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CHARACTERIZATION OF β-ENDORPHIN IMMUNOREACTIVE PEPTIDES IN RAT PITUITARY AND BRAIN BY COUPLED GEL AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

MICHAEL DENNIS*, CLAUDE LAZURE, NABIL G. SEIDAH and MICHEL CHRÉTIEN
Protein and Pituitary Hormone Laboratory, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal H2W 1R7 (Canada)

SUMMARY

A procedure which combines gel high-performance liquid chromatography (HPLC) and reversed-phase HPLC in two systems with radioimmunoassay was developed for the analysis of β-endorphin (βEND)-related peptides in rat pituitary and brain. The gel HPLC system allows rapid screening for relative amounts of precursors and products involved in βEND biosynthesis. Reversed-phase HPLC reveals minor post-translational modifications, such as acetylation, which greatly alter the biological activity of βEND. The application of this coupled procedure clearly demonstrates tissue-specific differences in the processing of βEND in pituitary and brain regions and indicates that the system may be generally applicable to the analysis of neuropeptide heterogeneity.

INTRODUCTION

Attempts to characterize peptides in brain extracts have relied heavily on the combined use of high-performance liquid chromatography (HPLC), especially in the reversed-phase mode, and radioimmunoassay (RIA)[1–3]. The success of this approach derives directly from the excellent resolving power, reproducibility and sample recovery attainable with HPLC, and from the high sensitivity and specificity inherent in RIA procedures. A suitable chromatographic system must be able to detect picomoles or less of a particular neuropeptide and must provide adequate separation of structurally related molecules, such as intermediates in the biosynthetic pathway, which can react in the detection procedure. This last point becomes particularly important in cases where minor post-translational modifications of peptide structure greatly alter its biological activity.

These considerations are illustrated very well by studies conducted in our laboratory on the opiate-active peptide β-endorphin (βEND). βEND is produced in cells of the anterior and intermediate pituitary by selective proteolysis at paired basic amino acids of a large precursor, pro-opiomelanocortin (POMC)[4–6]. In the anterior pituitary, this process yields principally β-lipotropin (β-LPH), and a small amount of βEND 1-31[7]. In the intermediate pituitary, however, β-LPH is completely converted

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into βEND[8] which can be further processed by α-N-acetylation and/or cleavage of the carboxy-terminal tetrapeptide[9]. Although all forms containing the midportion βEND sequence react with most antibodies to βEND, only those possessing the free N-terminal tyrosine are opiate-active[10].

βEND-like peptides have also been described in brain[11,12]. Numerous studies indicate that the precursor for βEND in brain is similar to or identical with that from pituitary[13,14] and that its maturation resembles that occuring in intermediate lobe cells[15,16]. Multiple forms of βEND immunoreactivity have been reported in brain, including the opiate-inactive N-acetyl derivatives[17], but there is evidence to suggest that these derivatives originate in the pituitary[18].

In order to pursue the characterization of βEND-immunoreactivity in brain, we have developed a chromatographic system to separate and quantitate βEND-related peptides by coupled HPLC–RIA procedures. The system utilizes gel HPLC under denaturing conditions to provide an initial separation on the basis of molecular weight. βEND-sized material detected by RIA on the eluate is then pooled and subjected directly to reversed-phase HPLC with two elution schemes and RIA detection. The application of this procedure to extracts of anterior and intermediate pituitary and [^{3}H]βEND produced by *in vitro* biosynthetic studies illustrates the validity of the method. The results of analyses of two selected rat brain regions representing sites of synthesis (arcuate nucleus) and terminals (periaquaductal grey) indicate that the system may be generally applicable to studies on neuropeptide heterogeneity.

EXPERIMENTAL

Materials

Molecular weight standards used to construct the gel permeation calibration curve were purchased from Sigma, Pharmacia, Schwarz/Mann or Pierce. β-MSH, ACTH 1-24, Met-enkephalin and corticotropin-like intermediate peptide (CLIP) were synthesized and kindly provided by S. St.-Pierre (Shorbrooke University), Ciba-Geigy, P. Schiller (Montreal) and P. Lowry (London) respectively. Synthetic ovine βEND 1-31, N-acetyl 1-31, βEND 1-27, N-acetyl 1-27 and α-MSH were generous gifts from N. Ling (Salk Institute) as was the βEND-antibody (βENDO2) used for the RIA.

Acetonitrile (Caledon) was of HPLC quality. Heptafluorobutyric acid (HFBA) was purchased from Pierce and trifluoroacetic acid (TFA) (redistilled) from Sigma. Guanidine hydrochloride was of ultra-pure grade (Schwarz/Mann), and the triethylamine (Fisher) was redistilled. All solvents were filtered through Millipore 0.2-μm filters and degassed before use.

Apparatus

Gel HPLC was performed on four Waters protein analysis columns (2 × I125 + 2 × I60), connected in series. Reversed-phase HPLC was carried out on μBondapak C_{18} columns (30 cm × 3.9 mm, Waters). A Beckman Model 100A liquid chromatograph, Varian UV-50 spectrophotometer and Waters Model 730 data module were used for all experiments.

Preparation of tissue extracts

Male Sprague-Dawley rats (250 g, Charles River) were decapitated and the anterior and neurointermediate pituitaries were dissected and frozen in plastic tubes on dry ice. The hypothalamic arcuate nucleus and midbrain periaquaductal grey were dissected from 1.5-mm slices of fresh brain with biopsy needles and placed in plastic tubes on dry ice. Frozen tissues from 50 rats were extracted in 1.0 ml 5 *M* acetic acid containing 0.3 mg/ml iodoacetamide and phenylmethylsulfonyl fluoride by sonication for 30 sec in a Kontes cell-disrupter. The homogenates were heated at 70°C for 5 min and insoluble material was removed by centrifugation at 89,000 g_{av} for 3 h at 4°C in a Beckman L8-80 ultra-centrifuge. The supernatants were dried in a Speedvac evaporating centrifuge (Savant Co.).

Preparation of tritiated peptides

Tritiated marker peptides were obtained by the *in vitro* incorporation of [^{3}H]leucine (Amersham) into neosynthesized proteins of rat neurointermediate pituitary, followed by Sephadex G-75 chromatography as previously described[19]. Radioactive material corresponding in size to β-END, β-LPH and POMC was pooled and repurified[20] for gel-permeation studies. β-END-containing fractions were also used directly for reversed-phase HPLC without repurification.

Gel permeation HPLC

Molecular weight standards (10 μg) and samples were dissolved in 100 μl 6 *M* guanidine–HCl/0.2 *M* triethylamine phosphate/0.5% β-mercaptoethanol. The solutions were subjected to gel HPLC at 1.0 ml/min in the same buffer but lacking β-mercaptoethanol. The absorbance of the eluate was monitored at 230 nm, and 0.5-ml fractions were collected and stored at −40°C. In the case of tritiated samples, 100-μl aliquots of the fractions were counted in 2.5 ml Aquasol (New England Nuclear). To determine the positions of immunoreactive peptides, duplicate 10-μl aliquots of the fractions were subjected to RIA for βEND as described previously[19].

Reversed-phase HPLC

Radioactive and immunoreactive fractions with the apparent molecular weight of βEND 1-31 were pooled, and duplicate 1.0-ml aliquots were subjected directly to reversed-phase HPLC in two elution schemes. In the first, 0.13% HFBA–35% acetonitrile was passed through the column for 5 min isocratically and then peptides were eluted with a gradient of 35–40% acetonitrile over 50 min. The other scheme consisted in isocratic elution with 0.1% TFA–25% acetonitrile for 5 min, followed by a gradient of 25–35% acetonitrile over 50 min. In both schemes the elution positions of synthetic ovine βEND standard peptides were determined in separate experiments by applying 1–2 μg of the standards in 1.0 ml 6 *M* guanidine HCl–0.2 *M* triethylamine phosphate (pH 3.0). Thereafter, fractions of 0.5 ml were collected between 25 and 65 min and stored at −40°C. Radioactivity was measured in 2.5 ml Aquasol for tritiated samples and, in the case of tissue extracts, RIA was performed on duplicate 100-μl aliquots of fractions which had been dried under vacuum and reconstituted in 0.5 ml RIA buffer (0.05 *M* Na_2HPO_4, 150 m*M* NaCl, 0.5% human serum albumin, 0.2% Triton X-100, 1 m*M* EDTA at pH 8.0).

Radioimmunoassay

The antiserum used in this study recognizes the midportion of βEND and cross-reacts on an equimolar basis with POMC, β-LPH, βEND 1-31, βEND 1-27 and the N-acetylated derivatives of βEND. At the dilution used (1/80,000 final) it has a sensitivity of 30–50 pg/ml. Details of the RIA procedure have been described previously[19].

RESULTS

Gel permeation HPLC

Analysis of standard and tritiated peptides. The characteristics of the gel HPLC system used in this study are illustrated by the calibration curve (Fig. 1), calculated according to Himmel and Squire[21]. The system yielded a linear relationship between F(V) and $M_r^{1/3}$ (r^2 = 0.975) for proteins of M_r 500–20,000 daltons and thus provides accurate molecular weight estimates in this range. Larger proteins eluted after the void volume (*ca.* 40,000 daltons) deviate from linearity, and this leads to underestimation of their molecular weights.

The useful fractionation range, although quite limited in comparison with previous studies[19], is adequate to separate the major size classes of βEND-related peptides. As shown in Fig. 2A, tritiated rat POMC, β-LPH and βEND, isolated from rat neurointermediate lobe following incorporation of [^{3}H]leucine, were clearly resolved in this system with recoveries exceeding 90%. Apparent molecular weights of βEND (3600) and β-LPH (12,000) were in good agreement with those determined by classical methods. POMC was eluted with an apparent molecular weight of 21,000

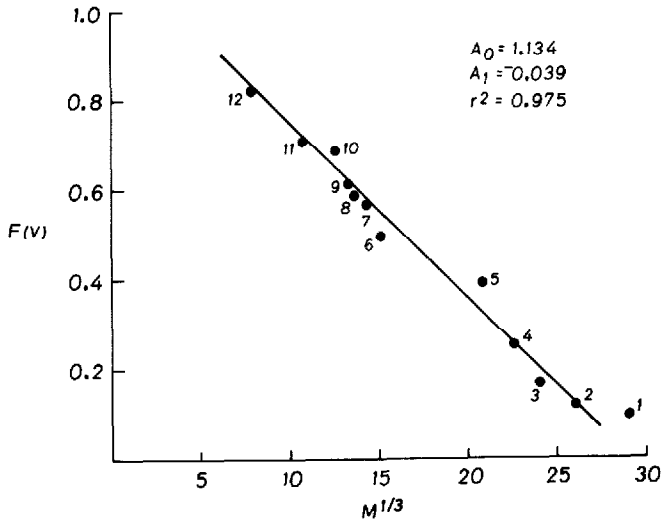

Fig. 1. Calibration curve for gel permeation. The curve was constructed as previously described[19]. Proteins used were chymotrypsinogen A (1), myoglobin (2), cytochrome *c* (3), human β-LPH (4), lima bean trypsin inhibitor (5), insulin B (6), ACTH 1-24 (7), insulin A (8), human CLIP (9), rat β-MSH (10), α-MSH (11) and Met-enkephalin (12). The V_0 and V_e were determined with thyroglobulin and DNP-glycine, respectively.

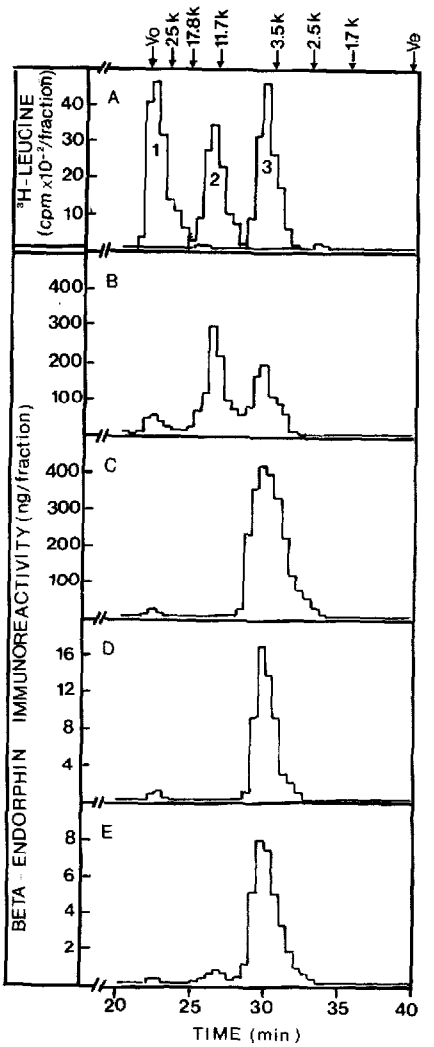

Fig. 2. Gel permeation elution profiles obtained with (A) [^{3}H]leucine-labeled POMC (1), β-LPH (2) and βEND (3), (B) βEND-immunoreactive peptides extracted from five rat anterior pituitaries, (C) five rat neurointermediate pituitaries, (D) 50 rat arcuate nuclei and (E) 50 rat midbrain periaquaductal grey. Arrows at the top of the figure indicate the elution times of selected standard proteins the molecular weights of which are expressed in daltons.

daltons, considerably lower than the true molecular weight (M_r = 31,000), because it was eluted outside the linear range of the system.

Analysis of βEND immunoreactivity in tissue extracts. The analysis of βEND immunoreactivity in extracts of rat pituitary and brain regions by gel HPLC–RIA produced the profiles shown in Fig. 2B–E. All tissues exhibited immunoreactive material corresponding in size to βEND and this represented the sole or major form in neurointermediate pituitary and the two brain regions. In the case of anterior pituitary, however, the majority of the immunoreactivity was eluted in the position of

β-LPH; a small amount of β-LPH-sized material was also observed in the periaquaductal grey. A minor peak eluted near the void volume in the position of POMC was also noted in all tissues.

Reversed-phase (RP)-HPLC

Analysis of standard and tritiated peptides. Two RP-HPLC systems, employing acetonitrile as the organic modifier and either TFA or HFBA as the ion-pairing reagent, were applied to the fractionation of βEND-related peptides. As indicated at the top of Figs. 3 and 4, each of the systems partially resolved a mixture of four synthetic ovine standards, composed of βEND 1-31 (1-31), α-N-acetyl βEND 1-31 (Ac 1-31), βEND 1-27 (1-27) and α-N-acetyl βEND 1-27 (Ac 1-27). In TFA, Ac 1-31 and 1-27 were eluted in close proximity but were well resolved by HFBA; conversely, 1-31 and Ac 1-27 were not separated by HFBA but were clearly separated by TFA. By exploiting the properties of these two ion-pairing reagents it was thus possible to obtain a complete separation of the four standard peptides, which correspond to the opiate-active form of βEND (1-31) and peptides resulting from previously demonstrated post-translational reactions that greatly reduce or abolish opiate activity. However, either system alone was insufficient to completely resolve these forms.

Tritiated βEND-sized peptides obtained following a 4-h pulse incorporation of [^{3}H]leucine into rat neurointermediate lobe cells were analyzed by reversed-phase HPLC in these two systems and the radioactivity in the eluate was monitored. The major peak of radioactivity was eluted in the position of ovine βEND 1-31 by both TFA (Fig. 3A) and HFBA (Fig. 4A), along with a minor peak in the position of Ac 1-31. These findings are in agreement with the results of kinetic studies on βEND biosynthesis in rat neurointermediate pituitary[9]. The observed chromatographic behavior of the ovine standard and the radiolabeled rat βEND 1-31 indicates that the ovine peptides are appropriate markers for these studies. It was previously reported that rat βEND differs from the ovine homolog in the substitution of a valine for an alanine unit at position 26[22]. This should have produced a shift in the elution positions in our system. This was not observed, and the amino acid substitution has not been confirmed by direct analysis of the peptide[23,24].

Analysis of βEND-immunoreactivity in tissue extracts. Immunoreactive material, corresponding in size to βEND, was pooled following gel HPLC and analyzed directly by reversed-phase HPLC–RIA with TFA and HFBA. As expected from previous reports[9,17], immunoreactivity eluted as a single peak in HPLC exhibited considerable heterogeneity in the two reversed-phase systems. The majority of immunoreactive forms resolved by RP-HPLC were common to all regions studied, but there was considerable variation among tissues in the relative amounts of the various forms.

Anterior pituitary exhibited a major peak of immunoreactivity, which migrated with 1-31/Ac 1-27 in the HFBA system (Fig. 3B), accompanied by a smaller peak in the position of Ac 1-31. A minor, early peak was also observed in this system. Analysis of the same material in the TFA system confirmed this interpretation and demonstrated the presence of a small amount of Ac 1-27, which could not be separated by HFBA.

Neurointermediate pituitary exhibited an immunoreactive profile in the HFBA

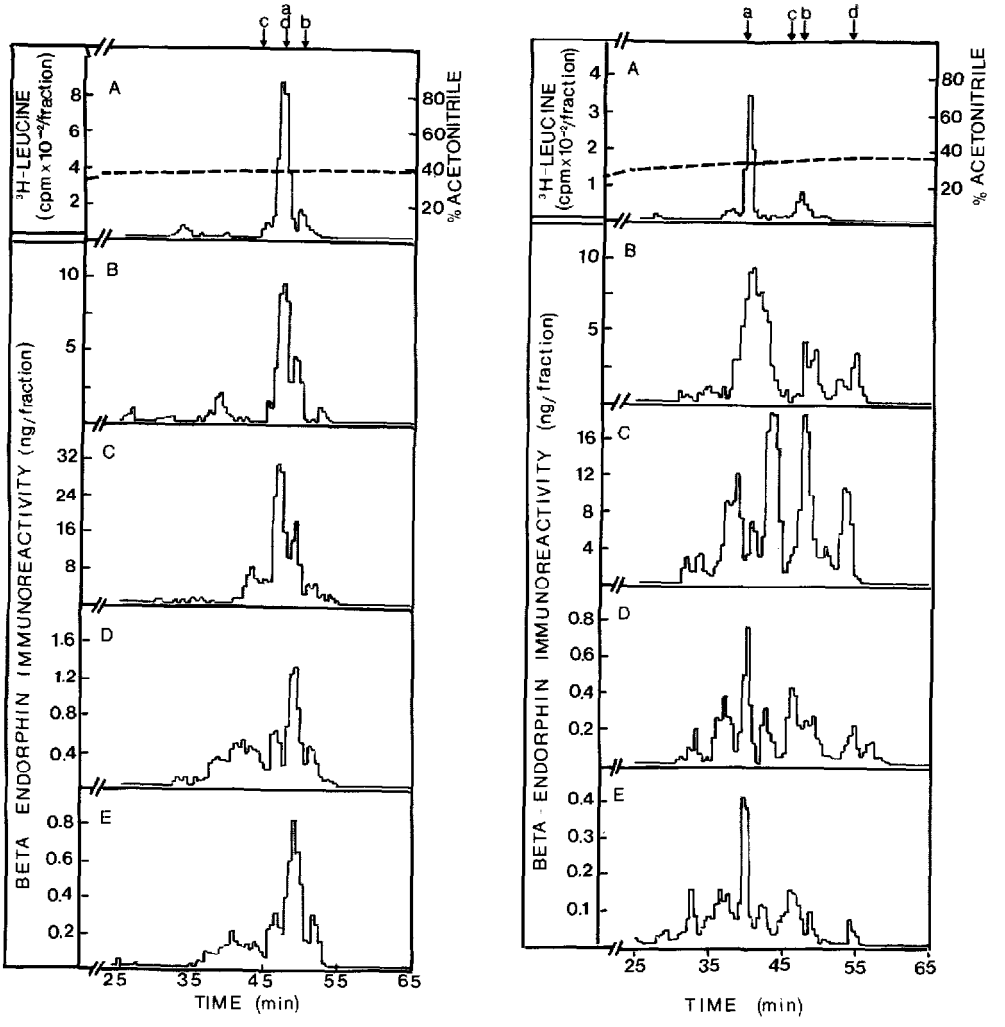

Fig. 3. HFBA-reversed-phase elution profiles of (A) tritiated βEND-sized peptides from G-75 chromatography of [^{3}H]leucine-labeled rat neurointermediate lobe and (B–E) βEND immunoreactivity in 1.0-ml aliquots of βEND-sized material pooled from gel HPLC of tissue extracts. The tissues are the same as in Fig. 2. Arrows at the top of the figure denote the positions of synthetic ovine standard peptides: βEND 1-31 (a), N-acetyl βEND 1-31 (b), βEND 1-27 (c) and N-acetyl βEND 1-27 (d). The acetonitrile gradient used is indicated in (A).

Fig. 4. TFA-reversed-phase elution profiles of (A) [^{3}H]leucine-labeled peptides and (B–E) βEND immunoreactivity. The tissues and marker peptides are as described in Fig. 3. The acetonitrile gradient used is indicated in (A).

system (Fig. 3C) similar to that observed in anterior lobe. The major peak was eluted with ovine 1-31/Ac 1-27, along with a minor peak in the position of Ac 1-31. An additional immunoreactive form migrating with 1-27 was also present in this tissue. However, elution by the TFA system revealed striking differences between the neurointermediate and anterior lobes in their content of βEND-related peptides.

Major peaks of immunoreactivity were observed in the elution positions of Ac 1-31 and Ac 1-27; material migrating with ovine 1-31 represented a relatively minor component in this lobe of the pituitary. In addition, a considerable proportion of the total immunoreactivity was eluted between 1-31 and 1-27 and could not be identified with any of the standard peptides used in this study.

Arcuate nucleus and periaquaductal grey exhibited very similar patterns of immunoreactivity. With the HFBA system (Fig. 3D and E), a major peak was observed in the position of Ac 1-31, and minor peaks migrated with 1-31/Ac 1-27 and 1-27. Additional, unidentifiable material, common to the two brain regions, was also observed in the chromatograms.

Analysis of the brain extracts with the TFA system produced puzzling results (Fig. 4D and E). The major peak of immunoreactivity in both regions was eluted in the position of ovine 1-31. Minor peaks were observed which migrated with 1-27, Ac 1-31 and Ac 1-27. In addition, a considerable proportion of the immunoreactive material in brain was eluted earlier than 1-31 by this system, along with a small amount eluted between 1-31 and 1-27. These unidentified peptides were present in both brain regions.

The patterns observed with HFBA thus appear to be inconsistent with those obtained with the TFA system. With the former, 1-31 appears to be a minor form, whereas with the latter it represents the predominant βEND-related species in both brain regions. This apparent discrepancy is probably due to the fact that the immunoreactive peptides which are resolved by TFA are not separated by HFBA. The identities of the unresolved species can be guessed by comparing the amount of immunoreactivity recovered with HFBA and TFA. For the arcuate nucleus (Figs. 3D and 4D) approximately 2.1 ng of βEND-immunoreactivity was eluted with 1-31/Ac 1-27 in the HFBA system as compared to 2.1 ng for the sum of the two resolved peaks of 1-31 and Ac 1-27 in TFA. The major peak migrating with Ac 1-31 in HFBA contained about 4.5 ng of immunoreactive material. This compares well with the sum of immunoreactivities present in Ac 1-31 (1.9 ng) and in the unidentified peaks (2.4 ng) observed in the TFA system. Similar calculations for the periaquaductal grey indicate that βEND 1-31 is indeed the major species in both brain regions and that the discrepant results obtained with HFBA are caused by the failure to separate several different immunoreactive peptides. This interpretation should be tested by pooling the HFBA fractions believed to contain the unresolved peptides and re-analyzing this material with the TFA system.

DISCUSSION

Application of the coupled gel HPLC–reversed-phase HPLC system described here permitted an extensive analysis of the molecular species comprising total βEND immunoreactivity in extracts of rat pituitary and brain regions. Gel permeation–RIA showed that βEND-sized material was the major immunoreactive form in neurointermediate pituitary and the two brain regions studied, but that β-LPH is a predominant species in anterior pituitary. These results indicate that POMC maturation in the brain resembles that in neurointermediate pituitary, as previously reported[16], although the small amount of β-LPH-like material in the periaquaductal grey may suggest regional differences in this process in brain. This technique alone may prove

very useful in screening for tissue-specific differences in the maturation of peptide precursors.

Reversed-phase HPLC of material obtained following gel HPLC demonstrated the heterogeneous nature of βEND immunoreactivity in the various tissues studied and revealed striking differences not evident from the molecular weight profiles. βEND from the anterior lobe was shown to be comprised principally of the opiate-active 1-31, accompanied by small amounts of the acetylated forms Ac 1-31 and Ac 1-27. The neurointermediate lobe, in contrast, possessed relatively little 1-31, most of the immunoreactivity being associated with the acetylated forms which are inactive as opiates. These findings are in agreement with previous studies in which different techniques were employed to study βEND heterogeneity in rat pituitary[9,17].

Neurointermediate lobe also contained immunoreactive peptides that did not correspond to any of the ovine standards used in this study. It has been reported previously[9] that this tissue contains significant amounts of α-N-acetyl βEND 1-26 produced by enzymatic removal of the C-terminal histidine of Ac 1-27. However, the increase in peptide hydrophobicity expected from such a modification is difficult to reconcile with the relative elution positions of the unidentified components. The identification of this material must await further characterization. Our inability to demonstrate the presence of the Ac 1-26 form, which should be eluted at the end of the chromatogram[25], may indicate that the antibody used in this study requires the presence of the His_{27} for binding.

The arcuate nucleus and periaquaductal grey exhibited strikingly similar contents of βEND-related peptides when analyzed by reversed-phase HPLC. Problems in interpreting results caused by failure of peptides to be separated by the HFBA system were resolved by analyzing the same material with TFA, and this should serve to caution against the use of a single chromatographic system in neuropeptide characterization. Our results indicate that the major βEND-related species in both brain regions is the opiate-active form βEND 1-31, accompanied by small amounts of βEND 1-27 and acetylated derivatives of these peptides. The presence of immunoreactive forms which could not be identified with standard peptides may be the result of oxidation of methionine residues during analysis[3] or it may indicate the presence in brain of βEND-related peptides not produced in pituitary, perhaps via the action of extracellular proteases[26].

It has been proposed[17] that βEND 1-31, synthesized in neurons of the arcuate nucleus, is slowly converted into its acetylated, opiate-inactive derivatives during transport to terminal regions in the periaquaductal grey. Our results do not support this assumption but indicate instead that these two brain regions have identical contents of βEND-related peptides and that the relative concentrations of the various forms are unchanged during axonal transport. The hypothesis that the acetylated derivatives of βEND found in brain are derived by retrograde transport from the pituitary[18] is appealing but cannot be addressed in this study.

The chromatographic system described in this report offers a very useful procedure for the analysis of peptides that share a common biosynthetic pathway. Gel HPLC–RIA provides a rapid screening procedure to assess the relative amounts of precursors and products that contain common antigenic determinants. This technique has clearly demonstrated differences in the processing of POMC to βEND in rat anterior and neurointermediate pituitary and has indicated that POMC maturation

in brain resembles that in the neurointermediate lobe. Reversed-phase HPLC of material separated by gel HPLC reveals small differences in peptide structure resulting from minor post-translational modifications, such as acetylation, which affect peptide hydrophobicity. In the case of βEND, such modifications greatly alter biological activity. It is therefore extremely important to resolve the various forms of βEND in order to obtain physiologically relevant information. The combined application of two reversed-phase HPLC schemes in this study showed that the major form of βEND in rat anterior pituitary, arcuate nucleus and periaquaductal grey is the opiate active 1-31, whereas in neurointermediate pituitary most of the immunoreactivity is associated with inactive species. The combined use of these procedures should prove very useful in studying regional differences in the processing of neuropeptide precursors in brain.

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