BIFUNCTIONAL ENKEPHALIN ANALOGUES FOR AFFINITY SEPARATION PURPOSES

A. KOMAN and L. TERENIUS

Department of Pharmacology, University of Uppsala, Biomedicum, Box 573, 751 23 Uppsala, Sweden

Received 14 July 1980

1. Introduction

Affinity separation of neural membranes or membrane constituents by the use of receptor probes could well be developed into an important tool in studying the complex structure of the CNS. This idea was also suggested in [1]. As a probe of opioid receptors, a bifunctional Leu^5-enkephalin analogue was thought to be plausible. A 'sandwich' technique was preferred to direct coupling to a separation matrix, enabling versatility of possible separation methods. Leu^5-enkephalin was coupled to biotin via two spacers of different lengths: Lys and Gly–Gly–Lys. The biotin–avidin interaction ($K_D < 10^{-14}$ M) as a second coupling function has already been used in other systems [2–4] as suggested in [5]. Both analogues had the same IC_{50} value against (D-Ala^2-Leu^5)-[^{3}H]enkephalin as indicator ligand in receptor-binding experiments. The affinities of both derivatives were lowered in the presence of avidin, but the decrease was less for the ligand with the longer spacer, indicating steric hindrance by avidin.

2. Materials and methods

(+)-Biotin and avidin (10–12 units/mg) were purchased from Sigma, amino acid derivatives and peptides from Bachem, 1,1′-carbonyldiimidazole and *N*-hydroxysuccinimide from Aldrich-Europe, thin-layer chromatography plates from Merck, CPK space-filling molecule models from Schwarz/Mann. The iodinated precursor of (D-Ala^2-Leu^5)-[^{3}H]enkephalin was synthesised by Ulf Ragnarsson, (Department of Biochemistry, Uppsala University) and tritiated at The Radiochemical Centre, Amersham (spec. act. 33 Ci/mmol). Biotin measurement was done colorimetrically according to [6]. Amino acid analysis was performed at the Department of Biochemistry, Uppsala University. HPLC was performed on a Whatman M9 semi-preparative Partisil ODS column, using 30 min linear gradients of 30–80% methanol containing 0.1% ammonium acetate, at pH 6.2. Synthesis progress was followed by thin-layer chromatography.

Abbreviations: DMF, dimethylformamide; CDI, 1,1′-carbonyldiimidazole; TFA, trifluoroacetic acid; DCCI, dicyclohexylcarbodiimide; HBT, 1-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography

2.1. *Synthesis of [(+)-biotinyllysyl^5]-Leu^5-enkephalin amide (BL)*

N^{α}-Boc-lysine amide (I): NH_3 gas was bubbled through N^{α}-Boc-N^{ϵ}-Z-lysine-*N*-hydroxysuccinimide ester in DMF for 2 h and the mixture left at 4°C overnight. The solvent was removed and the product recrystallized from ethanol–water. Z was removed by catalytic hydrogenation with Pd-coal in methanol and (I) was recrystallized from ethanol–water.

(+)-Biotinyl-*N*-hydroxysuccinimide ester (II): This compound was synthesised as in [7] using CDI and *N*-hydroxysuccinimide, and recrystallized from 2-propanol.

N^{ϵ}-Biotinyllysine amide (III): (I) + (II) were stirred overnight at 4°C in DMF. The product was purified by recrystallization from 2-propanol and by adsorption chromatography on silica gel for removal of contaminating N^{α}-Boc-lysine amide, and finally by DEAE-Sephadex chromatography subsequent to removal of Boc by treatment with TFA + anisole.

[(+)-Biotinyllysyl^5]-Leu^5-enkephalin amide (BL): Boc–Tyr–Gly–Gly–Phe–Leu + (III) were coupled in DMF by CDI. Boc was removed by treatment with TFA + anisole after precipitation of the reaction mixture by dilution with water. Purifica-

tion was achieved by HPLC. The product's composition was verified by biotin measurement and amino acid analysis.

2.2. *Synthesis of [(+)-biotinyldiglycyllysyl^5]Leu^5-enkephalin amide (BGL)*

N^{α}-Boc-N^{ϵ}-diglycyllysine amide (IV): Z-Gly–Gly was dissolved in DMF, equimolar CDI added and stirred at room temperature for 1 h after CO_2 evolution had ceased, whereupon (I) was added. Stirring was continued overnight at 4°C. The product was recrystallized from ethanol. Z was removed by catalytic hydrogenation.

N^{ϵ}-(+)-Biotinyldiglycyllysine amide (V): (II) + (IV) were dissolved in DMF and stirred overnight at 4°C. The product was recrystallized 3 times from ethanol at −20°C and Boc was removed with TFA + anisole.

[(+)-Biotinyldiglycyllysyl^5]-Leu^5-enkephalin amide (BGL): Boc-Tyr–Gly–Gly–Phe–Leu + (V) were coupled in DMF by DCCI + HBT and triethylamine. BGL was purified by HPLC and verified by biotin measurement and amino acid analysis.

2.3. *Binding studies*

Rat brain synaptic plasma membrane (SPM) preparations and binding studies were performed essentially as in [8]. SPM (200 μl) in physiological Hepes buffer pH 7.4 (1–2 mg protein/ml) was incubated for 15 min at 25°C with radioligand (to give 0.35 nM final conc.), competing ligand and eventual additions to 425 μl total vol. The incubation was terminated by cooling to 0°C and centrifugation for 5 min on a Beckman Microfuge. Non-specific binding of radioligand measured as binding in the presence of 10^{-5} M Leu^5-enkephalin (0% in displacement curves) was subtracted from all experimental values. When avidin was to be added, dilutions were made from an initial solution of bifunctional ligand + avidin in calculated molar ratios bifunctional ligand:avidin of 4:1 (most experiments) or 1:1. In a control experiment, biotin binding sites of avidin were blocked by preincubation with a 25% calculated excess of (+)-biotin.

3. Results

The two bifunctional ligands were tested in receptor displacement assays against (D-Ala^2-Leu^5)-[^{3}H]enkephalin, a metabolically stable analogue of Leu^5-enkephalin, as shown in fig.1. Both BL and BGL had an IC_{50} value of 5×10^{-8} M in the absence of avidin, to be compared with 5×10^{-9} M for the parent compound. In the presence of avidin, however, the affinities decreased, the IC_{50} of BL + avidin changing to ~10^{-5} M, of BGL + avidin to ~2×10^{-6} M.

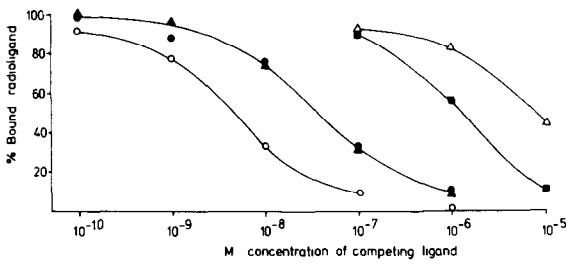

Fig.1. Displacement curves of Leu^5-enkephalin (○); BL (▲); BGL (●); BL + avidin 4:1 (△); and BGL + avidin 4:1 (■); using (D-Ala^2-Leu^5)-[^{3}H]enkephalin as indicator ligand.

Avidin blocked by an excess of biotin did not influence the IC_{50} value of BGL, nor did biotin (fig.2). Changing the ligand:avidin molecular ratio from 4:1 to 1:1 did not seem to cause a corresponding change in the IC_{50} value of BGL + avidin (fig.3).

4. Discussion

The control experiments with biotin-blocked avidin or biotin alone show that neither avidin nor

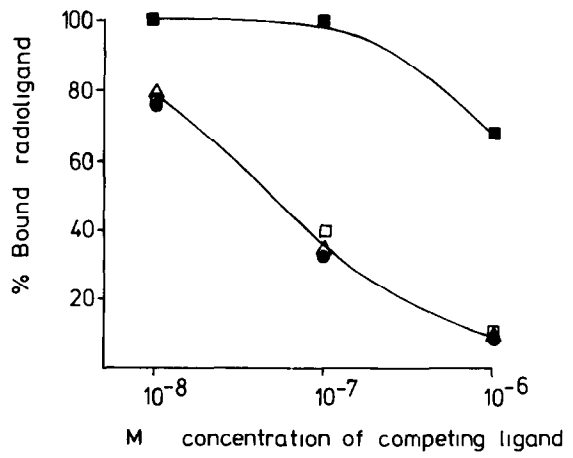

Fig.2. Effect of biotin-blocked avidin or biotin alone on BGL displacement of (D-Ala^{2}Leu^5)-[^{3}H]enkephalin: BGL (●); BGL + avidin 4:1 (■); BGL + biotin-blocked avidin 4:5:1 (△); BGL + biotin 4:5 (□).

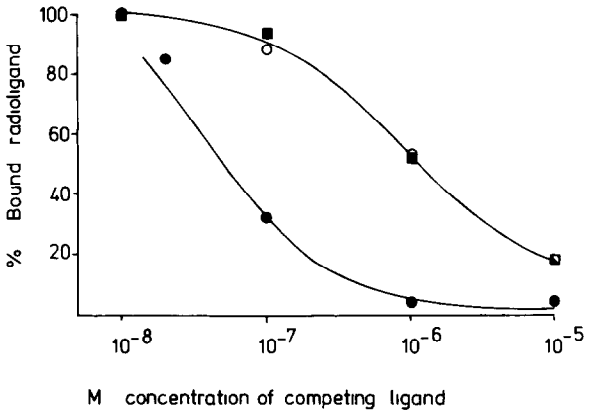

Fig.3. Lack of effect of changing ligand-avidin molecular ratio from 4:1 to 1:1 on BGL + avidin displacement of (D-Ala^{2}Leu^5)-[^{3}H]enkephalin: BGL (•); BGL + avidin (4:1) (▪); BGL + avidin 1:1 (○).

biotin interferes with (D-Ala^2-Leu^5)-[^{3}H]enkephalin binding to receptors.

The effect of different spacer lengths on receptor affinity in the presence of avidin suggests that the large avidin molecule (67 000 M_r) disturbs the interaction of ligand with receptor and thus, that the receptor site is embedded in an environment that prevents close contact with the avidin–ligand complex. The fully extended lengths of the ligands are 22–27 Å for BL and 30–35 Å for BGL. The conformation of enkephalin in solution is not well defined [9]. Little is known about the conformation necessary for receptor interaction of the enkephalin moiety; therefore it is hard to discuss molecular dimensions compatible with formation of the sandwiches. Avidin allows interaction of biotin with its binding sites if it is connected to a bulky molecule by a spacer of ⩾8–9 Å [5]. The length of lysine allows this interaction and a diglycine residue adds another 8–9 Å. It might be possible to achieve even higher receptor affinity in the presence of avidin by increasing the spacer length, unless the secondary structure becomes unfavorable. The lack of effect of changing the ligand:avidin ratio is of interest, one possible implication being that all the 4 ligands bound to one avidin molecule could interact with receptors assumed to be embedded in membranes. Four ligands should be able to bind simultaneously to avidin since studies of space-filling molecular models of the ligands gave a molecular radius of maximum 10 Å, well below the 20 Å allowed for simultaneous binding [5].

The applicability of the described bifunctional ligands to affinity separation experiments remains to be tested. It may be necessary to use steady-state conditions since the receptor affinities are low in the presence of avidin.

Acknowledgements

We thank Dr Ulf Ragnarsson and Mr Peter Lind of the Department of Biochemistry, Uppsala University for gifts of chemicals and helpful discussions. This study was supported by the Fortia Research Foundation and the Swedish Medical Research Council.

References

[1] Dvorak, D. J., Gipps, E. and Kidson, C. (1978) Nature 271, 564–566.
[2] Hofmann, K. and Kiso, Y. (1976) Proc. Natl. Acad. Sci. USA 73, 3516–3518.
[3] Hofmann, K., Finn, F. M., Friesen, H. J., Diaconescu, C. and Zahn, H. (1977) Proc. Natl. Acad. Sci. USA 74, 2697–2700.
[4] Meier, K. E. and Ruoho, A. E. (1978) J. Supramol. Struct. 9, 243–252.
[5] Green, N. M. (1975) Adv. Prot. Chem., 29, 85–133.
[6] Green, N. M. (1970) Methods Enzymol. 18A, 414–424.
[7] Jasiewicz, M. L., Schoenberg, D. R. and Mueller, G. C. (1976) Exp. Cell. Res. 100, 213–217.
[8] Terenius, L. (1974) Acta Pharmacol. Toxicol. 34, 88–91.
[9] Teschemacher, H. J. (1978) Developments in Opiate Research (Herz, A. ed), pp. 67–151, Dekker, Basel, New York.