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DISTRIBUTION OF METHIONINE- AND LEUCINE-ENKEPHALIN WITHIN THE RAT PITUITARY GLAND MEASURED BY HIGHLY SPECIFIC RADIOIMMUNOASSAYS

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SUMMARY: The distribution of methionine- and leucine-enkephalin within the rat pituitary gland was measured using highly specific antisera in conjunction with purification by high performance liquid chromatography. The highest concentrations were found in the pars intermedia (7 pmole/mg for methionine-enkephalin, 4 pmole/mg for leucine-enkephalin), whilst the pars nervosa contained 2.2 pmole/mg of each and the pars anterior the least (methionine-enkephalin: 0.51 pmole/mg, leucine-enkephalin: 0.36 pmole/mg).

The distribution within the brain of methionine-enkephalin (met-enk) and leucine-enkephalin (leu-enk), two pentapeptides with opiate-like activity, is well documented by numerous bioassays (1,2), radioimmunoassays (3-9) and immunohistochemical studies (10-12). There appears to be, however, a discrepancy in the literature concerning the existence of the enkephalins in the pituitary. Although the majority of the investigators found little or no enkephalins in the pituitary gland, three groups did report significant levels (9,13,14). The present paper also provides evidence for the existence of high concentrations of the enkephalins in the rat pituitary. By using highly specific radioimmunoassays the distribution of met-enk and leu-enk within the rat pituitary has been measured.

MATERIALS AND METHODS

Generation of met-enk and leu-enk directed antisera

Met-enk and leu-enk were conjugated to bovine thyroglobulin (Tg) using 1-ethyl-3(3-diemthyl-aminopropyl)-carbodiimide (CDI) as a coupling agent (15). 10 mg met- or leu-enk were dissolved with 50 mg Tg in 2.0 ml destilled water containing 2.3 mg CDI. After shaking, the solutions were incubated for 24 h at 5°C.

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Thereafter, an equal volume of 1 M hydroxylamine was added and the mixture further incubated for 5 h at room temperature. Then the solutions were dialyzed against 14 liter of 0.9% saline for 24 h at 5°C. About 20 molecules of each of the enkephalins had been incorporated into one molecule of the carrier protein as tested by adding tracer amounts of ^{3}H-met-enk or ^{3}H-leu-enk to the reaction mixture. The enkephalin/Tg conjugate was then diluted with 0.9% saline to a final volume of 6.5 ml and the solution was divided into two 1.0 ml aliquots for the initial injections and eight 0.5 ml aliquots which were frozen until used for the booster injections. The conjugate fractions were emulsified with an equal volume of complete Freunds' adjuvant and 100 µl aliquots were injected intradermally at multiple sites in the back of male New Zealand rabbits, each weighing about 2000 g. The rabbits simultaneously received an i.m. injection of 0.5 ml crude Bordetella pertussis vaccine (16). Collection of the antisera was performed after 4 booster injections at 3 week-intervals.

Radioimmunoassay (RIA) procedure

Appropriate dilutions of the antisera (1:1000 - 1:2000) were added in 0.1 ml aliquots to 1.5 ml polypropylene vials containing 0.3 ml sodium phosphate buffer (50 mM, pH 7.4) plus 0.05 ml solutions of standard concentrations of enkephalins, other peptides or of tissue extracts. The reaction was started by adding 10000 cpm ^{3}H-met-enk (about 0.2 pmole) or 20000 cpm ^{3}H-leu-enk (about 0.4 pmole) in 0.05 ml aliquots. After incubation for 2-4 h at 0°C bound enkephalins were separated from free by retaining the antibody-enkephalin complex on Millipore filters (0.45 µ) (3). The filters were washed with 5 ml standard buffer, dried and placed into scintillation vials containing 10 ml scintillation fluid (Aqua Lumac, Belgium). After shaking radioactivity was counted with an efficiency of 54%.

RIA for ß-endorphin

The RIA for ß-endorphin was conducted according to a protocol as recently described (17). Briefly, antibodies were raised in rabbits using human ß-endorphin coupled thyroglobulin as immunogen. Antibodies were directed against the c-terminus of the ß-endorphin; thus, met-enk, α- and γ-endorphin showed no cross-reactivity. Human ß-lipotropin, however, was recognized by the antiserum with an only twofold lower avidity than human ß-endorphin (on a molar basis). In the text, the immunoreactivity which refers to ß-endorphin and ß-lipotropin is expressed as ß-endorphin. Sensitivity of the assay for human ß-endorphin was 5 femtomole/tube.

Tissue extraction

Male Sprague Dawley rats (220-250 g) were decapitated and whole pituitaries were dissected in situ under a miscroscope into anterior lobe and posterior lobe. In one set of experiments the posterior lobe was carefully separated from the intermedia lobe on a precooled plate of 0°C within 2 min after decapitation. The lobes were immediately transferred to tared 1.5 ml plastic vials and weighed to the nearest tenth of a milligram. To each anterior lobe 25 volumes, and to each intermediate/posterior lobe 125 volumes of hot 0.1 N HCl (96%) was added. The samples were then incubated for 15 min at 96°C. The samples were homogenized and centrifuged in a Beckman Microfuge B for 3 min. Thereafter,

the supernatants were adjusted to pH 7.4 using saturated Na_2HPO_4' solution and 1 N NaOH. After recentrifuging 50 µl aliquots were used in the RIAs.

Chromatography

In one part of the experiments enkephalins in the acid supernatants were extracted according to Hughes et al. (2). The samples were applied to 0.7 x 4 cm columns containing XAD-2 neutral polystyrene beads (Amberlite) and adsorption of the enkephalins performed at a flow rate of 0.5 ml/min. After washing the column with 20 ml 0.1 N HCl and 20 ml H_2O, the enkephalins were eluted with 10 ml methanol. The eluate was evaporated under reduced pressure at 60°C and the residue redissolved with 400 µl 0.01 M sodium acetate (pH 4.0) and chromtographed by high performance liquid chromatography (HPLC). 100 µl aliquots were injected into a Waters 6000 A HPLC system and separation performed using a µ-Bondapak C_{18} (39 x 300 mm) column (Waters). The column was eluted with a 30 min non-linear gradient (see Fig. 2) from 0.01 M sodium acetate/30% (vol/vol) acetonitrile at a flow rate of 2 ml/min, lyophilized, redissolved in 250 µl volume of sodium phosphate buffer (0.05 M, pH 7.4) and 50 µl aliquots measured in the RIA for enkephalin content.

Substances

^{3}H-met-enk (spec. act. 43 Ci/mmole) and ^{3}H-leu-enk (spec. act. 43 Ci/mmole): Amersham, Braunschweig, F.R.G.; mehtionine-enkephalin, leucine-enkephalin, Tyr-Gly-Gly-Phe-Nor-Leu, Arg-Tyr-Gly-Gly-Phe-Leu, Gly-Gly-Phe-Met, Gly-Gly-Phe-Nor-Leu, Tyr-Gly-Gly, Fly-Gly-Phe, Phe-Met, and Phe-Leu: a gift from Dr. Moroder, Munich, F.R.G.; Tyr-Gly-Gly-Phe, Gly-Gly-Phe-Leu: a gift from Dr. Fasold, Frankfurt/Main, F.R.G.; ß-lipotropin 61-69 and 61-75: a gift from Drs. Wahlström and Terenius, Uppsala, Sweden; human ß-, α-, and γ-endorphin, Paesel, Frankfurt/Main, F.R.G.; all other chemicals were obtained from Merck, Darmstadt, F.R.G.

RESULTS AND DISCUSSION

Figure 1A shows the high avidity of the met-enk-directed antiserum for met-enk (sensitivity limit of the RIA about 50 fmole/tube). Leu-enk displays only a 0.25% cross-reactivity whilst the cross-reactivities of the other ß-lipotropin fragments (61-69, 61-75, 61-76 and 61-91) were negligible. The leu-enk-directed antiserum (Fig. 1B) recognizes leu-enk with a similarly high avidity (sensitivity limit of the RIA 50 fmole/tube). Met-enk shows a 0.33% cross-reactivity, whereas the other ß-lipotropin fragments virtually did not bind to this antiserum.

Table 1 illustrates that the tetra-, tri- and di-peptid-fragments of the enkephalins display no significant affinity for both of the antisera.

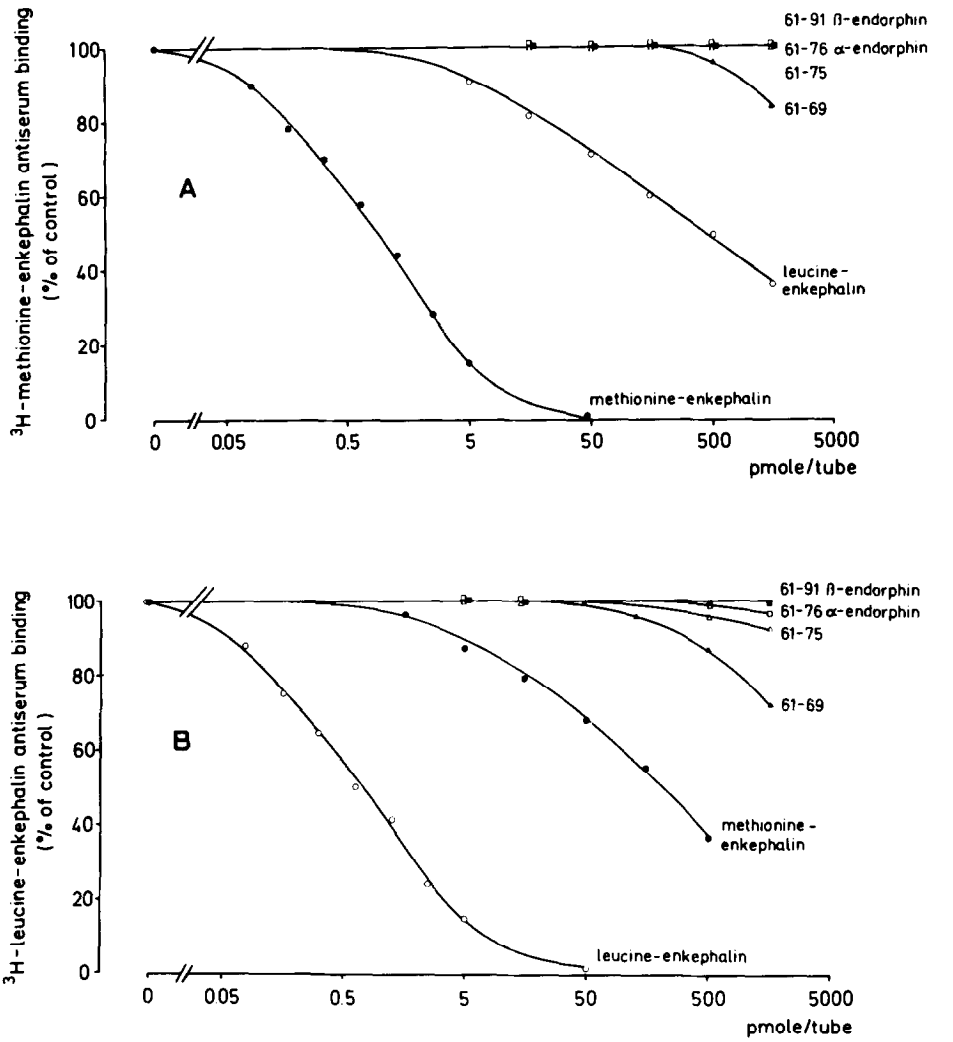

FIGURE 1: A: Specificity of the methionine-enkephalin-directed antiserum. Control binding represents the binding of the binding of 0.25 pmole ^{3}H-methionine-enkephalin to the antiserum/diluted (1:2000) in the absence of nonlabelled peptides. Under this condition 30% of the total radioactivity was bound.
B: Specificity of the leucine-enkephalin-directed antiserum. Control binding represents the binding of 0.4 pmole ^{3}H-leucine-enkephalin to the antiserum/diluted (1:1200). Under this condition 25% of the total radioactivity was bound.
Each point represents the mean of 2 experiments performed in triplicate.

The low cross-reactivities of the enkephalins to each other's antisera suggest that the antibodies are directed against the C-

TABLE I: Antiserum-cross-reactions of enkephalin fragments

Peptides	Cross-reactivity (%) Met-Enk Antiserum	Leu-Enk Antiserum
Tyr-Gly-Gly-Phe-Met	100.00	0.33
Tyr-Gly-Gly-Phe-Leu	0.25	100.00
Tyr-Gly-Gly-Phe-Nor-Leu	7.30	2.10
Arg-Tyr-Gly-Gly-Phe-Leu	0.34	60.00
Tyr-Gly-Gly-Phe	$\leq$ 0.01	$<$ 0.01
Gly-Gly-Phe-Met	0.81	$<$ 0.01
Gly-Gly-Phe-Leu	$\leq$ 0.01	0.16
Gly-Gly-Phe-Nor-Leu	$\leq$ 0.01	0.02
Tyr-Gly-Gly	$\leq$ 0.01	$<$ 0.01
Gly-Phe-Leu	$\leq$ 0.01	0.03
Phe-Met	$\leq$ 0.01	$<$ 0.01
Phe-Leu	$<$ 0.01	$<$ 0.01

The cross-reactivities have been calculated from the concentrations of the unlabelled peptides which decrease the binding of ^{3}H-met-enk and ^{3}H-leu-enk to the respective antisera by 50%. The values represent the affinity of the peptides for the antisera relative to met-enk or leu-enk in percent.

terminus of the enkephalin (in which they structurally differ). This is also evident in the fact that replacement of the amino-acid leucine in leu-enk by nor-leucine (which is structurally more similar to methionine) remarkably decreases the affinity of the peptide for the leu-enk antiserum, but considerably increases that for the met-enk antiserum (see Table 1).

On the other hand, the C-terminal specific antisera also appear to recognize enkephalins with amino-terminal extensions. This is at least true for the leu-enk antiserum which recognizes Tyr-Gly-Gly-Phe-Leu and Arg-Tyr-Gly-Gly-Phe-Leu to a similar extent.

Since putative amino-terminal extension analogues in rat pituitary tissue would be co-determined by our radioimmunoassays, we measured met- and leu-enk concentrations in the anterior lobe and the combined intermediate/posterior lobes after extraction

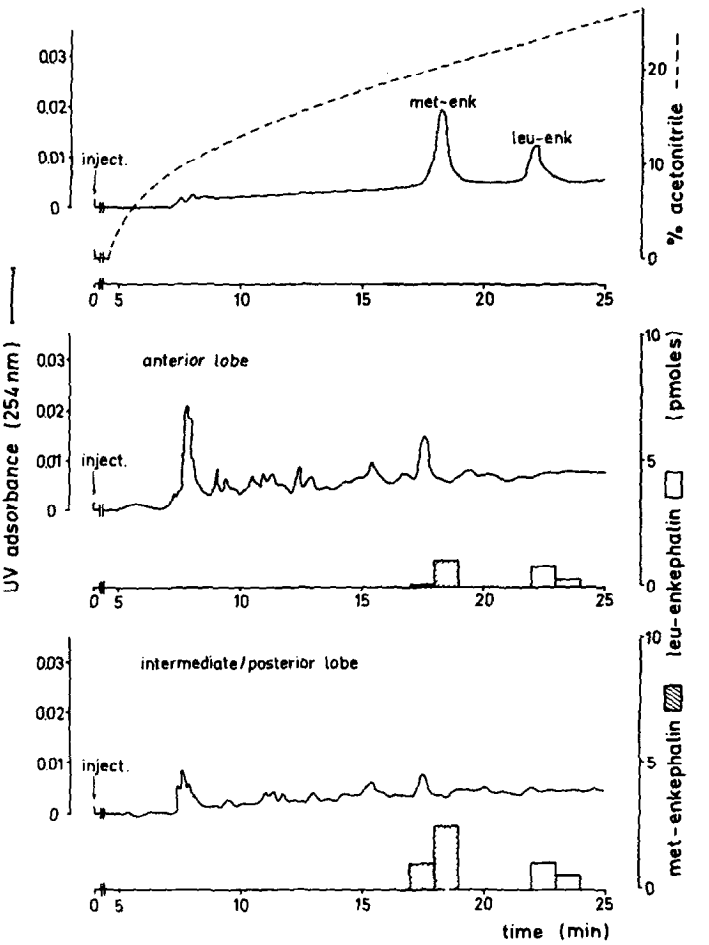

FIGURE 2: Chromatography of XAD-2 eluate from the extract of anterior and intermediate/posterior lobes of rat pituitary on a μ-Bondapak C_{18} column (3.9 x 300 mm). 10 anterior lobes and 10 intermediate/posterior lobes were extracted as described under Methods. The column was eluted with a 30 min nonlinear gradient from 0.01 M sodium acetate to 0.01 M sodium acetate/30% (vol/vol) acetonitrile at a flow rate of 2 ml/min. Immunoreactive met-enk and leu-enk content of aliquots representing a half of an anterior lobe (5 mg) or a half of an intermediate/posterior lobe (0.8 mg) are shown. Upper panel: UV absorbance of 10 μg methionine- and 5 μg leucine-enkephalin.

by XAD-2 polysterene beads and separation by reversed phase chromatography. Figure 2 shows that met-enk and leu-enk can be separated by the chromatographic system used (top panel). Enkephalin-like immunoreactivity in the extracts of anterior lobes (intermediate panel) and of intermediate/posterior lobe (bottom

panel) elutes with the same retention time as synthetic met- and leu-enk. The enkephalin concentrations in the pituitary lobes after reversed phase chromatography were close to those found by direct measurements (met-enk: 0.4 c.f. 0.5 pmole/mg anterior lobe and 6.6 c.f. 7.4 pmole/mg intermediate/posterior lobe; leu-enk: 0.3 c.f. 0.4 pmole/mg anterior lobe and 2.1 c.f. 2.9 pmole/mg intermediate/posterior lobe). This finding largely excludes the possibility that substantial amounts of compounds other than the enkephalins were co-determined by our radioimmunoassays.

Table 2 shows that immunoreactive met- and leu-enk are differently distributed within the rat pituitary when compared with immunoreactive ß-endorphin. Both the enkephalins and ß-endorphin are predominantly concentrated in the pars intermedia, but whereas the lowest levels of ß-endorphin are found in the posterior lobe, those of enkephalin are lowest in the anterior lobe.

The concentrations of met-enkephalin (about 0.7 pmole/mg) and of leu-enkephalin (about 0.4 pmole/mg) in the pars intermedia of the rat pituitary are of the same order of magnitude as those in the hypothalamus of the rat (met-enk: 0.5-0.8; leu-enk: 0.1-0.3 pmole/mg), and in the corpus striatum (met-enk: 0.8-1.5; leu-enk: 0.2-0.4 pmole/mg), brain areas, in which the highest enkephalin concentrations in the brain have been found (1,5,6).

These high enkephalin concentrations in the pituitary are in contradiction to bioassay- (2), radioimmunoassay- (7) and immunohistochemical studies (9,10), but, however, in agreement with results recently published by Rossier et al. (12), Rubinstein et al. (13) and Gramsch et al. (9). This discrepancy might be due the different extraction conditions used. The preboiling of the pituitary tissue before homogenizing used in our experiments, which has been reported to reduce the enzymatic degradation of

TABLE II: Distribution of immunoreactive met-enk, leu-enk and ß-endorphin within the rat pituitary gland

	Pars anterior	Pars intermedia	Pars nervosa
Met-enk pmoles/mg tissue	0.51 ± 0.13	6.94 ± 2.48	2.18 ± 0.79
Leu-enk pmoles/mg tissue	0.36 ± 0.19	3.95 ± 1.12	2.18 ± 1.19
ß-Endorphin pmoles/mg tissue	43.19 ± 8.67	243.89 ± 65.75	8.63 ± 1.37

Peptide concentrations have been measured by RIA as described in Methods. Each value represents the mean ± S.D. of 6 determinations.
Tissue weight: Pars anterior 10.9 ± 1.4 mg; pars intermedia 1.0 ± 0.2 mg; pars nervosa 0.8 ± 0.2 mg.

ß-endorphin (17), might also be effective in preventing the break down of the enkephalins. The different distribution of met-enkephalin compared to that of ß-endorphin suggests that the pentapeptide might not be regarded as a break down product of the larger peptides. The possibility that met-enk might be generated from ß-endorphin by our extraction procedure has been ruled out by control experiments: After the addition of ß-endorphin to the pituitary tissues prior to boiling and extraction no formation of any met-enk could be observed. Another argument against the hypothesis that enkephalins in the pituitary represent an epiphenomenon of the ß-endorphin break down is the presence of leucine-enkephalin, for which no ß-endorphin analogue has been found in rat pituitary (19). The much higher concentrations of the enkephalins found in the posterior lobe as compared to the anterior lobe correspond very well with the distribution of the density of opiate receptors in the pituitary gland (3). Although the physiological role of the high density opiate receptors in the posterior lobe is not known as yet, one is tempted to speculate that the enkephalins are candidates as endogenous opioid ligands for these receptors.

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