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PREPARATION OF TRITIUM LABELED β-ENDORPHIN SUITABLE FOR STUDYING BRAIN RECEPTOR INTERACTIONS[1]

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SUMMARY: The opioid peptide (porcine) β-endorphin has been tritiated using reductive methylation to prepare a derivative containing mainly [^{3}H]dimethyllysine. The tritiated β-endorphin has a specific activity of 9.8 Ci/mmol and is stable for an extended period of time. The labeled peptide binds reversibly to rat brain membrane preparations with a dissociation constant of 0.4 ± 0.1 nM and a receptor content of 23 ± 2 pmol/g brain. Under the conditions used, there is evidence for only one class of receptors. The technique employed for tritium labeling of β-endorphin should also be applicable to various other peptides including α-endorphin, γ-endorphin, and C'-fragment that have been found in brain and pituitary.

Most work involving peptide binding to opiate receptors has been done with iodinated or tritiated enkephalin analogues which are amenable to solid phase synthetic techniques. Consequently, little is known from direct binding studies of β-endorphin or the other opioid peptides such as α-endorphin, γ-endorphin, or the C'-fragment, i.e., lipotropin residues 61-76, 61-77, and 61-87, respectively (1,2,3).

Two groups have recently reported methods for radioactively labeling human β-endorphin while retaining its ability to bind to rat brain receptors. Cuatrecasas and coworkers iodinated the analogue [D-Ala^2]β-endorphin and found an apparent K_d of ca. 2.5 nM (4). Houghten and Li catalytically re-

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Abbreviations: Me, methylated lysine; SDS, sodium dodecyl sulfate.

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duced the diiodotyrosine analogue of β-endorphin at position 27 with tritium gas to yield a product with a specific activity of about 50 Ci/mmol (5). Using [^{3}H-Tyr^{27}]β-endorphin as ligand, one report described a single class of receptors characterized with a K_d of 0.3 nM (6), but a later communication indicated the presence of two classes of receptors with K_d's of 0.8 nM and 6.8 nM (7). Although the iodinated analogue is functional in terms of receptor binding, one cannot help but be concerned about the possible conformational change which may accompany the substitutions of D-Ala for Gly and of iodotyrosine for tyrosine at positions 2 and 1, respectively. The catalytic reduction procedure, although yielding a more innocuous derivative, necessitates the use of several Ci of tritium gas thus making it impractical for most laboratories.

We have taken a different approach to tritiate porcine β-endorphin which differs from human β-endorphin in three of the 31 positions: the porcine peptide contains Val, His, and Gln at residues 23, 27, and 31, respectively, while the corresponding residues in the human peptide are Ile, Tyr, and Glu (8,9). This approach involves reductive methylation which uses formaldehyde and high specific activity sodium [^{3}H]borohydride to form, under mild aqueous conditions, the monomethyl and dimethyl derivatives of amino groups (10,11, 12). Methylation by this technique preserves the charge of ε-amino groups at neutral pH since the pK_a of lysyl groups is altered only slightly by the modification (11). The methyl groups introduce much less steric volume than most modifying groups and may in fact protect to some extent against endopeptidase action at the methylated lysyl residues (11). Using a modification of a method developed in this laboratory to reductively methylate proteins with high specific activity [^{3}H]borohydride (13), we have prepared stable [^{3}H-Me]β-endorphin with a specific activity of 9.8 Ci/mmol which is sufficient for studies on receptor interactions.

MATERIALS AND METHODS

Materials. Tritiated sodium borohydride was purchased from either Amersham Searle (9-15 Ci/mmol) or from Research Products International (15-25

Ci/mmol); the latter product was prepared by La Centre d'Energie Atomique, France. Formaldehyde, sodium borate, acetic acid, and glycerol were Fisher ACS reagent grade, and urea was from Mallinckrodt. Dialysis tubing was the 1000 molecular weight cut-off Spectrapor 6 from Spectrum Medical Industries. Bovine serum albumin (Cohn fraction V), o-phthalaldehyde, poly-L-lysine (40,000 average molecular weight), bacitracin, and sodium azide were from Sigma Chemical Co. Acrylamide and bis-acrylamide were from Aldrich; NCS and ^{3}H-water (reference standard) were from Amersham-Searle. [D-Ala^{2}]methionine enkephalinamide was purchased from Calbiochem. Porcine β-endorphin was synthesized by the solid phase method and purified and characterized as described elsewhere (14).

Electrophoresis. Polyacrylamide gel electrophoresis in 8 M urea and 0.1% SDS was according to the procedure of Swank and Munkres (15). Radioactivity was measured by eluting 2 mm slices with 0.5 ml of 1:7 NCS in water overnight with shaking at room temperature in 7 ml plastic minivials and counting with 5 ml of Tritosol.

Determination of Methylated Lysines. Amino acid analysis of 3 N mercaptoethanesulfonic acid hydrolysates (110°C, 20 hours) for monomethyllysine and dimethyllysine used a 0.9 x 12 cm column of Aminex A-5 (Bio-Rad) developed at 30°C with filtered (0.45 micron Amicon) 0.35 M sodium citrate, pH 6.48, at a flow rate of 14.9 ml/hour. The column effluent was combined at a Durrum 3-way valve with a solution of 0.1 M sodium borate, 0.085 M sodium hydroxide, and 0.1 mM o-phthalaldehyde (freshly prepared each day) at a flow rate of 20 ml/hour and immediately passed to an Aminco flow fluorometer and finally to a fraction collector. One minute fractions were collected and 0.1 ml aliquots counted with 2 ml of Tritosol.

Reductive Methylation. The procedure used for tritiating β-endorphin was modified from that described by Ascoli and Puett (13). All steps preceding gel filtration were conducted in a fume hood. The peptide (1.0 mg, 0.3 μmole) was dissolved in 0.3 ml ice cold 0.1 M sodium borate, pH 9.0, in a conical 1.5 ml polypropylene tube. A small teflon stir bar was placed in the tube and 35 μl of a 0.4% formaldehyde solution in borate buffer added. The tube was sealed with a serum stopper and a nitrogen flush was begun through needles inserted through the septum. The nitrogen flush passed through a glass column containing finely powdered cupric oxide preactivated by heating with an open flame and heated during labeling to a temperature of 400°C using heat tape, then through a column of anhydrous calcium chloride, next through a sulfuric acid trap, and finally was vented into the fume hood. With this method the tritium gas released from hydrolysis of the [^{3}H]borohydride is trapped as water. Five minutes after addition of formaldehyde, 25 μl of a solution containing 25 mCi of tritiated sodium borohydride in 0.1 ml of 10 mM sodium hydroxide was added through the serum stopper using a microsyringe. Three more equal aliquots of borohydride, a 25 μl wash of the borohydride container, and finally 10 μl of glacial acetic acid were added at 5 minute intervals.

The nitrogen flush was stopped 5 minutes after addition of acetic acid and the nitrogen inlet removed. After bubbling from the sulfuric acid trap had stopped, the serum stopper was removed and the reaction mixture transferred to dialysis tubing using a silanized borosilicate glass pasteur pipette. The dialysis tubing was prepared for use by rinsing briefly in distilled water and soaking in the dialysis solution, 5% acetic acid in 50 mM KCl, until needed. Two changes of 200 ml each for 2 hours removed all of the dialysable radioactivity. Aliquots of each of the dialysates were taken to determine radioactivity and the dialysates pooled and stored in screwtop plastic jars until disposal.

The retentate was applied to a 0.8 x 46 cm column of Bio-Gel P-6 which was equilibrated and developed with 5% acetic acid in 50 mM KCl. The column was preconditioned on the same day by applying a mixture of 50 mg bovine serum albumin and 2 mg of poly-L-lysine in 1 ml of buffer and eluting 50 ml of

buffer. Fractions of 1 ml were collected in siliconized borosilicate glass tubes and 10 µl aliquots counted in 2 ml Tritosol for 0.1 minutes.

The fractions containing the peak activity were pooled and an ultraviolet absorption spectrum determined. Aliquots were removed from the cuvette and counted under conditions of known efficiency with no intermediate dilutions. The absorbance spectrum was corrected for light scattering and the concentration of [^{3}H-Me]β-endorphin was estimated using the extinction coefficient calculated from the amino acid composition, E(0.1%, 1 cm, 276 nm) = 0.42. The specific activity of [^{3}H-Me]β-endorphin was estimated from these two measurements.

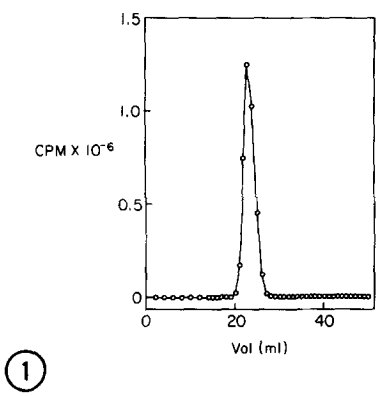
Preparation of Rat Brain Membranes. Brains from 180-250 g male Sprague-Dawley rats were removed and decerebellated immediately following decapitation. The tissue was homogenized in 50 mM Tris-HCl, pH 7.5, using a Brinkman Polytron PT-10 and then centrifuged at 12,000 xg for 20 minutes. The pellet was washed twice and finally suspended in the same buffer containing 20% (v/v) glycerol (0.1 g brain/ml); the membrane fractions were stored at -70°C. Protein contents of the membrane fractions were estimated using the Coomassie blue G-250 assay (16).

Binding of [^{3}H-Me]β-Endorphin to Brain Membranes. Details of the binding assay are given elsewhere (17). The incubation mixture consists of 1.05 ml of the membrane solution (the thawed stock diluted to about 0.46 mg protein/ml with 50 mM Tris-HCl, pH 7.5) containing sodium azide and bacitracin at concentrations of 0.2 mg/ml and 0.1 mg/ml, respectively. (The azide acts as an antibacterial agent and bacitracin is very effective in preventing proteolytic degradation of [^{3}H-Me]β-endorphin.) [^{3}H-Me]β-endorphin is added (25 µl in 50 mM acetic acid) with 25 µl of either buffer or a displacing dose of [D-Ala^2]methionine enkephalinamide (to ascertain nonspecific binding) to 1.5 ml polypropylene centrifuge tubes (Sarstedt). Incubation is for 2 hours at 30°C in a shaking water bath. This gives reversible equilibrium binding with no degradation of the labeled peptide (17).

Bound and free counts are separated by centrifugation (12,000 xg, 1 minute) in a Brinkmann Microfuge; the pellet was then washed, resuspended in buffer, and 0.1 ml of 10% (w/v) SDS was added to dissolve the membranes. The samples were counted at ca. 30% efficiency in a scintillation spectrometer using 10 ml of Tritosol (18).

RESULTS AND DISCUSSION

We found that the final yield of [^{3}H-Me]β-endorphin was vastly improved by using polypropylene tubes for labeling, acidification of the reaction mixture prior to transfer for dialysis, the low molecular weight cut-off dialysis tubing, and the use of a fairly concentrated acetic acid solution with a (protein/basic polypeptide) precoated column of relatively small total volume. Storage at -70°C of concentrated [^{3}H-Me]β-endorphin was optimized using siliconized borosilicate glass; alternatively, samples diluted 100-fold with 0.05 M acetic acid were successfully stored in 1.5 ml polypropylene centrifuge tubes. Storage at neutral pH resulted in near quantitative loss of [^{3}H-Me]β-endorphin after 24 hrs at 4°C in a variety of tubes. The diluent



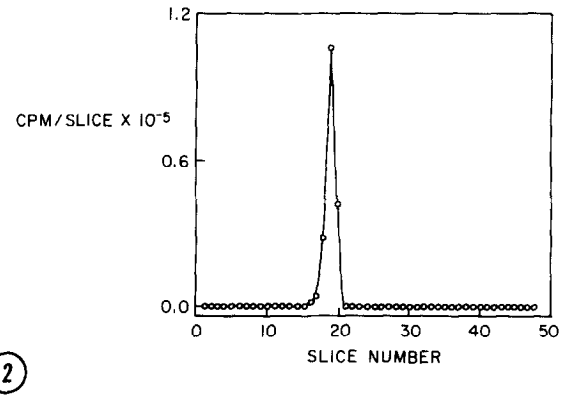


Figure 1. Gel exclusion chromatography of dialyzed [^{3}H-Me]β-endorphin on a 0.8 x 46 cm Bio-Gel P-6 column equilibrated and developed with 5% acetic acid containing 50 mM KCl at 25°C. Fractions of 1 ml were collected and 10 µl aliquots were counted.

Figure 2. Polyacrylamide gel electrophoresis of [^{3}H-Me]β-endorphin (pooled three tubes containing the highest radioactivity in Figure 1) in 0.1% SDS, 8 M urea. The gel was sliced into 2 mm sections and counted as described in the text.

suggested by Guillemin et al. (19) completely eliminated adsorption loss but was incompatible with the binding assay.

Figure 1 shows the elution pattern on a Bio-Gel P-6 column of the dialyzed reaction mixture. A single peak is observed, and there is no evidence of any labeled high molecular weight aggregates or low molecular weight components. The peak tube was pooled with the adjacent tubes on the ascending and descending sides to give the stock solution of [^{3}H-Me]β-endorphin in 5% acetic acid containing 50 mM KCl. The specific activity of the tritiated peptide was 9.8 Ci/mmol, and this value was consistently obtained when fresh [^{3}H]borohydride was used. The use of [^{3}H]borohydride which was stored for 2 months at 4°C resulted in a specific activity of only 0.5 Ci/mmol; however, this material could be relabeled to the higher activity using fresh [^{3}H]borohydride.

[^{3}H-Me]β-Endorphin migrated as a single component on SDS/urea polyacrylamide gel electrophoresis (Figure 2). These results testify to the size homogeneity of [^{3}H-Me]β-endorphin.

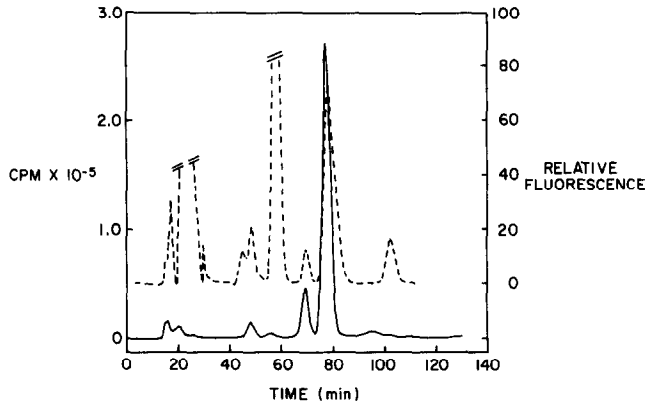


Figure 3. Ion exchange chromatogram of acid hydrolyzed [^{3}H-Me]β-endorphin on a 0.9 x 12 cm Aminex A-5 column equilibrated and developed with 0.35 M sodium citrate, pH 6.48, at 30°C. Both fluorescence from o-phthalaldehyde (---) and radioactivity (——) were determined on 0.1 ml aliquots from each fraction (0.25 ml). The retention times of standard lysine, ε-N-methyllysine, and ε-N,N-dimethyllysine were 57, 69, and 78 minutes, respectively.

Amino acid analysis of hydrolyzed [^{3}H-Me]β-endorphin demonstrated that ca. 88% of the recovered radioactivity was present as dimethyllysine, ca. 4% as monomethyllysine, and ca. 8% as unidentified components (Figure 3). The fluorescence data from the same chromatogram indicated that about two-thirds of the lysines remain unmodified suggesting that higher specific activities could be attained.

The equilibrium binding of [^{3}H-Me]β-endorphin to the brain membrane fraction is shown in Figure 4. A nonlinear least-squares analysis of these data using simple mass action binding showed that the binding was adequately fit with a single class of receptors characterized by a K_d of 0.4 nM. In independent assays, K_d was (mean ± SE) 0.4 ± 0.1 nM and the total receptor level was 23 ± 2 pmol/g brain or 0.8 ± 0.2 pmol/mg membrane protein. A Scatchard plot of these results was linear and there was no evidence in this system of a second class of receptors as reported by Law et al. (7). The reason(s) for this discrepancy is not known, although the buffer system used by Law et al. (25 mM Hepes, 0.1% bovine serum albumin fraction V, pH 7.7, containing 2 mM o-phenanthroline) differs considerably from that used herein which is almost identical to the buffer used by Hazum et al. (4).

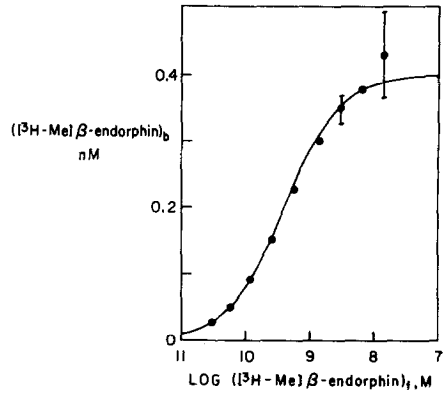


Figure 4. Equilibrium binding curve of [^{3}H-Me]β-endorphin to brain membranes using the conditions described in the text. The data are plotted as concentration of specifically bound [^{3}H-Me]β-endorphin vs. log concentration of free [^{3}H-Me]β-endorphin. Circles denote means and the standard deviations of replicates are indicated. The solid line represents the theoretical curve for a single class of receptors which binds [^{3}H-Me]β-endorphin in a non-cooperative fashion with a K_d of 0.4 nM.

Lastly, we wish to point out that the technique of reductive methylation described above should be applicable to β-endorphin from other species and to the various carboxyl-terminal deletion fragments of β-endorphin which have been found in brain and pituitary (cf. reference 20).

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