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IMMUNOREACTIVE DYNORPHIN IN THE RAT ADENOHYPOPHYSIS CONSISTS EXCLUSIVELY OF 6000 DALTON SPECIES

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SUMMARY: Immunoreactive dynorphin in the rat adenohypophysis exhibited an apparent molecular size of approximately 6 kilodaltons (6 kDal) upon characterization by gelfiltration. Essentially no dynorphin-related peptides with a molecular size of dynorphin-(1-17) or dynorphin-(1-8), which constitute the great majority of ir-dynorphin in the rat neurointermediate pituitary and brain, could be detected in the adenohypophysis. The possible presence of putative aggregations of smaller peptides were largely excluded by rechromatography under denaturating conditions in 4 M guanidine-HCL. SDS-gelelectrophoresis revealed that 6 kDal dynorphin consisted of at least three components of similar molecular size. From the predominant form of 6 kDal dynorphin, leucine-enkephalin could be liberated by sequential enzymatic cleavage with trypsin and carboxypeptidase B.

Dynorphin is a opioid peptide containing the leucine-enkephalin sequence at its N-terminus (1). Although the complete structure of the heptadecapeptide (dynorphin-(1-17)) was only recently ascertained (2,3), the initially characterized incomplete peptide (dynorphin-(1-13)) had already been shown to exhibit an extraordinarily high opiate-like potency on the guinea-pig ileum bioassay (1). Immunoreactive dynorphin-related peptides* (ir-dynorphin) have been shown to be widely distributed in brain, pituitary, spinal cord and gut with highest concentrations found in the neurointermediate pituitary and anterior pituitary (adenohypophysis) (4,5,6,7,8). Immunohistochemical studies revealed a pathway of ir-dynorphin fibres within the hypothalamo-posterior pituitary pathway, very similar to that found for the neurosecretory peptides vasopressin and oxytocin (8). In fact, the peptide pools of vasopressin

*All studies so far performed on the distribution and characterization of dynorphin-related peptides have been carried out with antibodies, directed against synthetic dynorphin-(1-13) (4-12). However, it should be noted out, that dynorphin-(1-13) and the complete peptide dynorphin-(1-17) are equally well recognized by the dynorphin antiserum "Goldy", used in our laboratory.

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and dynorphin in the neurohypophysis appear to be similarly modified in response to various endocrine manipulations such as dehydration, adrenalectomy and dexamethasone treatment (9,10). These treatments, do not, however affect the concentrations of ir-dynorphin in the adenohypophysis, indicative of a different mode of regulation of dynorphin pools in the particular lobes of the pituitary.

The present paper reports another difference between ir-dynorphin in the pituitary lobes, in showing that ir-dynorphin in the rat adenohypophysis consists almost exclusively of higher molecular weight forms, in contrast to the rat neurointermediate pituitary, where ir-dynorphin has been shown to be predominantly composed of a highly opiate-active peptide with a molecular size similar to that of dynorphin-(1-17), and of the opioid octapeptide dynorphin-(1-8) (4,11).

METHODS AND MATERIALS

Extraction. Male Sprague-Dawley rats (200-220 g) were decapitated and their pituitaries divided, in situ, into anterior lobes (adenohypophyses) and neurointermediate lobes. 50 or 100 adenohypophyses (6.0 ± 0.3 mg, mean ± SEM, n = 10, wet weight) were incubated in 0.75 or 1.5 ml of 0.1 M HCl for 10 min at 96°C. 250 or 500 µl methanol was added to prevent ir-dynorphin from adsorption to the tube walls according to Ghazarossian et al. (12). The tissue was homogenized and centrifuged (140.000 x g, 45 min, 4°C).

The supernatant was subjected to gelfiltration chromatography.

Gelfiltration-chromatography. Gelfiltration-chromatography was performed with Sephadex G-50 superfine columns (0.9 x 90 cm), eluting either in 10% (v/v) acetic acid or 4 M guanidine-HCL at 6°C at a flow rate of 5 ml/h. 1 ml-fractions were collected. The recovery of ir-dynorphin from the rat adenohypophysis on gelfiltration chromatography was >90%.

Sodium-dodecyl-sulfate (SDS)-polyacrylamide-gelelectrophoresis. SDS-gelelectrophoresis was performed with 12% polyacrylamide gels (Bio-Rad, Richmond, CA, USA) as described previously (13). The gels were cut into 2 mm-slices and the peptides eluted with 1.5 ml buffer D (according to Guillemin et al. (14)) for 16 hrs at 24°C. 350 µl aliquots were used for the dynorphin-radioimmunoassay (RIA), performed as described below. The recovery of ir-dynorphin on SDS-polyacrylamide-gelelectrophoresis was >90%.

High-performance liquid chromatography (HPLC). HPLC was performed with a Waters µ-Bondapak C 18 reverse phase column (3.9 x 300 mm). The column was eluted with 1 M acetic acid (pH 2.5) with a linear gradient of acetonitrile from 5% to 100% within 95 min at a flow rate of 2 ml/ min. 1 ml or 2 ml-fractions were collected. The recovery for endogenous ir-dynorphin from the rat adenohypophysis on HPLC was ∿25%. The recoveries for synthetic standards, used on HPLC, was >90%.

Radioimmunoassay (RIA) procedures. The dynorphin-RIA was performed with antiserum "Goldy", directed against synthetic porcine-dynorphin-(1-13) as described previously (5). The antiserum exhibits an identical avidity for dynorphin-(1-13) and dynorphin-(1-17), but has no avidity for leucine-enkephalin, methionine-enkephalin (Bachem, Bubendorf, Switzerland), camel ß-endorphin,

dynorphin-(1-6), dynorphin-(1-7), α-neo-endorphin-(1-10), BAM-12P and BAM-22P (Peninsula, San Carlos, USA) (see also 5,10).

The leucine-enkephalin-RIA was performed by the use of the same protocol and highly specific antiserum as described elsewhere (15). [^{125}I]-monoiodinated leucine-enkephalin (NEN, Dreieich, FRG) was used as the radioactive tracer. Cross-reactivities to other opioid peptides such as camel ß-endorphin, porcine-ß-lipotropin (gift from Dr. Gráf, Budapest, Hungary), methionine-enkephalin, dynorphin-(1-6) and dynorphin-(1-13) were negligible (see also (15)).

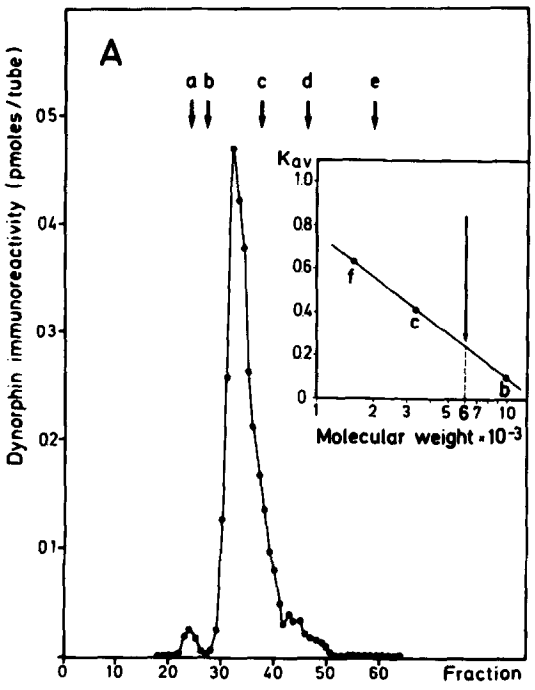
Immunoprecipitation procedures. The main dynorphin-related ir-species obtained from 100 rat adenohypophyses after pre-purification on gelfiltration chromatography and HPLC, were subjected to immunoprecipitation with 10 µl of purified dynorphin antiserum "Goldy" as described previously (4). The immunoprecipitate was disaggregated in 10% (v/v) acetic acid as described (4), and rechromatographed on a Sephadex G-50 column to separate ir-dynorphin from the immunoglobulins. The fractions of the column eluate containing ir-dynorphin, were pooled and lyophilized prior to enzymatic cleavage. The recovery of ir-dynorphin for immunoprecipitation and subsequent gelfiltration chromatography was ∿50%.

Enzymatic cleavage. Ir-dynorphin, pre-purified by gelfiltration chromatography, HPLC and immunoprecipitation as described above, was enzymatically treated with 4 µg bovine trypsin (Sigma, Taufkirchen, FRG) and subsequently with 500 ng carboxypeptidase B (Boehringer, Mannheim, FRG) by the use of the method previously described (4). The cleavage products were separated on HPLC and assayed for ir-leucine-enkephalin as described above.

RESULTS

Figure 1A depicts the immunoreactive dynorphin components found in the rat adenohypophysis and separated by use of a Sephadex G-50 column in 10% acetic acid. The vast majority of ir-dynorphin exhibited an apparent molecular weight of 6 kilodaltons (6 kDal). Virtually no immunoreactive components could be detected in the molecular range of dynorphin-(1-17) and dynorphin-(1-8), where the two major dynorphin-related opioid peptides from the rat neurointermediate pituitary elute (4,11). In order to largely exclude the possible presence of aggregations of smaller peptides, 6 kDal dynorphin was rechromatographed under denaturating conditions on a Sephadex G-50 column, eluting in 4 M guanidine-HCL. The molecular weight of about 6 kDal could be confirmed (Figure 1A, inset).

Analysis of 6 kDal dynorphin on SDS-gelelectrophoresis revealed that 6 kDal dynorphin displays a molecular heterogenity. Figure 1B shows the occurence of several different compounds, one major species with an apparent molecular weight of 12.5 kDal, and two additional minor immunoreactive compounds with apparent molecular weights of 10 kDal and 15 kDal on SDS-gelelectrophoresis. However, the molecular weights of these three dynorphin-related compounds might be overestimated on SDS-gelelectrophoresis, in analogy to those of other peptides



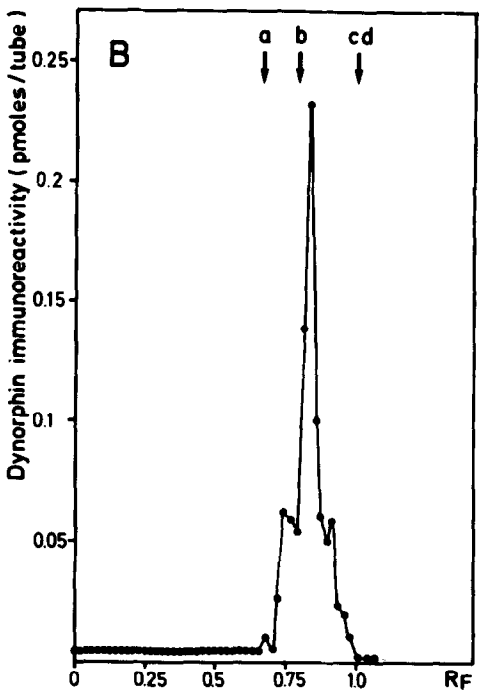


Figure 1A

Gelfiltration chromatography of dynorphin-related compounds from the rat adenohypophysis on a Sephadex G-50 column

The extract of 50 rat adenohypophyses was chromatographed on a Sephadex G-50 superfine column (0.9 x 90 cm), eluting in 10% (v/v) acetic acid. 1 ml-fractions were collected, 5% aliquots were assayed for ir-dynorphin. Inset: 100 rat adenohypophyses were chromatographed as described above, 85% of the eluate fractions containing ir-dynorphin were pooled and rechromatographed on a Sephadex G-50 superfine column, eluting in 4 M guanidine-HCL. 1.5% aliquots of the 1 ml-fractions were assayed for ir-dynorphin. The arrow indicates the position of endogenous ir-dynorphin. Markers for standardization: a = ^{14}C-BSA (Void volume) (Amersham, Braunschweig, FRG); b = porcine-ß-lipotropin; c = camel-ß-endorphin; d = porcine dynorphin-(1-17); e = leucine-enkephalin (total volume); f = porcine dynorphin-(1-13);

$K_{av} = \frac{V_e - V_o}{V_t - V_o}$, V_e = elution volume; V_o = void volume, V_t = total volume

Figure 1B

SDS-polyacrylamide-gelelectrophoresis of 6 kDal dynorphin from the rat adenohypophysis

50 rat adenohypophyses were chromatographed on a Sephadex G-50 column, eluting in 10% acetic acid as described above. 90% of the ir-dynorphin-containing fractions were pooled and 10% aliquots subjected to SDS-gelelectrophoresis as described in the Methods.

The gels were cut into 2 mm-slices and eluted with 1.5 ml RIA buffer. 350 µl aliquots were assayed for ir-dynorphin.

Markers for standardization: a = soybean trypsin inhibitor (MW:20.100); b = α-lactalbumin (MW:14.400) (both from Pharmacia, Freiburg i. Br., FRG); c = camel-ß-endorphin (MW:3.500); d = bromphenol blue (tracking-dye); R_F = relative mobility to the tracking-dye bromphenol blue.

with molecular sizes similar to that of 6 kDal dynorphin, as, for example, rat ß-lipotropin (13,16).

For further purification, 6 kDal dynorphin, pre-purified on gelfiltration chromatography, was chromatographed on a HPLC C 18 reverse phase column. One major and at least two minor dynorphin-related compounds were obtained (figure 2A) Dynorphin is known to contain the leucine-enkephalin sequence, followed by two basic amino acids (1,2,3). In order to demonstrate that 6 kDal dynorphin in the rat adenohypophysis also contains leucine-enkephalin, the following experiments were performed. For further purification, the major component of 6 kDal dynorphin on HPLC was immunoprecipitated with dynorphin antibodies which did not recognize α-neo-endorphin-(1-10), another recently isolated leucine-enkephalin containing opioid peptide (17). After rechromatography on a Sephadex G-50 column, in order to separate ir-dynorphin from the immunoglobulins, the peptide was enzymatically treated with trypsin and subsequently carboxypeptidase B. The cleavage products were separated on a HPLC C 18 reverse phase column and finally analyzed by a specific leucine-enkephalin-RIA. Figure 2B demonstrates that ir-leucine-enkephalin, comigrating with synthetic leucine-enkephalin on HPLC, could be liberated from the major form of 6 kDal dynorphin.

DISCUSSION

The present investigation indicates that ir-dynorphin in the rat adenohypophysis consists of peptides in the molecular range of about 6 kDal, the main form of these dynorphin-related peptides containing the leucine-enkephalin sequence. No dynorphin-related peptides in the molecular range of dynorphin-(1-17) and dynorphin-(1-8), which constitute the great majority of ir-dynorphin in the rat neurointermediate pituitary (4,11), rat brain and gut (5, Höllt et al., in preparation), could be detected in the rat adenohypophysis. One possible explanation for this unique pattern of ir-dynorphin is that the rat adenohypophysis contains a different opioid peptide system, which is only immunologically related to dynorphin by sequence analogies. Another possible explanation is a different post-translational processing of the, as yet unknown, dynorphin-precursor(s)

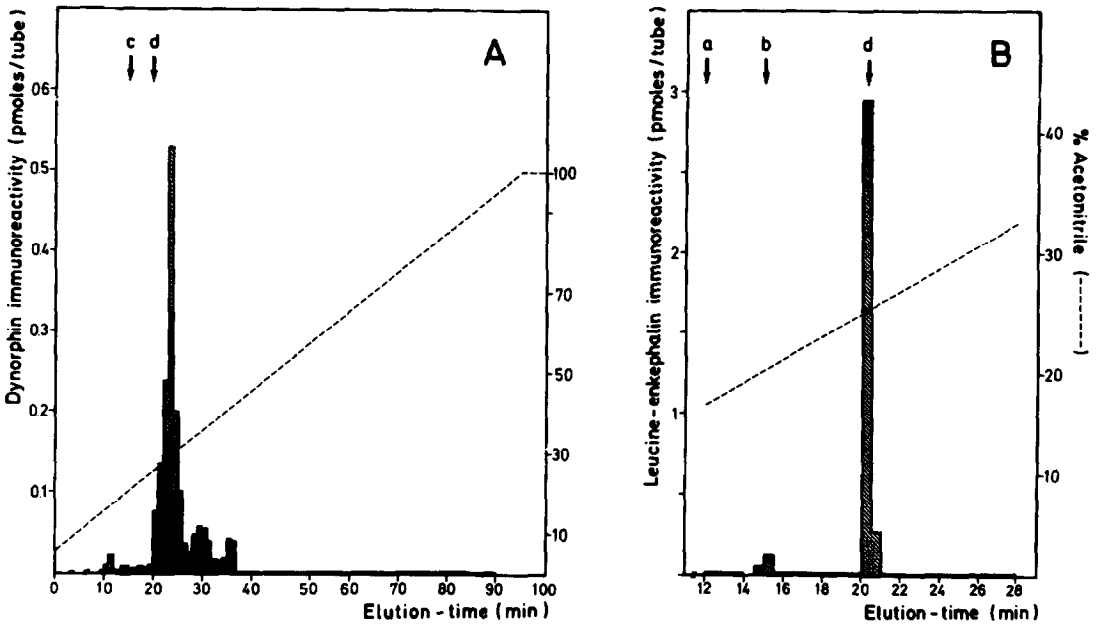


Figure 2A:

High-performance liquid chromatography of 6 kDal dynorphin from the rat adenohypophysis

100 rat adenohypophyses were chromatographed on Sephadex G-50 columns (10% acetic acid as eluting buffer) as described in the Methods.

90% aliquots of the ir-dynorphin-containing eluate fractions were pooled and chromatographed on a HPLC C 18 reverse phase column as described in the Methods. 10% aliquots of the 2 ml fractions of the column eluate were assayed for ir-dynorphin. Dynorphin-immunoreactivities are not corrected for recoveries.

Figure 2B:

High-performance liquid chromatography of immunoreactive leucine-enkephalin, liberated from the main form of 6 kDal dynorphin from the rat adenohypophysis upon enzymatic cleavage

80% of the HPLC eluate, containing the major ir-dynorphin peak (see figure 2A) was pooled and subjected to immunoprecipitation with a purified dynorphin antiserum. After rechromatography on a Sephadex G-50 column, ir-dynorphin was subjected to enzymatic cleavage with trypsin and subsequently carboxypeptidase B. The cleavage products were separated on HPLC C 18 reverse phase column and 50% aliquots of the 1 ml fractions of the column eluate were finally analyzed by a specific leucine-enkephalin RIA (for technical details see Methods). Immunoreactivities are not corrected for recoveries.

Markers for standardization: a = dynorphin-(1-7); b = dynorphin-(1-6); c = methionine-enkephalin; d = leucine-enkephalin.

within different tissues. Different processing of a prohormone in particular tissues has already been demonstrated for pro-opiomelanocortin (18), which is enzymatically processed into the higher molecular weight forms ß-lipotropin and adrenocorticotropic hormone within the adenohypophysis, while these two

peptides are further processed into the smaller molecular weight forms (α-N-acetyl-) ß-endorphin and α-melanocyte stimulating hormone within the intermediate pituitary (16,18,19,20).

The biological significance of the presence of high-molecular weight forms of ir-dynorphin in the rat adenohypophysis remains in need of elucidation. Preliminary studies have indicated that these high molecular weight forms of ir-dynorphin, after purification by gelfiltration and quantitative immunoprecipitation with dynorphin-antibodies, exhibited opiate-like activity on the guinea-pig ileum bioassay, but are, however, much less potent than that comparable amounts of immunoprecipitated dynorphin(1-17) (Seizinger, to be published). It might be, however, that the 6 kDal dynorphins, if they are released from the adenohypophysis into the blood stream, are enzymatically converted into dynorphin-like sequences with much higher opiate-activity during the transport to putative target tissues. It can not, however, be excluded that the high molecular weight forms of ir-dynorphin in the rat adenohypophysis may even be of a biological significance unrelated to opiate-like functions.

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REFERENCES

1. Goldstein, A., Tachibana, S., Lowney, L.I., Hunkapiller, M., and Hood, L. (1979) Proc. Natl. Acad. Sci. USA 76, 6666-6670.
2. Tachibana, S. (1981) INRC-Meeting, Kyoto, in press.
3. Goldstein, A., Fischli, W., Lowney, L.I., Hunkapiller, M., and Hood, L. (1981) Proc. Natl. Acad. Sci. USA, in press.
4. Seizinger, B.R., Höllt, V., and Herz, A. (1981b) Biochem. biophys. Res. Commun., in press.
5. Höllt, V., Haarmann, I., Bovermann, K., Jerlicz, M., and Herz, A. (1980a) Neurosci. Lett. 18, 149-153.
6. Goldstein, A., and Ghazarossian, V.E. (1980) Proc. Natl. Acad. Sci. USA 77, 6207-6210.
7. Millan, M.J., Tsang, Y.F., Przewłocki, R., Höllt, V., and Herz, A. (1981) Neurosci. Lett. 24, 75-79.
8. Watson, S.J., Akil, H., Ghazarossian, V.E., and Goldstein, A. (1981) Proc. Natl. Acad. Sci. USA 78, 1260-1263.
9. Höllt, V., Haarmann, I., Seizinger, B.R., and Herz, A. (1980b) Europ. J. Pharmacol. 68, 239-240.

10. Höllt, V., Haarmann, I., Seizinger, B.R., and Herz, A. (1981) Neuroendocrinology, in press.
11. Seizinger, B.R. (1981) Naunyn-Schmiedeberg's Arch. Pharmacol., Suppl. 316, R250.
12. Ghazarossian, V.E., Chavkin, C.H., and Goldstein, A. (1980) Life Sci. 27, 75-86.
13. Höllt, V., Haarmann, I., and Herz, A. (1981a) J. Neurochem. 31, 619-626.
14. Guillemin, R., Ling, N., and Vargo, T. (1977) Biochem. biophys. Res. Commun. 77, 361-366.
15. Duka, T., Höllt, V., Przewłocki, R., and Wesche, D. (1978) Biochem. biophys. Res. Commun. 85, 1119-1127.
16. Mains, R.E., and Eipper, B.A. (1979) J. Biol. Chem. 254, 7885-7894.
17. Kangawa, K., Minamino, N., Chino, N., Sakakibura, S., and Matsuo, H. (1981) Biochem. biophys. Res. Commun. 99, 871-878.
18. Crine, P., Gossard, F., Seidah, N.G., Blanchette, L., Lis, M., and Chrétien, M. (1979) Proc. Natl. Acad. Sci. 76, 5085-5089.
19. Mains, R.E., Eipper, B.A., and Ling, N. (1977), Proc. Natl. Acad. Sci. USA 74, 3014-3018.
20. Seizinger, B.R., and Höllt, V. (1980), Biochem. biophys. Res. Commun. 96, 535-543.