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Isolation and characterization of α-endorphin and γ-endorphin from single human pituitary glands

J. Peter H. Burbach and Victor M. Wiegant

Rudolf Magnus Institute for Pharmacology, Medical Faculty, University of Utrecht, Vondellaan 6, 3521 GD Utrecht, The Netherlands

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α-Endorphin and γ-endorphin, two closely related peptides of the pro-opiomelanocortin family with characteristic biological activities, were purified to homogeneity from single human pituitary glands and chemically identified. Isolation of the peptides was based on size fractionation by Sephadex G-75 chromatography followed by two HPLC steps using reverse-phase and paired-ion reverse-phase systems and was monitored by radioimmunoassay. During the isolation procedure α- and γ-endorphin-sized material behaved chromatographically and immunologically indistinguishably from synthetic α- and γ-endorphin. The amino acid composition and NH_2-terminus of isolated peptides demonstrated their identity as authentic α-endorphin and γ-endorphin. Acetylated forms were absent. In addition, evidence is provided that large forms with α- and γ-endorphin immunoreactivity detected during gel filtration are human lipotropin-(1–74) and -(1–75), respectively. The data substantiate that α-endorphin and γ-endorphin exist as endogenous peptides in the human pituitary gland.

α-Endorphin *γ-Endorphin* *Human pituitary gland* *β-Endorphin fragment* *Peptide isolation*

1. INTRODUCTION

The β-endorphin fragments γ-endorphin (β-endorphin-(1–17)) and α-endorphin (β-endorphin-(1–16)) have originally been identified as opioid peptides in an extract of hypothalamic-neurohypophyseal origin (Pitressin Intermediate) [1]. Now, γ- and α-endorphin have been established as neuropeptides with distinct behavioural and pharmacological activities independent of their opioid properties [2–4]. The endogenous presence of γ-endorphin and α-endorphin in pituitary and brain tissue has been indicated in studies using radioreceptor assays and immunological methods [5–9] and more directly demonstrated with combined chromatographic and immunological techniques [10–12]. However, the chemical nature of these peptides has not been elucidated so far. We have now achieved the complete characterization of native human γ-endorphin and α-endorphin purified from single pituitary glands. These data demonstrate that γ-endorphin and α-endorphin are naturally occurring peptides in the human pituitary gland.

2. MATERIALS AND METHODS

2.1. *Extraction procedure*

Two human pituitaries were obtained by autopsy at 7 h and 0.5 h post mortem, frozen on dry ice and stored at −80°C. The pituitary glands were extracted separately by boiling in 5 ml of 1 M acetic acid for 10 min, cooling to 0°C and homogenization in a Teflon-glass homogenizer. The homogenate was centrifuged at 15000 × g_{av} for 20 min and the pellet re-extracted with 5 ml of 1 M acetic acid and centrifuged again. The supernatants from the two centrifugations were combined and lyophilized.

2.2. *Gel filtration*

Each lyophilized pituitary extract was

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reconstituted in 600 μl of 50% (v/v) acetic acid and particles were removed by centrifugation at 15000 $\times$ g_{av} for 15 min. The clear extract was loaded on a Sephadex G-75 superfine column (dimensions 100 cm $\times$ 1.6 cm) in 50% (v/v) acetic acid and eluted at a flow rate of 1.5–2.0 ml/h. Fractions of about 1 ml were collected and aliquots were taken for radioimmunoassay determination. Guided by the radioimmunoassay data fractions were pooled and dried in vacuo at 60°C.

2.3. *High-performance liquid chromatography*

Two HPLC systems based on gradient elution of μBondapak C18 reverse phase columns (30 cm $\times$ 0.39 cm; 10 μm particle size; Waters Associates) were used. Samples in 0.1 M acetic acid, were first chromatographed with 10 mM ammonium acetate, pH 4.15 (solvent A) and methanol containing 1.5 ml acetic acid/l (solvent B) in the mobile phase. The linear gradient ran from 25 to 80% B in 55 min with a flow rate of 2.0 ml/min. Final purification of peptides was achieved by gradient elution of a μBondapak C18 column with 0.13% (v/v) heptafluorobutyric acid (HFBA) in water (solvent X) and 0.05% (v/v) HFBA in methanol (solvent Y). For γ-endorphin the gradient ran from 45 to 55% Y in 10 min; for α-endorphin from 40 to 55% Y in 10 min with a flow rate of 1.5 ml/min. In each HPLC step fractions of 30 s were collected and aliquots taken for radioimmunoassay determination.

2.4. *Amino acid analysis*

Peptide (100–300 pmol) was hydrolyzed in 6 M hydrochloric acid containing 0.5% (v/v) thioglycollic acid as in [13]. Amino acid analysis

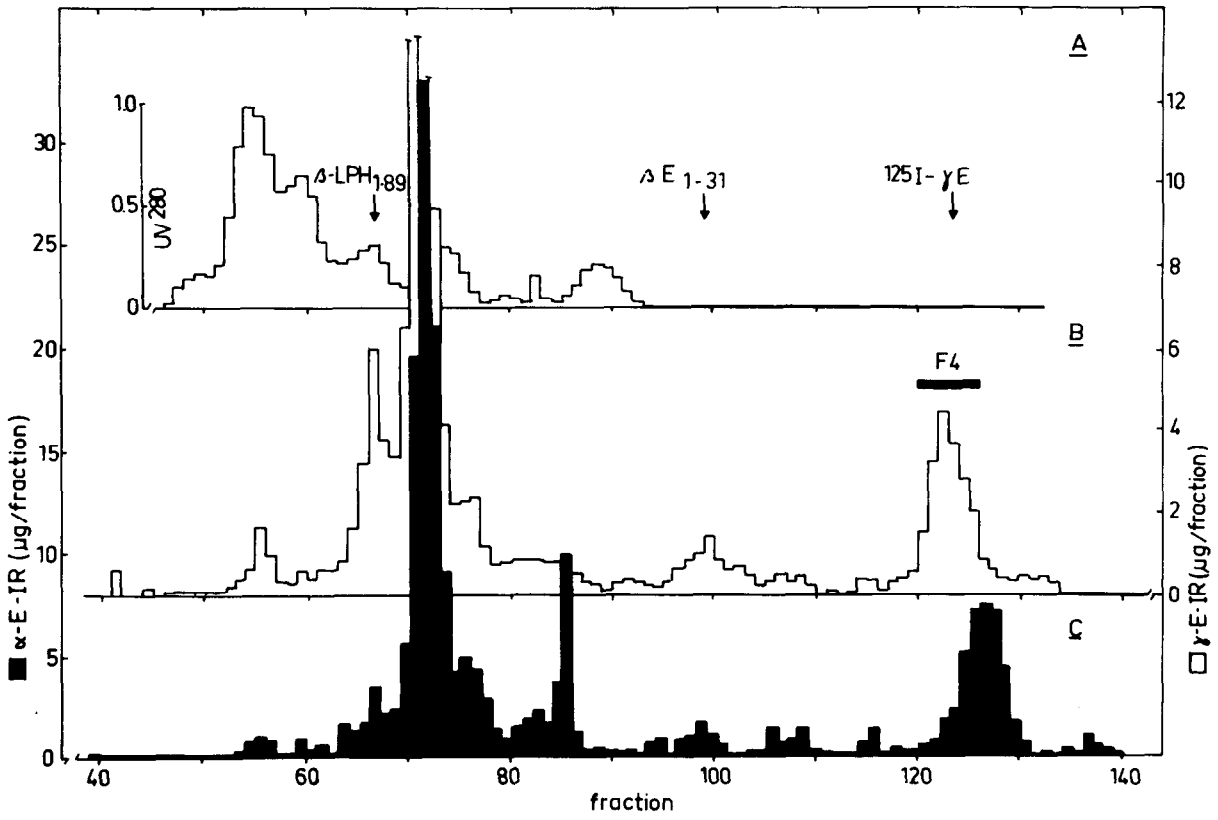

Fig.1. Gel filtration of a human pituitary extract on Sephadex-G75 superfine and profiles of γ-endorphin-like (B) and α-endorphin-like (C) immunoreactive components. (A) shows the profile of UV^{260} absorbing material. The arrows indicate the elution position of human β-lipotropin (β-LPH-(1–89)) and β-endorphin (βE-(1–31)), as determined by a radioimmunoassay system for β-endorphin, and of γ-[^{125}I]endorphin. Fraction F4 was used for further purification.

was performed by reverse-phase HPLC of *o*-phthaldialdehyde derivatives on a CP-Spher C8 column (25 cm × 0.46 cm; 7 μm particle size, Chrompack), eluted by a gradient of 100 mM sodium citrate (pH 6.7) containing 2% (v/v) tetrahydrofuran, and methanol [13]. End groups were determined by dansylation.

2.5. *Radioimmunoassay systems*

Two separate radioimmunoassay systems for γ- and α-endorphin, respectively, were used to monitor the purification of peptides. The radioimmunoassay for γ-endorphin employed antiserum L_2 which specifically recognized the sequence β-endorphin-(9–17) with free COOH-terminus. The cross-reaction of antiserum L_2 on weight basis was 8% with α-endorphin and 4% with β-endorphin-(1–31). In the radioimmunoassay for α-endorphin antiserum A_2 was used, specifically recognizing the COOH-terminal sequence β-endorphin-(9–16); cross-reaction with γ-endorphin was 2% and with β-endorphin 1%. Details of the specificity of the antisera and of the assay procedures have been described [14,15].

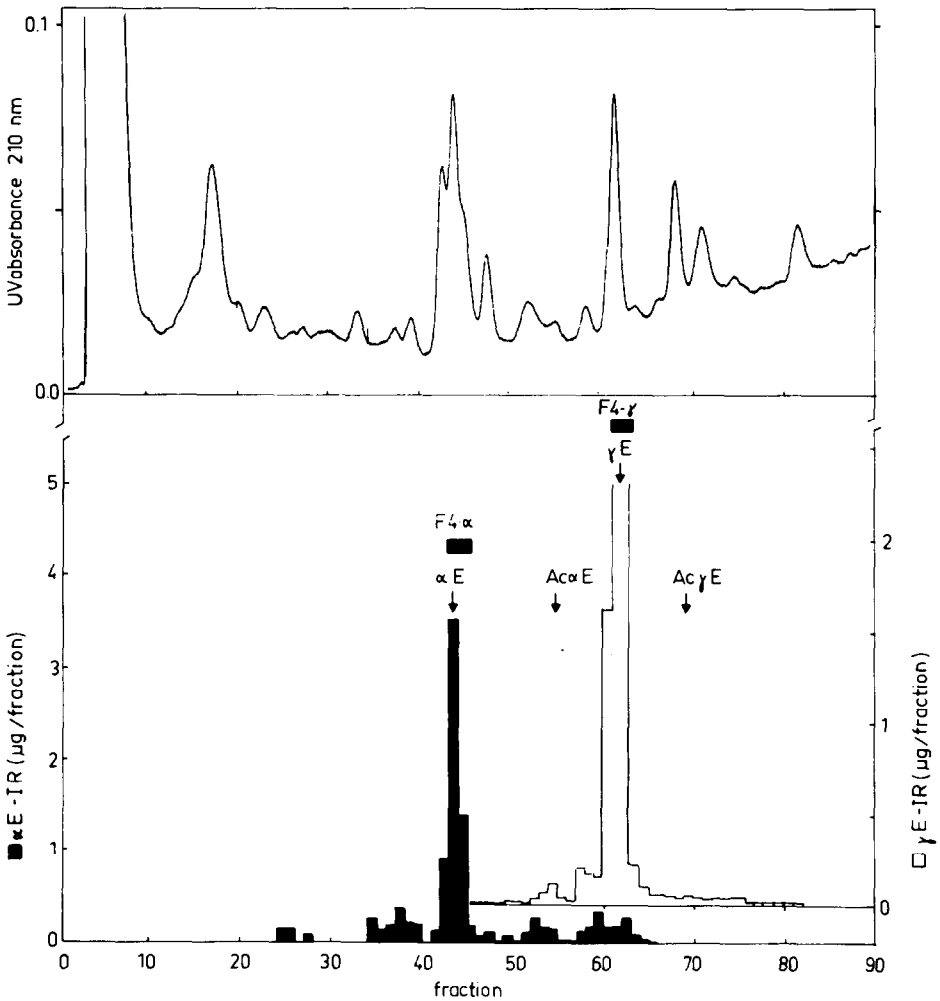

Fig.2. Fractionation of fraction F4 by HPLC using ammonium acetate in the mobile phase. (A) UV profile of eluting material and elution position of synthetic α-endorphin (αE), γ-endorphin (γE) and their N^{α}-acetylated forms (AcαE, AcγE) (B). Distribution of α-endorphin-like (dark area) and γ-endorphin-like (open area) immunoreactive peptides. The lag time between UV detection and fraction collection was about 10 s. Fraction F4-α (tubes 44,45) and F4-γ (tubes 63,64) were subjected to further purification.

3. RESULTS

Gel filtration on Sephadex G-75 superfine of an acid extract of a single human pituitary resulted in separation of two γ-endorphin immunoreactive components with different molecular size and two with α-endorphin immunoreactivity (fig.1). The larger γ-endorphin immunoreactive component (top: fraction 71; fig.1B) eluted 5 fractions later than human β-lipotropin-(1–89). The smaller γ-endorphin immunoreactive form co-eluted with γ-[^{125}I]endorphin. The large α-endorphin immunoreactive component had the top in fraction 72 (fig.1C), while the smaller component eluted slightly later than γ-[^{125}I]endorphin.

Fractions 121–126 having the smaller γ-endorphin immunoreactive component were pooled (coded F4, fig.1), and contained about half of the total quantity of the smaller α-endorphin immunoreactive form. HPLC fractionation of fraction F4 yielded single peaks of γ-endorphin- and α-endorphin-like immunoreactivity (coded F4-γ and F4-α, respectively; fig.2). Final purification of peptides F4-γ and F4-α was achieved by HPLC with HFBA in the mobile phase (fig.3). The amino acid composition and end group of peptides F4-γ and F4-α indicated the high purity of the isolated peptides and demonstrated that they represented authentic γ-endorphin and α-endorphin, respectively (table 1). Their identity was in agreement with their co-migration with synthetic γ- and α-endorphin in all analytic systems used.

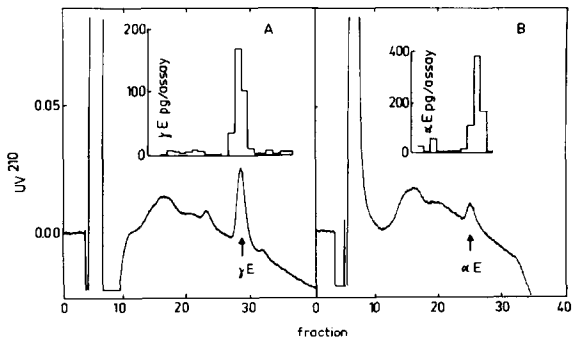

Fig.3. Final purification of fraction F4-γ (γ-endorphin; A) and F4-α (α-endorphin, B) by HPLC using HFBA in the mobile phase. The inset shows the profile of immunoreactivity. The arrows indicate the elution position of synthetic γ-endorphin (A) and α-endorphin (B).

Table 1

Amino acid analysis of native human γ-endorphin and α-endorphin isolated from single human pituitary glands

Amino acid[a]	Peptide F4-γ			Peptide F4-α		
Asp	0.04	0.01	(0)	b.d.	b.d.	(0)
Glu	2.01	1.83	(2)	2.24	2.08	(2)
Ser	2.23	1.82	(2)	1.87	1.91	(2)
His	b.d.	b.d.	(0)	b.d.	b.d.	(0)
Arg	b.d.	b.d.	(0)	0.05	b.d.	(0)
Gly	1.93	1.87	(2)	2.01	2.13	(2)
Thr	2.66	2.96	(3)	2.82	2.81	(3)
Ala	0.01	0.01	(0)	0.05	0.05	(0)
Tyr	1.22	1.22	(1)	1.14	1.09	(1)
Met	0.94	1.03	(1)	0.88	0.93	(1)
Val	1.03	1.09	(1)	1.03	0.95	(1)
Phe	0.93	1.03	(1)	1.06	1.05	(1)
Ile	0.02	b.d.	(0)	b.d.	b.d.	(0)
Leu	2.00	2.18	(2)	0.98	1.06	(1)
Lys	1.00	0.96	(1)	1.04	1.11	(1)
End group	Tyr			Tyr		
Proposed sequence	β-endorphin-(1–17) γ-endorphin			β-endorphin-(1–16) α-endorphin		

[a] Trp and Pro are not determined by the amino acid analysis technique [13]

b.d., below detection

Values represent molar ratios obtained from peptides isolated from two individually processed pituitary glands. Values in brackets represent the theoretical number of residues

The isolation of γ- and α-endorphin was undertaken twice and yielded 2.2 nmol γ-endorphin and 0.7 nmol α-endorphin from a human pituitary obtained 7 h post mortem and 3.0 nmol and 0.9 nmol, respectively, from a pituitary obtained only 0.5 h post mortem as estimated by UV absorption of the purified peptide in comparison with synthetic peptide. The values of α-endorphin represent about half of the maximal yield of α-endorphin due to the cut of fraction F4 (fig.1).

4. DISCUSSION

There is considerable evidence for the endogenous presence of neuropeptides related to γ- and α-endorphin in rat pituitary and brain. Firstly,

opioid peptides with the size of γ- and α-endorphin have been detected in rat anterior and neurointermediate pituitary tissue [5,6]. Secondly, using antisera for γ- or α-endorphin, immunoreactive peptides have been detected by radioimmunoassay [7–9] or by immunohistochemical techniques [9,16–18]. Thirdly, by combining radioimmunoassay and HPLC we measured peptides in rat pituitary and brain with properties indistinguishable from those of authentic γ- and α-endorphin [10–12]. Recently, we found that in addition to these peptides N^{α}-acetylated γ- and α-endorphin are present in the rat neurointermediate pituitary and extrahypothalamic brain areas [12]. The characterization of native γ-endorphin and α-endorphin isolated from human pituitary reported here demonstrates the chemical nature of these peptides being the free NH_2 terminal 1–17 and 1–16 sequence of β-endorphin, respectively. We failed to detect N^{α}-acetylated forms of these peptides in the HPLC eluate by using radioimmunoassays for γ- and α-endorphin (fig.2) and a radioimmunoassay specifically recognizing the N^{α}-acetylated terminus of endorphins (antiserum Nancy Beth 51, donated by Drs Watson and Akil). Thus, we conclude that γ- and α-endorphin with free NH_2 termini are naturally occurring fragments of β-endorphin in the human pituitary gland. Biological activities of γ- and α-endorphins in the central nervous system have been demonstrated [2–4]. The γ-endorphin isolated here is biologically active in a behavioural test (not shown). However, the physiological significance of these peptides in the human pituitary remains unknown as yet.

Recently, the isolation of β-endorphin-(1–18) from human pituitary glands has been reported [19]. In our experiments we did not detect significant amounts of this peptide.

We have achieved the chemical characterization of γ- and α-endorphin from a single human pituitary by combining effective HPLC procedures that allow good recovery of peptides with sensitive analytical techniques. We have chosen gel filtration as the initial step to separate the α/γ-endorphin sized material from a large bulk of polypeptides with larger size. By gel filtration large forms of γ- and α-endorphin-like immunoreactivity shortly eluting after human β-lipotropin (β-lipotropin-(1–89)) were detected. After trypsin digestion of the large γ-endorphin immunoreactive component we isolated and identified the tryptic fragment β-endorphin-(10–17). The specificity of antiserum L_2 for the free COOH terminal sequence β-endorphin-(9–17) and the isolation of β-endorphin-(10–17) demonstrates that this large form contains β-endorphin-(10–17) as COOH terminal sequence. The data indicate that this component is human β-lipotropin-(1–75). Indeed, formation of its porcine equivalent β-lipotropin-(1–77) by a porcine anterior pituitary homogenate has been reported [20].

Similarly, the α-endorphin-like immunoreactive component might represent human β-lipotropin-(1–74). Assuming approximately equal cross-reaction in the radioimmunoassay systems, the molar quantities of these peptides exceed those of γ- and α-endorphin about 10-fold. A similar ratio for β-lipotropin-(1–89) and β-endorphin exists in the anterior pituitary [5,21]. This suggests that in the human pituitary β-lipotropin-(1–75) and -(1–74) serve as precursors for α- and γ-endorphin, respectively. Alternatively, α- and γ-endorphin could be produced directly from β-endorphin by proteolytic processing [22,23].

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