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CHARACTERIZATION OF THE κ-SUBTYPE OF THE OPIATE RECEPTOR IN THE GUINEA-PIG BRAIN

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1 In homogenates of guinea-pig brain, the characteristics of the binding of [^{3}H]-ethylketazocine, an agonist for the putative κ-receptor, were determined by estimation of the affinity and capacity of binding, by competitive inhibition for the binding site by unlabelled ligands and by selective protection of the binding site from alkylation by phenoxybenzamine.

2 At 25°C the maximum number of binding sites for [^{3}H]-ethylketazocine was about 14 pmol/g fresh brain, of which about 50% were high affinity sites.

3 In competition experiments, the high affinity binding of [^{3}H]-ethylketazocine to the κ-binding site was readily displaced by several κ-agonists but not by the selective μ-ligand, D-Ala^2, MePhe^4, Gly-ol^5-enkephalin or the selective δ-ligand, D-Ala^2, D-Leu^5-enkephalin. In contrast, the κ-agonists tested so far exhibit a high degree of cross-reactivity with the μ-binding site but somewhat less with the δ-binding site. Similar specificities were observed in protection experiments.

4 The approximate proportions of the three subtypes of opiate receptor in the guinea-pig brain are 25% μ-binding sites, 45% δ-binding sites and 30% κ-binding sites.

5 The endogenous opioids, Met-enkephalin, Leu-enkephalin and porcine β-endorphin have only a low affinity for the κ-binding site.

Introduction

On the basis of their different pharmacological profiles and different potencies in binding assays, considerable evidence has accumulated for the fact that opiates and opioid peptides act on separate μ-(morphine) and δ-(enkephalin) receptors (Lord, Waterfield, Hughes & Kosterlitz, 1976; 1977; Kosterlitz, McKnight, Waterfield, Gillan & Paterson, 1978; Kosterlitz, Lord, Paterson & Waterfield, 1980; Gillan, Kosterlitz & Paterson, 1980). In addition it has been shown that opiates and opioid peptides selectively protect the μ- and δ-receptors from inactivation by the alkylating agents, phenoxybenzamine (Robson & Kosterlitz, 1979) and N-ethylmaleimide (Smith & Simon, 1980).

The unique pharmacological pattern of some of the benzomorphans is of interest because they are potent antinociceptive agents in rodents but do not suppress signs of withdrawal in morphine-dependent monkeys and have little or no antagonist activity (Villarreal & Seevers, 1972; Swain & Seevers, 1974; 1976). In the chronic spinal dog, behavioural and neurophysiological observations have suggested that the benzomorphans interact with a receptor that is different from the μ-receptor and has been called the κ-receptor (Martin, Eades, Thompson, Huppler & Gilbert, 1976; Gilbert & Martin, 1976). In the guinea-pig ileum and mouse vas deferens, these benzomorphans are pure opiate agonists but require more naloxone for antagonism than morphine (Hutchinson, Kosterlitz, Leslie, Waterfield & Terenius, 1975).

The availability of [^{3}H]-ethylketazocine has enabled us, in this paper, to examine directly its binding to the μ-, δ- and putative κ-subtypes of opiate receptor. Some of the results have been described at meetings of the Royal Society (Kosterlitz & Paterson, 1980) and Physiological Society (Kosterlitz, Paterson & Robson, 1980).

Methods

Binding assays

Binding assays were performed in homogenates of guinea-pig brain as described previously (Gillan *et al.*, 1980). The brain tissue, after removal of the cerebellum, was homogenized in Tris Buffer (pH 7.4 at 0°C), centrifuged at 49,000 *g* for 10 min, the pellet resuspended in Tris buffer, incubated at 37°C for 45 min and centrifuged again. For the assay, 1.9 ml final homogenate without addition of Na^+ and corresponding to 19 mg of brain tissue, was used and the volume made up to 2.0 ml with solutions of the inhibitory cold ligand and of the labelled primary ligand. The mixture was incubated for 40 min at 25°C or 150 min at 0°C, filtered through Whatman GF/B

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glass filter discs and washed three or four times with 4 ml ice-cold Tris buffer solution. Specific binding was obtained for each concentration of tritiated 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 100–500 times the K_I values obtained for Mr 2266; they were 150–250 nM for [^{3}H]-dihydromorphine, 500 nM for [^{3}H]-(±)-ethylketazocine and 1700–2500 nM for D-Ala^2, D-Leu^5-enkephalin.

For the calculation of the kinetic parameters of the binding of labelled and unlabelled ligands, the equilibrium dissociation constant (K_D), inhibition constant (K_I) and maximum number of binding sites were determined from multiphasic saturation curves and competition experiments (Hill, 1910; Scatchard, 1949; Cheng & Prusoff, 1973; Gillan *et al.*, 1980). When the ratio of K_I against [^{3}H]-(±)-ethylketazocine to the K_I against [^{3}H]-dihydromorphine is larger than 1, it indicates selectivity of the compound for the μ-binding site and, when it is less than 1, selectivity for the κ-binding sites. Similar considerations hold for other pairs of tritiated ligands. Since the deviation from unity gives a measure of the degree of selectivity of the compound for the μ-, δ- or κ-binding site, it is called the discrimination ratio.

Protection assays

The potency of ligands in protecting the binding sites from inactivation by the irreversible alkylating agent, phenoxybenzamine, was tested in homogenates of guinea-pig brain, using a modification of the method previously described (Robson & Kosterlitz, 1979); the 300 *g* fraction of the homogenate was not discarded. The homogenate was incubated, in the presence of unlabelled protecting ligands, for 25 min at 37°C. During the latter 15 min, phenoxybenzamine was added at a concentration which gave 60–80% inhibition of unprotected binding. The binding of [^{3}H]-ethylketazocine, [^{3}H]-dihydromorphine or [^{3}H]-D-Ala^2, D-Leu^5-enkephalin was tested after removal of the protecting ligands by washing. The degree of protection was expressed as the percentage of that amount of specific binding that had been inhibited by phenoxybenzamine. In each experiment, the protecting potencies of cold ligands were compared in one and the same homogenate.

Labelled primary ligands

The following primary ligands were used: [^{3}H]-dihydromorphine (50–81 Ci/mmol) and [^{3}H]-D-Ala^2, D-Leu^5-enkephalin (25–48 Ci/mmol; Radiochemical Centre, Amersham); [^{3}H]-(−)-ethylketazocine (19 Ci/mmol; National Institute on Drug Abuse) and [^{3}H]-(±)-ethylketazocine (15 Ci/mmol; New England Nuclear). The purity of all ligands used was more than 95%, achieved, if necessary, by high-pressure liquid chromatography on a μBondapak C_{18} column.

Drugs and peptides

The following drugs were used: etorphine hydrochloride (Reckitt & Colman), morphine hydrochloride and normorphine base (Macfarlan Smith); naloxone hydrochloride and naltrexone hydrochloride (Endo Laboratories); phenoxybenzamine hydrochloride (Smith, Kline & French); (±)-ethylketazocine methanesulphonate ((±)-α-9-methyl-5-ethyl-8-oxo-2-cyclopropylmethyl-2′-hydroxy-6,7-benzomorphan), (−)-ethylketazocine base and (+)-ethylketazocine base (Dr W.F. Michne, Sterling-Winthrop); (−)-bremazocine hydrochloride ((−)-5-ethyl-9,9-dimethyl-2-(1-hydroxy-cyclopropylmethyl)-2′-hydroxy-6,7-benzomorphan; Dr D. Römer, Sandoz); Mr 2034 as the tartrate ((−)-(1R,5R,9R,2″S)-5,9-dimethyl-2-tetrahydrofurfuryl-2′-hydroxy-6,7-benzomorphan), Mr 2266 as free base ((−)-α-5,9-diethyl-2-(3-furylmethyl)-2′-hydroxy-6,7-benzomorphan) and Mr 2267 the (+)-isomer of Mr 2266 as free base (all Mr compounds from Dr H. Merz, Boehringer-Ingelheim).

All peptides were synthetic: Tyr-D-Ala-Gly-Phe-D-Leu (Dr S. Wilkinson, Wellcome Laboratories), Tyr-D-Ala-Gly-MePhe-Met(0)-ol (Dr D. Römer, Sandoz), Tyr-Gly-Gly-Phe-Leu, Tyr-Gly-Gly-Phe-Met, Tyr-D-Ala-Gly-MePhe-Gly-ol (Dr B.A. Morgan, Reckitt & Colman), Tyr-D-Ala-Gly-Phe-Leu-NH_2 (Dr J.S. Morley, I.C.I.), and porcine β-endorphin (Dr R. Guillemin).

Stock solutions of the peptides 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 the addition of HCl when necessary. To minimize loss of β-endorphin or etorphine by adsorption, glassware and Hamilton syringes were siliconized. When β-endorphin was used, bovine serum albumin (1 mg/ml) was present in the homogenate.

Results

Binding of triated (−)- and (±)-ethylketazocine

Since the supply of [^{3}H]-(−)-ethylketazocine was limited, its binding characteristics were compared to those of [^{3}H]-(±)-ethylketazocine (Table 1). In guinea-pig brain, the (+)-moiety present in racemic

Table 1 Binding characteristics of the isomers of tritiated ethylketazocine in homogenates of guinea-pig brain

Affinity	*Isomer*	*Equilibrium dissociation constant* K_D (nM) 25°	0°C	*Number of binding sites* (pmol/g wet brain) 25°	0°C
High	(±)	0.64 ± 0.04	1.28 ± 0.30*	7.3 ± 0.5 (6)	5.3 ± 0.7 (3)
	(−)	0.59 ± 0.04	–	6.6 ± 0.8 (3)	–
Low	(±)	3.2 ± 0.4	4.2 ± 1.2	13.9 ± 0.5 (5)	10.6 ± 1.4 (3)
	(−)	3.1 ± 0.5	–	14.0 ± 0.8 (3)	–

The values were obtained from biphasic Scatchard plots and are the means ± s.e.mean. The number of observations is given in parentheses. *Significance for difference between 0° and 25°C in the same homogenate; $P < 0.05$ (paired analysis).

[^{3}H]-ethylketazocine did not interfere with the binding; unlabelled (+)-ethylketazocine (K_I = 2200 nM) had no significant effect on the binding of [^{3}H]-(±)-ethylketazocine. Furthermore, the specific binding of [^{3}H]-(±)-ethylketazocine was the same when unlabelled Mr 2266, (−)-ethylketazocine or (±)-ethylketazocine were used as inhibitors. At a concentration of 0.65 nM of [^{3}H]-(±)-ethylketazocine, the specific binding was 85% of the total binding. It should be noted that, so far, only homogenates of guinea-pig and rat brain have been used for the binding of [^{3}H]-(±)-ethylketazocine and [^{3}H]-(−)-ethylketazocine.

When the saturation curve of [^{3}H]-ethylketazocine was determined in homogenates of guinea-pig brain, it was shown that the Hill or Scatchard plots were not monophasic, leading to binding of varying affinities (Figure 1; Table 1). The maximum number of binding sites was 14 pmol/g at 25°C and 11 pmol/g brain tissue at 0°C. These sites were of a heterogeneous

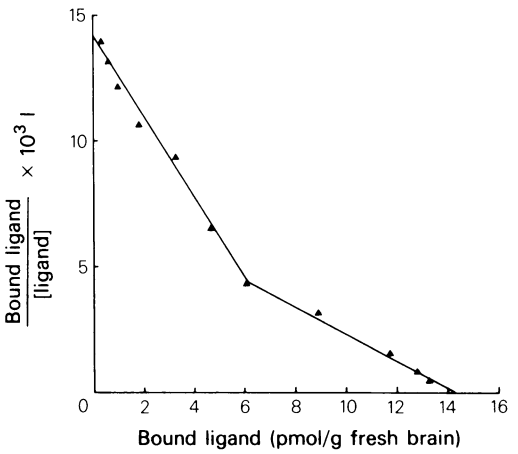

Figure 1 Scatchard plot of the specific binding of [^{3}H]-(±)-ethylketazocine. The K_D values were 0.62 nM and 2.2 nM.

nature, being based on the binding to μ-, δ- and κ-subtypes. The apparent high-affinity binding of the Scatchard plot corresponded to a K_D value of 0.59 nM for the (−)-isomer and 0.64 nM for the racemate and a binding capacity of 7.3 pmol/g brain tissue (Kosterlitz & Paterson, 1980). The absence of a difference between the two K_D values is difficult to explain since the radiochemical purity of both the ligands was > 95%.

Competitive inhibition by κ-agonists of the binding of tritiated dihydromorphine, D-Ala^2, D-Leu^5-enkephalin and ethylketazocine

The ability of unlabelled ethylketazocine, Mr 2034 and bremazocine to displace tritiated ligands from the μ-, δ- and κ-binding sites was tested (Table 2). These three compounds readily inhibited the binding of [^{3}H]-dihydromorphine and [^{3}H]-ethylketazocine; the K_I values were 1.32 and 1.00 nM for ethylketazocine, 0.66 and 0.51 nM for Mr 2034, and 0.36 and 0.22 nM for bremazocine. Thus, there was almost complete cross-reactivity between the μ- and κ-binding sites. At the δ-binding site, there was less cross-reactivity; the ratios of K_I against [^{3}H]-D-Ala^2, D-Leu^5-enkephalin to K_I against [^{3}H]-ethylketazocine were 5.5 for unlabelled ethylketazocine, 11.4 for Mr 2034 and 3.3 for bremazocine. In the rat, bremazocine inhibited the binding of [^{3}H]-naloxone almost as well as that of [^{3}H]-bremazocine (Römer, Büscher, Hill, Maurer, Petcher, Welle, Bakel & Akkerman, 1980).

Competitive inhibition of the binding of [^{3}H]-(±)-ethylketazocine by opiates and analogues of opioid peptides

Since κ-agonists of the benzomorphan group had a high degree of cross-reactivity with the μ-binding site (Table 2), it was necessary to determine whether or not μ-ligands and δ-ligands showed significant in-

Table 2 Inhibition by unlabelled κ-agonists of the binding of tritiated ligands for the μ-, δ- and κ-binding sites at 25°C

Unlabelled κ-agonists	*[^{3}H]-dihydromorphine* (μ-site, 0.3–0.55 nM) K_I (nM)	Hill coefficient	*[^{3}H]-D-Ala^2,D-Leu^5-enkephalin* (δ-site, 1–1.8 nM) K_I (nM)	Hill coefficient	*[^{3}H]-(±)-ethylketazocine* (κ-site, 0.65 nM) K_I (nM)	Hill coefficient
(−)-Ethylketazocine	1.32 ± 0.33	0.84 ± 0.09 (3)	5.5 ± 0.60	0.97 ± 0.06 (4)	1.00 ± 0.12	0.99 ± 0.06 (6)
Mr 2034	0.66 ± 0.12	0.84 ± 0.04 (3)	5.8 ± 0.8	0.96 ± 0.03 (4)	0.51 ± 0.13	0.97 ± 0.08 (3)
Bremazocine	0.36 ± 0.08	1.43 ± 0.25 (3)	0.72 ± 0.11	1.21 ± 0.04 (3)	0.22 ± 0.03	1.41 ± 0.20 (3)

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

teraction with the κ-binding site. Only if μ- and δ-ligands could not displace the binding of [^{3}H]-ethylketazocine, would it be permissible to postulate a separate κ-binding site. In this context, we examined displacement curves for three types of agonists, μ-selective agonists, δ-selective agonists and non-selective agonists (Table 3).

μ-Selective agonists. The inhibition curves against the binding of [^{3}H]-ethylketazocine resulted in Hill coefficients which were low for dihydromorphine, morphine and normorphine (0.59–0.63) and even more so for D-Ala^2,MePhe^4,Met(0)-ol^5-enkephalin and D-Ala^2,MePhe^4,Gly-ol^5-enkephalin (0.35–0.46). The K_I values of the morphine alkaloids were 73, 84 and 68 nM while those of the two enkephalin analogues were as high as 165 and 784 nM, respectively.

The Hill plot of the inhibition of [^{3}H]-ethylketazocine binding by D-Ala^2,MePhe^4,Gly-ol^5-enkephalin was biphasic (Figure 2a). The first phase (2–18 nM) and the second phase (4600–37000 nM) were connected by a horizontal line (36–1200 nM) and were readily separated into Hill plots with slopes of unity; the K_I values were 4.6 nM and 4960 nM (Figure 2b).

Biphasic curves were also obtained for D-Ala^2,MePhe^4,Met(0)-ol^5-enkephalin, morphine and normorphine. Of all these compounds, D-Ala^2,MePhe^4,Gly-ol^5-enkephalin had the highest K_I value against [^{3}H]-ethylketazocine binding, indicating that it was the most selective μ-ligand (Table 3a); it had also a high inhibition constant (820 nM) for the binding of [^{3}H]-D-Ala^2,D-Leu^5-enkephalin (Kosterlitz & Paterson, 1981). When tritiated ethylketazocine was used at 0.65 nM, the first phase of inhibition represented about 40% of the total binding.

δ-Selective agonist. The competition curve for the δ-ligand D-Ala^2,D-Leu^5-enkephalin against [^{3}H]-ethylketazocine binding was, as before, biphasic with a horizontal component between 700 and 5600 nM (Figure 3a). The two Hill plots had slopes of unity; the K_I values were 37.8 nM and 27900 nM (Figure 3b).

Non-selective agonists. We also tested two non-selective agonists (Table 3c). Etorphine had low inhibition constants (0.48–1.1 nM) against tritiated dihydromorphine (μ), D-Ala^2,D-Leu^5-enkephalin (δ)

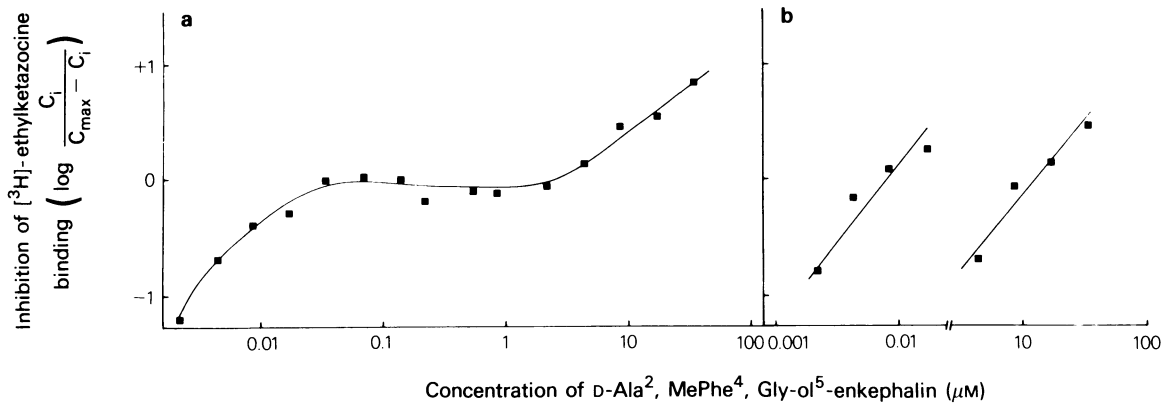

Figure 2 The inhibitory effect of D-Ala^2,MePhe^4,Gly-ol^5-enkephalin (μ-ligand) on the binding of [^{3}H]-(±)-ethylketazocine (0.65 nM) in a homogenate of guinea-pig brain. (a) Biphasic inhibition by D-Ala^2,MePhe^4,Gly-ol^5-enkephalin. (b) Inhibition calculated on the basis of two separate Hill plots: K_I = 3.3 nM, Hill coefficient = 1.34, and K_I = 6580 nM, Hill coefficient = 1.13. Typical result of four experiments. C_i, inhibited counts; C_{max}, maximal counts.

Table 3 Comparison of the inhibitory effects of agonist opiates and analogues of opioid peptides on the binding of [^{3}H]-(±)-ethylketazocine (0.65 nM) and [^{3}H]-dihydromorphine (0.3–0.55 nM) at 25°C

	Inhibition of [^{3}H]-(±)-ethylketazocine binding			*Inhibition of [^{3}H]-dihydromorphine binding*	*Discrimination ratios*	
Unlabelled compounds	K_I (nM)	Hill coefficient	Separation of the two components of the Hill plot K_I (nM)	K_I (nM)	K_I for κ / K_I for μ	K_I for κ / K_I for δ
a) *μ-Selective agonists*						
Dihydromorphine	73 ± 19	0.63 ± 0.05 (3)	–	1.4 ± 0.08 (3)*	52	–
Normorphine	83 ± 17	0.60 ± 0.03 (3)	6.3 ± 1.8 520 ± 150	5.0 ± 0.44 (3)*	104	–
Morphine	64 ± 10	0.59 ± 0.03 (3)	3.3 ± 1.0 410 ± 150	2.8 ± 0.62 (3)*	146	–
D-Ala^2,MePhe^4,Met(0)-ol^5-enkephalin	165 ± 75	0.46 ± 0.04 (3)	2.2 ± 1.2 1340 ± 180	1.3 ± 0.3 (3)	1030	–
D-Ala^2,MePhe^4,Gly-ol^5-enkephalin	784 ± 335	0.35 ± 0.03 (4)	4.6 ± 1.6 4960 ± 860	3.6 ± 0.9 (4)	1380	–
b) *δ-Selective agonists*						
D-Ala^2,D-Leu^5-enkephalin	5230 ± 1500	0.42 ± 0.04 (3)	37.8 ± 9.7 27900 ± 4160	16.8 ± 3.8 (5)*	–	15,500
c) *Non-selective agonists*						
Etorphine (μ + δ + κ)	0.48 ± 0.04	1.11 ± 0.11 (4)	–	1.1 ± 0.1 (4)*	0.44	0.86
D-Ala^2,L-Leu-NH$_2$^{5}-enkephalin (μ + δ)	361 ± 173	0.47 ± 0.05 (3)	10.2 ± 1.0 2450 ± 980	3.7 ± 0.6 (5)*	661	1750

The values are the means ± s.e.mean; the number of observations is given in parentheses. When the Hill coefficient was 0.6 or less, the K_I values for the two components were calculated separately; the Hill coefficients for the separated components were between 0.8 and 1.2. The readily displaced component was 37(35–40)% for the μ-agonists and 31% for the δ-agonists. The discrimination ratios were calculated for the less easily inhibited portion of [^{3}H]-(±)-ethylketazocine binding. The K_I values against [^{3}H]-D-Ala^2,D-Leu^5-enkephalin binding were 0.56 ± 0.10 nM(4) for etorphine, 1.8 ± 0.34 nM(10) for D-Ala^2,D-Leu^5-enkephalin and 1.4 ± 0.04 nM(4) for D-Ala^2, L-Leu-NH$_2$^{5}-enkephalin. *From Gillan *et al.* (1980). The Hill coefficients for inhibition of [^{3}H]-dihydromorphine binding varied between 0.8 and 1.5.

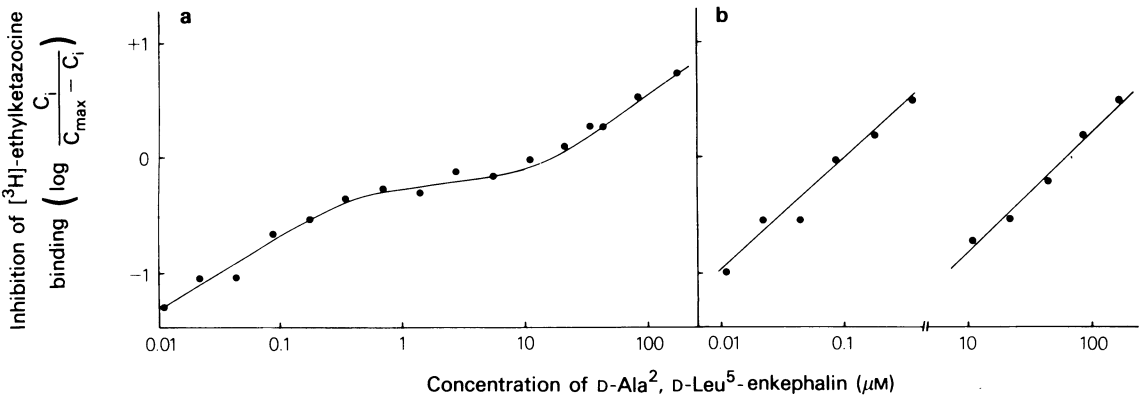

Figure 3 The inhibitory effect of D-Ala^2,D-Leu^5-enkephalin (δ-ligand) on the binding of [^{3}H]-(±)-ethylketazocine (0.65 nM) in a homogenate of guinea-pig brain. (a) Biphasic inhibition by D-Ala^2,D-Leu^5-enkephalin. (b) Inhibition calculated on the basis of two separate Hill plots: K_I = 52 nM, Hill coefficient = 0.77, and K_I = 33600 nM, Hill coefficient = 0.97. Typical result of three experiments. C_i, inhibited counts; C_{max}, maximal counts.

and ethylketazocine (κ) and may thus be considered to be a universal ligand. On the other hand, D-Ala^2,L-Leu-NH$_2$^{5}-enkephalin did not readily displace [^{3}H]-ethylketazocine but had low K_I values against dihydromorphine and D-Ala^2,D-Leu^5-enkephalin.

Discrimination ratios. The K_I values for the more easily inhibited portion of [^{3}H]-ethylketazocine binding were of the same order as the K_I values for the inhibition of [^{3}H]-dihydromorphine binding (Table 3). This observation indicates that the first component of the inhibition of [^{3}H]-ethylketazocine binding is due to displacement of the tritiated ligand from the μ-binding site and not from the κ-binding site.

The selectivity of the various agonists is measured by the discrimination ratios, K_I for κ/K_I for μ, or K_I for κ/K_I for δ (Table 3); ratios of 3 to 4 orders of magnitude were found for D-Ala^2,MePhe^4,Gly-ol^5-enkephalin as a μ-agonist and for D-Ala^2,D-Leu^5-enkephalin as a δ-agonist.

Antagonists. The inhibitory effects of three opiate antagonists were examined (Table 4). As far as naloxone and naltrexone were concerned, the K_I values were 4 to 6 times larger against the binding of [^{3}H]-ethylketazocine than against the binding of [^{3}H]-dihydromorphine. In contrast, Mr 2266 was equipotent against both tritiated ligands. None of the antagonists showed useful selectivity at the μ- and κ-binding sites or at the δ-binding sites (Gillan *et al.*, 1980).

Competitive inhibition of the binding of [^{3}H]-ethylketazocine by endogenous opioid peptides at 0°C

The peptides were tested for inhibition of the binding of [^{3}H]-dihydromorphine, [^{3}H]-D-Ala^2,D-Leu^5-enkephalin and [^{3}H]-ethylketazocine (Table 5). Leu-enkephalin and Met-enkephalin were more potent inhibitors of the binding of [^{3}H]-D-Ala^2,D-Leu^5-enkephalin (K_I = 1.0 and 0.7 nM) than of [^{3}H]-

Table 4 The inhibitory effects of opiate antagonists on the binding of [^{3}H]-(±)-ethylketazocine (0.65 nM) and [^{3}H]-dihydromorphine (0.30–0.55 nM) at 25°C

Unlabelled compounds	*Inhibition of binding of* [^{3}H]-(±)-*ethyl ketazocine* K_I (nM)	[^{3}H]-*dihydro-morphine** K_I (nM)	*Discrimination ratio* K_I for κ / K_I for μ
Mr 2266	1.06 ± 0.09 (4)	1.37 ± 0.36 (4)	0.8
Naloxone	10.8 ± 1.8 (4)	2.65 ± 0.73 (5)	4.1
Naltrexone	6.9 ± 1.0 (4)	1.08 ± 0.17 (3)	6.4

The values are the means ± s.e.mean; the number of observations is given in parentheses. The Hill coefficient for naloxone or naltrexone did not differ significantly from 1 but was 1.21 ($P < 0.05$) for Mr 2266. *From Gillan *et al.* (1980).

dihydromorphine (K_I = 21 and 11 nM). They were considerably less potent at inhibiting the binding of [^{3}H]-ethylketazocine; the K_I values were 857 nM for Leu-enkephalin and 508 nM for Met-enkephalin. Since the Hill coefficients were as low as 0.50 and 0.56, it was possible to separate the biphasic curves into their two components. The K_I values for Leu-enkephalin were 57 and 14800 nM and those for Met-enkephalin 25 and 3500 nM. When the K_I values (57 and 25 nM) for the more readily inhibited portion of [^{3}H]-ethylketazocine binding were compared to the K_I values (21 and 11 nM) of [^{3}H]-dihydromorphine binding, the inhibition constants were sufficiently similar to indicate that they are mainly due to displacement of binding from the μ-site rather than from the δ-site.

A somewhat different pattern was found for the inhibition of [^{3}H]-ethylketazocine binding by porcine β-endorphin. Although the Hill coefficient was as low as 0.44, the inhibition curve was not clearly biphasic and therefore it was not possible to separate its two components. In view of the low slope the apparent K_I value (9.4 nM) was considerably underestimated.

Selective protection of the binding sites of [^{3}H]-(±)-ethylketazocine from the inactivating effect of phenoxybenzamine

Phenoxybenzamine had an inhibitory effect on the binding of [^{3}H]-(±)-ethylketazocine similar to that on the binding of [^{3}H]-dihydromorphine and [^{3}H]-D-Ala^2,D-Leu^5-enkephalin (Figure 4). The IC_{50} values for this effect were 0.66 ± 0.09 μM, 0.29 ± 0.01 μM and 0.50 ± 0.04 μM (n = 3) and inhibitions of 60–80% were obtained at 1.8, 0.7 and 1.5 μM, respectively; the latter concentrations were used in the protection experiments.

The potencies of (−)-ethylketazocine, dihydromorphine and D-Ala,D-Leu^5-enkephalin to protect the binding of [^{3}H]-(±)-ethylketazocine were tested in the same homogenate (Figure 5). The regression line for dihydromorphine, and particularly that for D-Ala^2,D-Leu^5-enkephalin, was shallower than that for (−)-ethylketazocine. About 30 times more dihydromorphine than (−)-ethylketazocine was required to protect 50% of the binding against the inactivating effect of phenoxybenzamine while concentrations of up to 4900 nM of D-Ala^2,D-Leu^5-enkephalin protected only 20–40% of the binding. In 2 experiments in which the (−)-isomer of tritiated ethylketazocine was used, the same results were obtained.

Since the experiments with competitive inhibition of binding showed cross-reactivity of ethylketazocine with the δ-sites and particularly the μ-sites, it was important to establish how well ethylketazocine could protect the binding of [^{3}H]-D-Ala^2,D-Leu^5-

Table 5 The inhibitory effects of endogenous opioid peptides on the binding of [^{3}H]-dihydromorphine (0.6 nM), [^{3}H]-D-Ala^2,D-Leu^5-enkephalin (1.2 nM) and [^{3}H]-(±)-ethylketazocine (1.3 nM) at 0°C

	[^{3}H]-dihydromorphine		[^{3}H]-D-Ala^2,D-Leu^5-enkephalin		[^{3}H]-ethylketazocine		Inhibition constants of the two components
Peptide	K_I (nM)	Hill coefficient	K_I (nM)	Hill coefficient	K_I (nM)	Hill coefficient	K_I (nM)
Leu-enkephalin	21.3 ± 3.5	0.93 ± 0.09	1.01 ± 0.17	0.94 ± 0.08	857 ± 270	0.50 ± 0.09	57 ± 13 14800 ± 1200
Met-enkephalin	10.8 ± 0.6	0.74 ± 0.03	0.72 ± 0.02	1.01 ± 0.03	508 ± 120	0.56 ± 0.06	25 ± 9.0 3500 ± 580
Porcine β-endorphin	2.10 ± 0.23	0.89 ± 0.05	1.09 ± 0.06	1.26 ± 0.06	9.4 ± 1.8	0.44 ± 0.03	-

The values are the means ± s.e.mean of three observations. Since the Hill coefficient was less than 0.6, the K_I values for the two components of the Hill plot were calculated separately; the Hill coefficients for the separated components varied between 0.8 and 1.1. The readily displaced portion was about 50% of the total. The discrimination ratios, K_I for κ/K_I for δ, were 14,650 for Leu-enkephalin and 4,860 for Met-enkephalin. The ratios, K_I for κ/K_I for μ, were 700 for Leu-enkephalin and 325 for Met-enkephalin. Inhibition of the binding at the δ- and κ-sites by β-endorphin was determined simultaneously in the same homogenate (0°C, 150 min).

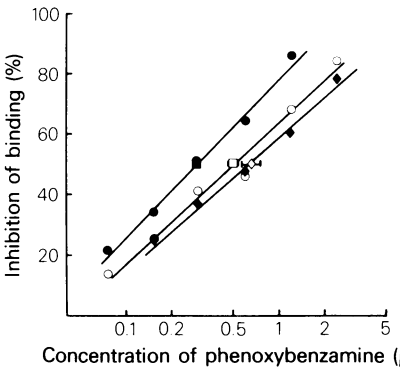

Figure 4 Inhibition by phenoxybenzamine of the binding of (♦) [^{3}H]-(±)-ethylketazocine (0.53 nM); (●) [^{3}H]-dihydromorphine (0.52 nM) and (○) [^{3}H]-D-Ala^2,D-Leu^5-enkephalin (1.0 nM) in homogenates of guinea-pig brain. The regressions of log of concentration on percentage inhibition were calculated from three experiments, each point representing two or three observations. The mean concentrations of phenoxybenzamine required to inhibit 50% of the binding are indicated by (◇), (■) and (□), the horizontal bars indicating s.e.mean.

enkephalin and [^{3}H]-dihydromorphine. In agreement with the findings on cross-reactivity it was found that ethylketazocine was equipotent with dihydromorphine in protecting the binding of [^{3}H]-dihydromorphine, and only a little less potent than D-Ala^2,D-Leu^5-enkephalin when the binding of [^{3}H]-D-Ala^2,D-Leu^5-enkephalin was examined (Figure 6).

Discussion

The evidence presented in the experimental section indicates that, in addition to the μ- and δ-subtypes of the opiate receptor, a third subtype is present in guinea-pig brain. Ethylketazocine, which was used in pharmacological investigations to characterize the then newly postulated κ-receptor (Martin *et al.*, 1976; Gilbert & Martin, 1976), showed saturable stereospecific binding in the brain homogenates, consisting of a high-affinity phase with a capacity of about 7 pmol/g wet tissue and a low-affinity phase with similar capacity.

Since ethylketazocine and other similar compounds cross-react to a large extent with the μ-binding site and to a smaller extent with the δ-binding site (Table 2), the main evidence for a separate third receptor rests on two observations. Firstly, there is the almost complete inability of a highly selective μ-ligand, D-Ala^2,MePhe^4,Gly-ol^5-enkephalin, or of a similarly selective δ-ligand, D-Ala^2,D-Leu^5-enkephalin, to displace tritiated ethylketazocine from the postulated κ-binding site. Secondly, in experiments in which the binding of [^{3}H]-ethylketazocine is protected against the alkylating action of phenoxybenzamine by adding varying concentrations of unlabelled ligands, dihydromorphine has less than 3% of the potency of ethylketazocine; D-Ala^2,D-Leu^5-enkephalin is even less active. Moreover, the cross-reactivity of ethylketazocine with the μ- and δ-binding sites can be demonstrated by experiments in which ethylketazocine readily protects the binding of [^{3}H]-dihydromorphine and [^{3}H]-

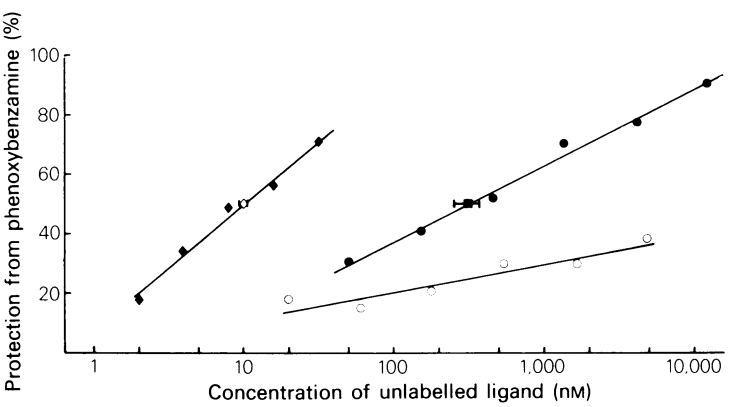

Figure 5 Protection of the binding in homogenates of guinea-pig brain of [^{3}H]-(±)-ethylketazocine (0.6 nM) from the inhibitory effect of phenoxybenzamine (1.8 μM). Each point is the mean of two or three observations. The regressions of percentage protection on log concentration of unlabelled ligand were calculated from three experiments. (♦) (−)-Ethylketazocine; (●) dihydromorphine; (○) D-Ala^2,D-Leu^5-enkephalin. The mean concentrations of the unlabelled ligands required to protect 50% of the binding are indicated by (◇) and (■), the horizontal bars indicating s.e.mean. Hill coefficients were 0.78 ± 0.03 and 0.52 ± 0.06 for the protection by (−)-ethylketazocine and dihydromorphine, respectively. The Hill coefficient for the regression line for D-Ala^2,D-Leu^5-enkephalin was 0.24. The inhibition of binding by phenoxybenzamine was 68.0 ± 5.4%.

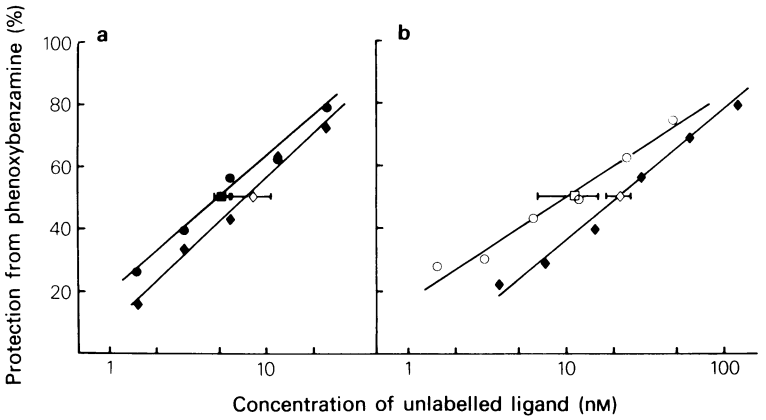

Figure 6 Protection of the binding in homogenates of guinea-pig brain of [^{3}H]-dihydromorphine (0.6 nM) (a) and [^{3}H]-D-Ala^2,D-Leu^5-enkephalin (1.1 nM) (b) from the inhibitory effects of phenoxybenzamine (0.7 μM and 1.5 μM, respectively). Each point is the mean of two or three observations. The regression of percentage protection on log concentration of unlabelled ligand was calculated from three experiments. (◆) (−)-Ethylketazocine; (●) dihydromorphine; (○) D-Ala^2,D-Leu^5-enkephalin. The mean concentrations of the unlabelled ligands required to protect 50% of the binding are indicated by (◇), (■) and (□), the horizontal bars indicating s.e.mean. Hill coefficients were 0.85 ± 0.13 for protection by dihydromorphine, 0.70 ± 0.03 for that by D-Ala^2,D-Leu^5-enkephalin, and 1.00 ± 0.21 and 0.93 ± 0.15 for protection by (−)-ethylketazocine of the binding of [^{3}H]-dihydromorphine and [^{3}H]-D-Ala^2,D-Leu^5-enkephalin, respectively. The inhibition of binding by phenoxybenzamine was 62.9 ± 4.2% for [^{3}H]-dihydromorphine and 74.8 ± 1.4% for [^{3}H]-D-Ala^2,D-Leu^5-enkephalin.

D-Ala^2,D-Leu^5-enkephalin from inactivation by phenoxybenzamine.

The affinity of the μ-agonists to the κ-binding site varies, D-Ala^2,MePhe^4,Gly-ol^5-enkephalin having negligible activity and morphine having an inhibition constant of about 70 nM. Etorphine, which is equally potent at μ- and δ-binding sites, has a high activity also at κ-binding sites.

The relationship between the three types of binding sites and the three types of ligands are shown in Table 6. The κ-binding site responds only to κ-ligands, of which ethylketazocine is a representative, the δ-binding site to δ-ligands and to ethylketazocine, and the μ-binding site to μ-ligands, to ethylketazocine and, to a smaller extent, to D-Ala^2,D-Leu^5-enkephalin. The question remains whether, as far as the structure of the ligands is concerned, the μ-binding site is less demanding than the κ-binding site and also the δ-binding site; an alternative is the possibility that the κ-ligands available at present are not sufficiently selective to prevent cross-reactivity with the μ-binding site. Furthermore, an event that is associated with binding could be of an agonist or antagonist nature. These considerations may be of importance in resolving the dilemma that most κ-agonists do not substitute for morphine in morphine-dependent monkeys (Villarreal & Seevers, 1972; Swain & Seevers, 1974; 1976) and yet interact with μ-binding sites, at least in the guinea-pig brain.

As far as the antagonists are concerned, the pattern of inhibition of the binding of [^{3}H]-dihydromorphine and [^{3}H]-ethylketazocine is similar to that found in pharmacological assays on isolated tissues (Hutchinson *et al.*, 1975). On the one hand, naloxone and

Table 6 Relative inhibitory potencies of prototype ligands at μ-, δ- and κ-binding sites

Prototype unlabelled ligands	*Inhibition of binding of tritiated ligands* Relative potencies at μ	δ	κ
μ: D-Ala^2,MePhe^4,Gly-ol^5-enkephalin	1	<0.01	<0.01
δ: D-Ala^2,D-Leu^5-enkephalin	0.1	1	<0.01
κ: (−)-Ethylketazocine	0.8	0.2	1

The potency of each unlabelled ligand in inhibiting binding at its own site is taken to be 1. The following tritiated ligands were used: for the μ-site, dihydromorphine, for the δ-site, D-Ala^2,D-Leu^5-enkephalin, for the κ-site, (±)-ethylketazocine.

Table 7 The number of binding sites for prototype ligands in guinea-pig brain at 25°C

Ligand	*Number of binding sites* (pmol/g wet tissue)
D-Ala^2,MePhe^4,Gly-ol^5-enkephalin	3.0*
D-Ala^2,D-Leu^5-enkephalin	6.1**
(−)-Ethylketazocine	6.6*** 14.0
Etorphine	14.6**

*Kosterlitz & Paterson (1981); **Gillan *et al.* (1980); ***From Table 1: high affinity and total number of binding sites.

naltrexone have a higher affinity for the μ- than for the κ-binding site and, on the other, antagonize μ-agonists more readily than κ-agonists. The benzomorphan antagonist, Mr 2266, behaves differently in that it is equipotent at the two binding sites and antagonizes the effects of μ- and κ-agonists equally well. None of the compounds known so far are specific μ-, δ- or κ-antagonists; in addition, they are less active at δ- than at μ-binding sites (Lord *et al.*, 1976; 1977).

As far as the endogenous opioid peptides are concerned, the potencies of Met-enkephalin, and particularly of Leu-enkephalin, in displacing [^{3}H]-ethylketazocine from the κ-binding site were negligible, having less than 0.3% of the activity at the μ-binding site and less than 0.05% of that at the δ-binding site. Thus, the pentapeptides can be excluded as endogenous ligands interacting at the κ-site. At the κ-binding site the profile of β-endorphin was different from that of the two enkephalins. Firstly, the K_I value for β-endorphin against [^{3}H]-ethylketazocine binding was about ten times larger than that against [^{3}H]-dihydromorphine or [^{3}H]-D-Ala^2,D-Leu^5-enkephalin binding. Secondly, the inhibition curve for β-endorphin against [^{3}H]-ethylketazocine was not biphasic although the Hill coefficient was as low (0.44) as that for Met-enkephalin and Leu-enkephalin. Therefore, it may be concluded that these factors favour the view that the affinity of β-endorphin for the κ-binding site is low.

It seems likely that there may be species differences in the distribution and density of κ-binding sites. In brains of the rat and guinea-pig, all authors have demonstrated that the putative unlabelled κ-ligands, ethylketazocine, bremazocine, Mr 2034 and Mr 2266, have high affinities for the μ- and κ-binding sites and somewhat lower affinities for δ-binding sites (Hiller & Simon, 1979; 1980; Chang, Hazum & Cuatrecasas, 1980; Harris & Sethy, 1980; Römer *et al.*, 1980; Snyder & Goodman, 1980; Woods, Charleson, Lane & Hudgin, personal communication). In contrast, the observations obtained in rat brain for the inhibition of [^{3}H]-ethylketazocine binding by unlabelled μ- and δ-ligands were inconsistent. Thus, Harris & Sethy (1980) showed that unlabelled morphine, naloxone and naltrexone inhibited [^{3}H]-ethylketazocine, binding more readily than did unlabelled ethylketazocine, while Snyder & Goodman (1980) found no difference in the inhibitory effects of morphine, naloxone and ethylketazocine against the binding of [^{3}H]-dihydromorphine and [^{3}H]-ethylketazocine. On the other hand, Hiller & Simon (1979) showed that the inhibitory effects of unlabelled morphine and Leu-enkephalin against [^{3}H]-ethylketazocine were only 1.6 and 6.6% of that of unlabelled (±)-ethylketazocine; qualitatively similar results were obtained by Woods *et al.*, (personal communication). Further analysis of the data on the rat brain is therefore required.

It has now become possible to give a first approximation of the proportion of the three subtypes of opiate receptors present in the guinea-pig brain (Table 7). The μ-agonist D-Ala^2,MePhe^4,Gly-ol^5-enkephalin is sufficiently selective to assume that the capacity of the μ-site is about 3 pmol/g wet brain tissue. Since the δ-agonist, D-Ala^2,D-Leu^5-enkephalin, cross-reacts with the μ-binding site somewhat more than 10%, the capacity of the δ-binding site may be assumed to be only 5.5 pmol/g wet brain tissue. The apparent capacity of the high-affinity κ-binding site is about 6.1 pmol/g wet brain tissue; in view of the cross-reactivity, particularly with the μ-binding site, not more than 4 pmol/g wet brain tissue may be allocated to the κ-binding site itself. The sum of the sites would be 12.5 pmol/g wet tissue, corresponding fairly well with the value obtained for the universal ligand, etorphine, of 14.6 pmol/g wet tissue. Thus, as a first approximation the proportions in guinea-pig brain would be 25% μ-binding sites, 45% δ-binding sites, and 30% κ-binding sites.

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