Br. J. Pharmac. (1985), **84**, 927–932

The use of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin as a highly selective ligand for the δ-binding site

R. Cotton, H.W. Kosterlitz*, S.J. Paterson[1]*, M.J. Rance & J.R. Traynor

ICI Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG. and Unit for Research on Addictive Drugs*, Marischal College, University of Aberdeen, Aberdeen, AB9 1AS

1 The characteristics of the binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin were determined in homogenates of guinea-pig and rat brain.

2 In the guinea-pig, the maximum binding capacity for [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin was 4.19 pmol g^{-1} and the K_D 1.61 nM. In the rat, the corresponding values were 2.47 pmol g^{-1} and 5.42 nM. In both species, the maximum binding capacity and the affinity were not altered when μ-binding was suppressed with [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin.

3 The μ-agonists, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin and morphine, displaced a small portion of the binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin with high affinities.

Introduction

The investigation of the interaction of opioids with their binding sites has been hindered by the lack of ligands of high selectivity. Recently, [D-Pen^2,D-Pen^5]-enkephalin has been shown to be such a ligand for the δ-binding site (Mosberg *et al.*, 1983; Corbett *et al.*, 1984). In this paper we describe the characteristics of the binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin in homogenates of guinea-pig and rat brain.

Methods

Binding assays

Brain homogenates from male Dunkin-Hartley guinea-pigs, 400 to 600 g, and from male hooded Lister rats, ICI Alderley Park, 150 to 200 g, were prepared as described previously (Gillan *et al.*, 1980; Gillan & Kosterlitz, 1982). The specific binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin was determined as the difference in the counts obtained in the presence and in the absence of unlabelled diprenorphine (1.2 μM) in guinea-pig brain or naloxone (10 μM) in rat brain. The parameters of the binding of labelled and unlabelled ligands, the equilibrium dissociation constant (K_D) and the inhibition constant (K_i) were determined from saturation or competition experiments.

The binding capacity and the equilibrium dissociation constants were determined for [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin or [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin in the presence or absence of unlabelled μ-ligand, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin, at a constant ratio of 10 or 30 nM to 1 × K_D (nM) of the tritiated ligand. In competition experiments in guinea-pig brain, the δ-binding site was labelled with [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin (1.6 nM), or with [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (0.7 nM) in the presence of 30 nM unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin. In competition experiments on rat brain, the ligand was [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin (1.5–2.2 nM). The ratio of specific to total binding was 0.49 in rat brain and 0.84 in guinea-pig brain.

Labelled ligands

The primary ligands were: [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin (45 Ci mmol^{-1}) and [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (41–56 Ci mmol^{-1}; Amersham International). In guinea-pig experiments, the radiochemical purity of the ligands was >95%; this was achieved by high performance liquid chromatography on a μBondapak C_{18} column.

Peptides and drugs

The drugs used were: [D-Pen^2,D-Pen^5]enkephalin (Dr H. Mosberg, University of Arizona and Dr R. Cotton,

[1] Author for correspondence

ICI), [D-Ala^2,D-Leu^5]enkephalin (Sigma and Cambridge Research Biochemicals), N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (Aib = α-aminoisobutyric acid; ICI 174864), [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (Dr D. Römer, Sandoz and Cambridge Research Biochemicals), normorphine hydrochloride (Wellcome Foundation), morphine hydrochloride (MacFarlan Smith), naloxone hydrochloride (Endo Laboratories), diprenorphine base (Reckitt & Colman), (−)-ethylketazocine base (Dr W.F. Michne, Sterling Winthrop), (−)-bremazocine hydrochloride (Dr D. Römer, Sandoz), Mr 2266 base ((−)-α-5,9-diethyl-2-(3-furylmethyl)-2′-hydroxy-6,7-benzomorphan; Dr H. Merz, C.H. Boehringer Sohn), U-50,488H base (*trans*-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)-benzeneacetamine; Upjohn Company).

Stock solutions were prepared in distilled water with the addition of HCl when necessary for dissolving bases. They were stored at − 25°C.

Results

Binding characteristics of [^{3}H]-[D-Pen^2,D-Pen^5] enkephalin in guinea-pig and rat brains

When the saturation curves obtained with [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin were analysed by the methods of Scatchard and Hill, the plots were linear and the values of affinity and maximum binding capacity were greater in the guinea-pig brain than in rat brain. In the Scatchard plot shown in Figure 1, the affinities were 0.65 and 0.15 $(K_D, \text{nM})^{-1}$ and the maximum binding capacities were 3.7 and 2.5 pmol g^{-1} in the brains of the two species. It is important to note that in both species neither the affinity nor the binding capacity was significantly changed when the residual μ-binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin was suppressed by the addition of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (Figure 1).

The values of the dissociation constants (K_D) and of the binding capacities (B_{max}) obtained with δ-ligands of different selectivity were compared in guinea-pig brain (Table 1). When [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin was tested, blocking the μ-binding sites had no effect on the values of K_D or B_{max}. In contrast, the binding capacities of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin or [^{3}H]-[D-Ser^2,L-Leu^5]enkephalyl-Thr were reduced by 36% or 28% when μ-binding was prevented by unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin. It should also be noted that after suppression of μ-binding the binding capacities of the three δ-ligands were identical.

In rat brain (Table 2) blocking the μ-binding sites did not affect the values of K_D or B_{max} of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin, thus confirming the findings in guinea-pig brain. The B_{max} values of δ-binding in the

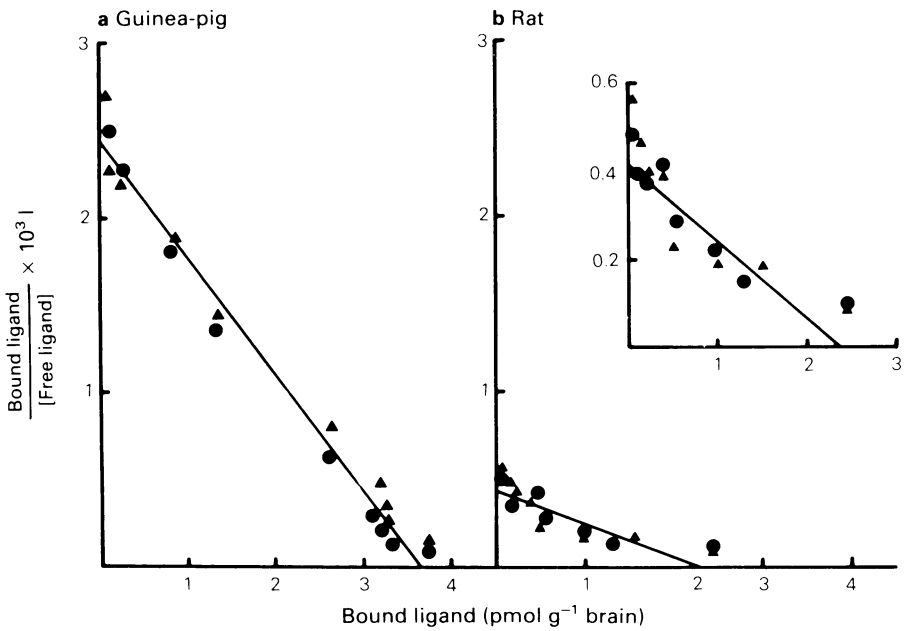

Figure 1 Scatchard plots of the specific binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin in the absence (▲) and in the presence (●) of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin in homogenates of guinea-pig (a) and rat brain (b). Suppression of μ-binding was obtained with a constant ratio of 10 nM (a) or 30 nM (b) to 1 × K_D (nM) of the tritiated ligand.

Table 1 Dissociation constants and binding capacities of three analogues of [Leu^5]enkephalin in homogenates of guinea-pig brain

Ligand	*μ-Binding not suppressed* Binding capacity (B_{max}, pmol g^{-1} brain)	*μ-Binding suppressed* Binding capacity at δ-site (B_{max}, pmol g^{-1} brain)	*μ-Binding suppressed* Dissociation constants (K_D, nM)
[^{3}H]-[D-Pen^2,D-Pen^5] enkephalin	4.19 ± 0.32 (4)	3.86 ± 0.30 (4)	1.61 ± 0.17 (4)
[^{3}H]-[D-Ser^2,L-Leu^5] enkephalyl-Thr	5.41 ± 0.72 (3)*	3.89 ± 0.44 (3)***	1.24 ± 0.15 (3)***
[^{3}H]-[D-Ala^2,D-Leu^5] enkephalin	6.1 ± 0.05 (5)**	3.92 ± 0.24 (3)***	0.59 ± 0.06 (3)***

The values are the means ± s.e.mean. Temperature 25°C. The binding capacities were calculated from saturation curves and the K_D values from Hill plots. Suppression of the μ-binding was obtained with a constant ratio of 10–30 nM of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin to 1 × K_D of the tritiated ligand. The K_D value of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin without suppression of μ-binding was 1.61 ± 0.16 nM (n = 4). *M.G.C. Gillan (unpublished observation). **From Gillan *et al.*, 1980. ***From Corbett *et al.*, 1984.

rat brain were about 60% of that in guinea-pig brain. However, in rat brain, the affinities for the δ-binding sites of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin and of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin with suppression of μ-binding were only about 30% of that found in guinea-pig brain (Tables 1 and 2).

Competitive inhibition of binding at the δ-site

The K_i values for a number of opioids at the δ-binding site were determined in homogenates of guinea-pig brain using as ligands [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin or, [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin with suppression of μ-binding (Table 3). The pattern of the K_i values obtained with the two tritiated ligands was of a similar order. Binding at the δ-site was selectively displaced by unlabelled [D-Pen^2,D-Pen^5]enkephalin (K_i = 2.56 nM) and [D-Ala^2,D-Leu^5]enkephalin (K_i = 1.35 nM). In contrast, the K_i value obtained with the κ-selective agonist U-50,488H was very high (12,000 nM). A similar pattern of selectivity was found in homogenates of rat brain (Table 4).

The effects of the μ-agonists normorphine, morphine and [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin, which have a cross-reactivity with the δ-site of 5, 2 and 0.5% (Magnan *et al.*, 1982; Corbett *et al.*, 1984), are more complex. Since unlabelled [D-Pen^2,D-Pen^5]enkephalin displays a cross-reactivity of only 0.4% with the μ-binding site (Corbett *et al.*, 1984), the inhibitory effect of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin on the binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin is of particular interest. It was found that in guinea-pig brain the interaction between these two peptide analogues is biphasic (Figure 2). In three out of ten experiments, the biphasic curve could be resolved into two separate components. The first phase was obtained with concentrations of [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin below 10 nM, while the second phase was obtained with concentrations between 100 and 6400 nM. Hill plots for the first component gave a K_i of

Table 2 Dissociation constants and binding capacities at the δ-binding site of two analogues of [Leu^5] enkephalin in homogenates of rat brain

Ligand	*μ-Binding not suppressed* Binding capacity (B_{max}, pmol g^{-1} brain)	*μ-Binding suppressed* Binding capacity at δ-site (B_{max}, pmol g^{-1} brain)	*μ-Binding suppressed* Dissociation constants (K_D, nM)
[^{3}H]-[D-Pen^2,D-Pen^5] enkephalin	2.47 ± 0.17 (5)	2.18 ± 0.08 (6)	5.25 ± 0.59 (6)
[^{3}H]-[DAla^2,D-Leu^5] enkephalin	—	2.20 ± 0.09 (3)	1.72 ± 0.25 (3)

The values are the means ± s.e.mean. Temperature 25°C. The binding capacities were calculated from saturation curves and the K_D values from Hill plots. Suppression of the μ-binding was obtained with a constant ratio of 10 or 30 nM of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin to 1 × K_D of the tritiated ligand. The K_D value of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin without suppression of μ-binding was 5.42 ± 0.69 nM (n = 5).

Table 3 Comparison of the inhibitory effects of opioids on the binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin (1.6 nM) and of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (0.7 nM) in the presence of 30 nM unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin to suppress μ-binding in homogenates of guinea-pig brain

Unlabelled compound	*[^{3}H]-[D-Pen^2,D-Pen^5]enkephalin* K_i (nM)	Hill coefficient	*[^{3}H]-[D-Ala^2,D-Leu^5]enkephalin in the presence of [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin* K_i (nM)	Hill coefficient
δ-Agonists				
[D-Pen^2,D-Pen^5]enkephalin	2.56 ± 0.33	0.94 ± 0.07 (4)	2.72 ± 0.17	0.97 ± 0.07 (3)
[D-Ala^2,D-Leu^5]enkephalin	1.35 ± 0.06	0.94 ± 0.04 (4)	2.06 ± 0.13	1.11 ± 0.04 (9)
μ-Agonists				
[D-Ala^2,MePhe^4,Gly-ol^5] enkephalin	407 ± 55	0.94 ± 0.04 (10)	345 ± 24	0.93 ± 0.08 (5)
Normorphine	238 ± 14	0.97 ± 0.02 (3)	116 ± 12	0.90 ± 0.04 (4)
Morphine	161 ± 25	0.98 ± 0.03 (3)	93 ± 11	0.81 ± 0.04 (4)
κ-Agonists				
U-50,488H	11600 ± 1500	0.95 ± 0.05 (4)	12900 ± 1400	0.93 ± 0.06 (5)
(−)-Ethylketazocine	6.62 ± 1.16	0.87 ± 0.04 (3)	4.62 ± 0.59	0.87 ± 0.03 (5)
(−)-Bremazocine	0.78 ± 0.10	1.20 ± 0.13 (3)	0.71 ± 0.09	1.20 ± 0.13 (5)
Antagonists				
N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864)	190 ± 27	1.10 ± 0.05 (4)	170 ± 25	1.02 ± 0.04 (3)
Naloxone	22.8 ± 2.3	0.91 ± 0.02 (4)	30.0 ± 2.8	0.94 ± 0.08 (5)
Diprenorphine	1.00 ± 0.10	1.13 ± 0.08 (4)	0.81 ± 0.14	1.13 ± 0.03 (4)
Mr 2266	2.68 ± 0.15	1.01 ± 0.06 (4)	3.71 ± 0.40	1.23 ± 0.05 (5)

The values are the means ± s.e.mean; the number of observations is given in parentheses. Temperature 25°C. The K_i values were calculated by the method of Cheng & Prusoff (1973). Aib = α-aminoisobutyric acid. Since the μ-agonists displaced a portion of the binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin with high affinity, the K_i values given were calculated for the δ-component as shown in Figure 2.

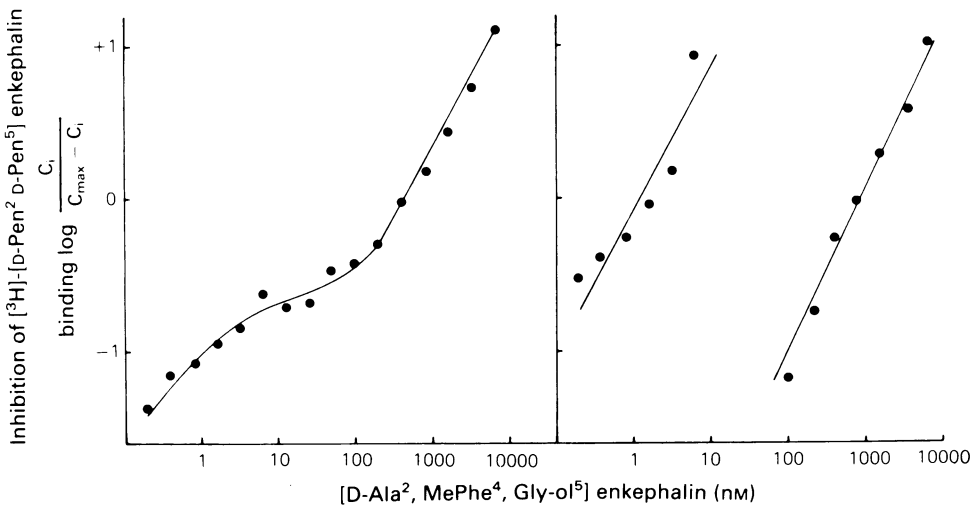

Figure 2 The inhibitory effects of [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin on the binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin (1.6 nM) in a homogenate of guinea-pig brain. (a) Biphasic inhibition curve. (b) Inhibition calculated on the basis of two binding sites: K_i = 0.63 nM, Hill coefficient = 0.89, and K_i = 481 nM, Hill coefficient 1.15. Typical result of three experiments. C_i, inhibited counts; C_{max}, maximal counts.

Table 4 Comparison of the inhibitory effects of opioids on the binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin (1.5–2.2 nM) in homogenates of rat brain

Unlabelled compound	K_i (nM)
[D-Ala^2,D-Leu^5]enkephalin	1.84 ± 0.20 (4)
N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864)	146 ± 15 (4)
[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin	153 ± 23 (7)
Morphine	213 ± 42 (4)
U-50,488H	4850 ± 910 (4)
(−)-Bremazocine	1.03 ± 0.01 (3)

The values are the means ± s.e.mean; the number of observations is given in parentheses. Temperature 25°C. The K_i values were calculated by the method of Cheng & Prusoff (1973). Aib = α-amino-isobutyric acid.

0.54 ± 0.10 nM and a Hill coefficient of 1.10 ± 0.20 ($n = 3$); for the second component, the K_i was 407 ± 55 and the Hill coefficient 0.94 ± 0.04 ($n = 10$). The values given for the δ-affinity of the μ-agonists in Table 3 were calculated for the inhibition of the second component. In 16 experiments, the first component represented between 5 and 25% of the specific binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin.

In this context, it is important to note that the widely used κ-agonists, ethylketazocine and particularly bremazocine, have significant affinities for the δ-binding site (Table 3).

As far as antagonists are concerned, the only selective compound is N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864) which interacts with the δ-receptor but unfortunately with a rather low potency (K_i = 190 nM). The other antagonists have affinity for the δ-site (Table 3) but also interact with μ- and κ-binding sites.

Discussion

The results presented in this paper confirm the high selectivity of [D-Pen^2,D-Pen^5]enkephalin for the δ-binding site (Mosberg *et al.*, 1983; Corbett *et al.*, 1984). Thus, in the guinea-pig brain the binding capacity of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin is not altered by suppression of μ-binding by unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin. Therefore, [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin will be of use for the study of δ-binding sites in regions where there is a high proportion of μ-sites.

Since the maximum binding capacity of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin (4 pmol g^{-1}) and its K_D (1.6 nM) are not altered by the addition of unlabelled μ-ligand, it is surprising that the μ-agonists [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin, morphine and normorphine have a high affinity for a portion of the binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin. It is so far not possible to assess the significance of this phenomenon. It may possibly be due to the residual affinity of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin for the μ-binding site or it may represent an interaction with a high affinity site. It is of interest that this interaction is not seen in saturation analysis but only in inhibitory binding assays. This discrepancy may be due to the difficulty in measuring the high affinity component. This is especially true, when the capacity of the high affinity component is small compared with the maximal binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin.

It is important to realize that there are species differences in the binding of opioid peptides. The binding capacity of the δ-sites in the brain of the guinea-pig (3.9 pmol g^{-1}) is greater than that in the hooded Lister rats from the ICI laboratories (2.2 pmol g^{-1}); the binding capacity found in the hooded rats of the Aberdeen colony was 6.7 pmol g^{-1} (Gillan & Kosterlitz, 1982). Furthermore, there are species differences in the binding affinities (K_D^{-1}) of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin and [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin as their values in the Lister rats are about 3 times lower than those in the guinea-pig (Tables 1 and 2).

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. J.R.T. is on study leave from Loughborough University.

References

CHENG, Y-C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymic reaction. *Biochem. Pharmac.*, **22**, 3099–3102.

CORBETT, A.D., GILLAN, M.G.C., KOSTERLITZ, H.W., McKNIGHT, A.T., PATERSON, S.J. & ROBSON, L.E. (1984). Selectivities of opioid peptide analogues as agonists and antagonists at the δ-receptor. *Br. J. Pharmac.*, **83**, 271–279.

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.

GILLAN, M.G.C., KOSTERLITZ, H.W. & PATERSON, S.J. (1980). Comparison of the binding characteristics of

tritiated opiates and opioid peptides. *Br. J. Pharmac.*, **70,** 481–490.

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.

(*Received October 26, 1984.*
Revised November 20, 1984.
Accepted November 21, 1984.)