Comparative binding properties of linear and cyclic δ-selective enkephalin analogues: [^{3}H]-[D-Thr^2, Leu^5] enkephalyl-Thr^6 and [^{3}H]-[D-Pen^2, D-Pen^5] enkephalin

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The range of δ-selectivity of linear and cyclic analogues of enkephalin in rat brain was found to be: [D-Pen^2, L-Pen^5] enkephalin (DPLPE) > [D-Pen^2, D-Pen^5] enkephalin (DPDPE) > [D-Thr^2, Leu^5] enkephalyl-Thr^6 (DTLET) > [D-Ser^2, Leu^5] enkephalyl-Thr^6 (DSLET). Saturation experiments performed with [^{3}H]DPDPE and [^{3}H]DTLET in NG108-15 cells and rat brain showed similar binding capacities for both the ligands, but the δ-affinity of [^{3}H]DTLET ($K_D \approx 1.2$ nM) was much better than that of [^{3}H]DPDPE ($K_D \approx 7.2$ nM). The rather low δ-affinity of DPDPE induced high experimental errors cancelling the benefit of its better δ-selectivity. Binding experiments in rat or guinea-pig brains showed, in both cases, the better δ-selectivity of [^{3}H]DTLET compared to [^{3}H]DSLET. The former peptide remains at this time the most appropriate radioactive probe for binding studies of δ-receptor.

δ-Enkephalin analogue *Opioid receptor binding* *Rat brain* *Guinea-pig brain* *NG108-15 hybrid cell*

1. INTRODUCTION

Considerable interest has been devoted to the biochemical and pharmacological properties of the δ opioid receptor type [1]. However, the lack of fully δ-selective ligands has led to controversy [2] about its molecular nature, i.e., whether it is an independent receptor [3,4] or μ-interconvertible conformation [5] or allosteric site [6,7]. From both extensive conformational studies performed by NMR and theoretical calculations, Fournié-Zaluski et al. [8] proposed structural requirements for ligands to preferentially interact with μ or δ sites. This led to the syntheses of Tyr-D-Ser-Gly-Phe-Leu-Thr (DSLET) and Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET), which have also been in tritiated form [9,10]. Owing to their enhanced δ-selectivity, these ligands have therefore been used, in preference to the poorly selective [D-Ala^2,D-Leu^5]enkephalin [10], to elucidate the pharmacological role of the δ-receptor [11–15], its distribution in the rat brain [16] and its binding properties [17]. Recently, Mosberg et al. [18] described two conformationally restricted cyclic enkephalin analogues, [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and [D-Pen^2,L-Pen^5]enkephalin (DPLPE), which show an even better selectivity for the δ-binding site than the linear peptides DSLET and DTLET. All of these analogues exhibit very low potencies at μ sites [22]. As one of the cyclic compounds, [^{3}H]DPDPE, is now commercially available, it was of interest to study its binding properties on rat

Abbreviations: DAGO, [D-Ala^2,(Me)Phe^4,Gly-ol^5]-enkephalin; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DSLET, [D-Ser^2,Leu^5]enkephalyl-Thr^6; DTLET, [D-Thr^2,Leu^5]enkephalyl-Thr^6; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; DPLPE, [D-Pen^2,L-Pen^5]enkephalin

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brain tissue which contains both μ and δ receptor types, with the aim of performing competition experiments with selective μ-agonists.

2. MATERIALS AND METHODS

2.1. *Chemicals*

DSLET [19] and DTLET [10] were prepared as previously described. DPDPE and DPLPE were a generous gift from Dr H.I. Mosberg or purchased from Bachem, AG. [^{3}H]DTLET (45 Ci/mmol) was obtained from its 2,5-dibromotyrosyl precursor [20]. [^{3}H]DSLET (30.5 Ci/mmol) was purchased from New England Nuclear and [^{3}H]DAGO (60 Ci/mmol) and [^{3}H]DPDPE (35 Ci/mmol) from Amersham.

2.2. *Preparation of crude membrane fractions and binding assays*

The experiments were performed on male Sprague-Dawley rats and guinea-pigs (Ruvel, France). NG108-15 cells were cultivated in Dulbecco's modified Eagle's medium. Crude membrane preparations and binding assays were previously described [17]. NG108-15 cells were homogenized with a Polytron grinder, prior to centrifugation at 100000 × g for 35 min. All binding assays were carried out in triplicate in a volume of 1 ml, at 35°C for 45 min unless otherwise indicated.

The K_I values were calculated from the relationship $K_I = IC_{50}/(1 + L/K_D)$ where L is the concentration of the labelled ligand and K_D its equilibrium dissociation constant. Linear regression analyses were done on a Hewlett-Packard HP85A desk calculator.

3. RESULTS

3.1. *Selectivities of bis-penicillamine enkephalins*

Selectivities of linear and cyclic enkephalin analogues for μ and δ sites of rat brain were indirectly determined by inhibition experiments using [^{3}H]DAGO (1 nM), a μ-selective ligand [21] and [^{3}H]DSLET (2 nM), a nearly δ-selective ligand [9,10] (table 1). The Hill coefficients accounted for the homogeneity of the displacement curves observed.

As shown in table 1, bis-penicillamine enkephalins DPDPE and DPLPE had higher K_I values at the μ-binding site than DSLET and DTLET. However, in agreement with a previous study [18], the conformationally restricted enkephalin analogues showed weaker inhibition potencies at δ-sites than the more flexible linear peptides. As illustrated by the ratio of their respective K_I values for δ and μ sites, the selectivity of the studied peptides (table 1) increased over the following range: DSLET, DTLET, DPDPE, DPLPE. These findings disagree with other papers [4,18,22] reporting a better selectivity for DSLET than DTLET.

Table 1

Inhibitory potencies of enkephalin analogues on the binding of [^{3}H]DAGO (1 nM) at μ-site and of [^{3}H]DSLET (2 nM) at δ-site in rat brain tissue

Analogues	μ-site		δ-site		$K_I(\delta)/K_I(\mu)$
	K_I[a] (nM)	n Hill[a]	K_I[a] (nM)	n Hill[a]	
DPLPE	873 ± 210	0.83 ± 0.09 (4)	10.9 ± 1.2	0.85 ± 0.05 (5)	0.0125
DPDPE	993 ± 151	0.80 ± 0.03 (3)	19.2 ± 1.4	0.82 ± 0.05 (4)	0.0193
DTLET[b]	25.3 ± 2.5	0.85 ± 0.03 (5)	1.35 ± 0.15	0.86 ± 0.02 (5)	0.053
DSLET[b]	31.0 ± 5.0	0.80 ± 0.05 (4)	4.80 ± 0.80	0.81 ± 0.04 (4)	0.15

[a] Means ± SE

[b] Values from [10]

Results were obtained at 35°C from the analysis of Hill plots using 8 concentrations of unlabelled compound. The number of observations is given in parentheses

While DPDPE was approx. 2.5-times more δ-selective than DTLET, the latter displayed a 14-times higher apparent affinity for the δ receptor type.

3.2. *Binding characteristics of [^{3}H]DPDPE and [^{3}H]DTLET on rat brain and NG108-15 cells membranes*

In the case of [^{3}H]DPDPE (10 nM), steady-state conditions were established from association kinetics on rat brain membranes at 35 and 4°C. Equilibrium was reached in 45 min at 35°C (k_{+1}(app) = 0.061 min^{-1}) and in 2 h at 4°C (k_{+1}(app) = 0.020 min^{-1}). However, the specific binding at 4°C was only 51% of that at 35°C. The binding kinetics of [^{3}H]DTLET have already been determined [10].

Saturation experiments were performed on membranes from rat brain and NG108-15 cells, at 35°C. The equilibrium dissociation constants (K_D) and binding capacities (B_{max}) were calculated from linear regression analysis of the saturation isotherms by either Scatchard or Eadie-Hofstee plots. The accuracy in the determination of binding parameters can be evaluated from the standard deviation of the error of the raw data, SD(Erad) [23]. As shown in table 2, in rat brain the SD(Erad) for [^{3}H]DTLET was around 5% while it reached 20% for [^{3}H]DPDPE. The range of error for the two radioligands is illustrated in fig.1 by the differences in B_{max} and K_D values derived from least-squares linear regression analyses of Scatchard and Eadie-Hofstee plots [24]. Since the specific binding of [^{3}H]DPDPE was greater in NG108-15 cells, its K_D values derived from the two analyses were similar, leading therefore to an accurate SD(Erad) estimate of 5%. As shown in table 2, [^{3}H]DPDPE had one sixth of [^{3}H]DTLET affinity. The binding capacity of [^{3}H]DPDPE in rat brain showed no significant difference from [^{3}H]DTLET binding capacity.

3.3. *Binding of [^{3}H]DSLET and [^{3}H]DTLET in rat and guinea-pig brains*

Saturation experiments of [^{3}H]DSLET and [^{3}H]DTLET showed that in both preparations [^{3}H]DTLET exhibited the highest affinity: K_D = 1.36 ± 0.12 nM against 3.49 ± 0.22 nM for [^{3}H]DSLET in rat brain and K_D = 1.08 ± 0.08 nM against 1.97 ± 0.16 nM for [^{3}H]DSLET in guinea-pig brain. In accordance with its higher cross-reactivity, the capacity for [^{3}H]DSLET was 29% greater than for [^{3}H]DTLET in rat brain (141 ± 9 and 109 ± 7 fmol/mg, respectively) and 23% greater in guinea-pig brain (86 ± 6 and 70 ± 5 fmol/mg, respectively). This higher cross-reactivity of [^{3}H]DSLET agrees with a recent report [22] and is in accordance with competition studies [10].

Table 2

Binding parameters of [^{3}H]DPDPE and [^{3}H]DTLET in crude membrane fractions of rat brain and NG108-15 cells at 35°C

^{3}H-labelled ligand (nM)[b]	Tissue	Scatchard analysis		Eadie-Hofstee analysis		
		K_D[a] (nM)	B_{max}[a] (fmol/mg)	K_D[a] (nM)	B_{max}[a] (fmol/mg)	SD(Erad)[a] (%)
DPDPE (1–20)	Rat brain	10.5 ± 2.4	118 ± 11	5.68 ± 0.63	86 ± 3	20.0 ± 2.9 (4)
	NG108-15	7.28 ± 0.05	419 ± 13	7.10 ± 0.14	410 ± 11	5.2 ± 1.3 (3)
DTLET (0.5–7)	Rat brain	1.19 ± 0.14	103 ± 5	1.12 ± 0.12	101 ± 4	5.1 ± 1.2 (4)
	NG108-15	1.25 ± 0.09	430 ± 10	1.19 ± 0.08	419 ± 9	4.7 ± 1.1 (3)

[a] Means ± SE

[b] Concentration range of tritiated ligand used (6 concentrations in a volume assay of 0.5 ml)

The number of observations is given in parentheses

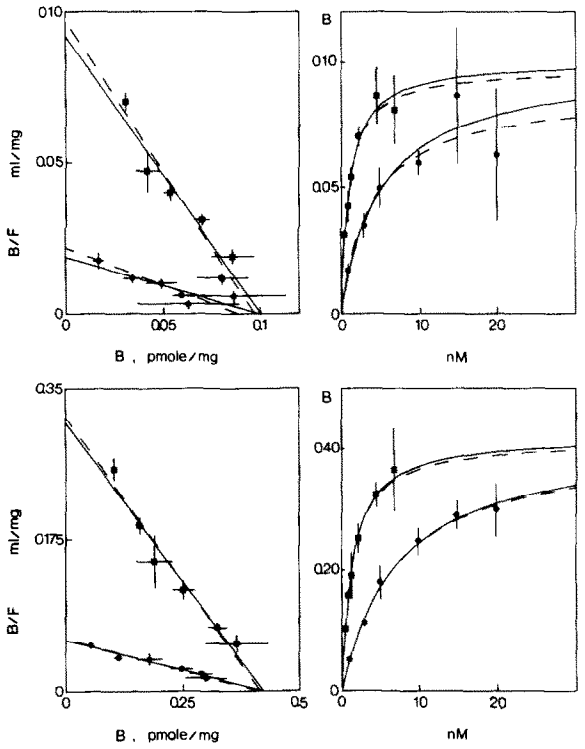

Fig.1. Scatchard (solid lines) and Eadie-Hofstee (dotted lines) analyses of [^{3}H]DTLET (■) and [^{3}H]DPDPE (●) specific binding: results from one experiment performed on the same homogenate of rat brain (upper plots) or NG108-15 cells (lower plots). In the Scatchard representation, B/F is plotted as a function of B ($B/F = B_{max}/K_D - (1/K_D) \cdot B$); in the Eadie-Hofstee representation, B is plotted as a function of B/F ($B = B_{max} - K_D \cdot (B/F)$) (inverted axes). Thus, the independent variables are, respectively, B and B/F. The corresponding saturation curves are shown on the right side. The concentration ranges were 25–84 and 9–67% of saturation for [^{3}H]DTLET and [^{3}H]DPDPE, respectively.

4. DISCUSSION

Two main molecular approaches for designing δ-probes were successfully used by Fournié-Zaluski et al. [8] and Mosberg et al. [25]. The highly conformationally restricted enkephalin analogues DPDPE and DPLPE exhibit an enhanced δ-selectivity but unfortunately this is associated with a large decrease in affinity. This could indicate that the loss of flexibility may not be favourable for the transconformational binding process [26]. The low specific binding of [^{3}H]DPDPE, 26% at 67% saturation, led to large standard deviations compared to [^{3}H]DTLET which displayed 79% specific binding at 63% saturation. The use of low temperature (4°C) does not improve the specific binding of the cyclic peptide. These features probably account for the large difference observed between the K_D and K_I values for DPDPE while these parameters were almost identical for DTLET. Therefore, [^{3}H]DPDPE cannot be safely used, either for competition studies or for acute determinations of the concentration of δ sites in brain following biochemical lesions or pharmacological manipulations.

In conclusion, [^{3}H]DTLET seems to be the most appropriate radioligand for binding studies and the cyclic analogue should be reserved for pharmacological experiments.

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