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DELTAKEPHALIN, TYR-D-THR-GLY-PHE-LEU-THR : A NEW HIGHLY POTENT AND FULLY SPECIFIC AGONIST FOR OPIATE δ-RECEPTORS

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Deltakephalin, Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET) was rationally designed as pure δ-probe from proposed models of μ and δ opiate receptors. On peripheral organs, deltakephalin displays a 3000 times higher inhibitory potency on the electrically stimulated mouse vas deferens (IC_{50} = 0.15 nM) as on the guinea pig ileum (IC_{50} = 460 nM). As expected [^{3}H]deltakephalin interacts at 35°C in rat brain tissue to a single class of binding sites (δ) (B_{max} = 0.115 pmole/mg protein) with a high affinity : K_D = 1.35 nM from equilibrium measurements and K_D = 0.43 nM from kinetic determinations. Deltakephalin occurs as the most specific ligand for δ-binding sites as shown by the following discrimination ratios $K_I(\mu)/K_I(\delta)$: 0.31 for D-Ala^2-D-Leu^5-enkephalin ; 0.15 for D-Ser^2-Thr^6-Leu-enkephalin and 0.05 for deltakephalin.

It is now well-admitted that Met-enkephalin and Leu-enkephalin interact with two kinds of binding sites called μ or morphine-receptor and δ or enkephalin receptor (1) whereas the longer peptide dynorphin (2) or a shorter fragment (3) behaves probably as the endogenous κ agonist. Data obtained from peripheral bioassays (4) binding studies (5) and autoradiographic measurements (6, 7) support such a classification. The occurrence of an heterogeneity of brain opioid peptides and related binding sites could explain the wide range of pharmacological effects elicited by administration of non-discriminant drugs as morphine. In this way, it is of great interest to notice that antinociceptive responses seem to be related to μ-receptors stimulation (8) a statement strongly reinforced by the lack of the naloxone-like effect of the recently described δ-antagonist ICI 154,129 (9). By contrast, according to their selective involvement in striatal dopamine release (10), δ-receptors could be implicated in behavioural responses (11). In spite of these features the question of whether μ and δ binding sites belong to different proteins or to a single

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opioid receptor complex characterized by allosteric coupling between μ and δ subsites (12,13) remains unresolved. Likewise the nature of the physiological effector (cyclase or ionic channel) associated with each kind of receptors is still unknown. Obviously, solutions to these problems require fully selective agonists allowing to perform competitive binding experiments and autoradiographic measurements in the absence of any significant cross-reactivity.

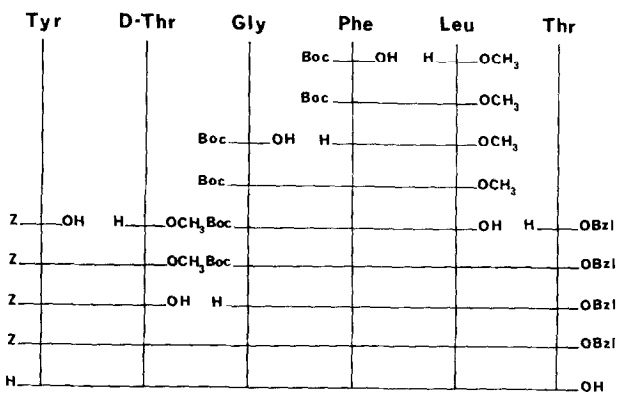
We reported in this paper the binding properties and δ-selectivity of "deltakephalin", Tyr-D-Thr-Gly-Phe-Leu-Thr, a new peptide designed from a proposed model of μ and δ binding sites (14) which should fulfill the above requirements.

MATERIAL AND METHODS

Chemicals. DAGO, Tyr-D-Ala-Gly-(Me)Phe-Gly-ol, was prepared according to Handa et al (15) and DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr, as previously reported (8). DADLE, Tyr-D-Ala-Gly-Phe-D-Leu, was purchased from BACHEM (Switzerland) and [^{3}H]DADLE (50.9 Ci/mmole) from New-England Nuclear, [^{3}H]DAGO (45 Ci/mmole), [^{3}H]DTLET (55 Ci/mmole) and [^{3}H]DTLET (35 Ci/mmole) were obtained from their 2,5-dibromotyrosyl precursors (16). [Tyr-D-Ala-Gly-Phe-NH$(CH_2)_6$]$_2$, DTE_{12}, was synthesized as described (17).

Synthesis. Deltakephalin, Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET) was prepared by liquid phase method as described in details for DSLET (8). The different steps of the synthesis are summarized in scheme 1. The structure of the final compound was confirmed by ^{1}H NMR (Bruker WH 270 MHz) and the purity checked both by TLC (single spot, Rf = 0.33, in BuOH/AcOH/H_2O ; 4/1/1) and by HPLC on a Waters apparatus (μ-bondapak C 18 colums ; detection at 210 nm ; solvent CH_3CN/NH_4AcO buffer 10^{-2}M, pH 4.2 ; 25/75, flow rate 1.2 ml/min, retention time of the single peak = 430 s). Aminoacid analysis of DTLET : Tyr = 1.01 ; Thr = 1.89 ; Gly = 0.98 ; Phe = 1.10 ; Leu = 1.04.

Binding assays. Crude rat brain membranes preparation and biochemical binding studies were done as previously reported in details (18,19). The specific binding was defined as the difference between the radioactivity bound in the presence and absence of Levorphanol (10 μM). Kinetic parameters of the binding of tritiated ligands were obtained from computer simulation of the Scatchard and Hill representations of the experimental data. The K_I values were calcula-



SCHEME 1

ted from the relation $K_I = IC_{50}/(1+L/K_D)$ where L is the concentration of the labelled ligand and K_D its equilibrium dissociation constant. The K_D values of DAGO = 3.3±0.28 nM and DSLET = 4.5±0.51 nM were obtained from Hill plots of the Scatchard analysis. All reported values are the means ± SEM of at least 3-4 independent experiments carried out in triplicate.

Pharmacological assays. Opioid activity was determined on the guinea-pig ileum and on the mouse vas deferens as previously described (4). Five different concentrations of each compound (3-4 assays for each) were tested for inhibition of electrically induced contractions. Methionine enkephalin was used in each assay as internal standard according to Kosterlitz et al (4).

RESULTS AND DISCUSSION

As previously shown with DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr (14), the introduction in enkephalin analogues of a D-hydrophilic aminoacid in position 2 and lengthening of the peptide sequence partialy inhibits μ-receptors recognition and lead to a strong increase in δ-receptor binding. Therefore, in order to still enhance the δ-selectivity of DSLET, we replaced the D-Ser[2] residue by a D-threonine. Such a substitution was assumed to increase the binding affinity through hydrophobic and Van der Waals interactions induced by the additionnal CH_3 group without change in the adverse recognition of the μ-receptor subsite.

Selectivity of deltakephalin on peripheral opiate receptors.

Pharmacological assays performed on guinea-pig ileum (GPI) and mouse vas deferens (MVD), two organs highly enriched respectively in μ and δ receptors, allow to determine the discrimination factor on peripheral opiate receptors from the ratio of IC_{50} values. As shown in Table 1, the δ-selectivity strongly increases from Tyr-D-Ala-Gly-Phe-D-Leu, DADLE, to deltakephalin, Tyr-D-Thr-Gly-Phe-Leu-Thr since this latter is 3000 times as potent on the MVD (IC_{50} = 0.15 nM) as on the GPI (IC_{50} = 460 nM). It must be observed that the enhanced selectivity of deltakephalin versus DADLE and DSLET is obtained from its better affinity for δ-receptors.

Cross reactivity of deltakephalin with μ and δ binding sites of rat brain tissue.

According to peripheral bioassays, a high δ-selectivity was also found for deltakephalin on brain tissue as evidenced from competition experiments performed at 35°C on crude rat brain membranes (Fig. 1) using [^{3}H] DAGO as μ-selective ligand (20) and [^{3}H] DSLET as nearly specific δ-agonist (18). As shown in Table 1, the K_I values of deltakephalin are 25.3±0.15 nM and 1.35± 0.15 nM respectively on μ and δ binding sites. The discrimination ratio K_I DSLET / K_I DAGO = 0.27 of the dimeric tetrapeptide, DTE_{12}, recently claimed (17) as the superior probe for δ-receptors shows that using a single tissue to determine preferential μ or δ recognition, this compound is 5 times less specific than deltakephalin ($K_I(\delta)/K_I(\mu)$ = 0.05). This result demonstrates

Table 1. Inhibitory effects of opioid peptides on the electrically induced contractions of guinea-pig ileum and mouse vas deferens and on the binding of [^{3}H]DAGO (1 nM) and [^{3}H]DSLET (2 nM) in crude rat brain homogenates.

	IC_{50} (GPI) nM	IC_{50} (MVD) nM	$\frac{IC_{50}\ (GPI)}{IC_{50}\ (MVD)}$
DAGO	11.50 ± 0.40	76.10 ± 6.70	0.15
DADLE	48.00 ± 7.20	0.55 ± 0.04	87.2
DSLET	406.00 ± 46.00	0.40 ± 0.04	1015.0
DTLET *(deltakephalin)*	460.00 ± 60.00	0.15 ± 0.012	3067.0

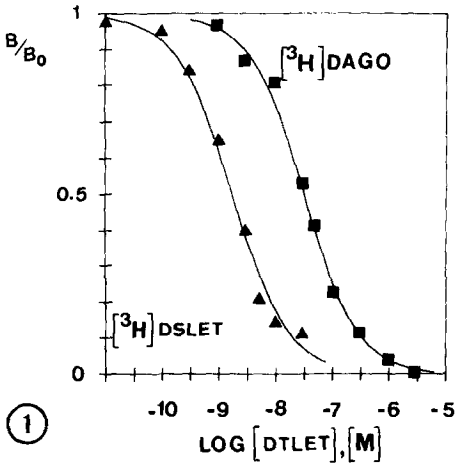
	K_I (nM)		$\frac{K_I\ DSLET}{K_I\ DAGO}$
	[^{3}H]DAGO	[^{3}H] DSLET	
DAGO	3.90 ± 0.80	700.00 ± 95.00	179.0
DADLE	7.70 ± 1.10	2.40 ± 0.31	0.31
DSLET	31.00 ± 5.00	4.80 ± 0.80	0.15
DTLET *(deltakephalin)*	25.30 ± 2.50	1.35 ± 0.15	0.053
DTE_{12} [a]	43.00 ± 7.70	11.50 ± 4.30	0.27
ICI 154,129 [b]	17,000 ± 2,000	840.00 ± 80.00	0.049

The IC_{50} values are the mean ± S.E.M. of 3-5 observations. Methionine enkephalin was used as internal standard in each assay. The K_I values are the mean ± S.E.M. of four independent determinations in triplicate. Each K_I value was obtained from computer analysis of Hill plots with 9 concentrations of unlabelled ligand. In the absence of inhibitor, specific binding amounted to 890 cpm for [^{3}H]DSLET and 1200 cpm for [^{3}H]DAGO.

a) *DTE_{12} = Tyr-D-Ala-Gly-Phe-NH-$(CH_2)_{12}$-NH-Phe-Gly-D-Ala-Tyr.*

b) *ICI 154,129 = $(allyl)_2$-Tyr-Gly-NH$(CH_2)_2$-S-CH$(CH_2\phi)$-CO-Leu-OH.*

that binding experiments performed on neuroblastoma cells to check δ-selectivity (17) cannot be surely extrapolated at the brain receptors level. K_I values reported in Table 1 were obtained from computer analysis of the displacement curves of [^{3}H] DAGO and [^{3}H] DSLET by the different probes assuming competitive inhibitions. As shown in Figure 1, such a model of independent two site-interaction fulfills the observed data indicating that, in the present conditions, putative allosteric interactions between μ and δ receptors do not significantly affect the observed results. The recently described (9) δ-antagonist ICI 154, 129 also exhibits a proper selectivity but its affinity is very weak as compared to deltakephalin. Furthermore, κ-affinity of deltakephalin was checked using guinea-pig brain membranes preparation and [^{3}H] ethylketocyclazocine (1 nM) as selective κ-agonist in the presence of DAGO (100 nM) and DADLE (100 nM) as respective μ and δ receptors blockers (20). In these conditions, IC_{50} value for deltakephalin was found superior to 10,000 nM.



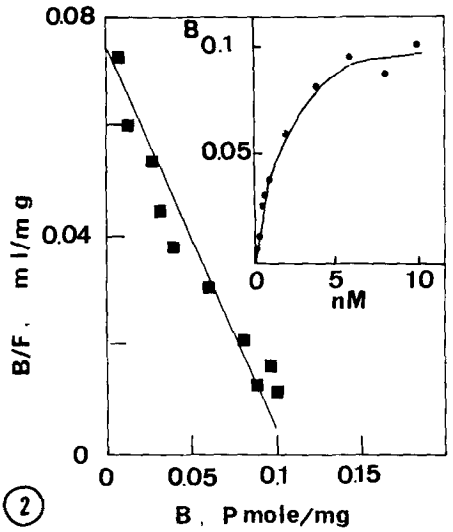


Figure 1. Inhibition curves of the specific binding of [^{3}H] DSLET (2 nM) and of [^{3}H] DAGO (1 nM) by DTLET. Each experimental value, expressed as percent of control, is the mean of three independent determinations performed in triplicate.

Figure 2. Scatchard analysis of the specific binding of [^{3}H] DTLET to rat brain membrane. The corresponding saturation curve are inserted. Each experimental value is the mean of three independent determinationsperformed in triplicate.

Binding characteristics of deltakephalin to rat brain tissue.

Equilibrium conditions. Saturation experiments performed at 35°C under steady state conditions show that, between 0.1 nM and 10 nM, tritiated deltakephalin, [^{3}H] DTLET, interacts with one single class of sites (Fig. 2) characterized by the following parameters : K_D = 1.35±0.19 nM ; B_{max} = 0.115±0.050 picomole/mg protein. As expected the binding capacity is similar to that observed with [^{3}H] DSLET and corresponds to the high affinity sites of [^{3}H] DADLE (18).

The Hill coefficient obtained from computer regression analysis was around unity showing no direct evidence of multiple sites or site-site interactions. It is interesting to notice that the K_D value is identical to the inhibition constant against [^{3}H] DSLET binding, K_I = 1.35±0.15 nM (Table 1). Similar binding parameters were obtained using cold deltakephalin instead of levorphanol to determine the non-specific bindings. The increased δ-receptors affinity of deltakephalin in regard to DADLE and DSLET was illustrated by comparative saturation experiments performed on the same membrane preparations. In such conditions the means of four independent determinations leads to the following data : K_D [^{3}H] DADLE = 3.3±0.28 ; K_D [^{3}H] DSLET = 4.5±0.51 ; K_D [^{3}H] DTLET = 1.35± 0.19 nM. These values agree with those obtained from displacement curves (Table 1).

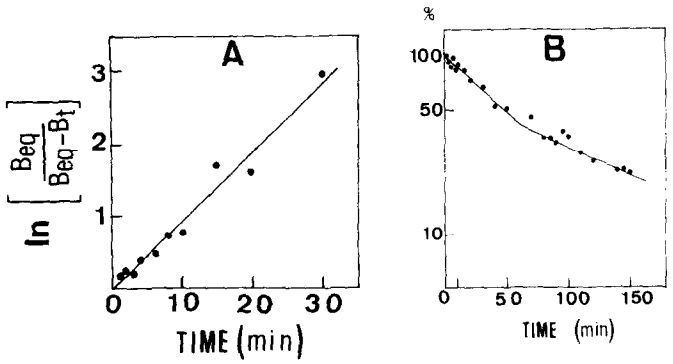


Figure 3.

A. Rate of association of [^{3}H] DTLET (1 nM) to rat brain membranes at 35°C. Amount of [^{3}H] DTLET bound at equilibrium, B_{eq}, or at a given time interval, B_t. Values are the mean of triplicate.

B. Rate of dissociation of ^{3}H DTLET at 35°C. Membranes were prelabeled with 1 nM [^{3}H] DTLET for 30 min at 35°C. At the end of this incubation levorphanal (final concentration : 10 µM) was added and the remaining binding determined at different time intervals. Each point represents the percentage at time t of the specific [^{3}H] DTLET total binding.

Finally at 4°C, maximal binding of [^{3}H] deltakephalin occurs after 180 min leading from Scatchard analysis to a K_D = 8.5±1.5 nM and a binding capacity similar to that computed at 35°C. According to the assumed role of the additional methyl group of the D-Thr^2 residue in the binding process, this decrease in δ-receptor affinity could indicate a significant loss of favourable hydrophobic interactions with the receptor site at low temperature. Since deltakephalin as DSLET are protected from enkephalin degrading enzymes (21) it is therefore more appropriate to perform studies at a physiological temperature.

Binding kinetics to rat brain tissue.

The association kinetics of [^{3}H] DTLET at 1 nM is very slow with binding reaching a steady-state in 25 min at 35°C. At equilibrium, less than 10 percent of the peptide is bound. In this condition, the on-rate constant, k_{+1}, can be determined from the pseudo-first order plot : (Ln $B_{eq}/B_{eq}-B_t$ vs t) in which B_{eq} and B_t are the concentrations of [^{3}H] DTLET bound at equilibrium and at a given time interval. The slope of this plot is equal to k_{+1} app = (k_{+1} · L) + k_{-1} in which L is the total concentration of [^{3}H] DTLET and k_{-1} the off-rate constant. From Figure 3A, a values of 0.093 min^{-1} was derived from k_{+1} app. The dissociation curve of [^{3}H] DTLET is slightly biphasic (Figure 3B) with half-times of 26 min (k_{-1} = 4.6 10^{-4} s^{-1}) and 100 min (k_{-1} = 1.2 10^{-4} s^{-1}). The equilibrium dissociation constant computed from the ratio k_{-1}/k_{+1} = 0.43 nM was lower than the K_D obtained from Scatchard plots (K_D = 1.35 nM). Such discrepancies between equilibrium and kinetics derived K_D were also reported for Leu-enkephalin (22) and DADLE (23). These features are not observed in the

case of antagonists and could be due to the occurrence of a ternary ligand-receptor complex or to allosteric interactions. Further studies are now in progress to clarify these binding features.

CONCLUSION

The aim of this work was to design a highly selective agonist for δ-opiate receptors. The cross-reaction b_1/b_t of a ligand L as DADLE, DSLET or deltakephalin (DTLET) can be illustrated from computed curves using the relation [1] (19):

$$\frac{b_1}{b_t} = \frac{[R_1L]}{[R_1L] + [R_2L]} = \left[1 + \frac{R_2t}{R_1t} \times \frac{K_D1 + [L]}{K_D2 + [L]}\right]^{-1} \qquad [1]$$

where R_1 = μ sites and R_2 = δ sites. R_1L and R_2L are the concentrations of sites R_1 and R_2 occupied by L ; [L] is the concentration of the free ligand ; K_D1 and K_D2 are respectively the equilibrium dissociation constants for R_1 and R_2.

As clearly shown on Figure 4, when the peptides are used at the K_D concentrations, deltakephalin (DTLET) exhibits the strongest specificity with a cross reactivity remaining safe even for high proportion of μ sites. Therefore deltakephalin behaves at this time as the most selective δ-agonist, for the biochemical and pharmacological characterisation of δ-opiate binding sites.

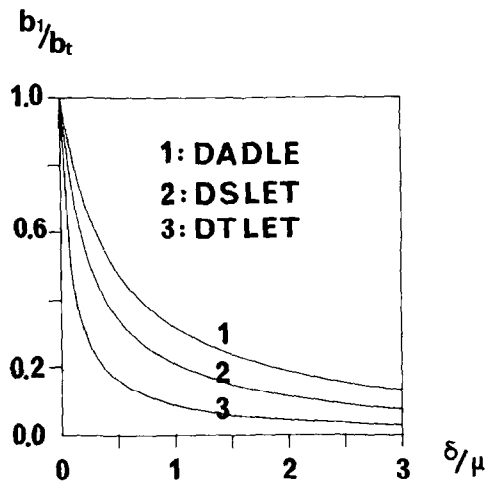


Figure 4. Computed cross-reaction (b_1/b_t) of DADLE (2.4 nM), DSLET (4.5 nM) and DTLET (1.35 nM) as a function of the capacity ratio δ/μ of the preparation.

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