D-TYR–SER–GLY–PHE–LEU–THR, A HIGHLY PREFERENTIAL LIGAND FOR δ-OPIATE RECEPTORS

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Received 11 July 1980

1. Introduction

Binding studies of radiolabeled enkephalins with brain homogenates [1–3] suggest that these peptides interact with at least two distinct classes of binding sites called μ and δ receptors. Enkephalins bind to δ receptors which constitute almost 20% of the total binding with a high affinity ($K_d \sim 0.5$ nM and to μ receptors with a lower affinity ($K_d \sim 5$ nM) [1,2]. Furthermore, displacement experiments with antagonists show that morphine and synthetic opiates interact strongly with μ receptors whereas peptides exhibit a high preference for δ receptors [1,2,4]. Such results were also found in peripheric organs and attributed to the preponderance of μ receptors in the guinea pig ileum (GPI) and δ receptors in the mouse vas deferens (MVD) [1,4]. Therefore, the difference in the pharmacological action of any compound on the two organs was used to evaluate its μ and δ specificity [4].

The biological significance of the presence of μ and δ receptors in the brain remains still unknown although it seems that μ receptors could be involved in analgesia [4] whereas δ receptors might be implicated in behavioral effects [5]. A possible dissociation of the pharmacological responses associated to multiple opiate receptors have incited a great number of studies in vivo [6,17] and in vitro [3,7] for the last months.

However, these studies were performed with D-Ala^2-D-Leu^5 enkephalin (DADL), a compound which exhibits only a partial specificity for δ receptors [4]. Therefore it was of great importance to search for a much higher specific ligand, in order to avoid multiple biological responses arising from simultaneous binding to the two kinds of states. Starting from conformational data and structure activity relationships in the enkephalin series, such a compound was prepared. This new hexapeptide Tyr–D-Ser–Gly–Phe–Leu–Thr exhibits a very high specificity for δ receptor, since its potency is 620-times greater in MVD ($IC_{50} = 0.58$ nM) than in GPI ($IC_{50} = 360$ nM).

2. Materials and methods

2.1. *Synthesis*

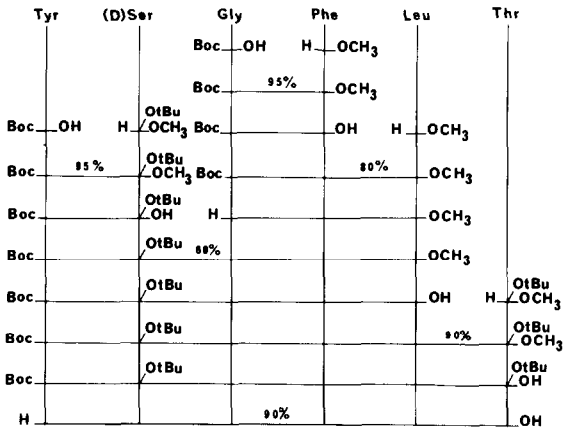
Protected aminoacids used in this synthesis were purchased from Bachem (Switserland). Met-enkephalin and Leu-enkephalin were prepared as in [8,9].

Tyr–D-Ser–Gly–Phe–Leu–Thr has been prepared by liquid phase method using terbutyloxycarbonyl (Boc) and methylesters as protecting groups and dicyclohexylcarbodiimide (DCC) with hydroxybenzotriazole as coupling reagents [8,10]. The deprotection steps were done using trifluoroacetic acid (TFA) for Boc group and saponification by NaOH for methyl esters. During the synthesis the structure of the different compounds was confirmed by NMR spectroscopy (Brüker WH 270 MHz) in $DMSOd_6$ and their purity was checked by thin-layer chromatography on silica-gel glass plate using: (A) *n*-BuOH/AcOH/H_2O, 4/1/1; (B) $CHCl_3$/MeOH, 9/1; or (C) $CHCl_3$/MeOH, 9/1, as solvents.

The different steps of the synthesis of Tyr–D-Ser–Gly–Phe–Leu–Thr are summarized in scheme 1.

The protection of D-Ser and Thr hydroxyl groups allowed us to obtain high yields in every step.

The final compound was obtained by deprotection of both N-terminal group and lateral chains, by stirring with HCl 1 N in TFA during 30 min. The solution



Scheme 1

was concentrated in vacuo and a white powder precipitated by addition of Et_2O. The crude product was purified by gel filtration on LH 20 (Pharmacia) using MeOH as solvent.

The hexapeptide is characterized by a single peak (retention times = 612 s) in high performance liquid chromatography (HPLC) on a reverse phase microbondapak C 18; solvent CH_3CN/10 mM, NH_4Ac buffer (pH 4.2) 20/80, flowrate 1.2 ml/min. The ^1H NMR chemical shifts (10^{-2} M in $DMSOd_6$ with HMDS as internal reference) for Tyr–D-Ser–Gly–Gly–Leu–Thr are: Tyr, OH = 9.17, H_m = 6.97, H_o = 6.61, H_α = 3.62; D-Ser, NH = 8.37, H_α = 4.14, OH = 5.33; Gly, NH = 8.20, CH_2 = 3.65; Phe, NH = 8.02, H_α = 4.41; Leu, NH = 8.32, H_α = 4.10, CH_3 = 0.78; Thr, NH = 7.23, H_α = 3.81, CH_3 = 0.86, OH = 4.96.

2.2. *Pharmacological assays*

Opioid activity was evaluated on GPI and on MVD as in [10]. Six different concentrations of each compound (6–8 assays for each) were tested for the inhibition of electrically induced contractions. IC_{50} were determined by regression analysis. According to [4], methionine-enkephalin was used in each assay as internal standard to avoid differences of sensitivity of each preparation.

2.3. *Degradation study*

The enzymatic stability of Tyr–D-Ser–Gly–Phe–Leu–Thr was checked by incubation with membranes from mouse striatum as in [10]. The peptide was dissolved in 1 ml P2 fraction (1 mg protein/ml corresponding to 10 mg tissue) at 10^{-6} M. Aliquots of 10 μl incubates were collected at intervals of 10 min and injected in the same conditions as in section 2.2. The detection was performed at 210 nm. No significant degradation seems to occur after 1 h as shown by the absence of change in the hexapeptide signal.

3. Results and discussion

We have shown that the phenylalanine moiety plays a crucial role in the differential recognition of enkephalins to μ and δ receptors [11]. Therefore the design of a specific ligand for δ receptors was based on the following considerations:

(i) As reported [12] the lengthening of Met-enkephalin by addition of a Thr residue on the C-terminal methionine enhances the potency on MVD and decreases the activity on GPI.

(ii) Leu-enkephalin shows a significant increased potency on MVD as compared to Met-enkephalin [1];

(iii) The replacement of Gly^2 by a D-amino acid inhibits the degradation of the peptide by aminopeptidases [13] and reinforces preferentially the binding to the μ receptor when the side chain of this D-amino acid is of hydrophobic nature [14].

These features lead to the synthesis of the hexapeptide Tyr–D-Ser–Gly–Phe–Leu–Thr. As shown in table 1, this compound exhibits a considerable increased potency on MVD as compared to Met-enkephalin which has been taken as a reference [4]. Moreover, at variance with Tyr–D-Ala–Gly–Phe–D-Leu, the introduction of D-Ser in the hexapeptide leads to a large decrease in activity on the GPI. Consequently the ratio of inhibitory potency on GPI (μ receptors) to MVD (δ receptors) is much higher for the hexapeptide (GPI/MVD = 620 than for the pentapeptide, GPI/MVD = 88.

From this large variation in activity on the two isolated organs, Tyr–D-Ser–Gly–Phe–Leu–Thr can be considered as a highly specific ligand for δ receptors and should allow a complete dissociation of the pharmacological responses according to its binding with a single class of receptors.

Furthermore, Tyr–D-Ser–Gly–Phe–Leu–Thr is not significantly degraded after 1 h incubation with mouse striatal membranes. This property results both from the replacement of Gly^2 by $D\text{-}Ser^2$ inhibiting the action of aminopeptidases [13] and from the addition of a threonine residue at the C-terminal part

Table 1
Comparison of the inhibitory effects on guinea pig ileum and mouse vas deferens between enkephalins, Tyr–D-Ala–Gly–Phe–D-Leu and Tyr–D-Ser–Gly–Phe–Leu–Thr

Peptide	Inhibition of contractions (IC_{50}, nM)		IC_{50}GPI/ IC_{50}MVD
	Guinea pig ileum	Mouse vas deferens	
Tyr–Gly–Gly–Phe–Met (Met-E)	200.0 ± 19 (6)	13.00 ± 1.50 (6)	15.4
Tyr–Gly–Gly–Phe–Leu (Leu-E)	455.0 ± 53 (6)	8.82 ± 1.20 (6)	51.6
Tyr–D-Ala–Gly–Phe–D-Leu[a]	47.8 ± 4.4 (4)	0.54 ± 0.09 (5)	88.5
Tyr–D-Ser–Gly–Phe–Leu–Thr	360.0 ± 28 (8)	0.58 ± 0.20 (8)	620.7

[a] From [4]

The values are the means ± SEM; the no. obs. is given in parentheses

of the peptide preventing its degradation by enkephalinase [15,16].

Therefore, this compound could be of great interest to confirm the very promising results on the lack of cross-tolerance between δ receptor agonists and μ receptor agonists [6], as well as to investigate the chemical differences between the two receptors at the molecular level of highly selective protection experiments [7].

Finally, despite the smaller concentration of δ receptors in the brain, Tyr–D-Ser–Gly–Phe–Leu–Thr could be used to evaluate the regional distribution of this kind of sites. For such purposes radiolabeling of this peptide is now in progress.

Acknowledgements

We thank Professor J. C. Schwartz (INSERM U 109) for his interest and Dr C. Llorens for the preparation of mouse striatal membranes. We are grateful to Dr X. Pascaud for helpful discussions on this subject and to A. Bouju for typing. This work was supported by grants from the Université René Descartes and the Délégation Générale à la Recherche Scientifique et Technique.

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