT of 28.3 min. Purity of the immunoreactive material (suckers, CNS) was established by capillary electrophoresis (Fig. 3). Quantification by ELISA at this step of purification indicated 3.75 ± 0.86 pmol of \(\alpha\)-NE-like material/CNS (recovery of ~60%) and of 9.86 ± 2.62 pmol of \(\alpha\)-NE-like material/sucker (recovery of ~64%).

In order to ensure that the \(\alpha\)-NE-like peptide purified either from CNS or from suckers were the same compound, a comparison on ODS C\(_{18}\) reversed-phase HPLC column of the two purified peptides was performed. A single peak was eluted at a RT of 28.3 min. Moreover, a comparison of the first derivatives of absorbance of the purified \(\alpha\)-NE-like peptide from CNS and from suckers at a same concentration revealed a total spectral overlapping between 190 and 300 nm and a ratio equal to 1, reflecting a very similar homology.

Characterization of the \(\alpha\)-NE-like Peptide—After the final purification step, a fraction aliquot of the immunoreactive material was evaluated by Edman degradation. The sequence, established on 139 pmol of purified \(\alpha\)-NE-like peptide with a sequencing yield of 95%, was Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys (Table II). The molecular mass of the CNS \(\alpha\)-NE-like peptide measured by electrospray mass spectrometry (m/z = 1228.04 ± 0.06 Da) is in agreement with the calculated isotopic mass (1228.68 Da) of the \(\alpha\)-NE (Fig. 4). The same result was obtained with the \(\alpha\)-NE-like peptide isolated from suckers (m/z = 1228.3 ± 0.35 versus 1228.68). Moreover, cation exchange on ODS C\(_{18}\) reversed-phase HPLC column of each purified peptides (CNS, suckers) and synthetic \(\alpha\)-NE revealed a single peak at a RT of 28.5 min. This procedure established that the primary structure of T. tessulatum \(\alpha\)-NE-like peptide is fully superposable on that of vertebrate \(\alpha\)-NE peptide. Its amount was estimated to 2.45 ± 1.15 pmol/CNS and 8.95 ± 2.15 pmol/sucker.

**DISCUSSION**

The present study demonstrates that to the peptide \(\alpha\)-NE isolated from the CNS and suckers of the leech T. tessulatum is structurally identical with the one identified in vertebrates (YGGFLRKYPK). The characterization of \(\alpha\)-NE in the leech constitutes the first report of the presence of a prodynorphin-derived peptide in an invertebrate. The same true \(\alpha\)-NE was isolated and identified both from CNS and suckers of mature T. tessulatum.

\(\alpha\)-Neoendorphin immunoreactivity is expressed by specific cells in CNS (Verger-Bocquet et al., 1987b, 1988), neuroendocrine cells of the forogut (Verger-Bocquet et al., 1987a), and by epithelial cells at the level of the suckers in this animal. Interestingly, this is not a novel observation in that in leeches certain monoclonal antibodies reacted with cells in the CNS and with epithelial cells (Hogg et al., 1983). Such an observation of a common localization among neurons and epithelial cells is very frequent in vertebrates.

The present study also shows that \(\alpha\)-NE immunoreactive cells in T. tessulatum suckers have morphological and ultrastructural characteristics close to the “releasing gland cells” of adhesive organs of the Branchiobdellids (Farnesi et al., 1981; Hogg et al., 1983; Gelder and Rowe, 1988; Weigl, 1994). These cells might constitute with the viscid gland cells a duo-gland adhesive organ (Tyler, 1976). In this regard, the sticky secretion of the viscid cells is used for substrate attachment and the secretion of the releasing cells may assist in detachment. However, we never found pores for these cells or releasing granules on the surface of the leech suckers. The same observation was made by Weigl (1994) in Branchiobdellids. The function of these releasing cells is currently not understood. Interestingly, they may be derived from nerve cells (Tyler, 1976; Gelder and Rowe, 1988), an observation that is based on similarities in structure and staining properties between them. In Branchiobdellids, the interpretation of Farnesi et al. (1981) is that these cells are neurons and the granules might be considered neurosecretory granules. Farnesi et al. (1981) suggest that the releasing cells are actually neurons with junctions on the viscid adhesive cell ducts to control the release of the viscid secretion granules. In T. tessulatum, the hypothesis of the nerve cell nature of these releasing cells is sustained by our observations showing that their intracytoplasmic granules (0.2–0.3 \(\mu\)m) contain \(\alpha\)-NE, identical to the one present in specific neurons. It is known that in leeches, neurons and epithelial cells are derived from the same blastomeres (Weisblat et al., 1980). In Hirudinea, Stewart et al. (1985) suggest that the epidermal cells recognized by monoclonal antibodies specific to the CNS are indeed peripheral neurons. Their study with the monoclonal antibody Lan 3–6 shows that during the embryonic development the same labeled epithelial cells possess an apical dendrite and a basal process; therefore, they are neurons. Stewart et al. (1985) suggest that the expression of the antigens in the axon, is either absent or below the level detectable with these technique in this cell. To answer this question anatomical experiences, e.g. anterograde transport, could be conducted. In T. tessulatum, as in Branchiobdellids (Weigl, 1994), continuity between these cells and necks of the viscid cells are scarce, which reinforces the improbable nervous control as proposed by

**Table II**

Automated Edman degradation of 139 pmol of the \(\alpha\)-neoendorphin-related peptide, with central nervous system of the leech T. tessulatum

| Cycle no. | PTH-Xaa | Yield (pmol) |
|----------|---------|-------------|
| 1        | Tyr     | 100.46      |
| 2        | Gly     | 121.24      |
| 3        | Gly     | 141.17      |
| 4        | Phe     | 124.6       |
| 5        | Leu     | 155         |
| 6        | Arg     | 43.95       |
| 7        | Lys     | 100.48      |
| 8        | Tyr     | 99.52       |
| 9        | Pro     | 63.64       |
| 10       | Lys     | 62.95       |
Farnesi et al. (1981). If the nerve nature of these \( \alpha \)-NE positive sucker cells is confirmed and considering their abundance, we could propose that they exert an important role either in the control of the adhesivity or in one of the multiple sensorial functions of the suckers. The existence of secretory granules suggests that \( \alpha \)-NE can be released in the intracellular spaces and acts via a paracrine mechanism. Although that relationship between Branchiobdellids and the other clitellates remains unclear (Holt, 1989; Brinkhurst and Gelder, 1989), the discovery in \textit{T. tessulatum} of cells resembling the releasing cells of Branchiobdellids argues in favor of a link between adhesive organs of Branchiobdellids and suckers of Hirudinea. Furthermore, in the CNS of the leech, the \( \alpha \)-NE secretion of neurons into the circulatory system at the neurohemal site suggests a hormonal role. Alternatively, an action as neurotransmitter or neuromodulator at the level of nerve endings located in the dorsal commissure or the neuropile is also possible, as in the presence of both mechanisms in these animals.

The fact that \( \alpha \)-NE is highly conserved in course of evolution from annelids to vertebrates suggests an essential function for this peptide. Actually we know that peptides conserved during evolution appear to keep an action on a same physiological function. This argument is sustained by two neuropeptides, acting on the osmoregulation, isolated in both \textit{T. tessulatum} and vertebrates, i.e. the lysine-conopressin (Salzet et al., 1993a) and the angiotensin II (Salzet et al., 1995b). In vertebrates, it is known that opioids are involved in variety of physiological functions and interact with endocrine system. The endogenous opioids peptides appear to have a role in the interaction between the CNS and the immune system (Scharrer, 1990; Scharrer et al., 1994). In invertebrates, until now, by contrast, nothing is known about the role of \( \alpha \)-NE. Consequently, the leech provides for a good model system in which to study this conserved peptide as well as the presence of a possibly conserved precursor, i.e. prodynorphin.

The existence of an ancestral proenkephalin gene is supported by the isolation of enkephalin peptides in invertebrate taxa, i.e. the crustacean \textit{Carcinus maenas} (Luschen et al., 1991), in the mollusk \textit{Mytilus edulis} (Leung and Stefano, 1984), and in the leech \textit{T. tessulatum} (Salzet et al., 1994). However, the ratio of Leu-enkephalin and Met-enkephalin in \textit{C. maenas} and \textit{T. tessulatum} is 3:1 and 2:1, respectively, whereas in vertebrates and \textit{M. edulis} Met-enkephalin is the major opioid peptide. Moreover, immunocytochemical studies performed at the level of \textit{T. tessulatum} brains revealed that Leu-enkephalin and Met-enkephalin are not detected in the same cells (Verger-Bocquet et al., 1987b). From these observations several hypothesis can be given. First, unlike in vertebrates, there are two separated genes, one coding for Met-enkephalin and the other for Leu-enkephalin. Second, the two pentapeptides come from a unique ancestral proenkephalin precursor and the expression of Met- or Leu-enkephalin is due to different posttranslation processing mechanisms; Leu-enkephalin is not expressed in cells expressing Met-enkephalin and vice versa. If we consider the first hypothesis, the question that is raised is: what can be the second opioid precursor, i.e. a prodynorphin or a POMC-like precursor?

Peptides yielded from POMC processing have been identified by immunocytochemistry and radioimmunoassay in invertebrates, i.e. ACTH and \( \beta \) endorphin in the mollusk \textit{Planorbarius corneus} (Franchesi and Ottaviani, 1992) and in insects (Duve and Thorpe, 1988), \( \beta \) endorphin in the flatworms (Reuter and...
peptides present in this multiple hormones precursor have different from POMC (Salzet et al., 1989) and MSH, and endorphins in annelids (Stefano, 1991). Recently, a peptide belonging to the POMC vertebrate family has been isolated in T. tessulatum brains. This peptide is related to the Vertebrate MSH (Franschis, C., and Ottaviani, E. (1992) J. Immunol. Res. 4, 53–55). It could also derive from a prodynorphin. Although epitopes of the different pro-dynorphin end-products are immunologically recognized in nervous system of different invertebrates, their chemical nature is unknown. However, in the leech T. tessulatum, α-NE and the dynorphin-like peptides are expressed in different neurons (Verger-Bocquet et al., 1987b, 1988). Only the characterization of the α-NE precursor would demonstrate precisely if a prodynorphin-like gene existed in annelids.

Our discovery of the α-NE confirms the very ancient stature of opioids in metazoans and the hypothesis emitted by Stefano et al. (1989) that the highly regulated vertebrate immune system probably had its origin in the invertebrates. These molecules would be used since the beginning of the evolution to start a type of integrated reply in order to maintained the body homeostasis (Franschis and Ottaviani, 1992) and notably in neuroimmunity reactions (Stefano, 1992), as well as documented neuroregulatory actions (Kream et al., 1980; Stefano, 1982).

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Purification, Sequence Analysis, and Cellular Localization of a Prodynorphin-derived Peptide Related to the α-Neo-endorphin in the Rhynchobdellid Leech Theromyzon tessulatum

Michel Salzet, Martine Verger-Bocquet, Philippe Bulet, Jean-Claude Beauvillain and Jean Malecha

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