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Isolation and characterization of the opioid peptides from rat pituitary: β-Endorphin*

(fluorescence/high-performance liquid chromatography/peptide maps)

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ABSTRACT **β-Endorphin was isolated from 200 rat pituitaries by means of high-performance column chromatography, using sensitive fluorometric methods and a radioreceptor assay for opioid activity. The β-endorphin was characterized as to molecular weight, amino acid composition, and mapping of tryptic peptides by a new microtechnique. It was found that rat β-endorphin is identical to camel and sheep β-endorphin. Furthermore, α-endorphin, Met-enkephalin, a nonpeptide morphine-like compound (MLC), and an additional unidentified opioid compound were detected in the extract of rat pituitary.**

Several structurally related peptides with opioid activity have recently been isolated, mainly from large animals after slaughter or from human cadavers. Initial physiological and pharmacological studies are typically carried out with laboratory animals such as the rat and, therefore, it is desirable to characterize the opioid compounds in this species. The first paper in this series described the isolation and characterization of β-lipotropin from rat pituitaries (1). In addition, several other compounds with opioid activity were observed. These included a precursor larger than β-lipotropin, as well as lower molecular weight substances.

The present work describes the purification to homogeneity and characterization of rat β-endorphin. Furthermore, evidence for the presence and identity of other low molecular weight opioid compounds is given. A novel procedure for obtaining a pattern of tryptic peptides by the use of high-performance liquid chromatography is also presented.

MATERIALS AND METHODS

Frozen male Wistar rat pituitaries were obtained from Pel-Freez Biochemicals, Rogers, AR. Camel β-endorphin and sheep β-lipotropin were contributed by C. H. Li of the Hormone Research Laboratory, University of California, San Francisco. Other peptides were obtained from Peninsula Laboratories, San Carlos, CA. Fluorescamine was from Hoffman-La Roche, Nutley, NJ. Water was purified by a system obtained from Hydro Services and Supplies, Durham, NC. The Partisil SCX column was obtained from Whatman, Clifton, NJ, and the Lichrosorb RP-18 column was obtained from Altex Scientific, Berkeley, CA. Pyridine and acetic acid were distilled twice over ninhydrin. All column buffers contained thiodiglycol (0.01%) and pentachlorophenol (0.0001%), both obtained from Pierce Chemical Co., Rockford, IL. Polypropylene tubes and silane-treated glassware were used for peptide solutions.

An automated fluorescence detection system was used for column monitoring of peptides (2), and amino acid analyses were performed on a fluorescamine analyzer as previously described (3, 4). The high-pressure liquid chromatography system for peptide chromatography included an LKB (Hicksville, NY) gradient mixer, a Milton Roy (Riviera Beach, FL) minipump, and a high-pressure sample injection valve, Altex Scientific (Berkeley, CA). Opioid activity was measured by a radioreceptor assay using neuroblastoma × glioma cells and [^{3}H]Leu-enkephalin.† The activity is reported in Leu-enkephalin equivalents.

RESULTS

Isolation and characterization of β-endorphin

Two hundred frozen pituitaries (2.8 g) were homogenized in 10 ml of cold 75% acetone/25% (vol/vol) 0.2 M hydrochloric acid containing 0.01% thiodiglycol and 0.001% phenylmethyl sulfonyl fluoride. The homogenate was centrifuged and the precipitate was extracted with an additional 5 ml of the acid/acetone mixture. Acetone was removed from the pooled extracts under a stream of nitrogen. Lipids were extracted four times with 5-ml volumes of ethyl acetate/ether (3:1 vol/vol). The aqueous phase was centrifuged and the supernatant was concentrated to 2 ml by lyophilization.

Initial fractionation was on Sephadex G-75 with 1 M acetic acid for elution. The bulk of the activity (3.5 nmol of Leu-enkephalin equivalent units) was present in fractions 44–54 (Fig. 1). This position was closer to the α-endorphin marker than to the β-endorphin marker. An additional peak was found in the salt volume. Following trypsin digestion (1) of aliquots from the G-75 column, a small peak of activity was detected in the β-lipotropin region.

Fractions 44–54 from the G-75 column were pooled and lyophilized. The residue was dissolved in 600 μl of 1 mM HCl and fractionated on a Lichrosorb RP-18 (reverse-phase) column. The major peak of activity (3 nmol in Leu-enkephalin equivalents) was eluted at the same position as the camel β-endorphin marker. Minor peaks of activity were also obtained (Fig. 2). Because α-endorphin and β-endorphin were well separated on the reverse-phase column, the major peak of activity was identified as β-endorphin rather than α-endorphin as it appeared in Fig. 1. To obtain the peptide pure enough for characterization, the material in fractions 23 and 24 (Fig. 2) was combined and lyophilized. The residue was dissolved in 600 μl of 1 mM HCl and separated on Partisil SCX. The opioid activity was localized in fractions 17–26 (Fig. 3), which were pooled and concentrated by lyophilization to a final volume of 0.8 ml. Because the SCX column did not achieve the expected resolution, an additional chromatography step was introduced.

The final purification was on Lichrosorb RP-18 with a shallower gradient than the one used in the previous run (Fig.

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* This is paper no. 2 in a series. Paper no. 1 is ref. 1.

† L. D. Gerber, S. Stein, M. Rubinstein, J. Wideman, and S. Udenfriend (1978) *Brain Res.*, in press.

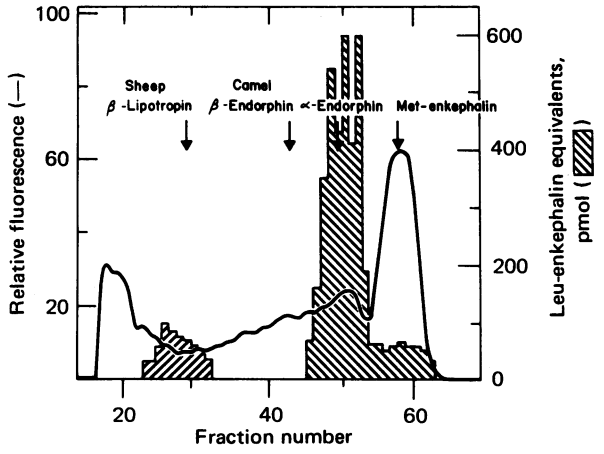

FIG. 1. Gel filtration of an acid/acetone extract of 200 pituitaries on a Sephadex G-75 column (1.6 × 40 cm) at 25°. One-molar acetic acid was used for elution, 1.5-ml fractions were collected, and 0.6% of the column effluent was directed to the fluorescamine monitoring system. Aliquots of fractions 15–40 (10 μl) were treated with trypsin and assayed for opioid activity. Aliquots of fractions 41–65 (10 μl) were assayed directly for opioid activity.

2) on this column. Two major peaks were observed, with most of the activity localized in fraction 34 (Fig. 4). This material (at least 800 pmol in Leu-enkephalin equivalent units) was used for chemical characterization.

The molecular weight of the purified peptide was determined on Bio-Gel P-30, using known linear peptides as standards. It was found to be 3500, very close to that of camel β-endorphin (Fig. 5). As shown in Table 1, the amino acid composition of the rat β-endorphin corresponded to that of camel β-endorphin. On the basis of amino acid analysis, 2.5 nmol of pure β-endorphin was present in fraction 34.

To further establish the identity of the two peptides, tryptic digests of the rat and camel β-endorphins were compared. Each peptide (600 pmol) was treated for 12 hr at 37° with trypsin [0.1 μg in 0.2 ml of *N,N*-bis(2-hydroxyethyl)glycine (Bicine) buffer, 50 mM, pH 8.5], and the resulting peptide mixture was chromatographed on a Lichrosorb RP-18 column. Identical patterns were obtained with the tryptic digests, as well as with an equimolar mixture of the two digests (Fig. 6), confirming the identity of camel and rat β-endorphin.

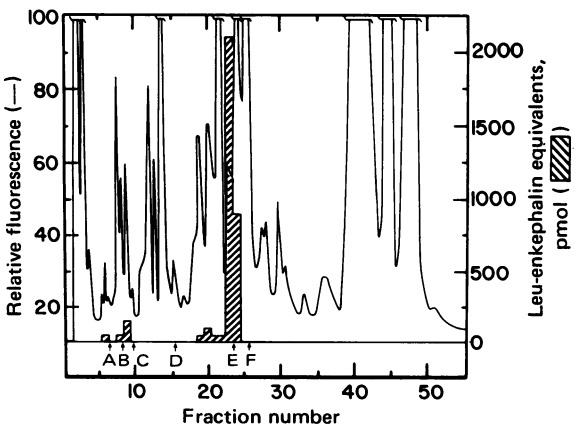

FIG. 2. Chromatography of fractions 44–54 from the Sephadex G-75 column on a Lichrosorb RP-18 column (3.2 × 250 mm) at 25°. The column was eluted with a 120-min linear gradient from 1 M pyridine/0.5 M acetic acid to 1 M pyridine/0.5 M acetic acid/40% (vol/vol) 1-propanol. A portion (5%) of the column effluent was directed to the fluorescamine monitoring system. The flow rate was 46 ml/hr and aliquots (50 μl) of each fraction (1.5 ml) were taken for the receptor binding assay. Known opioid peptides were used as markers: A, Met-enkephalin; B, Leu-enkephalin; C, α-endorphin; D, γ-endorphin; E, camel β-endorphin; F, sheep β-lipotropin.

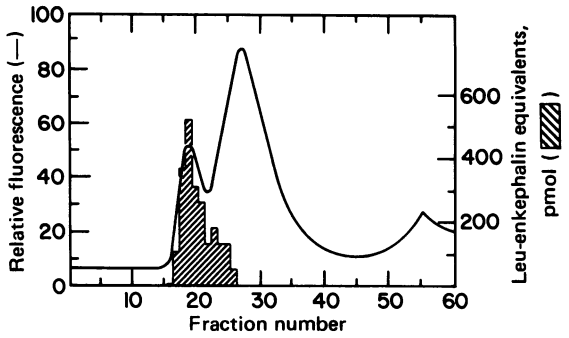

FIG. 3. Chromatography of fractions 23 and 24 from the Lichrosorb RP-18 column on a Partisil SCX column (4.6 × 250 mm) at 55°. The column was eluted with a 120-min linear gradient from 0.005 M pyridine/0.0025 M acetic acid to 3 M pyridine/1.5 M acetic acid. A portion (5%) of the column effluent was directed to the fluorescamine monitoring system. The flow rate was 50 ml/hr and aliquots (40 μl) of each fraction (1.65 ml) were taken for the receptor binding assay.

Low molecular weight opioid activity

To characterize the remaining opioid compounds, an extract from 40 fresh anterior pituitaries was prepared and fractionated on a Sephadex G-75 column as previously described. Fractions corresponding to a molecular weight of 4000 and lower were combined and lyophilized and the residue was separated on a Lichrosorb RP-18 column with the 1-propanol gradient (Fig. 7). In addition to β-endorphin (200 pmol),‡ four other peaks of activity were observed. Using known opioid peptides as markers, α-endorphin (130 pmol)‡ and Met-enkephalin (170 pmol)‡ were tentatively identified. A third peak of activity (12 pmol)‡ eluted in the void volume of the Lichrosorb RP-18 column. It was identified as the morphine-like compound (MLC)§ by its crossreactivity with antiserum to morphine. This material, which is not a peptide, has been found by Spector and his colleagues in brain (6) and urine (7). The fourth peak of activity (55 pmol),‡ apparently a peptide, did not comigrate with any of the peptide markers. Opioid activity corresponding to γ-endorphin was below the limits of detection of the methods that were used, while the activity corresponding to Leu-enkephalin was 5 pmol.‡

DISCUSSION

In the previous report rat β-endorphin was identified chromatographically in an extract of 40 rat anterior pituitaries that had been removed and frozen within 30 sec after death. However, only 80 pmol of β-endorphin (in Leu-enkephalin equivalent units) was detected. The present isolation procedure was scaled up in order to obtain sufficient material for chemical characterization. However, the relative proportions of the various components in the frozen glands was found to be quite different from that in the fresh glands. The β-endorphin content was increased 10-fold,¶ that of β-lipotropin was decreased to

‡ The values are reported in Leu-enkephalin equivalent units.

§ Aliquots from the same sample were analyzed with a morphine radioimmunoassay. The peak was found to contain 1.2 pmol of morphine equivalents. All other fractions had no activity in this assay.

¶ The difference found in the levels of β-endorphin may be explained, in part, by the fact that whole pituitaries were used in this study, whereas anterior pituitaries were used in the previous study.

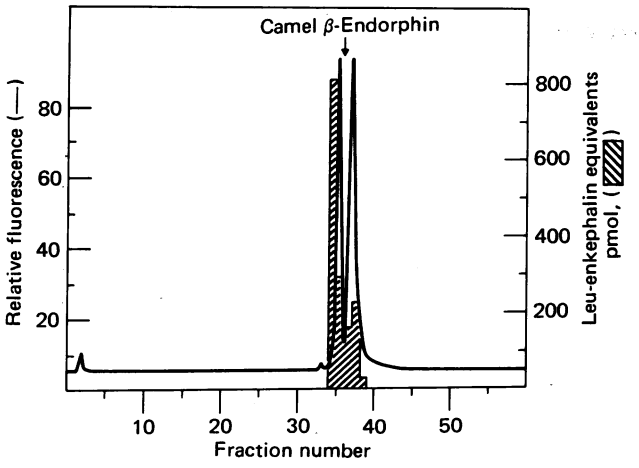

FIG. 4. Chromatography of fractions 17–26 from the Partisil SCX column on a Lichrosorb RP-18 column (3.2 × 250 mm) at 25°. The column was eluted with a 120-min gradient from 1 M pyridine/0.5 M acetic acid to 1 M pyridine/0.5 M acetic acid/20% (vol/vol) 1-propanol. A portion (5%) of the column effluent was directed to the fluorescamine monitoring system. The flow rate was 43 ml/hr and aliquots (20 μl) of each fraction (1.44 ml) were taken for the receptor binding assay. Arrow indicates camel β-endorphin position.

1/30, and the "large" opioid precursor was not detectable. In general, the fluorescence pattern of the Sephadex G-75 column (Fig. 1) showed a shift towards the lower molecular weight region. It was therefore concluded that at some time in its history the commercial pituitaries had been subjected to extensive autolysis. It should be borne in mind that such autolysis may generally take place when dealing with larger animals due to the rather long time required for the removal and workup of tissues from slaughterhouse material. Because most studies on pituitary factors have been performed with tissues from slaughterhouse animals, it is possible that much of the data accumulated over the last 40 years on peptide content is artifactual, at least in quantitative terms.

Although it was artifactual, the unexpected increase in the amount of β-endorphin in the frozen glands facilitated its isolation and characterization. All the analyses that were carried out show that rat β-endorphin is identical to camel, and hence sheep, β-endorphin. It therefore follows that the smaller fragments, e.g., α-endorphin, must also be identical in these species.

Using the high resolving power of reverse-phase chromatography, we were able to show that rat anterior pituitary

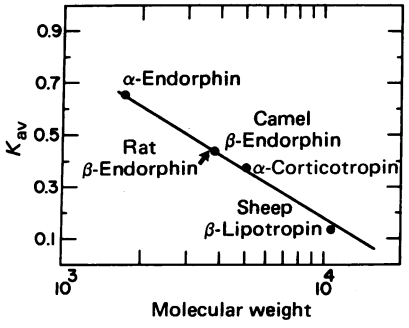

FIG. 5. Estimation of the molecular weight of rat β-endorphin by gel filtration. K_{av} is the partition coefficient. Samples (about 100 pmol each) of known peptides (corticotropin, ACTH, was human) and rat β-endorphin were applied to a Bio-Gel P-30 column (0.6 × 6.7 cm). The column was eluted with 1 M pyridine/0.5 M acetic acid, and about 50% of the column effluent was directed to the fluorescamine monitoring system. The flow rate was 6 ml/hr.

Table 1. Amino acid composition of camel and rat β-endorphins

Amino acid	Camel*	Rat†
Asx	2	2.11
Thr	3	3.01
Ser	2	1.58
Glx	3	3.08
Pro	1	1.28
Gly	3	2.81
Ala	2	2.13
Cys	0	0
Val	1	1.18
Met	1	0.94
Ile	2	1.86
Leu	2	2.15
Tyr	1	1.08
Phe	2	2.22
His	1	0.88
Lys	5	5.06
Arg	0	0

* See ref. 5.

† Average of four separate analyses on 150-pmol samples. The Ile value was obtained from duplicates after 72-hr hydrolysis. The precision was ±10%. Blank values were 20% for aspartic acid, <10% for the next three amino acids, and <5% for the remainder.

contains four major peaks of opioid activity, as well as several minor peaks. β-Endorphin, Met-enkephalin, α-endorphin, and an unknown material, which eluted between Leu-enkephalin and α-endorphin, were the major peaks. The minor peaks in-

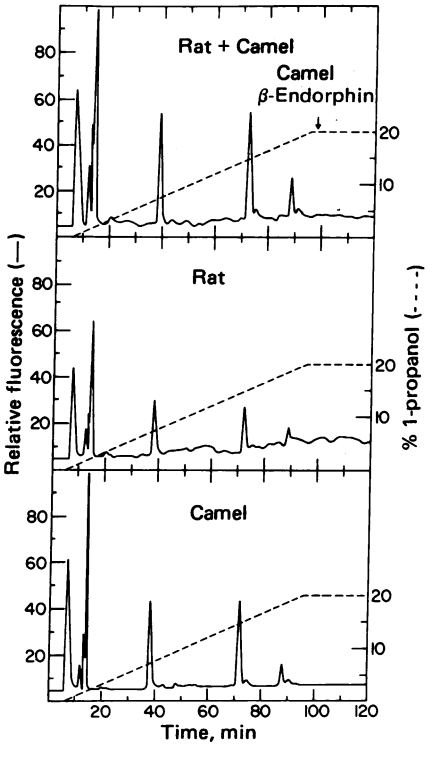

FIG. 6. Tryptic maps of rat and camel β-endorphins. Trypsin-treated samples (see *text* for details) were applied to a Lichrosorb RP-18 column (3.2 × 250 mm) at 25°. The column was eluted with a 120-min linear gradient from 1 M pyridine/0.5 M acetic acid to 1 M pyridine 0.5 M acetic acid/20% (vol/vol) 1-propanol. Essentially 100% of the column effluent was directed to the fluorescamine monitoring system. The flow rate was 14 ml/hr. Trypsin-digested β-endorphins were: (*Upper*) rat, 150 pmol, and camel, 150 pmol; (*Middle*) rat, 150 pmol; (*Lower*) camel, 250 pmol. The elution position of intact camel β-endorphin is indicated.

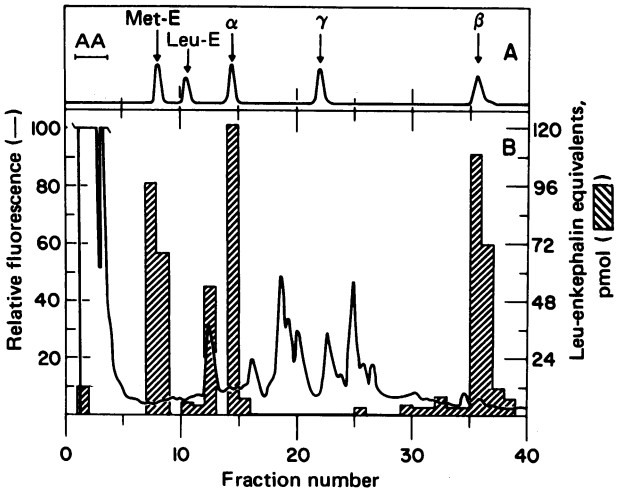

FIG. 7. Chromatography of opioid peptides on a Lichrosorb RP-18 column (3.2 × 250 mm): (*A*) A mixture of five synthetic opioid peptides. AA, amino acids; Met-E, Met-enkephalin; Leu-E, Leu-enkephalin; α, β, and γ, the respective endorphins. (*B*) Extract of 40 anterior pituitaries, from which the high molecular weight region (>4000) had been removed by gel filtration. The column was eluted with an 80-min linear gradient from 1 M pyridine/0.5 M acetic acid to 1 M pyridine/0.5 M acetic acid/16% (vol/vol) 1-propanol. A portion (5%) of the column effluent was directed to the fluorescamine monitoring system. The flow rate was 45 ml/hr and samples (0.2 ml) of each fraction (1.5 ml) were assayed for opioid activity.

cluded the morphine-like compound, as well as trace activity at the Leu-enkaphalin elution position. γ-Endorphin was not observed, even with the equivalent of five pituitaries being assayed for opioid activity.

The use of fluorescence techniques for ultra-microanalysis of peptides has once more been demonstrated and, in our laboratory, it is now a routine procedure for such applications as amino acid analysis, column monitoring, and molecular weight determination. Detailed descriptions of these procedures, all at the picomole range, were given in previous publications (1, 4).

In this study a new methodology, reverse-phase chromatography, was introduced for the comparison of tryptic digests. This method has many advantages over thin-layer chromatography and gel electrophoresis. High resolving power is possible with the columns now available. The RP-18 column is stable, i.e., there was no change in elution position of the marker peptides even after repeated use of the same column with the same set of buffers over a period of several months. Furthermore, the method is quantitative and thus relative peak heights may be compared. Finally, the resolved peptides can be collected and used for further characterization without the additional extraction steps required in the other two methods. Thus far, linear peptides up to a molecular weight of 11,000 have been resolved on this column. Recently, the method was used in our department for structural studies on the subunits (molecular weight ca 60,000 each) of prolyl hydroxylase. Over 30 discrete peaks were obtained from tryptic digests of 300 pmol of each protein (8).

We wish to thank Ms. Louise D. Gerber for carrying out the binding assay and Mr. Larry Brink for his excellent technical assistance. The morphine antibody binding assay was performed by Dr. J. Shorr of this Institute.

1. Rubinstein, M., Stein, S., Gerber, L. D. & Udenfriend, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3052–3055.
2. Böhlen, P., Stein, S., Stone, J. & Udenfriend, S. (1975) *Anal. Biochem.* **67**, 438–445.
3. Stein, S., Böhlen, P., Stone, J., Dairman, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 202–212.
4. Stein, S. (1977) in *Peptides in Neurobiology*, ed. Gainer, H. (Plenum Press, New York), pp. 9–37.
5. Li, C. H. & Chung, D. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1145–1148.
6. Ginzler, A. R., Levy, A. & Spector S. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2132–2136.
7. Blume, A., Shorr, J., Finberg, J. P. M. & Spector, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4928–4932.
8. Chen-Kiang, S., Cardinale, G. J. & Udenfriend, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4420–4424.