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Classification of multiple morphine and enkephalin binding sites in the central nervous system

(opiate receptor/naloxazone/μ receptor/δ receptor)

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ABSTRACT **Detailed competitive displacement curves of ^{3}H-labeled [D-Ala^2,Met^5]enkephalinamide, [D-Ala^2,D-Leu^5]enkephalin, and dihydromorphine by a series of opiates and enkephalins are biphasic, suggesting multiple sites. After treatment of tissue with naloxazone, the displacement of the three ^{3}H-labeled ligands by all opiates and enkephalins tested becomes monophasic, losing the high-affinity displacement seen with low concentrations of both opiates and enkephalins. Coupled with Scatchard analysis of saturation experiments, these findings suggest a common site that binds both opiates and enkephalins equally well and with highest affinity (K_d values, <1 nM). Termed the μ_1 site, it corresponds to the previously described high-affinity site and appears to be the site responsible for analgesia under normal circumstances. The low-affinity binding of [^{3}H]dihydromorphine (K_d, 3 nM) remaining after naloxazone treatment differs dramatically from low-affinity [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin binding (K_d, 5 nM). The μ_2 site, corresponding to the low-affinity [^{3}H]dihydromorphine receptor sites, binds morphine (K_i, 10 nM) and dihydromorphine (K_d, 3 nM) far better than [D-Ala^2,D-Leu^5]enkephalin (K_i, 50 nM). Low-affinity [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin receptor sites bind [D-Ala^2,D-Leu^5]enkephalin (K_i, 5–8 nM) more potently than morphine (K_i, 71 nM) and correspond to the previously established δ receptor.**

The binding of radiolabeled enkephalins directly to brain membranes was described (1) shortly after their discovery (2–4) and structural determination (5, 6). Although similar in many respects to opiate binding, significant differences do exist. Radiolabeled enkephalin binding is displaced more easily by enkephalins than by opiates and vice versa (7, 8). On the basis of these findings, it was proposed that enkephalins bind to an enkephalin-selective (δ) site in the central nervous system, whereas opiates such as morphine bind to a morphine-selective (μ) site. This proposal confirms the concept of discrete enkephalin and opiate mechanisms suggested by the marked differences in potency between opiates and enkephalins in the mouse vas deferens and the guinea pig ileum bioassays (1).

Multiple populations of opiate receptors also have been suggested on the basis of biochemical evidence (9, 10). By using differences in binding affinities, this method classified receptors as "high-" and "low-" affinity sites, the terms being in a relative sense because even the lower-affinity site binds opiates quite potently (K_d, <10 nM). Our understanding of these high- and low-affinity sites has been greatly expanded by the use of naloxazone (11–20), an irreversible ligand selective for the high-affinity site and active both *in vivo* and *in vitro*. In this study, we investigated the pharmacological properties of both enkephalins and a variety of opiates in an effort to correlate the high- and low-affinity sites with μ and δ receptors.

METHODS

[D-Ala^2,D-Leu^5]-[^{3}H]Enkephalin, [^{3}H]dihydromorphine, and [D-Ala^2,D-Met^2]-[^{3}H]enkephalinamide were obtained from New England Nuclear, as was Formula 963 scintillation fluor. Brain membranes were prepared from male Sprague–Dawley rats as described (21) and treated with naloxazone *in vitro* (16). In brief, naloxazone was dissolved in 1% glacial acetic acid (10 mg/ml), and 15 min later the solution was added to tissue to a final concentration of 2 μM. Tissue incubation was carried out at 25°C for 30 min, and followed by four washes. Each wash consisted of an incubation at 37°C for 10 min, followed by centrifugation and resuspension in buffer. This wash procedure effectively removes all reversible opiates at this concentration (13, 16). Control tissue in all naloxazone experiments went through all incubations and treatments without drug to permit an accurate comparison with naloxazone-treated tissue. Binding assays were performed as described (21), with 20 mg of tissue per ml.

RESULTS

Effects of Naloxazone on [D-Ala^2,Met^5]-[^{3}H]Enkephalinamide Binding. Naloxazone effectively and irreversibly blocked the high-affinity component of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide binding in tissue treated *in vitro* (Fig. 1). Analysis of the saturation data of control tissue by nonlinear, least-squares, weighted fit (unpublished data) showed two components. The high-affinity component (K_d, 0.2 nM) present in control tissue was eliminated in the tissue pretreated with naloxazone. By contrast, there was only a mild decrease (15%) in low-affinity binding between the control (K_d, 4.1 nM) and the naloxazone tissue (K_d, 6.9 nM), with no significant change in affinity.

Effects of Naloxazone on Displacement Studies by Opiates and Enkephalins. Displacement studies by a variety of drugs were then performed on [D-Ala^2,Met^5]-[^{3}H]enkephalinamide binding to compare their competitive interactions. Morphine inhibited radiolabeled enkephalin analogs in a biphasic manner (Fig. 2*A*), confirming previous reports (7, 8, 15). The initial displacement was quite sensitive to morphine, occurring at <1 nM morphine. The remaining binding, which comprises morphine's second displacement, required far larger doses. Treatment of this tissue with naloxazone eliminated the displacement of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide binding by low morphine concentrations. Thus, naloxazone selectively inhibited the high-affinity binding site for both [^{3}H]morphine (15) and [D-Ala^2,Met^5]-[^{3}H]enkephalinamide (Fig. 1) measured directly by Scatchard analysis in addition to blocking morphine's

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Abbreviation: IC_{50}, concentration causing 50% maximal inhibition.

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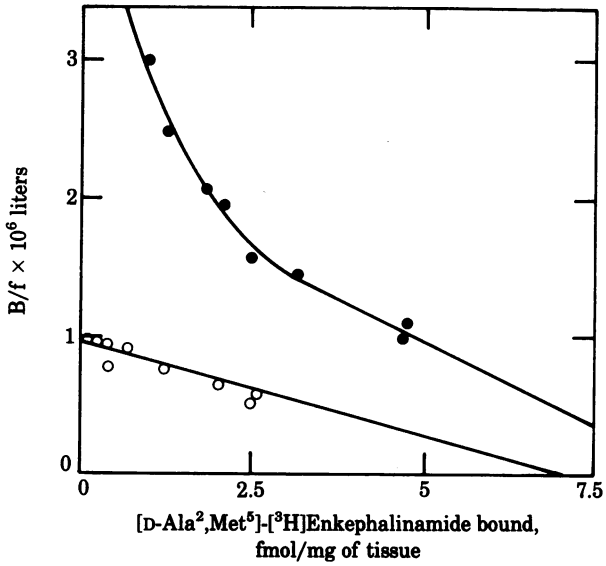

FIG. 1. Scatchard analysis of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide binding in naloxazone-treated and control tissue. Rat brain membranes were prepared and treated with no drug (●) or naloxazone (○) and assayed with [D-Ala^2,Met^5]-[^{3}H]enkephalinamide (0.1–4.7 nM). Saturation data were analyzed by a nonlinear, weighted, least squares fit (unpublished data). Control binding was broken into two components with different affinities (K_ds, 0.2 and 4.1 nM), whereas only a single component was demonstrated in naloxazone-treated tissue (K_d, 6.9 nM). The K_d value of the lower-affinity control tissue (4.1 nM), was not significantly different from that of naloxazone-treated tissue (6.9 nM). Naloxazone treatment eliminated the high-affinity site, causing only a small decrease (around 15%) in the lower-affinity site. The experiment has been replicated three times. B/f, bound/free.

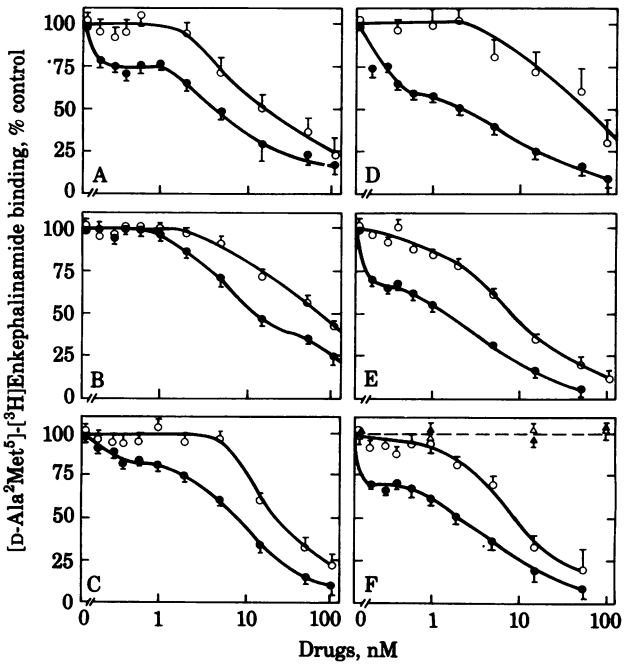

FIG. 2. Displacement of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide binding by opiates. Rat brain membranes were prepared and treated with no drug (●) or naloxazone (○). [D-Ala^2,Met^5]-[^{3}H]enkephalinamide (1 nM) binding was determined in the presence of the following unlabeled drugs: morphine (*A*), ketocyclazocine (*B*), SKF 10,047 (*C*), naloxone (*D*), naltrexone (*E*), and levallorphan (○, ●) and dextrallorphan (△, ▲) (*F*). Points are from one experiment and represent the mean ± SEM of triplicate determinations. Similar results have been replicated in three different experiments. The percentage of total binding that comprises the initial displacement can vary up to 10% from experiment to experiment. For this reason, a single preparation of brain membranes was divided into two fractions, treated with no drug or naloxazone, and assayed together with a single unlabeled ligand. Because the different unlabeled ligands were assayed in different experiments, variations in the initial displacement are probably not significant.

high-affinity competitive displacement of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide binding. Together, these results suggest that both drugs bind with highest affinity to the same site.

Scatchard analysis of saturation experiments shows that naloxazone also abolishes the high-affinity binding component for a number of other opiates, including the κ drug [^{3}H]ethylketocyclazocine (14), the σ drug [^{3}H]SKF 10,047 (17), and the antagonists [^{3}H]naloxone (12, 13) and [^{3}H]naltrexone (15). Therefore, we investigated naloxazone's actions on the displacement of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide binding by a variety of unlabeled ligands (Fig. 2*B–F*). [D-Ala^2,Met^5]-[^{3}H]Enkephalinamide binding was displaced in a biphasic manner by all of the unlabeled ligands tested, including ketocyclazocine, SKF 10,047, naloxone, naltrexone, and levallorphan. Dextrallorphan, the inactive stereoisomer of levallorphan, did not inhibit binding. As with morphine, naloxazone treatment eliminated the initial displacement by low concentrations of all the opiates, implying that μ, κ, σ opiates, antagonists, and enkephalin analogs all bind with highest affinity to a common receptor. Although both naloxone and naltrexone are considered pure antagonists and inhibited the naloxazone-sensitive binding present in control tissue about equally well, naltrexone was over 6-fold more potent than naloxone in inhibiting the binding remaining after naloxazone treatment of tissue.

The inhibition of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide binding by [D-Ala^2,D-Leu^5]enkephalin did not show clearly the biphasic curves seen with the opiates (Fig. 3*A*), but its shallow slope was suggestive of binding heterogeneity. [D-Ala^2,D-Leu^5]Enkephalin at 5 nM inhibited approximately 45% of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide binding in control tissue, whereas treating tissue with naloxazone eliminated any inhibition by [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin at this concentration, agreeing with previous findings utilizing I^{125}-labeled [D-Ala^2,D-Leu^5]enkephalin (18).

Because naloxazone treatment of membranes also abolished the high-affinity binding component of [^{3}H]dihydromorphine, we examined the inhibition of this μ agonist by [D-Ala^2,D-Leu^5]enkephalin (Fig. 3*B*). As with many of the other curves, [D-Ala^2,D-Leu^5]enkephalin inhibited [^{3}H]dihydromorphine binding in a biphasic manner. The initial displacement by [D-Ala^2,D-Leu^5]enkephalin appeared at concentrations less than 1 nM, with the secondary component requiring up to 100-fold greater concentrations. Naloxazone treatment blocked any inhibition of [^{3}H]dihydromorphine binding by [D-Ala^2,D-Leu^5]enkephalin at concentrations less than 10 nM.

To further investigate the ability of morphine to inhibit high-affinity enkephalin binding, we compared morphine directly with [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin (Fig. 4). The results were quite similar to those of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide. The initial displacement again was seen with concentrations far less than 1 nM, whereas the second displacement was even less sensitive to morphine displacement than that seen with [D-Ala^2,Met^5]-[^{3}H]enkephalinamide.

The Pharmacological Properties of [^{3}H]Dihydromorphine and [D-Ala^2,D-Leu^5]-[^{3}H]Enkephalin Binding in Naloxazone-Treated Tissue. The above studies suggest that all opiates and peptides tested bind with highest affinity to a common site. However, the binding remaining in naloxazone-treated tissue reflects striking differences between opiates and enkephalins.

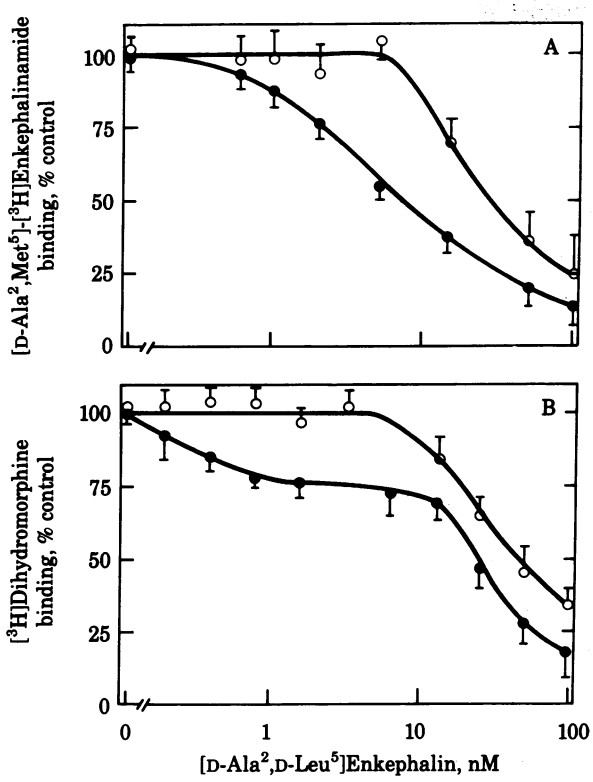

FIG. 3. Displacement of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide and [^{3}H]dihydromorphine binding by [D-Ala^2,D-Leu^5]enkephalin. Rat brain membranes were prepared and treated with no drug (●) or naloxazone (○), and were assayed with 1 nM [D-Ala^2,Met^5]-[^{3}H]enkephalinamide (*A*) or 1 nM [^{3}H]dihydromorphine (*B*) and increasing concentrations of unlabeled [D-Ala^2,D-Leu^5]enkephalin. Points are from one experiment and represent the mean ± SEM of triplicate determinations. This experiment has been replicated three times.

For example, 5 nM morphine inhibited the binding of [^{3}H]dihydromorphine in naloxazone tissue up to 40%, whereas it had no effect on [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin binding at this concentration. Similarly, [D-Ala^2,D-Leu^5]enkephalin inhibited [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin binding in naloxazone tissue with an approximate concentration causing half maximal

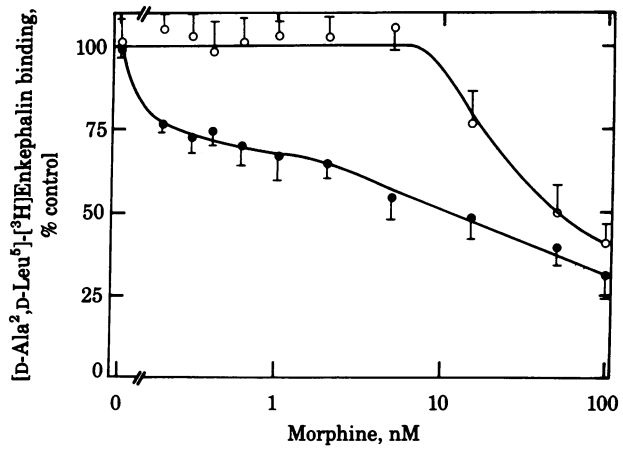

FIG. 4. Displacement of [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin by morphine. Rat brain membranes were prepared and treated with no drug (●) or naloxazone (○) and assayed with 1 nM [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin and increasing concentrations of morphine sulfate. Points are from one experiment and represent the mean ± SEM of triplicate determinations. The experiment has been replicated three times.

inhibition (IC_{50}) of less than 15 nM, whereas far greater concentrations were required to inhibit [^{3}H]dihydromorphine binding in similarly treated tissue. For these reasons, we examined the ability of a variety of peptides and opiates to inhibit both [^{3}H]dihydromorphine and [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin binding in naloxazone-treated tissue.

Unlabeled [D-Ala^2,D-Leu^5]enkephalin was more than 6-fold more potent in displacing [D-Ala^2,D-Leu^5]-[^{3}H] than [^{3}H]dihydromorphine (Table 1). Even greater differences in potency were seen with [D-Ala^2,Met^5]enkephalin. The synthetic enkephalin compound FK 33824 has been described as a μ or morphine-like peptide. In this assay system, it inhibited [^{3}H]opiate binding 3-fold more potently than [^{3}H]enkephalin binding, mimicking the 8-fold difference seen with morphine. Interestingly, the κ drug ketocyclazocine and the σ drug SKF 10,047 inhibited [^{3}H]enkephalin and [^{3}H]dihydromorphine binding approximately equally well.

DISCUSSION

Both the biphasic displacement curves and nonlinear Scatchard plots strongly suggest the presence of multiple classes of binding sites. It is clear that a portion of [D-Ala^2,Met^5]-[^{3}H]enkephalinamide binding is easily displaced equally well by low concentrations of both opiates and enkephalins. This initial displacement represents the higher-affinity binding of the unlabeled drugs because it occurs at their lowest concentrations. The loss of this initial competitive displacement by opiates and enkephalins of ^{3}H-labeled ligands in naloxazone-treated tissue, coupled with naloxazone's blockade of high-affinity μ ([^{3}H]morphine and [^{3}H]dihydromorphine), κ ([^{3}H]ketocyclazocine), σ ([^{3}H]SKF 10,047), and enkephalin ([D-Ala^2,Met^5]-[^{3}H]enkephalinamide, [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin, I^{125}-

Table 1. Inhibition of [^{3}H]dihydromorphine and [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin binding in naloxazone-treated tissue by opiates and enkephalins

Compounds	IC_{50}, nM: [D-Ala^2,D-Leu^5]-[^{3}H]-enkephalin δ binding	IC_{50}, nM: [^{3}H]Dihydromorphine μ_2 binding	IC_{50} ratio δ/μ_2 binding
Peptides			
[D-Ala^2,D-Leu^5]Enkephalin	10.0 ± 1.2	63.8 ± 7.4	0.15
[D-Ala^2,Met^5]Enkephalin	8.1 ± 1.4	97.0 ± 2.2	0.08
[D-Ala^2,*N*-MePhe^4-Met(O)-ol^5]-Enkephalin (FK 33,824)	50.9 ± 8.7	17.3 ± 5.8	2.9
Opiates			
Morphine	69.1 ± 3.2	10.8 ± 0.7	8.3
Ketocyclazocine	40.2 ± 8.4	51.6 ± 15.6	0.78
N-Allylnormetazocine (SKF 10,047)	20.0 ± 8.4	12.9 ± 2.6	1.6
Levallorphan	11.2 ± 1.7	2.5 ± 0.8	4.5
Naloxone	42.1 ± 6.6	18.8 ± 1.7	2.2

Rat membranes were prepared, incubated with 2 μM naloxazone, and extensively washed. The inhibition of both [^{3}H]dihydromorphine and [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin binding, assayed with 1 nM ^{3}H-labeled ligands at the same time with the same tissue, by opiates and enkephalins was then determined over a wide concentration range (0.2–100 nM). IC_{50} values were calculated by least squares fit to a logarithm-probit analysis. The results are expressed as the mean ± SEM of three determinations of the IC_{50} values (four determinations with [D-Ala^2,D-Leu^5]enkephalin). The IC_{50} values for [^{3}H]dihydromorphine and [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin are significantly different for both morphine and [D-Ala^2,D-Leu^5]enkephalin ($P < 0.001$) and for FK33824, [D-Ala^2,Met^5]enkephalin, and levallorphan ($P < 0.03$). No significant difference is seen with SKF 10,047 or ketocyclazocine.

labeled [D-Ala^2,D-Leu^5]enkephalin, [Met^5]-[^{3}H]enkephalin, and [Leu^5]-[^{3}H]enkephalin) binding measured directly in saturation experiments with Scatchard analysis, implies that all of the tested drugs bind with highest affinity to a common site. This receptor has been implicated in the analgesic properties of enkephalins and opiates by using both an *in vivo* naloxazone (11–15, 17–19) and a developmental (22, 23) model.

The displacement studies in naloxazone-treated tissue (Table 1), together with the actions of naloxazone on the biphasic competition curves (Figs. 2–4) and Scatchard plots (Fig. 1) are best explained by three classes of receptors (Table 2). The first site, which corresponds to the common high-affinity binding site described above and is sensitive to naloxazone's irreversible actions, binds opiates and enkephalins with approximately equal affinities (K_d, <1 nM). We propose naming this site μ_1. Binding opiates with slightly lower affinity than the μ_1 site, the μ_2 site (low-affinity [^{3}H]dihydromorphine site; K_d, 3 nM) selectively binds opiates such as morphine (K_d, 8–11 nM) markedly better than enkephalin analogs such as [D-Ala^2,D-Leu^5]enkephalin (K_i, 50 nM) or [D-Ala^2,Met^5]enkephalin (K_i, 75 nM). The lower-affinity [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin site (K_d, 5 nM) seen with Scatchard analysis fulfills the criteria previously proposed for a δ site (1, 7, 8), preferentially binding the prototypic δ ligand [D-Ala^2,D-Leu^5]enkephalin (K_d, 5–8) nM) far more potently than morphine (K_i, 70 nM). Of particular interest in this system is the unique properties of both the κ drug ketocyclazocine and the σ drug SKF 10,047. These drugs show little selectivity between the μ_2 and the δ site, agreeing with previous reports (24). Perhaps their similar potency at these two sites might explain some of their pharmacological actions.

At first glance, it is difficult to understand why binding to the higher-affinity sites represents a small fraction of total specific binding (Figs. 2–4) when using low ^{3}H-labeled ligand concentrations. Although the fractional occupancy of the higher-affinity site will be greater than the lower-affinity site, this difference is offset by the far greater numbers of low-affinity sites. For example, [D-Ala^2,Met^5]-[^{3}H]enkephalinamide Scatchard plots show two sites whose approximate K_d values (0.2 and 4 nM) and B_{max} (1 and 9 fmol/mg of tissue) differ significantly. At 1 nM, the fractional occupancy of the higher-affinity site is approximately 80%, whereas it is only about 20% for the low-affinity site (25). However, binding is the product of fractional occupancy and the number of sites. Therefore, high-affinity binding (0.8 fmol/mg of tissue) is only half of the low-affinity binding (1.8 fmol/mg of tissue) and only 30–35% of the total specific binding.

Table 2. Approximate dissociation constants for morphine and [D-Ala^2,D-Leu^5]enkephalin on μ_1, μ_2, and δ receptors

Ligand	Approximate K_d values, nM		
	μ_1	μ_2	δ
Morphine			
Saturation studies	0.4	11	—
Displacement studies	<1	8	71
[D-Ala^2,D-Leu^5]Enkephalin			
Saturation studies	0.5	—	5
Displacement studies	<1	50	8

Saturation study results use values obtained by computerized nonlinear, least-squares regression analysis of saturation studies with [^{3}H]morphine or [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin. Displacement results were calculated from the IC_{50} values in Table 1 by using the formula $K_i = (IC_{50})_i/(1 + C_A)$ where i is the unlabeled ligand, A is the radiolabeled ligand, and $C_A = [A]/K_{dA}$. The K_d values of [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin and [^{3}H]dihydromorphine used to calculate the K_i values in displacement studies in naloxazone-treated tissue (5 nM and 3 nM, respectively) were determined from saturation studies.

This classification helps to simplify much of the pharmacological and biochemical data currently in the literature. Enkephalins bind to both μ_1 and δ sites, and opiates such as morphine bind to the μ_1 and the μ_2 sites. Therefore, the morphine-like qualities described with enkephalins probably reflect their potent binding to the μ_1 site. Displacement experiments of [^{3}H]enkephalins by morphine illustrate the μ_1 and δ binding of enkephalins. The μ_1 binding of radiolabeled enkephalins is easily displaced by low morphine concentrations, whereas the δ binding is not. Previous studies (7, 8) have demonstrated the inability of opiates to displace enkephalin binding easily and vice versa. Our results (Table 1) imply that these studies were looking at differences between μ_2 and δ binding. The previously reported experiments utilized a single IC_{50} value. This single IC_{50} (Figs. 2–4) more closely approximates the value of the second displacement, which corresponds to μ_2 and δ interactions.

In summary, the evidence suggests three major types of morphine and enkephalin receptors. The μ_1 site appears to be quite distinct. It has a regional localization (20) that differs dramatically from that of the δ receptor, has a different ontological appearance (22, 23), appears to mediate opiate, enkephalin, and β-endorphin analgesia (11–15, 17–19, 22, 23), and is not present in lower species such as the goldfish (26). The μ_2 and δ sites also appear to be separate, differing from each other and the μ_1 sites both pharmacologically and biochemically (7, 8, 19, 21). The pharmacological properties of the μ_2 and δ sites are not yet fully known but would include many opioid actions not mediated by the μ_1 site, such as respiratory depression. It is easy to see how the enkephalins might serve as the endogenous ligand for both the μ_1 and δ sites. However, because they bind so poorly to the μ_2 sites, it seems unlikely that the enkephalins are natural ligands for all three receptor subtypes.

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