HORMONE–RECEPTOR INTERACTIONS: [4-CARBORANYLALANINE, 5-LEUCINE]-ENKEPHALIN AS A STRUCTURAL PROBE FOR THE OPIATE RECEPTOR

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Received 11 July 1977
Revised version received 15 August 1977

1. Introduction

The new phenylalanine analogue, L-carboranylalanine (Car) [1] was recently found to be a good probe for investigating some special properties of the aromatic recognition site of chymotrypsin [2]. The icosahedral side chain of Car is somewhat larger than the phenyl ring of Phe, rotating about its 1,4 axis. Yet it is easily accomodated by chymotrypsin; the concomitant deformation of the recognition site propagated to the mechanistic site (especially Ser 195) is probably the reason that Car esters are not hydrolysed by the enzyme.

One of the aims of our laboratory is to study hormone–receptor interactions. After the promising results with chymotrypsin as a model, we are investigating the use of Car replacements of Phe, Tyr, and Trp in polypeptide hormones.

Phenylalanine in position 4 seems to be quite important for enkephalin action, because a number of analogues with replacements of this amino acid by others all show reduced or even missing opiate receptor affinity and biological activity (review [3]; see also [4–6]). An introduction of Car into this position seemed ideally suitable to see whether or not carboranylalanine analogues of peptide hormones can be expected to have exceptional features.

We have therefore prepared [4-carboranylalanine, 5-leucine]-enkephalin, H·Tyr–Gly–Gly–Car–Leu·OH and investigated its ability to displace [^{3}H]naloxone from rat brain opiate receptor preparations according to the technique developed in the laboratory of Snyder [7]. [5-Methionine]-enkephalin, [5-leucine]-enkephalin, and $N(\alpha)$-acetyl-[5-methionine]-enkephalin amide were synthesized for comparison.

It was found that [Car^4, Leu^5]-enkephalin binds better than [Leu^5]-enkephalin (about equally well as [Met^5]-enkephalin) and that $N(\alpha)$-acetyl-[Met^5]-enkephalin amide is virtually inactive. The implications of these findings in terms of enkephalin–opiate receptor interaction are discussed.

2. Materials and methods

[4-Carboranylalanine, 5-leucine]-enkephalin was synthesized by a classical procedure (to be published, Helv. Chim. Acta) via the following analytically pure intermediates:

Boc·Car–Leu·OMe (m.p. 176°C; $[\alpha]_D^{20}$ –38.1°, *c* 0.53 in methanol),

Boc·Tyr–Gly–Gly–Car–Leu·OMe (crystals from chloroform/petroleum ether, m.p. 125–130°C) and

Boc·Tyr–Gly–Gly–Car–Leu·OH (m.p. 140°C).

The final product, HCl, H·Tyr–Gly–Gly–Car–Leu·OH, had m.p. 147°C (decomposition) and $[\alpha]_D^{20}$ –2.05° (*c* 0.44 in methanol).

$N(\alpha)$–Acetyl-[5-methionine]-enkephalin amide was synthesized in a similar manner (report for Helv. Chim, Acta, in preparation). Crystallized from ethyl acetate/ether as colorless platelets, m.p. 192°C, $[\alpha]_D^{20}$ 3.2° (*c* 1.0 in methanol).

Opiate receptor affinities were determined by a modified version of the binding assay described by Pasternak et al. [7]. Male Sprague-Dawley rats (250–300 g) of the Canadian Breeding Laboratories

were decapitated and after removal of the cerebellum the brains were homogenized in 30 vol ice-cold standard buffer (50 mM Tris–HCl, pH 7.7). The combined homogenates were centrifuged at 30 000 × *g* for 30 min at 4°C and the membranes reconstituted in the original volume of standard buffer. After incubation at 37°C for 30 min and subsequent centrifugation the pellet was again suspended in the initial volume of fresh standard buffer to yield the final membrane suspension. Aliquots (2 ml) of the membrane preparation were incubated for 1 h at 0°C [8] with 1 ml standard buffer containing the peptide to be tested and [^{3}H]naloxone (17.7 Ci/mmol; New England Nuclear Corp.) at a final concentration of 0.5 nM. At the end of the incubation the reaction mixtures were filtered through Whatman GF/B filters under vacuum at 4°C; the filters were then washed with two 5 ml portions ice-cold standard buffer. Subsequently the filters were transferred to scintillation vials and treated with 1 ml Soluene (Packard) for 30 min, whereupon 0.5 ml acetic acid and 10 ml Aquasol (New England Nuclear Corp.) were added. After shaking for 30 min the vials were counted at an efficiency of 40–45%. Stereospecific binding as determined by displacement of [^{3}H]naloxone with excess (10 μM) cold [Met^5]-enkephalin accounted for 60–70% of total binding. Values of half-maximal inhibition (IC50) of the stereospecific binding were obtained graphically from semi-logarithmic plots. Each compound was tested at least three times and [Met^5]-enkephalin was included in each binding experiment for comparison.

3. Results and discussion

According to our experience with corticotropin and melanotropin derivatives, naloxone displacement [7] is possibly a more reliable indication for specific interaction of opioid agonists and antagonists with the opiate receptor than dihydromorphine displacement [9]. (A number of non-analgesic hormone derivatives were found to be active in the latter, but not in the former test: Peter Schiller and Alex Eberle, unpublished observations.) We therefore used the former assay system without as yet looking into the effects of sodium, potassium, manganese and calcium ions, which are supposed to differentiate between opioid agonists and antagonists [10,11].

A typical experiment for the displacement of [^{3}H]naloxone by [Met^5]-enkephalin and [Car^4,Leu^5]-enkephalin is plotted in fig.1. In table 1, the average values of half-maximal specific displacement (IC50 [12]) are expressed as molar affinities (1/IC50) in percent [Met^5]enkephalin molar affinity.

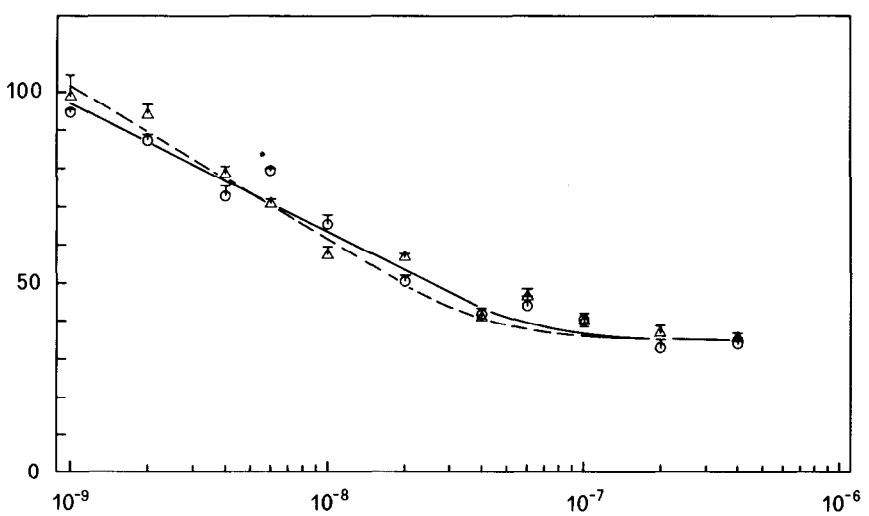

Fig.1. Displacement of [^{3}H]naloxone by [Met^5]-enkephalin (○——○) and [Car^4, Leu^5]-enkephalin (△– – –△) from rat-brain opiate receptors. Naloxone concentration, 0.5 nM. Each point represents the mean of 3 measurements ± SEM. Abscissa: molar concentration of the enkephalin peptides. Ordinate: percent naloxone bound.

Table 1
Relative molar affinities of enkephalin analogs

Analog	% Receptor affinity relative to [Met^5]-enkephalin
[Met^5]-enkephalin	100[a]
[Leu^5]-enkephalin	38 ± 6[b]
H·Tyr–Gly–Gly–Car–Leu·OH	116 ± 18[b]
Ac·Tyr–Gly–Gly–Phe–Met·NH_2	0.1

[a]IC50 1.2×10^{-8} M in this assay system
[b]Mean of three determinations ± SEM

Whereas the affinity of [Leu^5]-enkephalin was found to be about 1/3 of that of [Met^5]-enkephalin, a result that is in good agreement with those of other authors [12,13], [Car^4, Leu^5]-enkephalin is approx. equally potent, but *N*(α)-acetyl-[Met^5]-enkephalin amide only about 1/1000.

3.1. *The nature of the Phe^4-recognizing site of the opiate receptor*

Obviously, the replacement of the aromatic phenyl ring of phenylalanine by the slightly bulkier, but more lipophilic, carboranyl system results in a tighter association of the peptide with the opiate receptor. The situation is quantitatively similar to that of the interaction of Z·Ala–Ala–Car·OH and Z·Ala–Ala–Phe·OH with chymotrypsin: the former is an approx. 3 times better inhibitor (K_i 0.3 mM) than the latter (K_i 1 mM) [2]. It appears that the opiate receptor carries a phenyl recognition site comparable to the corresponding 'pocket' of chymotrypsin in that, like the enzyme, it is capable of accomodating the carboranyl icosahedron. Whether or not this accomodation is accompanied by a deformative adaptation of the recognition site and affects the ability of the receptor to be 'triggered' by the enkephalin is presently under investigation. Despite the similarities, at least one difference between the two recognition sites remains: whereas tyrosine is about as easily adapted by chymotrypsin as are phenylalanine and tryptophan, the analogue, [Tyr^4, Leu^5]-enkephalin retains only about 3% of the dihydromorphine displacing potency [6]. If this is true, the opiate receptor molecule, unlike chymotrypsin, must lack the possibility of adapting the *p*-hydroxyl group.

3.2. *The α-amino group of the enkephalins and the opiate receptor*

The very low level of activity found here for *N*(α)-acetyl-[Met^5]-enkephalin amide agrees with the findings of Bradbury et al. [4] that a positive charge at the N-terminus is required for binding. The virtual inactivity of the compound is certainly not due to the C-terminal carboxyamide group, because [Met^5]-enkephalin amide [4] and [D-Ala^2, Met^5]-enkephalin amide [5,14] belong to the most active compounds hitherto known.

Acknowledgements

We thank the Swiss National Science Foundation and the Medical Research Council of Canada for grants, Mr Willy Hübscher and Miss Claudia Petermann for expert technical assistance, and Miss Ruth Kuhn for secretarial help.

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