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SPECTRUM OF THE μ-, δ- AND κ-BINDING SITES IN HOMOGENATES OF RAT BRAIN

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1 In homogenates of rat brain, the binding characteristics of tritiated opiates and opioid peptides were examined and the relative capacities of μ-, δ- and κ-binding sites of the opiate receptor determined by saturation analysis.

2 In competition experiments, binding of the selective μ-ligand [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin at the μ-site was displaced by [D-Ala^2,D-Leu^5]enkephalin with rather low affinity (K_I = 12.6 nM) and more readily by the ketazocine-like compounds (−)-ethylketazocine (K_I = 3.1 nM) and (−)-bremazocine (K_I = 0.32 nM), which also displaced the binding of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin from the δ-site. In contrast, the binding to the κ-site was easily displaced by ethylketazocine (1.0 nM) and bremazocine (0.37 nM) but not by the μ-ligand [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (K_I = 2000–3000 nM) or the δ-ligand [D-Ala^2,D-Leu^5]enkephalin (K_I > 20,000 nM).

3 The dissociation equilibrium constant (K_D) and the binding capacity (pmol/g) of the μ-binding site were determined with the selective μ-ligand [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin. For the δ-site, [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin was used in the presence of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin in order to suppress cross-reactivity to the μ-binding site. For the estimation of κ-binding, [^{3}H]-(±)-ethylketazocine or [^{3}H]-(−)-bremazocine were used in the presence of unlabelled μ- and δ-ligands for the suppression of cross-reactivities to the μ- and δ-binding sites.

4 In rat brain the capacity of the μ-binding site was 7.3 pmol/g brain, that of the δ-binding site 6.7 pmol/g brain and that of the κ-binding site 2.0 pmol/g brain. Thus, the κ-binding site had the lowest value whereas in the guinea-pig brain the capacity of the μ-binding site was lower than that of the δ- or κ-binding site.

Introduction

In recent papers from this laboratory it was shown that in homogenates of guinea-pig brain 30 to 40% of the total number of binding sites belong to the κ-subtype of the opiate receptor (Kosterlitz & Paterson, 1980; Kosterlitz, Paterson & Robson, 1981). For rat brain the reports have been inconsistent. In some laboratories, κ-binding sites could not be identified with certainty (Hiller & Simon, 1979; 1980; Snyder & Goodman, 1980; Harris & Sethy, 1980); in others, evidence for their presence was obtained (Wood, Charleson, Lane & Hudgin, 1981), the amounts being lower than in guinea-pig (Chang, Hazum & Cuatrecasas, 1981).

There are two possibilities which may explain the difficulties experienced in experiments on rat brain. The first is a high ratio of μ-binding sites to κ-binding sites and the second is the insufficient selectivity of the tritiated ligands used for estimation of the binding sites. To overcome these problems, we used [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin as a selective μ-ligand, [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin in the presence of an excess of unlabelled μ-ligand as a selective δ-ligand, and [^{3}H]-ethylketazocine or [^{3}H]-bremazocine in the presence of an excess of unlabelled μ- and δ-ligands as a selective κ-ligand.

Methods

Binding assays

Brain homogenates from hooded rats of the Aberdeen colony (250–375 g) were prepared as previously described (Gillan, Kosterlitz & Paterson, 1980). Specific binding was calculated from the difference of the counts in the absence and presence of Mr 2266; when [^{3}H]-(−)-bremazocine was the ligand, diprenorphine was used to suppress specific binding because it does not have a benzomorphan-like structure. The concentrations of the inhibitors were dependent on the tritiated ligands; they were 200 to 500 times higher than the K_I values of the inhibitors (0.25–1.5 μM). For the calculation of the kinetic parameters of binding of labelled and unlabelled

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ligands, the equilibrium dissociation constant (K_D), inhibition constant (K_I) and maximal number of binding sites were determined from multiphasic saturation curves and competition experiments (Kosterlitz *et al.*, 1981). In the estimation of binding constants, the concentration of unbound tritiated ligand was determined by subtracting the concentration of bound ligand from the concentration of total ligand.

The binding capacity and the equilibrium dissociation constant were determined with the following tritiated ligands. For the μ-binding site, [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin was sufficiently selective to prevent significant cross-reactivity to the δ- and κ-binding sites at concentrations giving saturation at the μ-binding site (Handa, Lane, Lord, Morgan, Rance & Smith, 1981; Kosterlitz & Paterson, 1981). For the δ-binding site, it was necessary to reduce the cross-reactivity of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin to the μ-binding site (Kosterlitz *et al.*, 1981) by the addition of the unlabelled μ-ligand [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin in concentrations which will occupy 90% of the μ-binding sites (10 nM for each 1.3 nM of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin); because of the high selectivity of the μ-ligand, there will be only a negligible reduction in the binding of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin to the δ-binding site. For the κ-binding site, the cross-reactivity of [^{3}H]-(±)-ethylketazocine, of [^{3}H]-(−)-bremazocine (Römer, Büscher, Hill, Maurer, Petcher, Welle, Bakel & Akkerman, 1980) or of [^{3}H]-etorphine to both the μ- and δ-binding sites was reduced by addition of unlabelled μ- and δ-ligands in concentrations which will occupy 99% of the μ- and δ-binding sites (100–200 nM for each 0.1 nM of free [^{3}H]-(−)-bremazocine or 0.2 nM free [^{3}H]-etorphine). Since the K_I values of the inhibitory effects of [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin and of [D-Ala^2,D-Leu^5]enkephalin against the binding to the κ-site of [^{3}H]-ethylketazocine, [^{3}H]-bremazocine or [^{3}H]-etorphine are at least 1000 greater than against the binding at the μ- and δ-sites, the concentrations used will not lead to a significant decrease in the binding of the three tritiated ligands at the κ-site.

Labelled primary ligands

The following primary ligands were used: [^{3}H]-(−)-bremazocine (24 Ci/mmol; Sandoz), [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin (26-47 Ci/mmol), [^{3}H]-D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (55 Ci/mmol), [^{3}H]-etorphine (36-51 Ci/mmol; Amersham International) and [^{3}H]-(±)-ethylketazocine (15 Ci/mmol; New England Nuclear). The radiochemical purity of all ligands was measured on receipt. If it was less than 95%, high pressure liquid chromatography (h.p.l.c.) on a μBondapak C_{18} column was used for re-purification. This was a precautionary measure which was repeated at intervals.

Drugs and peptides

The following drugs were used: (−)-bremazocine hydrochloride ((−)-5-ethyl-9,9-dimethyl-2-(1-hydroxycyclopropylmethyl)-2'-hydroxy-6,7-benzomorphan; Dr D. Römer, Sandoz); diprenorphine as free base (Reckitt & Colman); (±)-ethylketazocine methanesulphonate ((±)-α-5-ethyl-9-methyl-8-oxo-2-cyclopropylmethyl-2'-hydroxy-6,7-benzomorphan), (−)-ethylketazocine base (Dr W.F. Michne, Sterling Winthrop); Mr 2266 as free base ((−)-α-5,9-diethyl-2-(3-furylmethyl)-2'-hydroxy-6,7-benzomorphan; Dr H. Merz, C.H. Boehringer Sohn).

All peptides were synthetic: Tyr-D-Ala-Gly-Phe-D-Leu (Dr S. Wilkinson, Wellcome Laboratories), Tyr-D-Ala-Gly-MePhe-Gly-ol (Dr D. Römer, Sandoz).

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.

Results

Competitive inhibition of tritiated ligands in homogenates of rat brain

The K_I value of the inhibitory effect of the selective μ-ligand [D-Ala^2,MePhe^4, Gly-ol^5]enkephalin on the binding was low against [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (1.8 nM), with a Hill coefficient of close to 1, but it was high against [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (133 nM), [^{3}H]-ethylketazocine (66 nM) and [^{3}H]-bremazocine (64 nM), with Hill coefficients of as low as 0.4 (Table 1). These results indicate that [D-Ala^2,MePhe^4, Gly-ol^5]enkephalin is a selective ligand for the binding of the μ-site and agree with earlier findings on the guinea-pig brain (Kosterlitz & Paterson, 1981; Kosterlitz *et al.*, 1981).

In contrast, the K_I value of the putative δ-ligand, [D-Ala^2,D-leu^5]enkephalin (13 nM) against the μ-ligand, [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin was only 7 times larger than the K_I value of the unlabelled μ-ligand (1.8 nM). The K_I values of the inhibitory effect of [D-Ala^2,D-Leu^5]enkephalin were high against the binding of [^{3}H]-ethylketazocine (156 nM) and [^{3}H]-bremazocine (88 nM), and the Hill coefficients were correspondingly low (0.5). Thus, as has been found in the guinea-pig brain, unlabelled [D-Ala^2,D-Leu^5]enkephalin cross-reacts readily with the μ-binding site whereas the cross-reactivity of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin to the δ-binding site is low.

This interpretation was confirmed by the analysis

Table 1 Comparison of the inhibitory effects of opiates and opioid peptides on the binding of [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (0.8–1.8 nM), [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (1.0–1.6 nM), [^{3}H]-(±)-ethylketazocine (0.6–1.9 nM) and [^{3}H]-(−)-bremazocine (0.1–0.4 nM) in homogenates of rat brain

	[^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5] enkephalin		*[^{3}H]-[D-Ala^2,D-Leu^5] enkephalin*		*[^{3}H]-Ethylketazocine*		*[^{3}H]-Bremazocine*	
Unlabelled compound	K_I (nM)	Hill coefficient	K_I (nM)	Hill coefficient	K_I (nM)	Hill coefficient	K_I (nM)	Hill coefficient
[D-Ala^2,MePhe^4,Gly-ol^5] enkephalin	1.83 ± 0.09	0.99 ± 0.06	133 ± 14	0.38 ± 0.01	66 ± 10	0.44 ± 0.03	64 ± 7.9	0.44 ± 0.03
[D-Ala^2,D-Leu^5] enkephalin	12.6 ± 1.3	1.04 ± 0.07	6.4 ± 0.59	1.10 ± 0.03	156 ± 43	0.57 ± 0.02	88 ± 7.9	0.46 ± 0.07
(−)-Ethylketazocine	3.10 ± 0.35	1.01 ± 0.02	6.6 ± 1.15	0.99 ± 0.05	3.36 ± 0.39	1.07 ± 0.01	1.04 ± 0.06	0.98 ± 0.03
(−)-Bremazocine	0.32 ± 0.02	0.98 ± 0.03	0.74 ± 0.13	1.09 ± 0.07	1.68 ± 0.21	0.99 ± 0.05	0.37 ± 0.02	1.02 ± 0.09

The values are the means ± s.e.mean of 3–4 observations. The K_I values were calculated from the apparent K_D values obtained for the multiple binding sites (Table 2). The concentrations of the tritiated ligands were uncorrected for losses to the binding sites.

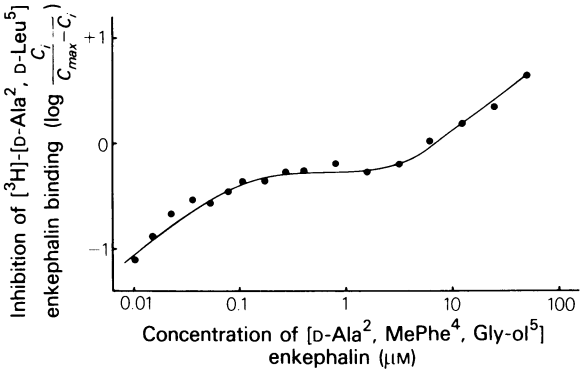

Figure 1 Homogenate of rat brain: the inhibitory effect of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (selective μ-ligand) on the binding of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (1.7 nM; δ-ligand with significant affinity to the μ-binding site). Typical result of 3 experiments. C_i, inhibited counts; C_{max}, maximal counts.

of the inhibitory dose-response curves. When the effects of increasing concentrations of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin were plotted against the binding of 1.7 nM of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (K_D at δ-site = 1.8 nM) a biphasic curve was obtained (Figure 1). The calculation of the K_I value for the first phase of the curve gave 2.01 ± 0.16 nM and that for the second phase 960 ± 215 nM ($n = 3$). Similarly, the curve showing the inhibition of 0.18 nM of [^{3}H]-(−)-bremazocine (K_D at κ-site = 0.07 nM) by [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin was also biphasic (Figure 2). The K_I value for the first phase was 7.8 ± 2.4 nM and for the second phase 2026 ± 492 nM ($n = 3$); corresponding values for the inhibition of [^{3}H]-(±)-ethylketazocine were 9.3 ± 0.57 nM and 3307 ± 498 nM. The Hill coefficients for the first and second phases varied

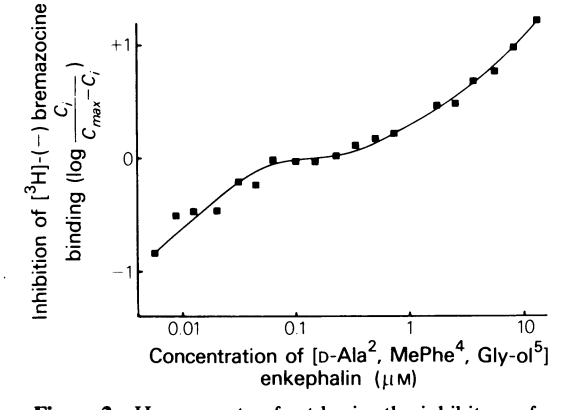

Figure 2 Homogenate of rat brain: the inhibitory effect of [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (selective μ-ligand) on the binding of [^{3}H]-(−)-bremazocine (0.18 nM; ligand with high affinities to the μ-, δ- and κ-binding sites). Typical result of 3 experiments. C_i, inhibited counts; C_{max}, maximal counts.

Table 2 Binding characteristics of tritiated opiates and opioid peptides in homogenates of rat brain

	Multiple binding sites			*Single binding site*	
Tritiated ligand	*Binding capacity* (pmol/g wet brain)	*Binding sites*	*dissociation constant* K_D (nM)	*Binding capacity* (pmol/g wet brain)	*Binding site*
[D-Ala^2,MePhe^4,Gly-ol^5] enkephalin			1.09 ±0.12	7.3 ±0.6	μ
[D-Ala^2,D-Leu^5] enkephalin	16.2 ± 1.3	$\mu + \delta$	1.78 ±0.18	6.7 ±0.9	δ*
(±)-Ethylketazocine	17.0 ± 0.2	$\mu + \delta + \kappa$	1.87 ±0.26	2.10 ± 0.15	κ**
(−)-Bremazocine	16.4 ± 1.3	$\mu + \delta + \kappa$	0.067 ± 0.006	1.84 ± 0.08	κ***
Etorphine	18.2 ± 0.5	$\mu + \delta + \kappa$	0.050 ± 0.104	2.13 ± 1.88	κ***

The values are the means ± s.e.mean of three observations. *Suppression of μ-binding was obtained by a constant ratio of 10 nM of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin to 1 K_D (1.3 nM)of free [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin. **Suppression of μ- and δ-binding was obtained by a fixed concentration of 200 nM of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin and by 200 nM unlabelled [D-Ala^2,D-Leu^5]enkephalin. ***Suppression of μ- and δ-binding was obtained by a constant ratio of 200 nM unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin and of 200 nM unlabelled [D-Ala^2,D-Leu^5]enkephalin to 0.1 nM of free [^{3}H]-(−)-bremazocine or to 0.2 nM of free [^{3}H]-etorphine. The correlation coefficients varied between 0.92 and 0.99.

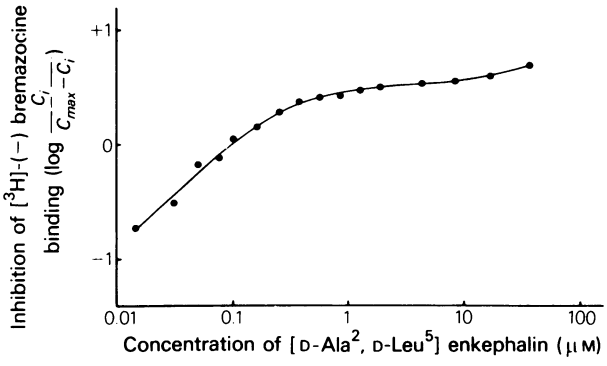

Figure 3 Homogenate of rat brain: the inhibitory effect of [D-Ala^2, D-Leu^5]enkephalin (δ-ligand with significant affinity to the μ-binding site) on the binding of [^{3}H]-(−)-bremazocine (0.12 nM; ligand with high affinities for the μ-, δ- and κ-binding sites). The curve is almost linear to about 70% inhibition at 500 nM [D-Ala^2,D-Leu^5]enkephalin (Hill coefficient of 1.1) and then is very shallow up to 50,000 nM. Typical result of 3 experiments. C_i, inhibited counts; C_{max}, maximal counts.

between 0.9 and 1.3. The inhibitory effect of [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin at the first phase is assumed to be mediated at the μ-binding site and that at the second phase at the κ-binding site, as will be discussed later.

The K_I values for the inhibition of unlabelled ethylketazocine and bremazocine against [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin were low, a finding that indicates that these compounds cross-react very readily with the μ-binding site. A similar pattern is obtained for the inhibition of the δ-ligand, [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (Table 1).

The inhibition by unlabelled [D-Ala^2,D-Leu^5]enkephalin of [^{3}H]-(−)-bremazocine differed from that caused by unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin in that the inhibition during the first phase reached 70% instead of about 50% and in the second phase was not completed by a concentration of as high as 50,000 nM (Figure 3). When [^{3}H]-(±)-ethylketazocine was used as ligand, similar results were obtained.

Binding characteristics of tritiated opiates and opiate peptides in rat brain

In Table 2, the values of the binding affinities of the opiates and opioid peptides are given as the dissociation equilibrium constants, K_D (nM), and those of the binding capacities as the maximum numbers of binding sites (pmol/g brain). When the selective ligand [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin was used, the Scatchard plot did not deviate from linearity and no unlabelled ligands were added. With the other, non-selective, ligands, the μ-binding site was blocked with unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin and the δ-binding site with unlabelled [D-Ala^2,D-Leu^5]enkephalin (Table 2).

With [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin, the K_D value was 1.1 nM and the capacity was 7.3 pmol/g. The other tritiated ligands, [D-Ala^2,D-Leu^5]enkephalin, ethylketazocine, bremazocine and etorphine, had much larger binding capacities, varying between 16 and 18 pmol/g. Since it was shown that [D-Ala^2,D-Leu^5]enkephalin cross-reacts to the μ-binding site (Table 1), it is likely that its high binding capacity is due to combined binding at the μ- and δ-sites. This view was confirmed by the fact that the presence of the unlabelled μ-ligand [D-

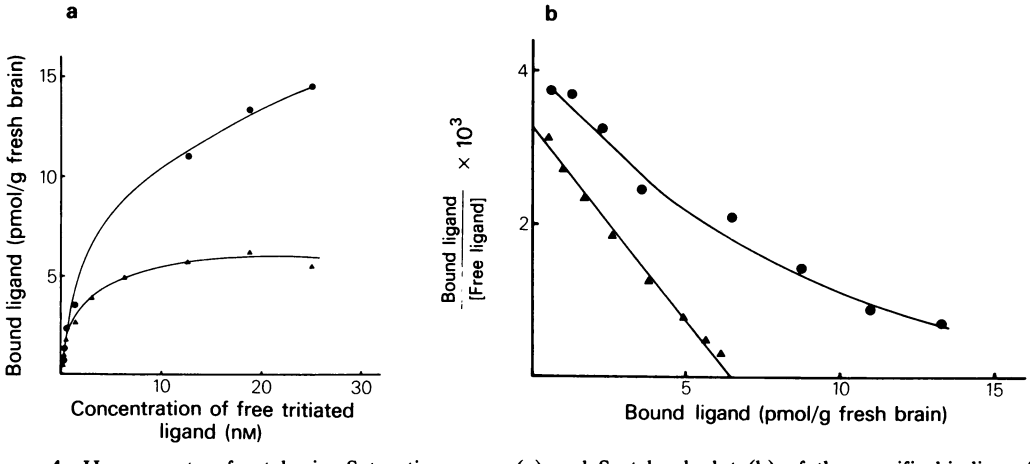

Figure 4 Homogenate of rat brain. Saturation curve (a) and Scatchard plot (b) of the specific binding of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin in the absence (●) and presence (▲) of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (μ-suppression at a ratio of 10 nM of the unlabelled to 1.5 nM of the labelled ligand, approx. 1 K_D).

Ala^2,MePhe^4,Gly-ol^5]enkephalin lowered the mean binding capacity by about 60% to 6.7 pmol/g brain and converted the curvilinear Scatchard plot to an almost straight line, giving a mean K_D value of 1.78 nM at the δ-binding site (Figure 4 and Table 2).

In homogenates of guinea-pig brain, [^{3}H]-ethylketazocine and [^{3}H]-bremazocine have been shown to bind to μ-, δ- and κ-sites (Kosterlitz *et al.*, 1981). In brain homogenates of the rat, the binding capacities of about 17 pmol/g brain were reduced by 88% to 2 pmol/g when unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin and [D-Ala^2,D-Leu^5]enkephalin were both present in ratios of 200 nM to 1 K_D of [^{3}H]-bremazocine, or at a constant concentration of 200 nM when [^{3}H]-ethylketazocine was used (Table 2). These effects of the unlabelled ligands on the saturation binding of [^{3}H]-bremazocine are shown in Figure 5a. Whereas in the absence of the unlabelled ligands the Scatchard plot was curvilinear, it became near-linear after the addition of the unlabelled μ- and δ-ligands (Figure 5b). It was found that the mean K_D value at the κ-site was 1.87 nM for [^{3}H]-(±)-ethylketazocine and 0.067 nM for [^{3}H]-(−)-bremazocine.

Etorphine, although it is not a benzomorphan, also binds at the μ-, δ- and κ-binding sites (Magnan, Paterson, Tavani & Kosterlitz, 1982). In the absence of suppressant unlabelled ligands, its binding capacity was slightly higher than that of the other three non-selective tritiated ligands but, in the presence of the unlabelled μ- and δ-ligands, the capacity of the binding at the κ-site was 2.1 pmol/g brain and the K_D 0.08 nM, values not different from those obtained for bremazocine (Table 2).

Table 3 Binding capacities of μ-, δ- and κ-binding sites in brain homogenates of rat and guinea-pig

Species	*Binding capacity* (pmol/g brain) μ-site	δ-site	κ-site
Rat	7.3	6.7	2.0
Guinea-pig	3.0	6.1	5.8

The values of rat brain are from Table 2 and the values of guinea-pig brain from Kosterlitz *et al.* (1981) and Magnan *et al.* (1982). Since the cross-reactivity of the δ-ligand to the μ-site is only 10% in guinea-pig brain, no allowance has been made for μ-binding. The ligand for the μ-site was [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin, for the δ-site [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin and for the κ-site [^{3}H]-(+)-ethylketazocine, [^{3}H]-(−)-bremazocine or [^{3}H]-etorphine after appropriate suppression by unlabelled ligands.

Discussion

The results presented in this paper confirm that homogenates of rat brain as well as of guinea-pig brain contain μ-, δ- and κ-binding sites although the relative proportions are different. As has been discussed in the Introduction, there have been reports suggesting that κ-binding sites cannot be identified in rat brain; however, the experiments described in the present paper indicate that this difficulty is due to the fact that, in this species, the κ-binding sites amount to only 12% of the sum of the μ-, δ- and κ-binding sites.

At present, the main problem encountered in the determination of the different binding sites, is the

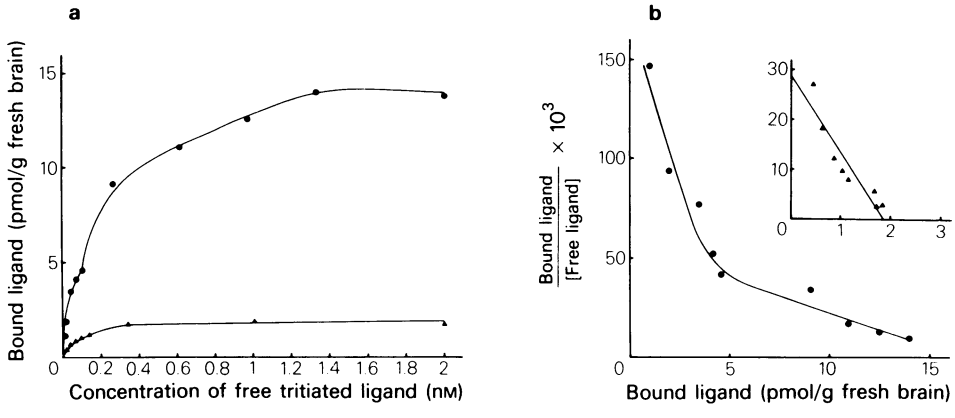

Figure 5 Homogenate of rat brain. Saturation curve (a) and Scatchard plot (b) of the specific binding of [^{3}H]-(−)-bremazocine in the absence (●) and presence (▲) of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (μ-suppression at a ratio of 200 nM to 0.1 nM [^{3}H]-bremazocine, approx. 1 K_D) and [D-Ala^2,D-Leu^5]enkephalin (δ-suppression at the same ratio as above). In the inset of (b), scale was increased 2.5 fold.

lack of selective ligands. Only the μ-ligand [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin has a degree of selectivity that is sufficient for reliable saturation binding. The putative δ-ligand [D-Ala^2,D-Leu^5]enkephalin binds not only to the δ-site but to a lesser degree also to the μ-binding site. When, as in guinea-pig brain, the number of μ-binding sites is low relative to that of the δ-binding sites, the error in assessing δ-binding sites is not large; however, when the number of μ-binding sites is high relative to the δ-sites as in rat brain, then assessment of the true capacity of binding to the δ-sites becomes difficult (Table 2 and Figure 4a, b). Since in rat brain the capacity of the μ-binding site is similar to that of the δ-binding site, cross-reactivity is relatively large. Therefore, in such circumstances the choice of the concentration of the unlabelled μ-ligand is critical. If it is too low there could be insufficient suppression of μ-binding; if, on the other hand, it is too high the binding at the δ-site may be depressed. In our experiments we used a constant ratio of unlabelled to labelled ligands, i.e. a concentration of 10 nM of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (10 K_D) when the concentration of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin was 1.7 nM (1 K_D). Under these conditions, the binding at saturation of the δ-binding site was about 40% of the capacity of the combined μ- and δ-binding sites (Figure 1).

The binding spectrum of [^{3}H]-ethylketazocine, [^{3}H]-bremazocine and [^{3}H]-etorphine showed affinity to all three of the μ-, δ- and κ-binding sites. However, since the K_I values for the inhibitory effects of the unlabelled μ-ligand, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin or the unlabelled δ-ligand, [D-Ala^2,D-Leu^5]enkephalin on the binding at the κ-binding site are about three orders of magnitude larger than the K_D values of the μ- or the δ-ligand, 100–200 nM of the unlabelled μ- and δ-ligands (100–200 K_D) can be used without causing interference with the binding at the κ-site, either at a constant concentration or a constant ratio of the concentrations of unlabelled to labelled ligands. Further evidence for the usefulness of this technique was obtained by the finding that the capacities at the κ-binding site varied only between 1.8 and 2.1 pmol/g brain whether the tritiated ligand was ethylketazocine, bremazocine or etorphine. The binding at the κ-site was about 12% of the combined binding capacity at the μ-, δ- and κ-sites.

There are problems which are encountered in estimations of the binding capacities of the different subtypes of the opiate receptor. One of them is the cross-reactivity discussed in the preceding paragraphs. Another dilemma is the difficulty of obtaining a standard of reference for the total number of binding sites since there may be variations in binding sites due to differences in the procedures used for the preparation of the homogenates. It is helpful to relate the number of subtypes of binding sites to the total number of the μ-, δ- and κ-binding sites. The usefulness of such a procedure is shown in Table 2, where the binding capacity of [D-Ala^2,D-Leu^5]enkephalin, ethylketazocine, bremazocine or etorphine varied only between 16 and 18 pmol/g rat brain, with an average of 17 pmol/g.

As far as the κ-binding sites in rat brain are concerned, Chang *et al.* (1981) have shown that, after suppression of μ-binding by morphiceptin and of δ-binding by [D-Ala^2,D-Leu^5]enkephalin, the residual binding by [^{3}H]-diprenorphine is smaller than the binding at the μ-sites or δ-sites, a finding which is in general agreement with results described in the present paper.

It is of interest to show that the relative binding capacities of the μ-, δ- and κ-binding sites are different in the brains of the guinea-pig and rat (Table 3). While the number of δ-binding sites is equal in the two species, the rat contains about 2.4 times more μ-sites than κ-sites and the guinea-pig 2.9 times more κ-sites than μ-sites. It would appear that, in the mouse, the number of κ-binding sites is as low as in the rat (Pasternak, 1980).

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