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EVIDENCE FOR THE OCCURRENCE OF THE OPIOID OCTAPEPTIDE DYNORPHIN-(1-8) IN THE NEUROINTERMEDIATE PITUITARY OF RATS

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SUMMARY: Evidence is provided for the existence of the opioid peptide dynorphin-(1-8) in the neurointermediate pituitary of rats. The octapeptide was isolated by immunoadsorption to antibodies directed against porcine dynorphin-(1-13) followed by a variety of chromatographic separation procedures. The identity of the purified material with dynorphin-(1-8) was indicated by the following criteria: comigration with synthetic dynorphin-(1-8) on gelfiltration chromatography and high-performance liquid chromatography systems and liberation of a peptide with the same chromatographic behavior as leucine-enkephalin after sequential cleavage with trypsin and carboxypeptidase B.
Radioimmunological estimations revealed that dynorphin-(1-8) is a major dynorphin-related opioid peptide in the pituitary of rats.

Recently, several "big" leucine-enkephalins have been detected in brain and pituitary (1-6). An extraordinary potent leucine-enkephalin-containing opioid peptide, dynorphin, was isolated from porcine pituitaries and partially sequenced (dynorphin-(1-13)) (2). Subsequently, an opioid peptide, comprising the N-terminal eight residues of dynorphin (dynorphin-(1-8)) was isolated from porcine hypothalamus (3). With the use of antisera directed against dynorphin-(1-13), the highest concentration of immunoreactive (ir-) dynorphin in the rat was found in the neurointermediate pituitary and consisted of several different molecular weight forms (7-9). The structure of these different forms of ir-dynorphin, as well as the existence and distribution of all other "big" leucine-enkephalins in the rat, however, remained to be elucidated.

Abbreviations: HPLC - high-performance liquid chromatography
ir- - immunoreactive
k - kilodalton(s)
RIA - radioimmunoassay

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The low amount of tissue makes isolation and sequence analysis of "big" leucine-enkephalins from the rat virtually impossible. Thus, the present paper intends to provide at least high evidence for the existence of a "big" leucine-enkephalin, dynorphin-(1-8), within the rat neurointermediate pituitary.

METHODS AND MATERIALS

Extraction

Male Sprague-Dawley rats (200-220 g) were decapitated and their pituitaries divided in situ, into anterior- and neurointermediate lobes. 100 rat neurointermediate pituitaries (1 neurointermediate pituitary: 1.2 ± 0.2 mg, mean ± S.D., n = 10, wet weight) were incubated in 500 µl of 0.1 M HCL for 10 min at 96°C. 500 µl methanol was added to prevent ir-dynorphin from adsorbtion to the tube walls according to Ghazarossian et al. (10). The tissue was homogenized, centrifuged (140.000 x g, 45 min, 4°C) and the supernatant was lyophilized prior to immunoprecipitation of ir-dynorphin.

Immunoprecipitation procedure

The lyophilized supernatant was redissolved in 700 µl of buffer D (according to Guillemin et al. (11)), 100 µl buffer E and 200 µl buffer F (according to Mains and Eipper (12)), adjusted to pH 7.4, divided into 4 equal aliquots and subjected to immunoprecipitation with antiserum "Goldy" (see below). "Goldy" was purified by a method described by Ey et al. (13), the immunoglobulin type eluting at pH 5.6 from the protein A-Sepharose column was used. Each of the fractions was incubated with 10 µl of purified antiserum for 12-16 hrs at 4°C, and subsequently incubated for 4 hrs at 4°C with 6.5 I.U. of goat-anti-rabbit-γ-globulin (Calbiochem, La Jolla, CA, USA). The pellets thus formed were disaggregated in 250 µl 10% (v/v) acetic acid for 10 min at 96°C, pooled and subjected to gelfiltration chromatography. The immunoprecipitation proved successful in that almost no ir-dynorphin remained in the supernatant.

Gelfiltration chromatography

Gelfiltration chromatography was performed with Sephadex G-50 superfine columns (0.9 x 90 cm), eluting either in 0.1 M acetic acid or under denaturating conditions in 4 M guanidine-HCL at a flow rate of 5 ml/hr at 6°C. 1 ml fractions were collected. The recovery for synthetic dynorphin-(1-8), as well as for endogenous ir-dynorphin was >90% on both columns.

High-performance liquid chromatography (HPLC)

For HPLC, a Waters µ-Bondapak C 18 reverse-phase column (3.9 x 300 mm) was used. The column was either eluted with 1 M acetic acid (pH 2.5) or 10 mM ammonium formate (pH 4) and a linear gradient of acetonitrile from 5% to 40% within 35 min. 1 ml fractions were collected at a flow rate of 2 ml/min. The recoveries for endogenous ir-dynorphin and for all synthetic standards was >90% on HPLC.

Enzymatic cleavage

The immunoreactive material which comigrated with synthetic porcine dynorphin-(1-8) on gelfiltration chromatography and two HPLC columns

(see above), was dissolved in 250 µl of 50 mM Tris-HCL (pH 8.5) and incubated with 4 µg bovine trypsin (Sigma, Taufkirchen, FRG). After shaking for 5 hrs at 37°C, 500 ng carboxypeptidase B (Boehringer, Mannheim, FRG) was added and the incubation continued for 20 min at 37°C. The mixture was boiled for 10 min at 96°C in order to inactivate the enzymes. Thereafter, the cleavage products were separated on HPLC and assayed for ir-leucine-enkephalin. The recovery of leucine-enkephalin-immunoreactivity liberated from dynorphin-immunoreactivity (calculated in terms of synthetic dynorphin-(1-8)), was ∿65%.

Radioimmunoassay (RIA) procedures

The dynorphin-RIA was performed with antiserum "Goldy" which was directed against synthetic porcine-dynorphin-(1-13) according to the protocol described previously (8).

Vasopressin, oxytocin, camel-ß-endorphin, porcine-ß-lipotropin, methionine-enkephalin, leucine-enkephalin, dynorphin-(1-6) and -(1-7), BAM-12P and BAM-22P exhibited no cross-reactivity in the RIA (see also 8,14,15). The cross-reactivity of dynorphin-(1-8) to the antiserum was 25%. Dynorphin-(1-13), -(1-12), -(1-11), -(1-10) and -(1-9) were equally well recognized by the antiserum.

Peptides: Dynorphin-(1-6), α-neo-endorphin (Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys), methionine-enkephalin: Bachem, Bubendorf, Switzerland; Dynorphin-(1-7), -(1-8), -(1-9), -(1-11), -(1-13), BAM-12P, BAM-22P and camel-ß-endorphin: Peninsula, San Carlos, USA; porcine-ß-lipotropin: gift from Dr. L. Gráf, Hungary; Leucine-enkephalin: gift from Dr. Wünsch, Munich). Dynorphin-(1-12) was obtained from dynorphin-(1-13), dynorphin-(1-10) from Dyn-(1-11) and ß-neo-endorphin (Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro) from α-neo-endorphin by enzymatic cleavage with carboxypeptide B and subsequent purification on HPLC.

The leucine-enkephalin-RIA was performed by the use of the same protocol and the same highly specific antiserum as described elsewhere (16). [^{125}]-monoiodinated leucine-enkephalin (NEN, Dreieich, FRG, 800 Ci/mmole) was used as radioactive tracer. Cross-reactivities to any other opioid peptides such as camel-ß-endorphin, porcine-ß-lipotropin, methionine-enkephalin, dynorphin-(1-6) and -(1-13) are negligible (see also 16).

RESULTS

Figure 1 shows the profile of dynorphin-related immunoreactive peptides in the rat neurointermediate pituitary that were immunoprecipitated with antiserum "Goldy", directed against dynorphin-(1-13), and separated on a Sephadex G-50 superfine column.

Four different molecular weight forms of ir-dynorphin were found. The two major dynorphin-related ir-species obtained (peak 3 and 4), exhibited a similar size as dynorphin-(1-13) (peak 3) and dynorphin-(1-8) (peak 4). Rechromatography of peak 4 under denaturating conditions on a Sephadex G-50 column, eluting in 4 M guanidine-HCL, again

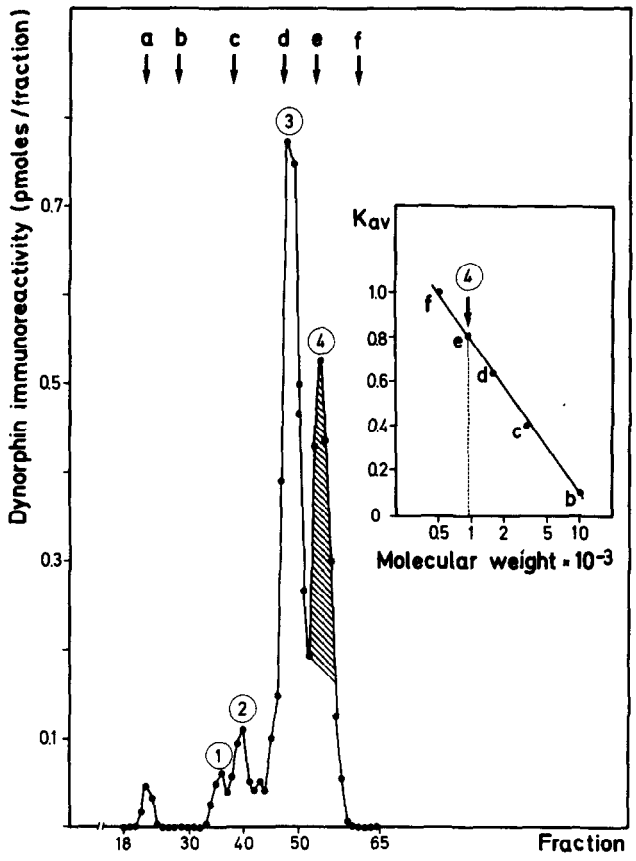

Figure 1

Gelfiltration chromatography of dynorphin-related peptides from the rat neurointermediate pituitary after immunoprecipitation

100 rat neurointermediate pituitaries were extracted and immunoprecipitated with purified dynorphin antibodies as described in the Methods. The dynorphin-related peptides were separated on a Sephadex G-50 superfine column, eluting in 0.1 M acetic acid. 5% aliquots of the 1 ml fractions were assayed for ir-dynorphin.

Inset figure

80% of the ir-peak 4 containing fractions (see above) were pooled and rechromatographed on a Sephadex G-50 superfine column, eluting in 4 M guanidine-HCL. 2% aliquots of 1 ml fractions were assayed for ir-dynorphin.

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$; Ve = Elution volume; Vo = Void volume; Vt = Total volume

Markers for standardization

a = ^{14}C-BSA (NEN, Dreieich, FRG)
b = porcine ß-lipotropin
c = camel ß-endorphin
d = porcine dynorphin-(1-13)
e = porcine dynorphin-(1-8)
f = ^{3}H-leucine-enkephalin (Amersham, Braunschweig, FRG)

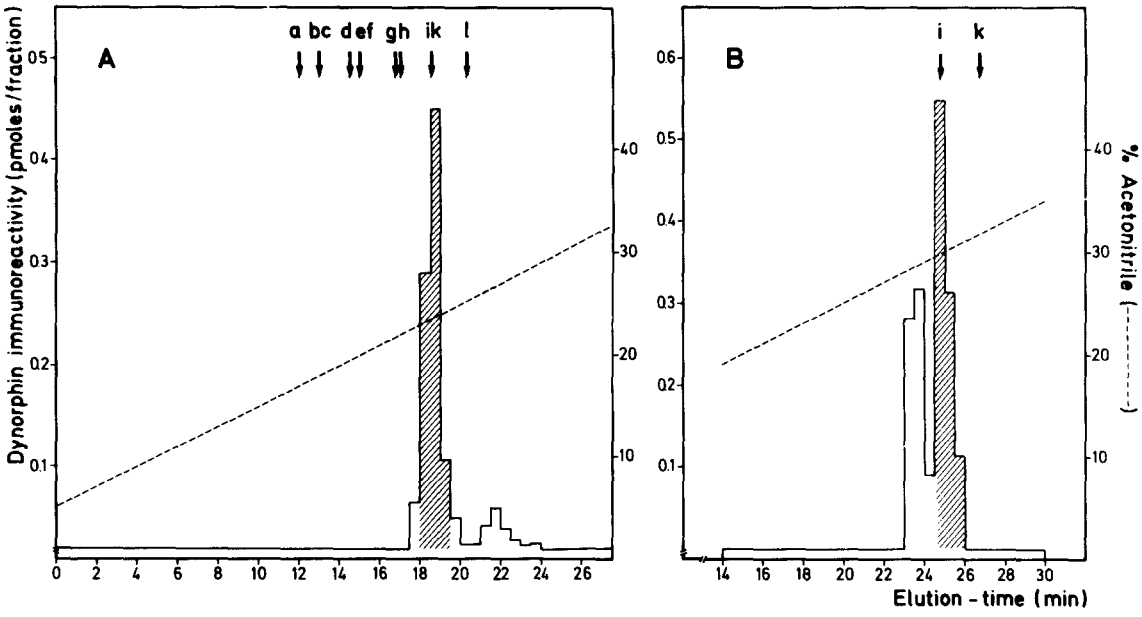

Figure 2

High-performance liquid chromatography of immunoreactive dynorphin-(1-8) from the rat neurointermediate pituitary pre-purified by immunoprecipitation and gelfiltration chromatography

A) 100 rat neurointermediate pituitaries were extracted, immunoprecipitated and chromatographed as described in the Methods and in the legend to figure 1. 75% of the ir-peak 4 (0.9 k ir-dynorphin) containing fractions (see figure 1) were pooled and chromatographed on a Waters HPLC C 18 reverse phase column by the use of 1 M acetic acid (pH 2.5) as eluting buffer and a linear gradient of acetonitrile from 5% to 40% within 35 min. 5% aliquots of the 1 ml fractions were assayed for ir-dynorphin.

B) 50% of the ir-dynorphin containing fraction, which coeluted with synthetic dynorphin-(1-8) on the first HPLC separation (see figure 2A) were subjected to a second HPLC separation by the use of the same column type and eluting conditions as described above, but with 10 mM ammonium formate (pH 4) as eluting buffer. 20% aliquots of the column eluate were assayed for ir-dynorphin.

Markers for standardization: (Dyn = dynorphin)

a = Dyn-(1-7); b = Dyn-(1-11); c = α-neo-endorphin (Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys); d = Dyn-(1-13); e = Dyn-(1-6); f = Dyn-(1-9); g = ß-neo-endorphin (Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro); h = Dyn-(1-10); i = Dyn-(1-8); k = Dyn-(1-12); l = leucine-enkephalin.

revealed an apparent molecular weight of 0.9 k (k = kilodaltons), similar of that of dynorphin-(1-8) (figure 1, inset).

Figure 2 exhibits the separation profile of 0.9 k ir-dynorphin on a HPLC C 18 reverse phase column (1 M acetic acid (pH 2.5)/acetonitrile). The vast majority of 0.9 k ir-dynorphin comigrated with synthetic dynorphin-(1-8). Virtually no dynorphin-immunoreactivity coeluted with

synthetic dynorphin-(1-9), -(1-10), -(1-11) and -(1-13), although these peptides would be recognized by the dynorphin antiserum (see Methods).

Synthetic α-neo-endorphin and ß-neo-endorphin, two "big" leucine-enkephalins, recently isolated from porcine hypothalamus (5,6), do not comigrate with dynorphin-(1-8) on this HPLC system. However,

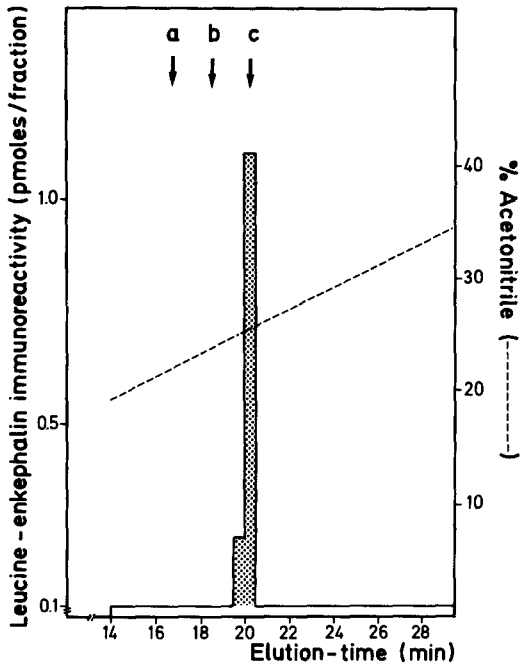

Figure 3

High-performance liquid chromatography of immunoreactive leucine-enkephalin, liberated from immunoreactive dynorphin-(1-8) upon enzymatic treatment

Ir-dynorphin-(1-8) was purified from 100 rat neurointermediate pituitaries by immunoprecipitation, gelfiltration chromatography and HPLC as described in the Methods and the legends to figure 1 and 2. 50% of the major ir-dynorphin species, comigrating with synthetic dynorphin-(1-8) on the second HPLC separation (see figure 2B), was subjected to enzymatic cleavage with trypsin and carboxypeptidase B as described in the Methods. The cleavage products were separated by the use of the same column and eluting conditions as described in the legend to figure 2A. 20% aliquots of the column eluate were assayed for ir-leucine-enkephalin.

Markers for standardization

a = methionine-enkephalin
b = dynorphin-(1-8)
c = leucine-enkephalin

dynorphin-(1-8) and dynorphin-(1-12) exhibit identical elution times. In order to exclude putative comigration of dynorphin-(1-8) with dynorphin-(1-12), the major 0.9 k ir-dynorphin species, obtain by the first HPLC separation, was subjected to a second HPLC C 18 reverse phase column (10 mM ammonium formate (pH 4.0)/acetonitrile). Two dynorphin-related ir-peaks were obtained (figure 2B). The major ir-peak again coeluted with synthetic dynorphin-(1-8). The identity of the minor ir-peak with a retention time shorter than dynorphin-(1-8), is still in need of clarification.

Since dynorphin-(1-8) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile) can be enzymatically cleaved into the opioid pentapeptide leucine-enkephalin by subsequent treatment with trypsin and carboxypeptidase B, the major ir-dynorphin species, expected to be dynorphin-(1-8), was demonstrated to also contain leucine-enkephalin: Figure 3 shows that material, comigrating with synthetic leucine-enkephalin on HPLC, could be liberated.

In consideration of the recovery of ir-dynorphin-(1-8) from 0.9 k ir-dynorphin and the cross-reactivity of the dynorphin antiserum to dynorphin-(1-8), the opioid octapeptide may occur in a concentration of almost 1 pmole/mg within the neurointermediate pituitary of rats.

DISCUSSION

The present investigation provides convincing evidence for the occurrence of dynorphin-(1-8) in the neurointermediate pituitary of rats. Ir-dynorphin-(1-8) occurred in a concentration similar to that found for leucine-enkephalin and methionine-enkephalin within the rat neurointermediate pituitary (16,17).

Dynorphin-immunoreactive material with a molecular weight corresponding to dynorphin-(1-8) within this tissue was not reported by Goldstein and Ghazarossian (7). However, this is most likely explained

by the very low cross-reactivity of the dynorphin-antiserum to dynorphin-(1-8) used in that study (see (10)).

Though dynorphin-(1-8) could already be isolated from porcine hypothalami by the use of an extraction procedure, different from ours (3), biosynthesis studies are necessary to exclude the possibility of an artificial generation of dynorphin-(1-8) from larger dynorphin-related peptides. Moreover, they have to elucidate the putative biosynthetic relationships of dynorphin-(1-8) to these larger dynorphin-related peptides and to leucine-enkephalin (dynorphin-(1-5)).

The biological significance of dynorphin-(1-8) in the neurointermediate pituitary is still in need of clarification. Dynorphin-(1-8) and leucine-enkephalin exhibit similar opiate-like activities in the guinea-pig ileum bioassay (Dr. R. Schulz, personal communication) and occur in similar concentrations within the rat neurointermediate pituatary.(the present investigation, 16,17).

Thus, dynorphin-(1-8) may also contribute to the regulation of the vasopressin- and oxytocin release from the neurohypophysis, which was recently shown to be influenced by opiates and opioid peptides (18,19).

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