Br. J. Pharmac. (1980), **70**, 481–490

COMPARISON OF THE BINDING CHARACTERISTICS OF TRITIATED OPIATES AND OPIOID PEPTIDES

MAUREEN G.C. GILLAN, H.W. KOSTERLITZ & S.J. PATERSON

Unit for Research on Addictive Drugs, University of Aberdeen, Aberdeen

1 Binding assays on homogenates of guinea-pig brain showed that the maximal number of binding sites was different for different tritiated ligands interacting with the opiate receptors.

2 At 25°C the binding capacity of morphine or dihydromorphine was only about 3 pmol/g fresh brain whereas etorphine and D-Ala^2-L-Leu^5- and D-Ala^2-L-Met^5-enkephalin amide had capacities of 13 to 15 pmol/g brain. D-Ala^2-D-Leu^5-enkephalin had an intermediate capacity of about 6 pmol/g brain.

3 The binding capacities of the natural methionine- and leucine-enkephalins measured at 0°C were 5 to 6 pmol/g brain. At this temperature, the binding capacity of dihydromorphine, D-Ala^2-D-Leu^5-enkephalin and of the two enkephalin amides was only slightly lower than at 25°C.

4 In assays in which unlabelled ligand competed with the same labelled ligand, the inhibition constants (K_I) were equal to or not more than twice as large as the equilibrium dissociation constant (K_D) determined in saturation assays. In contrast, the K_I of unlabelled dihydromorphine against [^{3}H]-D-Ala^2-D-Leu^5-enkephalin or of unlabelled D-Ala^2-D-Leu^5-enkephalin against [^{3}H]-dihydromorphine were about 20 times higher than the respective K_D values.

5 When for a given compound the ratio of the K_I value against [^{3}H]-D-Ala^2-D-Leu^5-enkephalin to the K_I value against [^{3}H]-dihydromorphine (discrimination ratio) is calculated, a high value indicates selectivity in favour of the μ-receptor and a low value selectivity in favour of the δ-receptor. The most selective μ-agonist known so far is normorphine with a discrimination ratio of 70 and the most selective δ-agonist is D-Ala^2-D-Leu^5-enkephalin with a ratio of 0.11. The selectivity of the known antagonists is in favour of the μ-receptor, since their discrimination ratios are larger than 1, varying between 10 for naloxone and 4 for Mr 2266.

Introduction

In the last 3 years, evidence has accumulated for the view that the enkephalins and β-endorphin interact with at least two different receptors, the μ- and δ-receptors (Lord, Waterfield, Hughes & Kosterlitz, 1976; 1977; Kosterlitz & Hughes, 1978; Kosterlitz, McKnight, Waterfield, Gillan & Paterson, 1978; Kosterlitz, Lord, Paterson & Waterfield, 1980). On the basis of four parallel assays using two pharmacological models, the guinea-pig ileum and the mouse vas deferens, and two models measuring the inhibition of binding of [^{3}H]-naltrexone and [^{3}H]-leucine-enkephalin in homogenates of guinea-pig brain, it was proposed that the enkephalins interacted in the mouse vas deferens mainly with δ-receptors and in the guinea-pig ileum with μ-receptors, although to a lesser extent, whereas β-endorphin was equipotent in interacting with δ- and μ-receptors in both preparations. This interaction with δ-receptors was mirrored by the inhibition of [^{3}H]-leucine-enkephalin binding in the brain and the interaction with μ-receptors by the inhibition of [^{3}H]-naltrexone binding.

In view of the degradation of leucine-enkephalin by peptidases, the binding assays had to be performed at or near 0°C but the advent of more stable analogues made it possible to conduct assays at 25°C. For the assay of the δ-binding sites, [^{3}H]-D-Ala^2-D-Leu^5-enkephalin was chosen because it had been shown to have retained an enkephalin-like pattern of activity (Kosterlitz *et al.*, 1980). Further support for the choice of this analogue was obtained by the finding that unlabelled D-Ala^2-D-Leu^5-enkephalin protected the binding of [^{3}H]-D-Ala^2-D-Leu^5-enkephalin against the alkylating action of phenoxybenzamine much better than unlabelled dihydromorphine, while the reverse was observed when the binding of [^{3}H]-dihydromorphine had to be protected (Robson & Kosterlitz, 1979).

In this paper, we have examined the dissociation

0007-1188/80/110481-10 $01.00

equilibrium constants and the maximal number of binding sites of various tritiated ligands having a high affinity for either the μ- or δ-receptors or both and the influence of temperature on this binding. Further, we have determined the constants of inhibition (K_I) of a number of unlabelled ligands against the binding of the tritiated ligands. Thus, information has been obtained on the specificity of the ligands and the cross reactivity between receptor binding sites. Some of these results have been communicated at a meeting of the British Pharmacological Society (Gillan, Kosterlitz & Paterson, 1979).

Methods

Binding assays

Guinea-pigs, weighing 400 to 600 g, were killed by cervical dislocation. The technique of the binding assays was the same as described previously (Kosterlitz *et al.*, 1980), except that the incubation periods were either 150 min at 0°C or 40 min at 25°C.

In the experiments, in which the equilibrium dissociation constant (K_D) and the maximal number of binding sites were determined, the specific binding was obtained for each concentration of labelled ligand by deducting the non-specific binding that was not inhibited by the opiate antagonist Mr 2266, from the total binding. The concentrations of Mr 2266 varied for the different tritiated ligands, being 100 to 300 times the K_I values of Mr 2266; they were 150 nM for [^{3}H]-dihydromorphine, 300 nM for [^{3}H]-etorphine, 500 nM for [^{3}H]-D-Ala^2-L-Leu^5-enkephalin amide and 1700 nM for [^{3}H]-D-Ala^2-D-Leu^5-enkephalin. That the inhibition of specific binding was complete, was confirmed by experiments in which in the same homogenate Mr 2266 was replaced by the respective unlabelled ligands in concentrations 100 times higher than their K_D values (see Table 1). There were no differences in the K_D values or the maximal number of binding sites.

For the calculation of the kinetic parameters of the binding of the tritiated ligands, the methods of Scatchard (1949) and Hill (1910) were used. In the latter method, the maximal number of binding sites was obtained directly from the saturation curves (Figure 1) while in the former method this value was obtained from the intersection with the abscissa scale of the line for the regression of the ratio of bound to free ligand on the amount of bound ligand. The presence of binding sites with different affinities for the same ligand was indicated when the Scatchard plot deviated from linearity and the slope of the Hill plot (Hill coefficient) from unity. Since in this investigation the accuracy of the estimation of the total number of binding sites was particularly important, the Hill plot was used routinely; log K_D was obtained when log occupied/free binding sites was 0. The linear correlation coefficient for the Hill plot was smaller than 0.96 only in one experiment which was discarded. No significant differences were found between the values of K_D and maximal binding obtained by the two methods.

Since the specific binding at the point of maximal binding may be low and therefore the estimation of the number of maximal binding sites difficult, it was important to establish whether or not this latter value was correlated with the degree of specific binding. The compounds with low values of maximal binding, dihydromorphine and D-Ala^2-D-Leu^5-enkephalin had values of specific binding at maximal occupation of 22.3 ± 1.6 and 42.2 ± 3.7% of total binding, respectively, and those with high values of maximal binding, etorphine and D-Ala^2-L-Leu^5-enkephalin amide had values of 53.0 ± 1.5 and 21.7 ± 0.7%, respectively. Thus, no correlation was found.

The inhibition of binding by cold ligands was determined from the regression of log percentage inhibition of specific binding on log concentration of cold ligand. The inhibition constant (K_I) was calculated from

$$K_I = \frac{IC_{50}}{1 + [L]/K_D},$$

where [L] is the concentration of the labelled ligand and K_D its equilibrium dissociation constant (Chang & Prusoff, 1973). When the ratio of the K_I against [^{3}H]-D-Ala^2-D-Leu^5-enkephalin to the K_I against [^{3}H]-dihydromorphine is larger than 1, it indicates selectivity of a compound for the μ-receptor and, when it is smaller than 1, selectivity for the δ-receptor. The magnitude of deviation from unity gives a measure of the degree of selectivity for one or the other receptor and is therefore called the discrimination ratio.

Labelled primary ligands

The following primary ligands were used: [^{3}H]-leucine-enkephalin (30 to 40 Ci/mmol), [^{3}H]-methionine-enkephalin (34.6 Ci/mmol), [^{3}H]-D-Ala^2-leucine-enkephalin amide (17 to 39 Ci/mmol), [^{3}H]-D-Ala^2-methionine-enkephalin amide (15 Ci/mmol), [^{3}H]-D-Ala^2-D-Leu^5-enkephalin (44 to 48 Ci/mmol), [^{3}H]-dihydromorphine (50 to 81 Ci/mmol), [^{3}H]-morphine (28 Ci/mmol) and [^{3}H]-etorphine (36 Ci/mmol). All were obtained from the Radiochemical Centre, Amersham.

Drugs and peptides

The following drugs were used: morphine hydrochloride (Macfarlan Smith); levorphanol tartrate (Roche

Products); naloxone hydrochloride and naltrexone hydrochloride (Endo Laboratories) and (−)-α-5,9-diethyl-2-(3-furylmethyl)-2′-hydroxy-6,7-benzomorphan as free base (Mr 2266, Dr H. Merz, Boehringer Sohn, Ingelheim).

All peptides were synthetic. Tyr-D-Ala-Gly-Phe-D-Leu was supplied by Dr S. Wilkinson (Wellcome Laboratories), Tyr-D-Ala-Gly-Phe-Leu NH_2 by Dr J. S. Morley (ICI) and Tyr-D-Ala-Gly-Phe-Met NH_2 by Dr B. A. Morgan (Reckitt & Colman).

Stock solutions of the peptides (1 mg/ml) were made in distilled water and kept in plastic tubes at −25°C. Stock solutions of the other compounds were made in distilled water, with addition of HCl when necessary.

Results

Binding characteristics of tritiated enkephalin analogues and opiates at 25°C

The results obtained with six tritiated ligands at 25°C are given in Table 1. The K_D values of the three peptides and of morphine and dihydromorphine varied between approximately 1 and 3 nM; etorphine had a lower value (0.3 nM) than any of the other compounds. The Hill coefficient for dihydromorphine was low which was in agreement with the low correlation coefficient found in the Scatchard analysis (0.892 ± 0.026) and indicated a high (K_D = 0.3 nM) and a low (K_D = 1.9 nM) affinity binding site at the μ-receptor. For the remaining compounds, however, the Hill coefficient was around unity and the correlation coefficients of the Scatchard plots varied between 0.942 and 0.986.

In contrast to this relative uniformity of the affinity values of dihydromorphine and the peptides, there were considerable differences in the maximal number of binding sites. Morphine and dihydromorphine had the lowest binding capacities of approximately 3 pmol/g brain whereas the two D-Ala^2-enkephalin amides and also etorphine had the highest capacities of 13 to 15 pmol/g brain. D-Ala^2-D-Leu^5-enkephalin had an intermediate capacity of about 6 pmol/g brain (Table 1). These differences were well illustrated when, in paired experiments in the same homogenate, a comparison was made of the saturation curves of specific binding of representative members of these three groups or of the Scatchard plots for D-Ala^2-D-Leu^5-enkephalin and D-Ala^2-L-Leu^5-enkephalin amide (Figures 1 and 2). In another series of such paired experiments it was found that the capacity of [^{3}H]-dihydromorphine was 3.8 ± 0.2 pmol/g brain and that of [^{3}H]-D-Ala^2-L-Met^5-enkephalin amide 13.9 ± 0.8 pmol/g brain ($P < 0.001$; $n = 4$).

Binding characteristics of the enkephalins, their analogues and dihydromorphine at 0°C

Because of the lability of natural enkephalins in brain homogenates, their binding characteristics had to be determined at 0°C (Kosterlitz *et al.*, 1979). It was therefore important to determine the effects of lowering the temperature of the assay medium on the binding of the more stable compounds (Table 2). The K_D values varied between about 1 and 4.4 nM, with only dihydromorphine and D-Ala^2-L-Leu^5-enkephalin amide having values apparently higher than at 25°C. This was confirmed when these two compounds were assayed in the same homogenate at 25° and 0°C; the K_D of dihydromorphine rose from 1.7 to 2.7 nM ($P < 0.025$; $n = 4$) and that of D-Ala^2-L-Leu^5-enkephalin amide from 3.7 to 6.0 nM ($P < 0.05$; $n = 3$). Similarly, lowering of the temperature from 25 to 0°C reduced the maximal number of binding sites for dihydromorphine from 3.8 to 2.8 pmol/g brain ($P < 0.025$; $n = 4$) and that of D-Ala^2-L-Leu^5-

Table 1 Binding affinities and capacities of tritiated opioid peptides and opiates at 25°C

Tritiated ligand	*Equilibrium dissociation constant*, K_D (nM)	*Hill coefficient*	*Maximal number of binding sites* (pmol/g brain)
D-Ala^2-D-Leu^5-enkephalin (5)	0.96 ± 0.12	1.07 ± 0.03	6.1 ± 0.5
D-Ala^2-L-Leu^5-enkephalin amide (5)	2.77 ± 0.67*	0.99 ± 0.04	13.0 ± 0.5*
D-Ala^2-L-Met^5-enkephalin amide (5)	2.63 ± 0.53	0.98 ± 0.04	13.9 ± 2.4
Dihydromorphine (4)	1.66 ± 0.29	0.85 ± 0.08	3.8 ± 0.4
Morphine (3)	0.86 ± 0.18	0.99 ± 0.03	2.6 ± 0.2
Etorphine (3)	0.29 ± 0.02	1.03 ± 0.02	14.6 ± 1.6

The values are the means ± s.e. mean; the number of observations is given in parentheses. K_D was calculated from Hill plots.

* The values from three experiments, paired with 0°C, were 3.73 ± 0.57 nM and 13.8 ± 0.2 pmol/g.

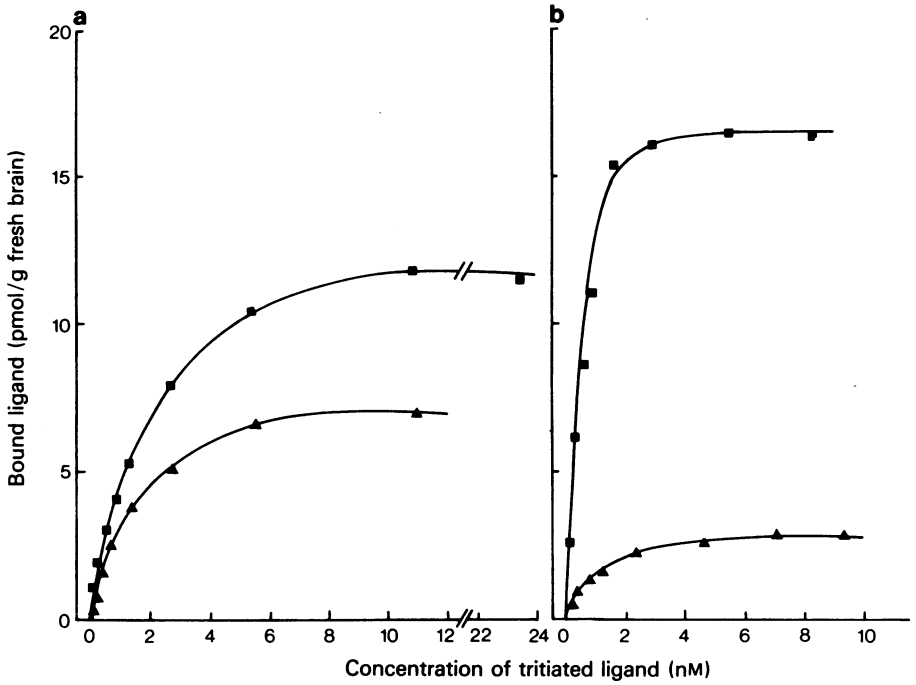

Figure 1 Saturation curves of the specific binding of tritiated D-Ala^2-L-Leu^5-enkephalin amide (■) and D-Ala^2-D-Leu^5-enkephalin (▲) (a) and of tritiated etorphine (■) and morphine (▲) (b). Each pair of ligands was examined in the same homogenate of guinea-pig brain.

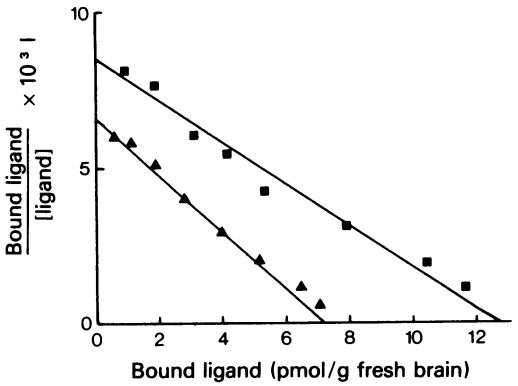

Figure 2 Scatchard plots of the specific binding of [^{3}H]-D-Ala^2-L-Leu^5-enkephalin amide (■) and [^{3}H]-D-Ala^2-D-Leu^5-enkephalin (▲). The K_D values in this experiment were 1.2 and 0.9 nM, respectively.

enkephalin amide from 13.8 to 10.5 pmol/g brain ($P < 0.01$; $n = 3$). Since the effects of lowering the temperature amounted to not more than 25% of the binding capacity at 25°C, it may be assumed that the values obtained for the natural enkephalins at 0°C are probably not far removed from those pertaining at more physiological temperatures.

Competitive inhibition of the binding of tritiated dihydromorphine, D-Ala^2-D-Leu^5-enkephalin, D-Ala^2-L-Leu^5-enkephalin amide and etorphine by the same or closely related unlabelled compounds

When an unlabelled ligand competes with the same tritiated compound, the inhibition constant, K_I, should equal the K_D value obtained from the saturation curve of the labelled ligand. It was found that, at 25°C, the K_I and K_D values for dihydromorphine were 1.4 and 1.7 nM, respectively, for D-Ala^2-D-Leu^5-enkephalin 1.8 and 1.0 nM, for D-Ala^2-L-Leu^5-enkephalin amide 3.0 and 2.8 nM and for etorphine 0.5 and 0.3 nM (Tables 3 and 4).

In contrast to this good agreement, the K_I values of morphine, whose K_D was 0.9 nM (Table 1), were 2.8 nM against dihydromorphine, 90 nM against D-Ala^2-D-Leu^5-enkephalin, 52 nM against D-Ala^2-L-Leu^5-enkephalin amide and 27 nM against etorphine (Table 3). Thus, morphine had a low affinity for the binding site of etorphine and even more so for the binding sites of the two analogues of leucine-enkephalin. It was of particular interest, that the lowest affinity was found

for D-Ala^2-D-Leu^5-enkephalin which has a lower binding capacity than D-Ala^2-L-Leu^5-enkephalin amide and probably represents the δ-receptor binding site.

When unlabelled D-Ala^2-D-Leu^5-enkephalin was the displacing ligand, it had K_I values of 6.2 nM against [^{3}H]-D-Ala^2-L-Leu^5-enkephalin amide, 16.8 nM against [^{3}H]-dihydromorphine and 125 nM against [^{3}H]-etorphine. It was therefore more potent in displacing [^{3}H]-dihydromorphine (discrimination ratio of 0.11; see Methods) than was unlabelled dihydromorphine in displacing [^{3}H]-D-Ala^2-D-Leu^5-enkephalin (discrimination ratio of 29; Table 4). Compared with dihydromorphine, normorphine was less potent in inhibiting the binding of [^{3}H]-dihydromorphine and, to an even greater degree that of [^{3}H]-D-Ala^2-D-Leu^5-enkephalin. Therefore, normorphine is at present the most selective agonist for the μ-binding site with a discrimination ratio of about 70 (Table 4).

The two compounds with high binding capacity showed quite a different pattern of competitive inhibition. D-Ala^2-L-Leu^5-enkephalin amide was a good inhibitor of the binding of [^{3}H]-dihydromorphine (discrimination ratio of 1.2), representing the μ-receptor, and of [^{3}H]-D-Ala^2-D-Leu^5-enkephalin (discrimination ratio of 2), representing the δ-receptor; against etorphine, however, it had a K_I value which was about five times higher than its K_D value. Etorphine was the only unlabelled ligand whose K_I values against all four tritiated ligands (0.5 to 1.1 nM) were sufficiently close to its K_D (0.3 nM).

Competitive inhibition of the binding of tritiated dihydromorphine, D-Ala^2-D-Leu^{5}enkephalin, D-Ala^2-L-Leu^5-enkephalin amide and etorphine by the antagonists naloxone, naltrexone and Mr 2266

All three antagonists were good inhibitors of [^{3}H]-dihydromorphine binding; Mr 2266 discriminated least and naloxone most between the binding of dihydromorphine and D-Ala^2-D-Leu^5-enkephalin; Mr 2266 was about twice and naltrexone 2.4 times more potent than naloxone in displacing [^{3}H]-dihydromorphine binding. This pattern was slightly different for the inhibition of [^{3}H]-D-Ala^2-D-Leu^5-enkephalin binding, with the result that naloxone had the highest discrimination ratio of 10 in favour of the μ-binding site. None of the antagonists was a good inhibitor of the binding of [^{3}H]-D-Ala^2-D-Leu^5-enkephalin to the δ-site (Table 5).

In contrast to naloxone and naltrexone, Mr 2266 showed only minor differences in the inhibition of the binding of [^{3}H]-dihydromorphine, [^{3}H]-D-Ala^2-L-Leu^5-enkephalin amide and [^{3}H]-etorphine. In this context, it may be important that, only with Mr 2266, was the Hill coefficient consistently larger than 1.

Discussion

The main aim of this investigation was to obtain further support for the hypothesis that the naturally occurring opioid peptides, methionine- and leucine-enkephalin and β-endorphin, can interact with at least two receptors, the μ-receptor and the δ-receptor. The evidence obtained so far is based, first, on the different rank order of potency of compounds in four parallel assays, two of which were pharmacological and two were binding assays (Lord *et al.*, 1976; 1977; Kosterlitz *et al.*, 1978; Kosterlitz *et al.*, 1980) and, second, on the ability of the appropriate cold ligands to protect the two receptors selectively against the alkylating action of phenoxybenzamine (Robson & Kosterlitz, 1979).

In the two binding assays, the inhibition of [^{3}H]-leucine-enkephalin and [^{3}H]-naloxone or [^{3}H]-naltrexone was measured at 0°C. In the present paper, the binding capacity of some of the tritiated ligands

Table 2 Binding affinities and capacities of tritiated opioid peptides and dihydromorphine at 0°C

Tritiated ligand	*Equilibrium dissociation constant* K_D (nM)	*Hill coefficient*	*Maximal number of binding sites* (pmol/g brain)
Leucine-enkephalin (3)	1.95 ± 0.32	1.04 ± 0.03	4.9 ± 0.5
Methionine-enkephalin (4)	1.63 ± 0.22	0.95 ± 0.04	6.1 ± 0.7
D-Ala^2-D-Leu^5-enkephalin (2)	1.12, 1.11	1.3, 1.1	6.9, 5.9
D-Ala^2-L-Leu^5-enkephalin amide (5)	4.39 ± 1.01*	1.05 ± 0.04	10.6 ± 0.5*
D-Ala^2-L-Met^5-enkephalin amide (5)	2.39 ± 0.23	1.01 ± 0.09	12.7 ± 2.0
Dihydromorphine (4)	2.71 ± 0.17	0.94 ± 0.11	2.8 ± 0.3

The values are the means ± s.e. mean; the number of observations is given in parentheses. K_D was calculated from Hill plots.

* The values from three experiments, paired with 25°C, were 5.99 ± 0.4 nM and 10.5 ± 0.9 pmol/g.

Table 3 The inhibitory effects (K_I) of morphine, D-Ala^2-D-Leu^5-enkephalin, D-Ala^2-L-Leu^5-enkephalin amide and etorphine on the binding of [^{3}H]-dihydromorphine (0.55 nM), [^{3}H]-D-Ala^2-D-Leu^5-enkephalin (1.8 nM), [^{3}H]-D-Ala^2-L-Leu^5-enkephalin amide (3.5 nM) and [^{3}H]-etorphine (0.52 nM)

Unlabelled compounds	*[^{3}H]-dihydromorphine*		*[^{3}H]-D-Ala^2-D-Leu^5-enkephalin*		*[^{3}H]-D-Ala^2-L-Leu^5-enkephalin amide*		*[^{3}H]-etorphine*	
	K_I (nM)	*Hill coefficient*	K_I (nM)	*Hill coefficient*	K_I (nM)	*Hill coefficient*	K_I (nM)	*Hill coefficient*
Morphine	2.8 ± 0.6	0.92 ± 0.14 (5)	90 ± 17	0.74 ± 0.10 (3)	52 ± 7.5	0.90 ± 0.07 (3)	27 ± 6	0.87 ± 0.13 (4)
D-Ala^2-D-Leu^5-enkephalin	16.8 ± 3.8	1.42 ± 0.07 (5)**	1.8 ± 0.34	1.13 ± 0.08 (10)	6.2 ± 0.68	0.87 ± 0.12 (6)	125 ± 10	0.60 ± 0.08 (3)
D-Ala^2-L-Leu^5-enkephalin amide	3.7 ± 0.6	1.54 ± 0.14 (5)**	1.4 ± 0.04	1.05 ± 0.19 (4)	3.0 ± 0.49	1.27 ± 0.15 (4)	14.8 ± 4.4	0.93 ± 0.14 (4)
Etorphine	1.1 ± 0.07	1.36 ± 0.15 (4)	0.56 ± 0.10	1.48 ± 0.16 (4)*	0.57 ± 0.05	1.41 ± 0.19 (4)	0.53 ± 0.03	1.22 ± 0.12 (4)

The values are the means ± s.e. mean; the number of observations is given in parentheses after the Hill coefficients.
P values: * <0.05, ** <0.01 for difference of the Hill coefficients from unity.

was up to 25% lower at 0° than at 25°C. Our values for methionine- and leucine-enkephalin at 0°C are in good agreement with those obtained at 37°C in the presence of the protease inhibitor, bacitracin (Meunier & Moisand, 1977). As far as K_D values were concerned, we found a minor rise of the K_D of dihydromorphine at 0°C while no change was observed by Law & Loh (1978). These data would indicate that K_D and binding capacity at 0°C give a useful indication of equilibria obtained at physiological conditions although rate constants have been shown in many laboratories to be greatly affected by changes in temperature.

The next two steps were the determination of the binding capacities of different tritiated ligands in homogenates of nervous tissue, of whole brain in the first instance, and the estimation of the constants of inhibition, K_I, of unlabelled ligands against tritiated ligands with the purpose of finding ligands which are able to discriminate between the μ-receptor and the δ-receptor.

Caution has to be used in the interpretation of Scatchard plots because the estimation of the specific binding near saturation is difficult, due to the low ratio of specific to total binding. Extrapolation of the Scatchard plot to the x-axis may give misleading results, particularly when the plots are biphasic. Similar considerations were recently raised for subtypes of the β-adrenoceptor when computerized curve fitting was recommended as an alternative (Hancock, De Lean & Lefkowitz, 1979). For our purpose, direct estimation of the maximum of saturable binding was found to be satisfactory.

As far as binding capacity is concerned, [^{3}H]-dihydromorphine and [^{3}H]-morphine, the two representatives of ligands for the μ-receptor, bound to the smallest number of binding sites, 3 pmol/g brain. Since these two compounds had a high ratio of discrimination of about 30 against [^{3}H]-D-Ala^2-D-Leu^5-enkephalin, the ligand for the δ-receptor, it may be assumed that the estimated binding capacity is probably close to the true value for the μ-receptor. It is likely that the [^{3}H]-dihydromorphine and [^{3}H]-morphine binding sites are composed of sites of higher and lower affinity because the linear correlation coefficient of the Scatchard plot and the Hill coefficient are both low. However, the binding capacity of 3 pmol/g brain comprises the total number of sites up to saturation of specific binding. High and low affinity binding sites for [^{3}H]-dihydromorphine have already been described (Wong & Horng, 1973; Pasternak & Snyder, 1975); these authors found K_D values of 0.3 and 3 nM, values which agree with those obtained by us when we were able to demonstrate biphasic Scatchard plots. If there are such high and low affinity binding sites at the μ-receptor, their significance is unknown. It seems very likely, however, that the low affinity sites are not situated at the δ-receptors. First, the discrimination ratio of dihydromorphine of 29 is larger than the ratios of the K_D values for the high and low affinity binding sites and, second, [^{3}H]-D-Ala^2-D-Leu^5-enkephalin has twice the binding capacity of [^{3}H]-dihydromorphine.

It has been found that the discrimination ratio of D-Ala^2-D-Leu^5-enkephalin of 0.11 deviates much less from unity than that of dihydromorphine, which is 29. This difference in degree of selectivity agrees well with that found for the selective protection against alkylation by phenoxybenzamine (Robson & Kosterlitz, 1979). Thus, D-Ala^2-D-Leu^5-enkephalin is not sufficiently specific for the δ-receptor to avoid cross-reactivity with the μ-receptor in higher concentrations. For this reason, the estimate of binding capacity of 6 pmol/g brain may overestimate the number of δ-receptors.

The finding that the two tritiated D-Ala^2-enkephalin amides have a larger binding capacity than either dihydromorphine or D-Ala^2-D-Leu^5-enkephalin is in good agreement with the earlier findings (Lord *et al.*, 1976; 1977; Kosterlitz *et al.*, 1978; Kosterlitz *et al.*, 1980), namely that amidation makes enkephalins more morphine-like. D-Ala^2-L-Leu^5-

Table 4 Comparison of the inhibitory effects (K_I, nM) of dihydromorphine, morphine, normorphine and D-Ala^2-D-Leu^5-enkephalin on the binding of [^{3}H]-dihydromorphine (0.55 nM) and [^{3}H]-D-Ala^2-D-Leu^5-enkephalin (1.8 nM)

Unlabelled compounds	*[^{3}H]-dihydromorphine (DHM)* K_I (nM)	*[^{3}H]-D-Ala^2-D-Leu^5-enkephalin* K_I (nM)	*Discrimination ratio (D-Ala^2-D-Leu^5/DHM)*
Dihydromorphine	1.4 ± 0.08 (3)	40 ± 5.4 (4)	29
Morphine	2.8 ± 0.62 (3)	90 ± 17 (3)	32
Normorphine	5.0 ± 0.44 (3)	360 ± 26 (3)	72
D-Ala^2-D-Leu^5-enkephalin	16.8 ± 3.8 (5)	1.8 ± 0.3 (10)	0.11

The values are the means ± s.e. mean; the number of observations is given in parentheses.

Table 5 Comparison of the inhibitory effects (K_I, nM) of antagonists on the binding of [^{3}H]-dihydromorphine (0.55 nM), [^{3}H]-D-Ala^2-D-Leu^5-enkephalin (1.8 nM), D-Ala^2-L-Leu^5-enkephalin amide (3.5 nM) and etorphine (0.52 nM)

Unlabelled compounds	[^{3}H]-*dihydromorphine* K_I (nM)	[^{3}H]-D-*Ala*^{2}-D-*Leu*^{5}-*enkephalin* K_I (nM)	[^{3}H]-D-*Ala*^{2}-L-*Leu*^{5}-*enkephalin amide* K_I (nM)	[^{3}H]-*etorphine* K_I (nM)	*Discrimination ratio* (D-*Ala*^{2}-D-*Leu*^{5}/*DHM*)
Naloxone	2.65 ± 0.73 (5)	27 ± 6.1 (4)	14.4 ± 3.9 (4)	6.0 ± 1.4 (4)	10
Naltrexone	1.08 ± 0.17 (3)	6.6 ± 0.8 (3)	5.4 ± 1.3 (4)	3.5 ± 0.71 (4)	6.1
Mr 2266	1.37 ± 0.36 (4)	6.0 ± 1.4 (6)	1.69 ± 0.41 (4)	1.11 ± 0.18 (4)	4.4

The values are the means ± s.e. mean; the number of observations is given in parentheses. The Hill coefficients for naloxone and naltrexone did not differ significantly from 1 but for Mr 2266 varied between 1.23 and 1.34 ($P < 0.05$).

enkephalin amide is a good inhibitor of binding of tritiated ligands to either the μ- or δ-receptors. It is of interest to note that the binding capacity of D-Ala^2-enkephalin amides of 13 pmol/g brain appears to be greater than the sum of the capacities of dihydromorphine and D-Ala^2-D-Leu^5-enkephalin binding.

The capacity of etorphine binding, which has been known to be large (Simon, Hiller & Edelman, 1973; Simon, Hiller, Groth & Edelman, 1975), is of the same order as that of the D-Ala^2-enkephalin amides. At the same time, unlabelled etorphine is equipotent in inhibiting the four tritiated ligands. In view of the high binding capacities of the D-Ala^2-enkephalin amides and particularly of etorphine, it is tempting to speculate whether or not there are opiate receptors with a different pattern of binding. A good candidate would be the κ-receptor demonstrated in neurophysiological investigations (Martin, Eades, Thompson, Huppler & Gilbert, 1976; Gilbert & Martin, 1976) and *in vitro* preparations (Hutchinson, Kosterlitz, Leslie, Waterfield & Terenius, 1975).

While the investigations presented in this paper were in progress, results were published which were obtained independently and which confirmed the concept of multiple receptors for opioid peptides and alkaloid opiates (Chang & Cuatrecasas, 1979). These authors used [^{125}I]-D-Ala^2-D-Leu^5-enkephalin instead of the tritiated compound used by us. The iodonated compound has the advantage that much lower concentrations can be used and the disadvantage that the iodonation causes a significant increase in molecular weight and may also lead to a change in the hydrophobic balance of the molecule. In fact, mono-iodonated D-Ala^2-D-Leu^5-enkephalin has only 30% of the potency of the parent compound in the guinea-pig ileum and 13% in the mouse vas deferens as shown by Miller, Chang, Leighton & Cuatrecasas (1978). Chang & Cuatrecasas (1979) found ratios of the K_I values against [^{125}I]-D-Ala^2-D-Leu^5-enkephalin to those against [^{3}H]-dihydromorphine of 96 for morphine, 26 for naloxone and 0.24 for D-Ala^2-L-Leu^5-enkephalin. These values are in fair agreement with our ratios of the K_I values against [^{3}H]-D-Ala^2-D-Leu^5-enkephalin to those against [^{3}H]-dihydromorphine of 32, 10 and 0.11 for morphine, naloxone and D-Ala^2-D-Leu^5-enkephalin, or 66, 21 and 0.22 if the K_D (0.3 nM) for the high affinity binding was used instead of its median K_D (1.7 nM). As far as the number of binding sites is concerned, Chang & Cuatrecasas (1979) found that the binding capacity of [^{3}H]-dihydromorphine was about twice that of [^{125}I]-D-Ala^2-D-Leu^5-enkephalin whereas in the present paper the number of [^{3}H]-D-Ala^2-D-Leu^5-enkephalin binding sites is about twice as high as that of [^{3}H]-dihydromorphine. This difference may be due to the use of the iodonated compound by Chang & Cuatrecasas (1979). Later, Chang, Cooper, Hazum & Cuatrecasas

(1979) examined the distribution in the brain of δ- and μ-binding sites with high affinities for [^{125}I]-D-Ala^2-D-Leu^5-enkephalin and [^{125}I]-D-Ala^2-MePhe^4-Met-(O)-ol^5-enkephalin as representatives of enkephalin- and morphine-like ligands, respectively. Finally, Pert & Taylor (1979) have recently classified opiate receptors by the differential effects of guanosinetriphosphate (GTP) on binding; it is possible that their type 1, which is GTP-sensitive, may be correlated to the μ-receptor, and their type 2, which is GTP-insensitive, to the δ-receptor. Both Chang *et al.* (1979) and Pert & Taylor (1979) have demonstrated regional differences in the proportions of μ or morphine or type 1 receptors and of δ or enkephalin or type 2 receptors.

Another possible explanation for the findings presented in this paper and of those of Chang *et al.* (1979) and Pert & Taylor (1979) has been suggested by Simantov, Childers & Snyder (1978) and Childers, Creese, Snowman & Snyder (1979) who propose that there are several points of attachment to a single receptor. It would appear that the final decision will have to await successful isolation of the receptor; the weight of the evidence available at present appears to be in favour of the hypothesis of multiple opiate receptors.

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

References

CHANG, K.-J., COOPER, B.R., HAZUM, A.A. & CUATRECASAS, P. (1979). Multiple opiate receptors: different regional distribution in the brain and differential binding of opiates and opioid peptides. *Mol. Pharmac.*, **16**, 91–104.

CHANG, K.-J. & CUATRECASAS, P. (1979). Multiple opiate receptors: enkephalin and morphine bind to receptors of different specificity. *J. biol. Chem.*, **254**, 2610–2618.

CHANG, 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 enzymatic reaction. *Biochem. Pharmac.*, **22**, 3099–3108.

CHILDERS, S.R., CREESE, I., SNOWMAN, A.M. & SNYDER, S.H. (1979). Opiate receptor binding affected differentially by opiates and opioid peptides. *Eur. J. Pharmac.*, **55**, 11–18.

GILBERT, P.E. & MARTIN, W.R. (1976). The effects of morphine- and nalorphine-like drugs in the nondependent, morphine-dependent and cyclazocine-dependent spinal dog. *J. Pharmac. exp. Ther.*, **198**, 66–82.

GILLAN, M.G.C., KOSTERLITZ, H.W. & PATERSON, S.J. (1979). Comparison of the binding characteristics of tritiated opiates and opioid peptides. *Br. J. Pharmac.*, **66**, 86–87P.

HANCOCK, A.A., DE LEAN, A.L. & LEFKOWITZ, R.J. (1979). Quantitative resolution of beta-adrenergic receptor subtypes by selective ligand binding: application of computerized model fitting technique. *Mol. Pharmac.*, **16**, 1–9.

HILL, A.V. (1910). The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J. Physiol.*, **40**, IV–VIII.

HUTCHINSON, M., KOSTERLITZ, H.W., LESLIE, F.M., WATERFIELD, A.A. & TERENIUS, L. (1975). Assessment in the guinea-pig ileum and mouse vas deferens of benzomorphans which have strong antinociceptive activity but do not substitute for morphine in the dependent monkey. *Br. J. Pharmac.*, **55**, 541–546.

KOSTERLITZ, H.W. & HUGHES, J. (1978). Development of the concepts of opiate receptors and their ligands. In *The Endorphins. Advances in Biochemical Psychopharmacology*, vol. 18. ed. Costa, E. & Trabucchi, M. pp. 31–44. New York: Raven Press.

KOSTERLITZ, H.W., LORD, J.A.H., PATERSON, S.J. & WATERFIELD, A.A. (1980). Effect of changes in the structure of enkephalins and of narcotic analgesic drugs on their interactions with μ- and δ-receptors. *Br. J. Pharmac.*, **68**, 333–342.

KOSTERLITZ, H.W., MCKNIGHT, A.T., WATERFIELD, A.A., GILLAN, M.G.C. & PATERSON, S.J. (1978). Assessment of analogues of opioid peptides in four parallel assays. *Rep. 40th meeting, Committee Probl. Drug Dependence*, **40**, 139–150.

LAW, P.-Y. & LOH, H.H. (1978). [^{3}H]-Leu^5-enkephalin specific binding to synaptic membrane—comparison with [^{3}H]-dihydromorphine and [^{3}H]-naloxone. *Res. Comm. Chem. Path. Pharmac.*, **21**, 409–434.

LORD, J.A.H., WATERFIELD, A.A., HUGHES, J. & KOSTERLITZ, H.W. (1976). Multiple opiate receptors. In *Opiates and Endogenous Opioid Peptides.* ed. Kosterlitz, H.W. pp. 275–280. Amsterdam: North-Holland Publishing Co.

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.

MARTIN, W.R., EADES, C.G., THOMPSON, J.A., HUPPLER, R.E. & GILBERT, P.E. (1976). The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dogs. *J. Pharmac. exp. Ther.*, **197**, 517–532.

MEUNIER, J.C. & MOISAND, C. (1977). Binding of Leu^5-enkephalin and Met^5-enkephalin to a particulate fraction from rat cerebrum. *FEBS Lett.*, **77**, 209–213.

MILLER, R.J., CHANG, K.-J., LEIGHTON, J. & CUATRECASAS, P. (1978). Interaction of iodinated enkephalin analogues with opiate receptors. *Life Sci.*, **22**, 379–388.

PASTERNAK, G.W. & SNYDER, S.H. (1975). Identification of novel high affinity opiate receptor binding in rat brain. *Nature, Lond.*, **253**, 563–566.

PERT, C.B. & TAYLOR, D. (1979). Type 1 and Type 2 opiate receptors: a subclassification scheme based upon GTP's differential effects on binding. In *Endogenous and Exogenous Opiate Agonists and Antagonists.* ed. Way, E.L. pp. 87–90. Oxford and New York: Pergamon Press.

ROBSON, L.E. & KOSTERLITZ, H.W. (1979). Specific protection of the binding sites of D-Ala^2-D-Leu^5-enkephalin (δ-receptors) and dihydromorphine (μ-receptors). *Proc. R. Soc. B*, **205**, 425–432.

SCATCHARD, G. (1949). The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660–672.

SIMANTOV, R., CHILDERS, S.R. & SNYDER, S.H. (1978). The opiate receptor binding interaction of [^{3}H]-methionine enkephalin: an opioid peptide. *Eur. J. Pharmac.*, **47**, 319–331.

SIMON, E.J., HILLER, J.B. & EDELMAN, I. (1973). Stereospecific binding of the potent narcotic analgesic [^{3}H]-etorphine to rat-brain homogenate. *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1947–1949.

SIMON, E.J., HILLER, J.B., GROTH, J. & EDELMAN, I. (1975). Further properties of stereospecific opiate binding sites in rat brain: on the nature of the sodium effect. *J. Pharmac. exp. Ther.*, **192**, 531–537.

WONG, D.T. & HORNG, J.S. (1973). Stereospecific interaction of opiate narcotics in binding of [^{3}H]-dihydromorphine to membranes of rat brain. *Life Sci.*, **13**, 1543–1556.

(Received November 13, 1979.)