Vol. 88, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 13, 1979 Pages 841-846

FLUORESCENT AND PHOTO-AFFINITY ENKEPHALIN DERIVATIVES: PREPARATION AND INTERACTION WITH OPIATE RECEPTORS

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Received April 17,1979

SUMMARY

The fluorescent and photo-affinity derivatives of enkephalin, Tyr-D-Ala-Gly-Phe-Leu-Lys-N^{ε}-Rhodamine (II) and Tyr-D-Ala-Gly-Phe-Leu-Lys-N^{ε}-nitro-azidophenyl (III), were prepared by conventional methods followed by chemical modification. The two peptides inhibit the binding of ^{125}I-labeled enkephalin to brain membrane preparations, with apparent IC_{50} values of 5.9 nM and 5.5 nM for peptides II and III, respectively. The iodinated derivative of peptide III binds specifically to brain membrane preparations with an apparent K_d of about 2.1×10^{-9}M.

INTRODUCTION

Since the discovery of the enkephalins, Tyr-Gly-Gly-Phe-Leu ([Leu] enkephalin) and Tyr-Gly-Gly-Phe-Met ([Met]enkephalin), as naturally occurring ligands of opiate receptors (1,2), hundreds of analogs of these pentapetides have been prepared and tested. Numerous workers have attempted to identify the postulated opiate receptors by biochemical techniques, using binding assays of radioactive opiates to brain membrane preparations and neuroblastoma cells. It has been found that opiate receptor binding is stereospecific and extremely sensitive to proteolytic enzymes, nonionic detergents and protein-modifying reagents (3-4).

Fluorescent derivatives of enkephalins represent potentially useful experimental tools for the microscopic visualization of opiate receptors and the exploration of ligand-receptor interactions, while photo-affinity derivatives can be utilized for identification of the membrane components comprising these opiate binding sites. We report here on the synthesis and binding properties of such two compounds (Fig. 1):

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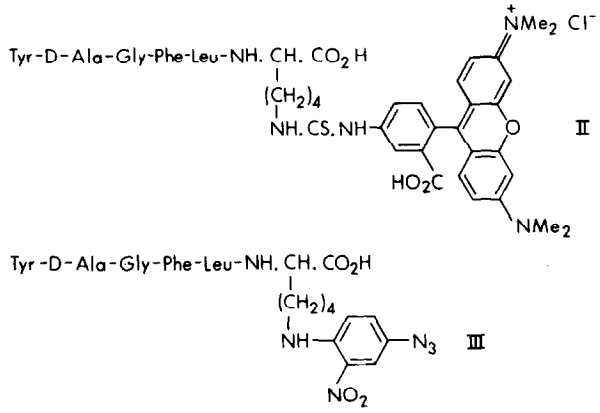

Fig. 1 The structures of [D-Ala^2, Leu^5]enkephalin-Lys-N$^{\varepsilon}$-Rhod (II) and [D-Ala^2, Leu^5]enkephalin-N$^{\varepsilon}$-NAP (III).

Tyr-D-Ala-Gly-Phe-Leu-Lys-N$^{\varepsilon}$-Rhodamine ([D-Ala^2, Leu^5]enkephalin-Lys-N$^{\varepsilon}$-Rhod) and Tyr-D-Ala-Gly-Phe-Leu-Lys-N$^{\varepsilon}$-nitro-azidophenyl ([D-Ala^2, Leu^5]enkephalin-Lys-N$^{\varepsilon}$-NAP).

MATERIALS AND METHODS

Synthesis

Boc-Tyr (OBu^t)-D-Ala-Gly-Phe-Leu-Lys (I) was prepared by conventional solution methods using tertbutyloxycarbonyl (Boc) and tertbutyl (Bu^t) as protecting groups and dicyclohexylcarbodiimide 1-hydroxybenzotriazole mediated coupling reactions.

Tyr-D-Ala-Gly-Phe-Leu-Lys-(N$^{\varepsilon}$-Rhod) (II) was prepared by reaction of I with two equivalents of rhodamine (Rhod) isothiocyanate (tetramethyl form, hereafter referred to simply as rhodamine) in methanol: dichloromethane (1:5, v/v), in the presence of 1.2 equivalents of triethylamine. After standing at 24°C for 24 hours, the reaction mixture was evaporated to dryness in vacuo and treated for 10 minutes with anhydrous trifluoroacetic acid (TFA). The deprotected product (II) was solidified by trituration with dimethylformamide (DMF)-petroleum ether, followed by extraction with 1 M acetic acid and lyophilization, and finally purified on thin layer chromatography plates (silica gel, precoated plastic sheets, Brinkmann Instruments); R_f = 0.6 in BuOH:AcOH:H_2O (4:1:4, v/v).

Tyr-D-Ala-Gly-Phe-Leu-Lys-(N$^{\varepsilon}$-NAP) (III) was prepared essentially as described for peptide II. I was reacted with three equivalents of 4-fluoro-3-nitro phenylazide (F-NAP) in dichloromethane:DMF (10:1), in the presence of 1.2 equivalents of triethylamine. After 24 hours at 24°C the reaction mixture was evaporated to dryness and deprotection was achieved by anhydrous TFA. III was solidified by DMF-petroleum ether, washed three times with ethylacetate and finally recrystallized in DMF-ethylacetate; R_f = 0.7 in BuOH:AcOH:H_2O (4:1:4).

Iodination

Peptide III was iodinated as described previously (5). Briefly, to 0.1 ml of 0.25 M sodium phosphate buffer, pH 7.6, containing 3 nmoles of III and 3 mCi of carrier-free iodine-125 were added: 20 µl of chloramine-T (0.5 mg/ml) followed after 20 seconds by 20 µl of phenol (5 mg/ml). The reaction mixture was applied immediately to a Biogel P-2 column (1 x 10cm) previously equilibrated and eluted with 0.25 M sodium phosphate buffer containing 0.1% bovine serum albumin.

Binding Assays

Brain membranes were prepared as described previously (5-7). Briefly, whole rat brains were homogenized with a Polytron homogenizer at 4°C in 0.32 M sucrose in 5 mM Tris·HCl buffer, pH 7.4. The nuclei and most of the mitochondria were removed by centrifugation for 15 minutes at 5000 x g and the supernatant was further centrifuged for 30 minutes at 40,000 x g and finally suspended in 50 mM Tris·HCl buffer, pH 7.4, at 4°C. Aliquots 0.2 ml of the homogenate, containing 1 mM Mn^{+2}, were incubated with radioactive enkephalin and the tested substances (as indicated) for 45 minutes at 25°C (equilibrium conditions). The homogenate was then filtered under vacuum through Whatman GF/C filters, washed with 10 ml of ice-cold incubation buffer and the filters counted in a γ-counter. Specific binding represents the bound radioactivity which can be displaced by simultaneous or prior addition of 10^{-6}M unlabeled [D-Ala^2, Leu^5]enkephalin.

RESULTS AND DISCUSSION

Two analogs of enkephalin were prepared by addition of a lysine residue at the carboxyl terminal and subsequent chemical modification: [D-Ala^2, Leu^5]enkephalin-Lys-N^{ε}-Rhod (II) and [D-Ala^2, Leu^5]enkephalin-Lys-N^{ε}-NAP (III) as shown in Fig. 1. The protected hexapeptide, Boc-Tyr(OBu^t)-D-Ala-Gly-Phe-Leu-Lys, was selected as the starting material since a) substitution of D-Ala in the second position of enkephalin results in a more potent and metabolically stable derivative (8) and b) the ε-amino group of lysine serves as a spacer for substitution reactions and thus the enkephalin sequence and conformation are less likely to be disturbed. The two peptides are homogeneous when checked by thin-layer chromatography and are stained positively by ninhydrin reagent. Spectrophotometric studies of the two peptides (Fig. 2) reveal spectral properties indentical to those of rhodamine, ε_{545}=80,000Mcm^{-1} (9), and azido-nitrophenyl derivatives, ε_{260}=28,000 and ε_{459}=4800 (10). The inhibition of binding of [^{125}I][D-Ala^2, D-Leu^5]enkephalin to brain membrane preparations by

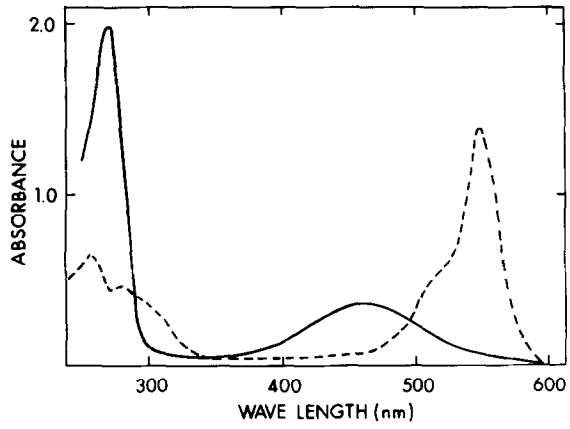

Fig. 2 The ultraviolet absorption spectra of Tyr-D-Ala-Gly-Phe-Leu-Lys-N^{ε}-Rhod (---) at 1.7×10^{-5} M in 0.1 M acetic acid and Tyr-D-Ala-Gly-Phe-Leu-Lys-N^{ε}-NAP (——) at 6×10^{-5} M in ethanol.

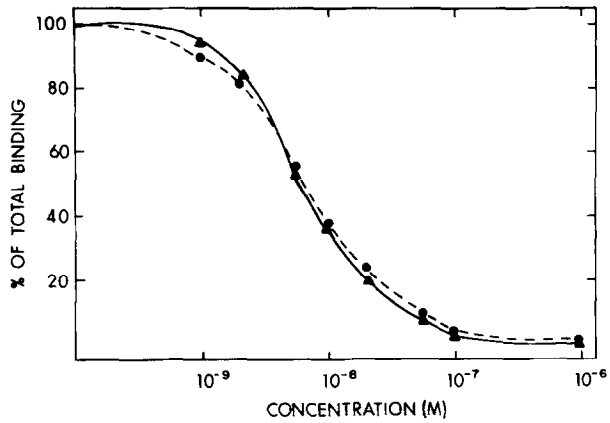

Fig. 3 Competition of binding of [^{125}I]D-Ala^2, D-Leu^5]enkephalin to brain membranes by [D-Ala^2, Leu^5]enkephalin-Lys-N^{ε}-Rhod (●---●) and [D-Ala^2, Leu^5]enkephalin-Lys-N^{ε}-NAP (▲——▲). The radioactive enkephalin (0.5 nM) was incubated with different concentrations of the tested compound for 45 minutes at 25°C in a final volume of 0.25 ml containing brain membranes (0.5 mg of protein/ml) and 1 mM Mn^{+2}, and the binding was measured as described in Materials and Methods. Values are means of duplicate incubations.

peptides II and III is shown in Fig. 3. The apparent IC_{50} values (the concentration of unlabeled ligand at which the maximum specific binding of labeled ligand is displaced by 50%) were found to be 5.9 nM and 5.5 nM for peptides II and III, respectively.

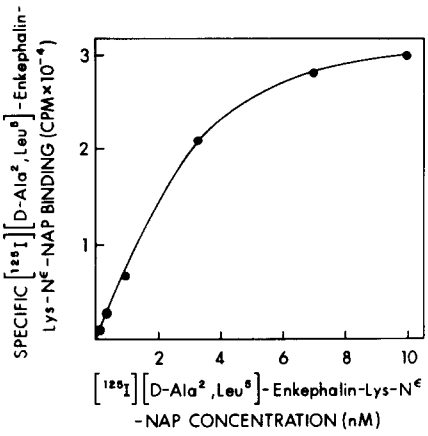

Fig. 4 Saturation binding curve for ^{125}I-labeled [D-Ala^2, Leu^5]enkephalin-Lys-N$^{\epsilon}$-NAP to opiate receptors of brain membranes. Binding assays were carried out as described in Materials and Methods. Values are means of duplicate incubations.

Peptide III was iodinated using chloramine-T and the reaction was terminated by the addition of phenol. The iodinated peptide shows specific binding to brain membrane preparations [Fig. 4]. The binding is readily saturable, reaching a plateau at about 10 nM. The apparent K_d from this data is about 2.1×10^{-9}M.

The data indicate that the two enkephalin derivatives can interact specifically with opiate receptors. Conventional methods appear inadequate for purification of opiate receptors since these are extremely sensitive to nonionic detergents (4). Preliminary experiments with iodinated peptide III reveal that after photoactivation it is bound covalently to brain membrane preparations. The advantage of photo-generated nitrene intermediates is that they are highly reactive and are capable of insertion into all protein amino acid side chains, and they can also react with other components of the membrane. The detailed interaction of this photo-affinity derivative with brain membrane preparations and neuroblastoma cells will be described in a subsequent report. Studies using the fluorescent derivative of enkephalin for visualization of the qualitative patterns and redistribution of cell surface opiate receptors by fluorescence microscopy will be published elsewhere.

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