Br. J. Pharmac. (1984), **83**, 271–279

Selectivities of opioid peptide analogues as agonists and antagonists at the δ-receptor

A.D. Corbett, M.G.C. Gillan, H.W. Kosterlitz, A.T. McKnight, S.J. Paterson & L.E. Robson

Unit for Research on Addictive Drugs, Marischal College, University of Aberdeen, Aberdeen, AB9 1AS

1 The endogenous opioid ligands interact with more than one of the μ-, δ- and κ-binding sites. By the use of binding assays and bioassays, enkephalin analogues have been assessed for their selectivity for binding at the δ-binding site and for their agonist and antagonist activities at the δ-receptor. The electrically-induced contractions of myenteric plexus-longitudinal muscle preparations of the guinea-pig ileum were inhibited by μ- and κ-receptor ligands. Inhibitions were seen with μ-, δ- and κ-receptor ligands in the mouse vas deferens, mainly with μ-receptor ligands in the rat vas deferens and only with κ-receptor ligands in the rabbit vas deferens.

2 From observations on a considerable number of [Leu^5] enkephalin analogues, it has been concluded that [D-Pen^2, D-Pen^5] enkephalin and [D-Pen^2, L-Pen^5] enkephalin are the most selective δ-agonists and that N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH is the most selective antagonist (Aib = α-aminoisobutyric acid). The binding of these peptides at the δ-site is 99% of the total binding. As to potency, the agonists are superior to the antagonists.

Introduction

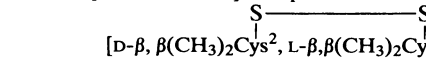
The characterization of the subtypes of the opioid receptor has been hampered by the lack of peptide or non-peptide ligands which are highly selective for μ-, δ- or κ-binding sites and which act either as agonists or antagonists on the receptors in the central and peripheral nervous systems. In this paper, we have assessed compounds which may be suitable agonists and antagonists for the δ-receptor.

In the series of δ-receptor agonists, the widely used [D-Ala^2, D-Leu^5] enkephalin has shown a degree of cross-reactivity with the μ-binding site which is unacceptable, particularly in the rat brain (Gillan & Kosterlitz, 1982). Other analogues of [Leu^5] enkephalin have been described which have enhanced selectivity for the δ-binding site. These are, [D-Ser^2, L-Leu^5] enkephalyl-Thr (Gacel *et al.*, 1980; David *et al.*, 1982); [D-Thr^2, L-Leu^5] enkephalyl-Thr (Zajac *et al.*, 1983); [D-Ala^2, Ser(OBz)^5] enkephalin (Shi *et al.*, 1981); Tyr-Gly-Gly-Phe-NH-$(CH_2)_{12}$-NH-Phe-Gly-Gly-Tyr (DTE_{12}, Shimohigashi *et al.*, 1982a); Tyr-Gly-Gly-Phe-Leu-NH-$(CH_2)_2$-NH-Leu-Phe-Gly-Gly-Tyr (DPE_2, Shimohigashi *et al.*, 1982b) and finally [D-Pen^2, L-Pen^5] enkephalin* and [D-Pen^2, D-Pen^5] enkephalin (Mosberg *et al.*, 1983).

As far as δ-antagonists are concerned, N,N-diallyl-Tyr-Gly-Gly-ψ-(CH_2S)-Phe-OH (ICI 154129, Shaw *et al.*, 1982) was one of the first compounds but it had a rather low potency. Since then a new analogue with higher potency has been obtained, N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864, Cotton *et al.*, 1984).

The results of the bioassays and binding assays of some of these compounds are presented in the following sections.

*[D-Pen^2,L-Pen^5] enkephalin =



[D-β, β(CH$_3$)$_2$Cys^2, L-β,β(CH$_3$)$_2$Cys^5] enkephalin

Methods

Binding assays

Binding of the peptide analogues was assayed in brain homogenates of guinea-pigs or rats as described previously (Magnan *et al.*, 1982; Gillan & Kosterlitz, 1982; Corbett *et al.*, 1982). The μ-binding site was selectively labelled with [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (1 nM), the δ-binding site with [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin (0.7 nM) in the presence of 10 or 30 nM unlabelled [D-Ala^2,

MePhe[4], Gly-ol[5]] enkephalin to suppress μ-binding and the κ-binding site with [^{3}H]-(−)-bremazocine (0.1–0.15 nM) in the presence of 100 nM each of unlabelled [D-Ala[2], MePhe[4], Gly-ol[5]] enkephalin and [D-Ala[2], D-Leu[5]] enkephalin to suppress μ- and δ-binding.

Pharmacological assays in isolated tissues

Preparations of the myenteric plexus-longitudinal muscle obtained from the small intestine of male guinea-pigs (Dunkin-Hartley, 400–500 g) were used for field stimulation with single, bipolar rectangular pulses of supramaximal voltage (75 mA, 0.5 ms pulse duration, 0.1 Hz). The siliconized organ bath contained 3 ml of Krebs-Henseleit solution. Stimulation was between the upper and lower ends of the bath and recording was isometric (Corbett *et al.*, 1982).

Vasa deferentia from hooded rats (250–350 g) of the Aberdeen colony, California rabbits (2–3 kg) and TO mice (30–35 g) were mounted in organ baths of 3 ml for rabbits and rats, or 0.5 ml for mice. Stimulation consisted of trains of 3 pulses of supramaximal voltage (20–25 mA, 0.5–1 ms pulse duration and 250 ms intervals between pulses); the trains were repeated at 0.1 Hz. When the mouse vas deferens was used, Mg^{2+} was omitted from the Krebs solution (Hughes *et al.*, 1975).

To minimize breakdown of the compounds by peptidases present in the tissues, the bath solution contained bestatin (10 μM, or 30 μM when the rat or rabbit vas deferens was used), L-leucyl-L-leucine (2 mM), thiorphan (0.3 μM) and captopril (10 μM) (Corbett *et al.*, 1982; McKnight *et al.*, 1983). The enkephalin analogues were first assayed without the addition of peptidase inhibitors which were added after two to three determinations of the effects of the peptides; the assays were then repeated after an interval of 1 to 2 h.

Labelled ligands

The following ligands were used: [^{3}H]-[D-Ala[2], MePhe[4], Gly-ol[5]] enkephalin (51–56 Ci mmol^{-1}), [^{3}H]-[D-Ala[2], D-Leu[5]] enkephalin (41–56 Ci mmol^{-1}, Amersham International), [^{3}H]-[D-Thr[2], L-Leu[5]] enkephalyl-Thr (27 Ci mmol^{-1}, Dr B. Roques, INSERM), [^{3}H]-[D-Ser[2],L-Leu[5]] enkephalyl-Thr (35 Ci mmol^{-1}, New England Nuclear) and [^{3}H]-(−)-bremazocine (24.1 Ci mmol^{-1}, Dr D. Römer, Sandoz). The purity of the ligands was >95%, achieved by high-performance liquid chromatography on a μBondapak C_{18} column.

Peptides and drugs

Analogues of enkephalin: [Leu[5]] enkephalin, [D-Ala[2], L-Leu[5]] enkephalin and [D-Ala[2], D-Leu[5]] enkephalin (Dr S. Wilkinson, Wellcome Research Laboratories), [D-Thr[2], L-Leu[5]] enkephalyl-Thr (Dr B. Roques, INSERM), [D-Ser[2], L-Leu[5]] enkephalyl-Thr and [D-Ala[2], Ser(OBz)[5]] enkephalin (Dr J. Morley, ICI), [D-Pen[2], L-Pen[5]] enkephalin and [D-Pen[2], D-Pen[5]] enkephalin (Dr H. Mosberg, University of Arizona), [D-Ala[2], MePhe[4], Gly-ol[5]] enkephalin (Dr D. Römer, Sandoz), Tyr-Gly-Gly-Phe-NH-$(CH_2)_{12}$-NH-Phe-Gly-Gly-Tyr (DTE_{12}) and Tyr-Gly-Gly-Phe-Leu-NH-$(CH_2)_2$-NH-Leu-Phe-Gly-Gly-Tyr (DPE_2) (Dr D. Rodbard, NIH).

Antagonists of δ-ligands: N,N-diallyl-Tyr-Gly-

Table 1 The inhibitory effects (IC_{50}, nM) of [Leu[5]] enkephalin and agonist enkephalin analogues on electrically-evoked contractions of the myenteric plexus-longitudinal muscle of the guinea-pig and the vasa deferentia of the mouse, rat and rabbit

Analogues	*Guinea-pig myenteric plexus*	*Mouse vas deferens*	*Rat vas deferens*	*Ratio IC_{50} in mouse vas deferens: IC_{50} in guinea-pig ileum*
[Leu[5]] enkephalin	35.6 ±7.3 (8)	1.73±0.27 (6)	550±62 (6)	0.049
[D-Ala[2], L-Leu[5]] enkephalin	21.1 ±6.6 (4)	1.37±0.17 (4)	156±41 (4)	0.065
[D-Ala[2], D-Leu[5]] enkephalin	8.9 ±0.76 (4)	0.73±0.12 (4)	134±22 (6)	0.082
[D-Thr[2], L-Leu[5]] enkephalyl-Thr	68±6.1 (4)	0.41±0.03 (4)	403±113 (3)	0.006
[D-Ser[2], L-Leu[5]] enkephalyl-Thr	112±9.2 (3)	0.59±0.03 (4)	202±80 (3)	0.005
[D-Pen[2], L-Pen[5]] enkephalin	2350±130 (3)	2.77±0.12 (4)	>10,000 (4)	0.001
[D-Pen[2], D-Pen[5]] enkephalin	3000±520 (3)	4.14±0.71 (4)	>10,000 (4)	0.001
[D-Ala[2], MePhe[4], Gly-ol[5]] enkephalin	4.50±1.17 (4)	32.8 ±3.72 (4)	105±17.4 (4)	7.3

The values are the means ± s.e.mean; the number of observations is given in parentheses. The Krebs solution contained the following peptidase inhibitors: bestatin (10 μM, or 30 μM for the rat and rabbit vas deferens), thiorphan (0.3 μM), captopril (10 μM) and L-leucyl-L-leucine (2 mM). Bath temperature was 37°C. None of these peptides has activity in the rabbit vas deferens (> 3000 nM) indicating a lack of κ-affinity.

Gly-ψ-(CH_2S)-Phe-Leu-OH (ψ-CH_2S replaces CO-NH; ICI 154129), N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (Aib = α-aminoisobutyric acid; ICI 174864).

Inhibitors of peptidases: bestatin and L-leucyl-L-leucine (Sigma Chemical Co), thiorphan (Dr D. Römer, Sandoz) and captopril (Squibb).

Stock solutions were prepared in water and stored at −25 °C. Dilutions for binding assays were made with 50 mM Tris buffer solutions at pH 7.4 and for bioassays with Krebs solution.

Results

Spontaneous changes in agonist potency of peptidase-resistant enkephalin analogues and of normorphine during prolonged incubation

It was important to know what changes in agonist potency of enkephalin analogues occur during incubation of an isolated tissue for 5 to 6 h. We used as test tissues the myenteric plexus of the guinea-pig ileum and the vasa deferentia of the mouse and the rat. The inhibitory effects (IC_{50}) of seven peptidase-resistant peptide analogues and of normorphine were assayed before and after addition of peptidase inhibitors; the ratio of the difference of the IC_{50} value before and after peptidase inhibition to the IC_{50} value before peptidase inhibition was used as the normalized ratio. The mixture of the inhibitors and the peptide analogues used are those in Table 1, with the exception of the peptidase-sensitive [Leu^5] enkephalin.

In the myenteric plexus the addition of the peptidase inhibitors did not cause a significant change in the mean IC_{50} value of the seven peptides, with a normalized ratio of $+0.06 \pm 0.063$ ($n = 25$; NS) but there was an increase in the normalized ratio of the non-peptide opioid normorphine ($+0.78 \pm 0.24$, $n = 8$; $P < 0.02$). In the vas deferens of the mouse the normalized ratio was -0.25 ± 0.05 ($n = 28$; $P < 0.001$) for the seven peptides and -0.32 ± 0.05 ($n = 12$; $P < 0.001$) for normorphine; the corresponding ratios in the rat vas deferens were -0.21 ± 0.07 ($n = 20$; $P < 0.01$) and -0.16 ± 0.065 ($n = 6$; NS). From these results it may be concluded that the changes observed were due to changes in the sensitivity of the test tissues to the peptides and normorphine during the course of an experiment.

Inhibitory agonist actions of enkephalin analogues in bioassays

In bioassays it is rarely possible to have tissues which interact only with one of the μ-, δ- and κ-receptor ligands. From previous experience, the myenteric plexus-longitudinal muscle of the guinea-pig ileum

Table 2 Inhibitory effects of agonist enkephalin analogues with relatively selective μ- or δ-activities on the binding at μ-, δ- and κ-opioid sites in homogenates of guinea-pig brain

Analogues	*Inhibitory effects* (K_i, nM) *at*			*Affinity at preferred site* (K_i, nM)$^{-1}$		*Relative affinity*	
	μ-site	*δ-site*	*κ-site*	*μ-site*	*δ-site*	*μ-site*	*δ-site*
[D-Ala^2, L-Leu^5] enkephalin	9.32 ± 0.99 (3)	1.91 ± 0.25 (4)	11900 ± 2320 (4)	—	0.52	0.17	0.83
[D-Ala^2, D-Leu^5] enkephalin	13.8 ± 1.3 (7)	2.06 ± 0.13 (9)	16000 ± 1120 (3)	—	0.49	0.13	0.87
[D-Thr^2, L-Leu^5] enkephalyl-Thr	33.9 ± 1.7 (4)	2.65 ± 0.41 (4)	14500 ± 730 (3)	—	0.38	0.073	0.927
[D-Ser^2, L-Leu^5] enkephalyl-Thr	38.9 ± 3.0 (6)	1.78 ± 0.35 (4)	6040 ± 374 (3)	—	0.56	0.045	0.955
[D-Pen^2, L-Pen^5] enkephalin	659 ± 65 (3)	2.80 ± 0.41 (3)	> 15000 (3)	—	0.36	0.004	0.996
[D-Pen^2, D-Pen^5] enkephalin	713 ± 13 (3)	2.72 ± 0.17 (3)	> 15000 (3)	—	0.37	0.004	0.996
[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin	1.86 ± 0.43 (4)	345 ± 24 (5)	6090 ± 1020 (5)	0.54	—	0.994	0.006

The values are the means ± s.e.mean; the number of observations is given in parentheses. Temperature 25 °C. The tritiated ligands used were as follows: for μ-binding [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (1 nM), for δ-binding [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin (0.7 nM) with 30 nM unlabelled [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin to suppress μ-binding and for κ-binding [^{3}H]-(−)-bremazocine (0.1–0.15 nM) with 100 nM unlabelled [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin and 100 nM [D-Ala^2, D-Leu^5] enkephalin to suppress μ- and δ-binding. Relative affinity is K_i^{-1} for μ, δ or κ/(K_i^{-1} for μ + K_i^{-1} for δ + K_i^{-1} for κ). The values for relative affinity at the κ-site are less than 0.0003 and have been omitted from the Table.

Table 3 Inhibitory effects of agonist enkephalin analogues with relatively selective δ-activities on the binding at μ- and δ-opioid sites in homogenates of rat brain

Analogues	*Inhibitory effects* (K_i, nM) *at* μ-site	δ-site	*Affinity at δ-site* $(K_i, nM)^{-1}$	*Relative affinity* μ-site	δ-site
[D-Ala², D-Leu⁵] enkephalin	12.6 ± 1.3	4.99 ± 0.52	0.20	0.28	0.72
[D-Thr², L-Leu⁵] enkephalyl-Thr	61.3 ± 9.4	5.57 ± 0.32	0.18	0.08	0.92
[D-Ser², L-Leu⁵] enkephalyl-Thr	49.8 ± 10.9	3.30 ± 0.32	0.30	0.06	0.94

The values are the means ± s.e.mean of three observations. Temperature 25 °C. The tritiated ligands used were as follows: for μ-binding [³H]-[D-Ala², MePhe⁴, Gly-ol⁵] enkephalin (0.8 nM) and for δ-binding [³H]-[D-Ala², D-Leu⁵] enkephalin (1.1 nM) with 30 nM unlabelled [D-Ala², MePhe⁴, Gly-ol⁵] enkephalin to suppress μ-binding.

interacts mainly with μ- and κ-receptor ligands, the mouse vas deferens with μ-, δ- and κ-receptor ligands, the rat vas deferens mainly but not exclusively with μ-receptor ligands and the rabbit vas deferens exclusively with κ-receptor ligands.

In Table 1, the IC_{50} values for the myenteric plexus of the guinea-pig ileum and the vasa deferentia of the mouse and rat are given. There are no values for the rabbit vas deferens because none of the enkephalin analogues used in the experiments of Table 1 had a depressant effect on the electrically-evoked contractions; this finding indicates that these enkephalins have no significant affinity for the κ-receptor. In this context, it is important to note that in the guinea-pig ileum the inhibition of the electrically-evoked contractions by opioids is not mediated by δ-receptors; the inhibitory effects of [Met⁵] enkephalin, [Leu⁵] enkephalin and of the analogues in Table 1 are mediated by the μ-receptor (Lord *et al.*, 1977). Therefore, the ratio of the IC_{50} value in mouse vas deferens to the IC_{50} value in guinea-pig ileum is an indication of the selectivity of the peptide for the δ-receptor. If the ratio is very low, e.g. 0.001 for [D-Pen², D-Pen⁵]enkephalin, the selectivity is high; if it is not so low, e.g. 0.082 for [D-Ala², D-Leu⁵] enkephalin, the selectivity is reduced by the affinity of the peptide to the μ-site. [D-Thr², L-Leu⁵] enkephalyl-Thr and [D-Ser², L-Leu⁵] enkephalyl-Thr had selectivities that are intermediate between the two extremes. As expected, the highly selective μ-receptor ligand, [D-Ala², MePhe⁴, Gly-ol⁵] enkephalin, had a high ratio of IC_{50} in mouse vas deferens to IC_{50} in guinea-pig ileum.

The effects of the enkephalin analogues were tested also in the rat vas deferens. It was of interest that the particularly selective δ-receptor ligands [D-Pen², D-Pen⁵] enkephalin and [D-Pen², L-Pen⁵] enkephalin were not active in this preparation, an observation indicating that these enkephalin analogues show no μ-activity. Furthermore, they are inactive in the rabbit vas deferens which interacts only with κ-receptor ligands (Oka *et al.*, 1981; 1982; Corbett *et al.*, 1982).

Inhibitory effects of enkephalin analogues on μ-, δ-, and κ-binding sites in guinea-pig brain

Table 2 shows the binding spectrum in guinea-pig brain homogenates of six enkephalin analogues with preference for the δ-binding site and one with preference for the μ-binding site. The K_i values for δ-binding of the analogues of [Leu⁵] enkephalin and of

Table 4 Dissociation constants and binding capacities at the δ-site of three analogues of [Leu⁵] enkephalin in homogenates of brains of guinea-pig and rat

Ligands	*Guinea-pig* K_D (nM)	B_{max} (pmol g^{-1} brain)	*Rat* K_D (nM)	B_{max} (pmol g^{-1} brain)
[³H]-[D-Ala², D-Leu⁵] enkephalin	0.59 ± 0.06	3.92 ± 0.24	1.60 ± 0.16	6.3 ± 0.81
[³H]-[D-Thr², L-Leu⁵] enkephalyl-Thr	—	—	1.02 ± 0.08	6.6 ± 0.91
[³H]-[D-Ser², L-Leu⁵] enkephalyl-Thr	1.24 ± 0.15	3.87 ± 0.44	1.44 ± 0.20	6.0 ± 0.46

The values are the means ± s.e.mean of three estimations. Temperature 25 °C. The B_{max} values were calculated from saturation curves and the K_D values from Hill plots. Suppression of μ-binding was obtained by a constant ratio of 10 to 30 nM of unlabelled [D-Ala², MePhe⁴, Gly-ol⁵] enkephalin to 1 × K_D (0.59–1.60 nM) of the tritiated ligands. When calculated as fmol mg^{-1} protein the mean B_{max} value of guinea-pig brain was 67 and that of the rat brain was 105.

the [Pen^2, Pen^5] enkephalins varied between 1.8 and 2.8 nM, corresponding to affinities of 0.56 and 0.36 nM^{-1}. Their K_i values at the μ-binding site showed a much greater variation, namely from 9.3 to 713 nM. There was no significant binding affinity at the κ-binding site. The relative affinities (Table 2) of the values at the δ-binding site were 0.83 for [D-Ala^2, L-Leu^5] enkephalin, 0.87 for [D-Ala^2, D-Leu^5] enkephalin, 0.93 for [D-Thr^2, L-Leu^5] enkephalyl-Thr and 0.96 for [D-Ser^2, L-Leu^5] enkephalyl-Thr. The values for [D-Pen^2, D-Pen^5] enkephalin and [D-Pen^2, L-Pen^5] enkephalin were not different from 1.0; these are, therefore, the ligands of choice for binding at the δ-binding site. In contrast, [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin had a relative affinity that was not different from unity at the μ-binding site.

Inhibitory effects of enkephalin analogues at the μ-, δ-, and κ-binding sites in rat brain

In the rat brain, the relative affinity of [D-Ala^2, D-Leu^5] enkephalin differed from that in the guinea-pig brain in that it was higher at the μ-binding site and correspondingly lower at the δ-binding site (Table 3), a species difference not found with the analogues [D-Thr^2, L-Leu^5] enkephalyl-Thr or [D-Ser^2, L-Leu^5] enkephalyl-Thr. There was, however, such a difference in the K_i values for the δ-binding site which were lower in the guinea-pig brain than in the rat brain; this finding indicates that the affinity of δ-binding is higher in the guinea-pig brain than in the rat brain.

Dissociation constants and binding capacities at the δ-binding site of guinea-pig and rat brain

Dissociation constants (K_D) and binding capacities (B_{max}) at the δ-binding site were obtained at 25 °C for three analogues; binding at the μ-site was suppressed by a constant ratio of unlabelled μ-receptor ligand, [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin to $1 \times K_D$ of free tritiated ligands (Table 4).

In the guinea-pig brain, the dissociation constant of [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin binding was about half that of [^{3}H]-[D-Ser^2, L-Leu^5] enkephalyl-Thr binding; there was no difference in the δ-binding capacities of the two ligands.

In the rat brain, the K_D values varied between 1.02 nM and 1.60 nM; the binding capacities of [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin, [^{3}H]-[D-Ser^2, L-Leu^5] enkephalyl-Thr and [^{3}H]-[D-Thr^2, L-Leu^5] enkephalyl-Thr varied between 6.0 ± 0.46 and 6.6 ± 0.91 pmol g^{-1} brain. This difference between the ligands was not significant. It should be noted that the binding capacity (pmol g^{-1} tissue) of the guinea-pig brain was less than that of the rat.

There is so far no information regarding the con-

Table 5 The apparent δ-binding at K_D and $10 \times K_D$ of three analogues of [Leu^5] enkephalin in brain homogenates of the rat and of the guinea-pig with and without suppression of μ-binding

		Binding (pmol g^{-1} brain) at $1 \times K_D$ concentration of tritiated ligand			*Binding (pmol g^{-1} brain) at $10 \times K_D$ concentration of tritiated ligand*		
Ligands	*Species*	*(μ + δ)-binding*	*δ-binding*	*δ-binding/ (μ + δ)-binding*	*(μ + δ)-binding*	*δ-binding*	*δ-binding/ (μ + δ)-binding*
[^{3}H]-[D-Ala^2, D-Leu^5] enkephalin	Rat	4.77 ± 0.41	3.03 ± 0.39	0.64	13.7 ± 0.84	6.4 ± 0.72	0.47
	Guinea-pig	2.13 ± 0.18	1.78 ± 0.07	0.84	5.9 ± 0.32	3.79 ± 0.32	0.64
[^{3}H]-[D-Thr^2, L-Leu^5] enkephalyl-Thr	Rat	3.17 ± 0.38	2.64 ± 0.49	0.83	8.3 ± 1.48	5.9 ± 0.71	0.72
[^{3}H]-[D-Ser^2, L-Leu^5] enkephalyl-Thr	Rat	3.46 ± 0.34	2.73 ± 0.18	0.79	9.5 ± 1.11	5.8 ± 0.46	0.61
	Guinea-pig	2.17 ± 0.28	1.76 ± 0.19	0.81	5.5 ± 0.47	3.76 ± 0.30	0.68

The values are the means ± s.e.mean of three estimations. Temperature 25 °C. The suppression of μ-binding by [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin was obtained as indicated in Table 4.

Table 6 Inhibitory effects of agonist enkephalin analogues with relatively selective δ-activity on the binding at μ-, δ- and κ-opioid sites in homogenates of guinea-pig brain

Analogues	*Inhibitory effects* (K_i, nM) *at*			*Affinity at the δ-site*	*Relative affinity*		
	μ-site	*δ-site*	*κ-site*	$(K_i, nM)^{-1}$	*μ*	*δ*	*κ*
[D-Ala^2, Ser (OBz)^5] enkephalin	12.2 ±1.03 (3)	2.07±0.33 (4)	11700±446 (3)	0.48	0.15	0.85	<0.001
[D-Ala^2, L-Leu-NH$_2^5$] enkephalin	4.25±1.20 (3)	2.29±0.20 (3)	1460±200 (3)	0.44	0.35	0.65	0.001
Tyr-D-Ala-Gly-Phe-Leu-NH—$(CH_2)_2$	5.5 ±1.09 (4)	0.66±0.02 (3)	32.5±5.4 (4)	1.52	0.10	0.88	0.02
Tyr-D-Ala-Gly-Phe-Leu-NH Tyr-D-Ala-Gly-Phe-NH—$(CH_2)_{12}$	38.3 ±4.57 (4)	10.0 ±1.67 (4)	87±7.8 (4)	0.10	0.19	0.73	0.08
Tyr-D-Ala-Gly-Phe-NH—							

The values are the means ± s.e.mean; the number of observations is given in parentheses. Temperature 25 °C. The binding procedure is the same as in Table 2. The IC_{50} values of [D-Ala^2, Ser(OBz)^5] enkephalin (Bz = benzoyl) were 25.6 ± 1.04 nM (n = 3) in the myenteric plexus of the guinea-pig ileum, 1.54 ± 0.19 nM (n = 4) in the mouse vas deferens and 182 ± 72 nM (n = 3) in the rat vas deferens.

centrations of the opioid ligands at the site of their action. It is, however, of interest to measure the ratio of δ-binding to(μ + δ)-binding at different concentrations of the tritiated ligands. In experiments presented in Table 5 the (μ + δ)-binding and δ-binding were determined at concentrations of K_D and 10 × K_D of the tritiated ligands (pmol g^{-1} brain); the δ-binding was estimated after suppression of the μ-binding by the method described in the legend of Table 4. The κ-binding of these ligands was negligible. As was expected from the data in Tables 2 and 3, [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin was the least selective δ-receptor ligand of the three enkephalin analogues; in the rat brain the δ-binding was only 64% of the (μ + δ)-binding at K_D concentration and 47% at 10 × K_D and the corresponding values in the guinea-pig brain were 84% and 64% (Table 5). The two analogues, [^{3}H]-[D-Thr^2, L-Leu^5] enkephalyl-Thr and [^{3}H]-[D-Ser^2, L-Leu^5] enkephalyl-Thr, were more selective in the rat brain where the mean δ-binding was 81% of the (μ + δ)-binding at a concentration of K_D and 66.5% at 10 × K_D. In the guinea-pig brain, the corresponding values were 81% and 68% which were similar to those found with [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin. Thus, the species difference was particularly marked with [D-Ala^2, D-Leu^5] enkephalin.

Inhibitory effects of some additional agonist enkephalin analogues purported to show preference for the δ-binding site and δ-receptor

One analogue, [D-Ala^2, Ser(OBz)^5] enkephalin has been estimated by Shi *et al.* (1981) to be 15,700 times more potent than morphine in the mouse vas deferens but only 1.34 times more potent in the guinea-pig ileum. We have assayed its binding properties in homogenates of the guinea-pig brain and found that its affinity for the δ-binding site is similar to [D-Ala^2, D-Leu^5] enkephalin (Table 6). Our IC_{50} value for the bioassay in the myenteric plexus of the guinea-pig ileum was 25.6 ± 1.04 nM and in the mouse vas deferens 1.54 ± 0.19 nM. For morphine, the corresponding values are 68 nM and 492 nM (Magnan *et al.* 1982). Thus, we confirm the data of Shi *et al.*, (1981) for the guinea-pig ileum but not those for the mouse vas deferens.

Shimohigashi *et al.* (1982a) found that, in the rat brain, the dimeric pentapeptide Tyr-D-Ala-Gly-Phe-Leu-NH-$(CH_2)_2$-NH-Leu-Phe-Gly-D-Ala-Tyr had a higher affinity for the δ-binding site than had the corresponding monomer, [D-Ala^2, L-Leu-NH$_2^5$]-enkephalin; the ratio for the dimer to the monomer was 8.0. We have found that in the guinea-pig brain this value is 3.5 (Table 6). It should be noted that these results are not strictly comparable since, apart from the species difference, different tritiated ligands

Table 7 The inhibitory effects of antagonist enkephalin analogues with relatively selective δ-activities on the binding at μ-, δ- and κ-opioid sites in homogenates of guinea-pig brain

Analogues	*Inhibitory effects* (K_i, nM) *at* μ-site	δ-site	κ-site	*Affinity at δ-site* $(K_i, nM)^{-1}$	*Relative affinity* μ-site	δ-site
N,N-diallyl-Tyr-Gly-Gly-ψ-(CH_2S)-Phe-Leu-OH (ICI 154129)	10100 ± 1020 (3)	778 ± 44 (3)	> 50000	0.0013	0.07	0.93
N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864)	24700;29700	193 ± 19 (4)	> 69000	0.0052	0.01	0.99

The values are the means ± s.e.mean; the number of observations is given in parentheses. Temperature 0°C. The μ-receptor ligand was [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (1 nM), the δ-receptor ligand was [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin (1 nM) and the κ-receptor ligand was [^{3}H]-(−)-bremazocine (0.3 nM) with the addition of unlabelled μ- and δ-ligands (100 nM each). The values for the δ-binding site are probably too high by about 15% since [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin was used without suppression of its μ-component. The inhibitory effect of ICI 174864 on the binding at the δ-site with suppression of the μ-binding by 30 nM [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin had a K_i of 169 ± 25 nM (n = 3) at 25°C. Its antagonist activity (K_e) against [D-Ser^2, L-Leu^5] enkephalyl-Thr in the mouse vas deferens was 36.4 ± 3.2 nM with a slope 1.05 ± 0.06 (n = 10). This activity was not affected by the peptidase inhibitors used in Table 1. Aib = α-aminoisobutyric acid.

were used by the two groups of authors. There is, however, no doubt that, in the guinea-pig brain also, the dimeric enkephalin analogue is more potent with an affinity of 1.52 nM^{-1} than is the monomeric analogue with an affinity of 0.44 nM^{-1}.

As far as the dimeric tetrapeptide analogue is concerned (Table 6), Shimohigashi *et al.* (1982b) have found that the ratio of IC_{50} for μ-binding of [^{3}H]-naloxone in rat brain to IC_{50} for δ-binding of [^{3}H]-[D-Ala^2, L-Leu^5] enkephalin in NG108-15 cells was 91. Our experiments with guinea-pig brain have given a value of 3.8 for the ratio of the K_i for μ-binding with [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin to the K_i for δ-binding with [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin. It should be noted that in these experiments the residual μ-binding of [D-Ala^2, D-Leu^5] enkephalin was suppressed as indicated in Table 2. Furthermore, we have found that in the guinea-pig brain the affinity of the dimeric tetrapeptide at the δ-site was only 6.6% of that of the dimeric pentapeptide and that it was also somewhat less selective.

Antagonist effects of [Leu^5] enkephalin analogues

Recently, antagonist analogues of [Leu^5]-enkephalin have been described (Shaw *et al.*, 1982; Cotton *et al.*, 1984). They have in common the addition of N,N-diallyl to the tyrosine of [Leu^5] enkephalin. In one of the analogues (ICI 154129) the —CO—NH— bond between Gly and Phe is replaced by —CH_2—S— while in the other (ICI 174864) the two glycines of [Leu^5] enkephalin have been replaced by α-aminoisobutyric acid. We have tested the two compounds in binding assays (Table 7). Both analogues had a high selectivity for the δ-binding site but their affinity was low. ICI 174864 was the more active of the two with an affinity at the δ-binding site of 0.0052 nM^{-1}; its relative affinity was high being 0.99 at the δ-site and 0.01 at the μ-site. Furthermore, it was stable at 25°C with a K_i at the δ-binding site of 169 ± 25 nM compared with a value at 0°C of 193 ± 19 nM.

Its antagonist activity (K_e) against [D-Ser^2, L-Leu^5] enkephalyl-Thr in the mouse vas deferens was 36.4 ± 3.2 nM (n = 10; slope = 1.05 ± 0.06). This activity was not affected by the peptidase inhibitors used in Table 1.

Discussion

The main requirement in the analysis of the mode of action of opioid peptides is the availability of highly selective agonists and antagonists of high potency at the μ-, δ- and κ-receptors and binding sites. This aim has been achieved with [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin which is a potent μ-receptor agonist resistant to the action of peptidases and which binds selectively to the μ-opioid site. In low concentrations naloxone binds selectively at the μ-site and antagonizes the μ-receptor. In higher concentrations, however, it also has antagonist actions at δ- and κ-receptors (Magnan *et al.*, 1982). With regard to agonist or antagonist compounds acting on the κ-receptors, a combination of high selectivity, potency and resistance to peptidases has so far not been achieved.

In this communication, we describe the activity of agonists and antagonists at the δ-receptor and δ-binding site. The most widely used agonist is [D-Ala^2, D-Leu^5] enkephalin which, however, has a considera-

ble cross-reactivity with the μ-binding site. This is particularly troublesome when the ratio of μ-binding sites to δ-binding sites is high, as has been found in rat brain (Gillan & Kosterlitz, 1982). In such a situation, it is essential to have δ-receptor ligands of high selectivity. In this respect, the best ligands are [D-Pen^2, D-Pen^5] enkephalin and [D-Pen^2, L-Pen^5] enkephalin (Mosberg *et al.*, 1983; this paper) which are peptidase-resistant and whose ratio of affinity for the μ-binding site to the affinity for the δ-binding site is very low (0.004; Table 2). This is reflected in the low value reported for the ratio of the IC_{50} for inhibition of [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin binding to the IC_{50} for inhibition of [^{3}H]-naloxone binding (Mosberg *et al.*, 1983). In bioassays, [D-Pen^2, D-Pen^5] enkephalin and [D-Pen^2, L-Pen^5] enkephalin have no measurable inhibitory effect in the rat vas deferens and have IC_{50} values in the guinea-pig ileum between 2,350 and 6,930 nM. In the mouse vas deferens, however, they are potent agonists with IC_{50} values between 2.19 and 4.14 nM (Mosberg *et al.*, 1983; Table 1 of the present paper). Unfortunately, at the time of writing, [^{3}H]-[Pen^2, Pen^5] enkephalins are not yet available. None of the other analogues of [Leu^5] enkephalin has this degree of selectivity in brain homogenates of either the rat or the guinea-pig. However, δ-receptor ligands [D-Ser^2, L-Leu^5] enkephalyl-Thr and [D-Thr^2,L-Leu^5] enkephalyl-Thr (Gacel *et al.*, 1980; David *et al.*, 1982; Zajac *et al.*, 1983) are more selective than [D-Ala^2, D-Leu^5] enkephalin.

It has been found that N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864) is an enkephalin derivative that is suitable as an antagonist at the δ-receptor (Cotton *et al.*, 1984; Table 7 of the present paper). The binding assays in homogenates of guinea-pig brain showed that it was stable at 25 °C and had a K_i value of 169 nM at the δ-binding site. Since the ratio of affinity for the μ-binding site to the affinity for the δ-binding site was 0.007, it was a highly selective δ-receptor ligand. In the bioassay in the mouse vas deferens, it was an antagonist with a K_e value of 34.6 nM against [D-Ser^2, L-Leu^5] enkephalyl-Thr as agonist. This K_e value was considerably lower than the K_i value found for inhibition of the δ-binding site in the homogenate of guinea-pig brain. The significance of this observation requires further analysis.

It is important to note that there are species differences in the binding of opioid peptides. When the binding in brain homogenates of three enkephalin analogues, [D-Ala^2, D-Leu^5] enkephalin, [D-Thr^2, L-Leu^5] enkephalyl-Thr and [D-Ser^2,L-Leu^5]-enkephalyl-Thr was studied, it was found that their mean B_{max} values are 3.9 pmol g^{-1} brain in the guinea-pig and 6.3 pmol g^{-1} brain in the rat.

The need to use selective ligands of high purity for assaying δ-binding sites is well illustrated when the ratio of δ-binding to ($\mu + \delta$)-binding at K_D is compared with that at $10 \times K_D$ of the tritiated ligand (Table 5). When these ratios were determined at K_D with [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin the value was 0.64 in rat brain and 0.84 in guinea-pig brain but, when they were determined at $10 \times K_D$, the value was 0.47 in rat brain and 0.64 in guinea-pig brain. It follows that, particularly in rat brain, [^{3}H]-[D-Ala^2, D-Leu^5] enkephalin cannot give reliable results for the δ-binding unless the μ-binding is suppressed with an unlabelled μ-receptor ligand. This necessity has been stressed earlier (Gillan & Kosterlitz, 1982); however, it is also of importance when [^{3}H]-[D-Thr^2, L-Leu^5] enkephalyl-Thr or [^{3}H]-[D-Ser^2, L-Leu^5] enkephalyl-Thr are used to determine binding at the δ-site since the cross-reactivity to the μ-binding site is still significant.

Supported by grants from the U.K. Medical Research Council and the U.S. National Institute on Drug Abuse (DA 00662). Acknowledgement is made of generous gifts of the compounds mentioned in Methods.

References

CORBETT, A.D., PATERSON, S.J., McKNIGHT, A.T., MAGNAN, J. & KOSTERLITZ, H.W. (1982). Dynorphin$_{1-8}$ and dynorphin$_{1-9}$ are ligands for the κ-subtype of opiate receptor. *Nature (Lond.)*, **299**, 79–81.

COTTON, R., GILES, M.B., MILLER, L., SHAW, J.S. & TIMMS, T. (1984). ICI 174864: a highly selective antagonist for the opioid δ-receptor. *Eur. J. Pharmac.*, **97**, 331–332.

DAVID, M., MOISAND, C., MEUNIER, J.-C., MOREAT, J.-L., GACEL, G. & ROQUES, B.P. (1982). [^{3}H]-Tyr-D-Ser-Gly-Phe-Leu-Thr: A specific probe for the δ-opiate receptor subtype in brain membranes. *Eur. J. Pharmac.*, **78**, 385–387.

GACEL, G., FOURNIÉ-ZALUSKI, M.C. & ROQUES, B.P. (1980). Tyr-D-Ser-Gly-Phe-Leu-Thr, a highly preferential ligand for δ-opiate receptors. *FEBS Lett.*, **118**, 245–247.

GILLAN, M.G.C. & KOSTERLITZ, H.W. (1982). Spectrum of the μ-, δ- and κ-binding sites in homogenates of rat brain. *Br. J. Pharmac.*, **77**, 461–469.

HUGHES, J., KOSTERLITZ, H.W. & LESLIE, F.M. (1975). Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics. *Br. J. Pharmac.*, **53**, 371–381.

LORD, J.A.H., WATERFIELD, A.A., HUGHES, J. & KOSTERLITZ, H.W. (1977). Endogenous opioid peptides: multiple agonists and receptors. *Nature (Lond.)*, **267**, 495–499.

McKNIGHT, A.T., CORBETT, A.D. & KOSTERLITZ, H.W. (1983). Increase in potencies of opioid peptides after peptidase inhibition. *Eur. J. Pharmac.*, **86,** 393–402.

MAGNAN, J., PATERSON, S.J., TAVANI, A. & KOSTERLITZ, H.W. (1982). The binding spectrum of narcotic analgesic drugs with different agonist and antagonist properties. *Naunyn Schmiedebergs Arch. Pharmac.*, **319,** 197–205.

MOSBERG, H.I., HURST, R., HRUBY, V.J., GEE, K., YAMAMURA, H.I., GALLIGAN, J.J. & BURKS, T.F. (1983). Bis-penicillamine enkephalins possess highly improved specificity towards δ-opioid receptors. *Proc. natn. Acad. Sci. U.S.A.*, **80,** 5871–5874.

OKA, T., NEGISHI, K., SUDA, M., MATSUMIYA, T., INAZU, T. & UEKI, M. (1981). Rabbit vas deferens: a specific bioassay for opioid κ-receptor agonists. *Eur. J. Pharmac.*, **73,** 235–236.

OKA, T., NEGISHI, K., KAJIWARA, M., WANATABE, Y., ISHIZUKA, Y. & MAISUMIYA, T. (1982). The choice of opiate receptor subtypes by neo-endorphins. *Eur. J. Pharmac.*, **79,** 301–305.

SHAW, J.S., MILLER, L., TURNBULL, M.J., GORMLEY, J.J. & MORLEY, J.S. (1982). Selective antagonists at the opiate delta-receptor. *Life Sci.*, **31,** 1259–1262.

SHI, P., NIU, J., WU, S. & ZOU, G. (1981). Enkephalin analogs with extremely high affinity for δ-receptor. *Kexue Tongbao*, **26,** 750–752.

SHIMOHIGASHI, Y., COSTA, T., CHEN, H-C. & RODBARD, D. (1982a). Dimeric tetrapeptide enkephalins display extraordinary selectivity for the δ opiate receptor. *Nature (Lond.)*, **297,** 333–335.

SHIMOHIGASHI, Y., COSTA, T., MATSUURA, S., CHEN, H-C. & RODBARD, D. (1982b). Dimeric enkephalins display affinity and selectivity for the *delta*-opiate receptor. *Molec. Pharmac.*, **21,** 558–563.

ZAJAC, J-M., GACEL, G., PETIT, F., DODEY, P., ROSSIGNOL, P. & ROQUES, B.P. (1983). Deltakephalin, Tyr-D-Thr-Gly-Phe-Leu-Thr: a new highly potent and fully specific agonist for opiate δ-receptors. *Biochem. biophys. Res. Commun.*, **111,** 390–397.

(*Received March 9, 1984.*
Revised April 17, 1984.)