δ and μ opiate receptor probes: fluorescent enkephalins with high receptor affinity and specificity

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The fluorescent amino acid, L-1-pyrenylalanine (Pya) was incorporated into [D-Ala^2,Leu^5]enkephalin and its methyl ester at position 4 or 5. Pya-enkephalins showed strong fluorescent intensity and displayed high binding affinity for opiate receptors. Pya^4-enkephalins showed high specificity for the μ receptors, while Pya^5-enkephalins showed high specificity and selectivity for the δ receptors. Particularly, [D-Ala^2,Pya^5]enkephalin was as potent as the most utilized δ-specific ligand of [D-Ala^2,D-Leu^5]enkephalin (DADLE), and yet its δ-selectivity was about 5-times greater than that of DADLE. Thus, Pya-enkephalins per se can be utilized as a fluorescent probe or tracer for the opiate receptor-binding assays.

Fluorescent enkephalin *Peptide synthesis* *Opiate receptor* *Receptor affinity* *Receptor selectivity*

1. INTRODUCTION

Fluorescent derivatives of biologically active peptides represent useful experimental tools for the visualization and exploration of ligand-receptor interactions. The fluorescent groupings are usually introduced into peptides by covalent bonding through the amino, carboxyl or thiol group in their sequence. However, such functional groups often play an important role in biological action, and thus it is necessary to incorporate a fluorescent group without changing or destroying conformational and biological characteristics of the parent peptide. If the fluorescent core can be incorporated into an amino acid as a constructive grouping, the usefulness of such an amino acid would become considerable when introduced into a biologically active peptide. We have developed a facile procedure to synthesize an optically pure fluorescent amino acid of L-1-pyrenylalanine (Pya) [1], which possesses considerably high quantum yield, long lifetime, high aromaticity, and high lipophilicity. Tryptophan is well-known as a fluorescent amino acid, but its quantum yield is not quite as high as compared with Pya. Here, we report the incorporation of Pya into the opiate peptide enkephalin.

Since the discovery of the multiplicity of the opiate receptors, which include at least 2 distinct sites of δ-enkephalin and μ-morphine receptors [2,3], the biological evaluation of synthesized enkephalin analogs has been performed by 2 different pharmacological experimental procedures: (i) receptor-binding assays using rat brain membrane preparations, and (ii) in vitro biological assays using isolated and electrically stimulated

Abbreviations: Abbreviations used are according to IUPAC-IUB Commissions (1984) Eur. J. Biochem. 138, 9–37. Other abbreviations: DADLE, [2-D-alanine,5-D-leucine]enkephalin; DAGO, [2-D-alanine, 4-*N*-methylphenylalanine,5-glycinol]enkephalin; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Pya, L-1-pyrenylalanine; Pya^4-Enk-OH and -OMe, [2-D-alanine,4-pyrenylalanine,5-leucine]enkephalin and its methyl ester; Pya^5-Enk-OH and -OMe, [2-D-alanine, 5-pyrenyl-alanine]enkephalin and its methyl ester; Pya^4- and Pya^5-enkephalins denote analogs of enkephalins containing Pya at position 4 and 5, respectively

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smooth muscle preparations. Binding assays, however, invariably require expensive radiolabelled enkephalin peptide or opiate derivatives and experimental restrictions because of their radioactivity. If the fluorescent enkephalin derivative possesses high receptor-binding affinity together with high fluorescence characteristics, it can be utilized much more easily as a fluorescent tracer for the binding assays. Thus, we have synthesized [D-Ala^2,Pya^4,Leu^5]- and [D-Ala^2,Pya^5]enkephalins and their methyl ester derivatives (fig.1), and examined the binding characteristics for the δ and μ receptors. Pya^4-enkephalins showed high specificity for the μ receptors, while Pya^5-enkephalins showed high specificity and selectivity for the δ receptors.

2. EXPERIMENTAL

2.1. *Syntheses of enkephalin analogs containing Pya*

Pya was synthesized by the asymmetric hydrogenation of cyclic dehydrodipeptide [4] derived from cyclo(-Gly-Ala-) and 1-pyrenecarboxyaldehyde; m.p. 220°C (dec); $[\alpha]_D^{20}$ −36° (*c* 0.2, AcOH).

Synthesis of [D-Ala^2,Pya^4,Leu^5]enkephalin (**1**) is described briefly as a representative example. Boc-Pya-OH was coupled to H-Leu-OMe by the DCC-HOBt method, and after removing the Boc group by HCl/dioxane, the dipeptide was coupled with Boc-Tyr-D-Ala-Gly-OH also by DCC-HOBt. Saponification (1 M NaOH) of the resulting Boc-pentapeptide methyl ester followed by treatment with trifluoroacetic acid to remove the Boc group afforded the desired Pya^4-enkephalin (Pya^4-Enk-OH), while Pya^4-enkephalin methyl ester (Pya^4-Enk-OMe) was obtained by treatment with trifluoroacetic acid without saponification. Purifications were performed by gel filtration with a Sephadex G-15 column in 30% AcOH. Their purity was confirmed by silica gel thin-layer chromatography using the following solvent systems: R_f^1, *n*-BuOH/AcOH/pyridine/H_2O (4:1:1:2); R_f^2, $CHCl_3$/MeOH/AcOH (25:5:1). Pya^4-Enk-OH, R_f^1 0.78, R_f^2 0.55; Pya^4-Enk-OMe, R_f^1 0.82, R_f^2 0.58. Pya^5-enkephalins were prepared similarly. The syntheses will be reported in detail elsewhere.

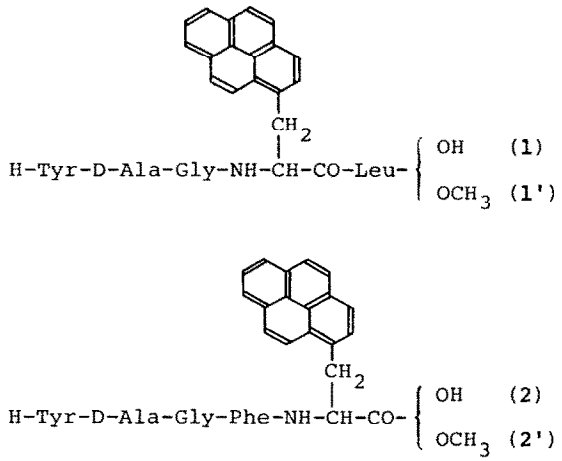

Fig.1. Chemical structures of Pya^4- and Pya^5-enkephalins.

2.2. *Fluorescence characterization*

The fluorescence spectra were recorded on a Hitachi 650-10S spectrofluorophotometer. The spectra were recorded in 50 mM Tris-HCl buffer (pH 7.4) at 25°C with various concentrations. Fluorescence spectra of Pya-enkepahlins (1×10^{-7} M) excited at 342 nm are shown in fig.2.

2.3. *Receptor-binding assays*

Receptor-binding assays using rat brain membrane preparations were carried out essentially as described [5]. [^{3}H]DADLE (40 Ci/mmol, New England Nuclear) and [^{3}H]DAGO (38 Ci/mmol, New England Nuclear) were used as tracers specific for δ and μ receptors, respectively, at a final concentration of 0.25 nM. Incubations were carried out for 60 min at 25°C in 50 mM Tris-HCl buffer (pH 7.4) containing bestatin (1 μM) and bacitracin (100 μg/ml) as enzyme inhibitors.

Dose-response curves were constructed utilizing 11 dose levels in duplicate. Results were analyzed by computer program [6] and the data used to construct the least-squares estimates of the logistic curves relating the binding of labelled ligand to concentrations of unlabelled ligand. The results are shown in table 1.

3. RESULTS AND DISCUSSION

The pyrenyl group has several interesting photophysical properties such as a long lifetime of yrene monomers and efficient formation of ex-

cimers associated with its high quantum yield [7,8]. The fluorescence spectra of Pya[4]- and Pya[5]-enkephalins measured in 50 mM Tris-HCl buffer (pH 7.4) are shown in fig.2. All the Pya-enkephalins exhibited the maximum intensity of fluorescence by exciting at 342 nm, which is one of the absorption maxima of the Pya residue per se. It is of note that fluorescence emission in Tris-HCl buffer, the same buffer employed in the binding assay, can be detected even below 10^{-9} M for all Pya-enkephalins. These results certainly demonstrate that Pya-enkephalins possess sufficient fluorescence properties even for utilizing them as a fluorescence probe in biological assay systems.

Receptor-binding affinities of Pya-enkephalins were evaluated for their ability to displace [^{3}H]DADLE and [^{3}H]DAGO for the δ and μ receptors in rat brain membrane, respectively (table 1). It is clear that all Pya-enkephalins possess considerably high binding potencies for both the δ and μ receptors. Pya[4]-enkephalins (**1** and **1'**) displayed almost the same affinity (16.3 and 11.9 nM, respectively) for the μ receptors as DADLE (20.1 nM), while Pya[5]-enkephalins (**2** and **2'**) displayed almost the same affinity (2.2 and 3.6 nM, respectively) for the δ receptors as DADLE (2.14 nM). DADLE is the most utilized δ-specific ligand to date. It should be noted that Pya[5]-enkephalins become quite δ receptor selective due to the considerably diminished affinity for the μ receptors (113–124 nM). When the ability of enkephalins to discriminate between δ and μ receptors is expressed as a ratio of the IC_{50} using [^{3}H]DAGO vs IC_{50} using [^{3}H]DADLE, both Pya[5]-enkephalins (**2** and **2'**) showed high δ-selectivity ratios of 51 and 34, respectively, which are much higher than that of DADLE (9.4) (table 1).

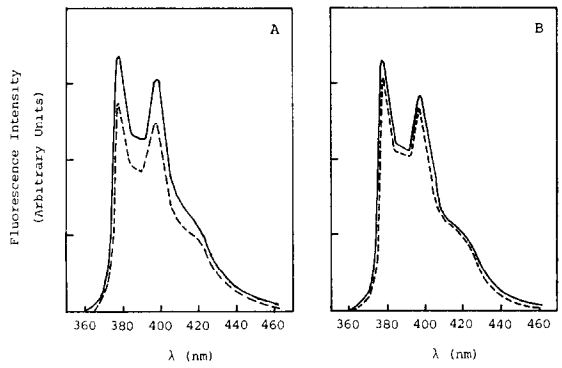

Fig.2. Fluorescence spectra of Pya-enkephalins in 50 mM Tris-HCl buffer (pH 7.4) at 1×10^{-7} M. (A) Pya[4]-enkephalins, (B) Pya[5]-enkephalins, (——) carboxylic acid, (– – –) methyl esters.

These results indicate that the Pya[5] residue may have very strong interaction with the corresponding subsites in the δ receptors or its environment, but not with the μ receptors.

Furthermore, this possible interaction should be emphasized due to the absence of a difference in affinity between Pya[5]-Enk-OH (**2**) and -OMe (**2'**) for both the δ and μ receptors. The C-terminal carboxyl group is one of the important enkephalin-like characteristics which result in greater specificity and selectivity for the δ receptors [9]. Thus, Pya[5]-Enk-OH (**2**) was strongly δ-selective. When the carboxyl group is esterified or amidated, the derivative usually increases its affinity greatly for the μ receptors, and becomes a non-selective ligand. However, Pya[5]-Enk-OMe (**2'**) displayed almost unchanged μ-affinity and thus sustains a very high δ-selectivity as mentioned above. This

Table 1

Receptor binding affinity and δ-selectivity of Pya-enkephalins

Enkephalin	IC_{50} (nM)		δ-selectivity[a]
	[^{3}H]DADLE	[^{3}H]DAGO	
DADLE	2.14	20.1	9.4
DAGO	80.2	1.45	0.018
Pya[4]-Enk-OH (**1**)	11.8	16.3	1.4
Pya[4]-Enk-OMe (**1'**)	73.9	11.9	0.16
Pya[5]-Enk-OH (**2**)	2.2	113.2	51
Pya[5]-Enk-OMe (**2'**)	3.6	123.7	34

[a] Calculated from IC_{50} (μ)/IC_{50} (δ)

may indicate that the Pya[5] residue has a very favorable interaction with δ receptors and unfavorable interaction with the μ receptors. Fauchére [10] has reported the synthesis and δ and μ activities of enkephalin analogs containing so-called fat amino acids in position 5 such as adamantylalanine and *tert*-butylglycine. These amino acid residues have considerably high hydrophobicity and steric bulkiness. However, those amidated enkephalins were non-selective, exhibiting a fair potency for both the δ and μ receptors. High aromaticity and/or high lipophilicity of the pyrene ring in Pya[5]-enkephalins may be responsible for the discriminative recognition of δ receptors.

Pya[4]-Enk-OH (**1**) is non-selective, exhibiting fair affinities for the δ and μ receptors. When compound **1** was esterified, the resulting methyl ester **1'** displayed an almost 6-fold decrease in δ-affinity and a slight increase in μ-affinity and thus become μ-selective. This suggests that the Pya[4] residue possesses a reversed effect of the Pya[5] residue in Pya[5]-enkephalins for the opiate receptors. These results, however, are comparable to the fact that the enkephalin amides containing fat amino acids, adamantylalanine or carboranylalanine in position 4, were μ receptor selective [10].

Some fluorescent enkephalins have been reported by other groups as probes for conformational studies [11,12] or studies of ligand-receptor interactions [13]. These include [Trp[4]]enkephalins [11] and dansylated enkephalin analogs [12,13]. However, their fluorescence intensity was moderate compared to the strong intensity of Pya-enkephalins. In addition, very high receptor specificity and selectivity have been found for Pya-enkephalins in the present study. Thus, Pya-enkephalins may represent a useful fluorescence probe or tracer to explore discriminatively the δ and μ receptors. Their applications to binding assays and studies on interactions with lipids are under our consideration.

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