Multiple Opiate Receptors

ENKEPHALINS AND MORPHINE BIND TO RECEPTORS OF DIFFERENT SPECIFICITY*

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The use of very low concentrations of $^{125}$I-$D$-Ala$^2$-$D$-Leu$^5$enkephalin, $[^3]$H]naloxone, and $[^3]$H]dihydromorphine under similar conditions enables the measurement of different opioid binding sites in a rat brain membrane preparation. In the absence of sodium ions, one such site binds enkephalins and their $D$-Ala$^2$-substituted analogs with higher affinity than morphine and naloxone (enkephalin receptor), while another site exhibits better affinity for naloxone and morphine (morphine receptor).

Morphine, naloxone, and enkephalin do not facilitate the dissociation of bound $^{125}$I-$D$-Ala$^2$-$D$-Leu$^5$enkephalin, suggesting that heterogenous or homogenous cooperativity does not exist for opioid receptors. Sodium ion decreases the high affinity binding of morphine and enkephalins to both receptor sites. Scatchard plots of concentration binding isotherms of $^{125}$I-$D$-Ala$^2$-$D$-Leu$^5$enkephalin indicate two binding sites with dissociation constants of 0.8 nM and 6 nM and capacities of 43 and 88 fmol/mg of membrane protein.

Many neuroblastoma cell lines bear only the enkephalin receptors. So far, cell lines containing only the morphine receptor have not been found. Homogenization or exposure to morphine for 24 h does not affect the binding characteristics of neuroblastoma cells.

$N$-Cyclopropylmethylnoretorphine, an analgesic about 100 times more potent than morphine with less propensity to induce physical dependence, competes for the binding of $^{125}$I-$D$-Ala$^2$-$D$-Leu$^5$enkephalin and $[^3]$H]naloxone with $IC_{50}$ values of 2.9 and 1 nM, respectively, and shows no "sodium shift" in the $[^3]$H]naloxone-binding assay. Partial agonists, pentazocine, butorphanol, and oxilorphan, also have a weak "sodium shift" and a low $IC_{50}$ ratio in competing with $[^3]$H]naloxone compared to $^{125}$I-$D$-Ala$^2$-$D$-Leu$^5$enkephalin binding.

Enkephalin and its stable analogs exhibit high affinity for the enkephalin receptors. The analog, Try-Gly-Gly-Phe, which retains significant intrinsic activity, is still able to bind to the enkephalin receptor better than to the morphine receptor. Tyr-ethyl ester binds to both receptor sites equally well, showing an $IC_{50}$ value of about 0.1 nM. These data may suggest that the hydrophobic group of the phenylalanine residue of enkephalin could be responsible for the major structural difference between enkephalin and morphine for receptor recognition. This may also explain why $N$-cyclopropylmethylnoretorphine, which contains a hydrophobic group at the C-19 position of oripavine, binds to the enkephalin receptor better than naloxone and morphine.

In 1975, several laboratories simultaneously reported that a morphine-like material could be extracted from brain and pituitary. Hughes et al. (1) and Hughes (2) first reported that their opiate-like material consisted of two similar pentapeptides with sequences of H-Tyr-Gly-Gly-Phe-Met-OH ($[Met^1]$enkephalin) and H-Tyr-Gly-Gly-Phe-Leu-Oh ($[Leu^1]$enkephalin). The existence of these two peptides was rapidly confirmed by Pasternak et al. (3) and Simantov and Snyder (4). The structure of $[Met^1]$enkephalin is contained within the sequence (residues 61 to 65) of 91 amino acids of $\beta$-lipotropin, a peptide isolated from the pituitary gland of several vertebrate species (5, 6). Bradbury et al. (7) and Cox et al. (8) reported that the 31 amino acid COOH-terminal fragment of $\beta$-lipotropin was as potent as $[Met^1]$enkephalin. Various COOH-terminal fragments of $\beta$-lipotropin known as $\alpha^+$, $\beta^+$, and $\gamma^+$-endorphins have now also been isolated from the pituitary (6, 9).

$[Met^1]$Enkephalin and $[Leu^1]$enkephalin are rapidly metabolized in vivo and in vitro (10-12). Many analogs of enkephalin have now been synthesized and tested for morphine-like activity and for stability. Replacement of glycine at position 2 by $D$-Ala together with substitution of leucine at position 5 by $D$-Leu has yielded a very stable and more potent pentapeptide, $[D$-Ala$^2$-$D$-Leu$^5]$enkephalin (13-15).

Utilizing labeled narcotic agonists and antagonists of high specific radioactivity, specific opioid binding sites (receptors) have been identified and characterized (16-18). $H$-labeled $[Leu^1]$enkephalin has recently been employed to study enkephalin-receptor interactions in brain membranes (19, 20) and cultured cells (21). $^{125}$I-labeled $[D$-Ala$^2$-$D$-Leu$^5]$enkephalin has been prepared successfully and is found to bind to opiate receptors stereospecifically and with high affinity (22-24).

Simultaneous comparison of the potency of narcotic agonists, antagonists, and enkephalin analogs in inhibiting the binding of $H$-labeled narcotics and enkephalin, or of $^{125}$I-labeled enkephalin, reveal that narcotics are more potent in inhibiting labeled narcotics than enkephalins (19, 20, 24). The contrary is true for enkephalins and their analogs. Good correlations exist between the relative potency of enkephalin analogs (13, 14) in the mouse vas deferens bioassay and in the opiate binding assay. However, the correlation of binding is not as good with activity in the guinea pig ileum assay (13, 14). All enkephalin analogs tested so far show considerably greater activity relative to morphine in the mouse vas deferens compared to the guinea pig ileum (13, 14). Certain narcotics are relatively more potent in the guinea pig ileum than on the...
mouse vas deferens (19). These data have been used to support the view that there are multiple opiate receptors. Martin and colleagues (25, 26) have postulated that there are at least three different opiate receptors.

By using $^{125}$I-labeled [D-Ala$^2$,D-Leu$^5$]enkephalin of high specific radioactivity, direct evidence has been obtained for at least two distinct types of opiate binding sites in the brain. One of these binds enkephalins with higher affinity than narcotics, while the other binds narcotics with higher affinity. Independent measurement of both sites (receptors) with minor interference due to cross-reactivity can be accomplished by using very low concentrations of $^{125}$I-[D-Ala$^2$,D-Leu$^5$]enkephalin and $^{125}$I-labeled [D-Ala$^2$,D-Leu$^5$]enkephalin. The hydrophobic moiety of the benzene ring of enkephalin appears to be one of the major factors differentiating these two binding sites. The binding sites which bind enkephalin with higher affinity probably contain a hydrophobic recognition area for the benzene side chain of enkephalin or the C-19 hydrophobic group of opioids derivatives.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enkephalins and their analogs were synthesized by Dr. S. Wilkinson, The Wellcome Research Laboratories, Beckenham, England (14). Naloxone was obtained from Endo Laboratories, Inc. Morphine sulfate was obtained from Mallinckrodt Chemical Works. Pentazocine and meperidine were obtained from Sterling-Winthrop Research Institute. Butorphanol and oxilorphan were obtained from Bristol Laboratories. N-Cyclopropylmethylnororphan was obtained from Reckitt and Colman Products, London, England. $^{[3}H]$Naloxone (specific activity, 23 Ci/mmol) was purchased from New England Nuclear. $^{[3}H]$Etorphine (specific activity, 32 Ci/mmol) and $^{[3}H]$Lydihydromorphine (specific activity, 70 Ci/mmol) were purchased from Amersham/Searle.

**Methods**—Crude brain membranes are prepared by the method of differential centrifugation in isotonic sucrose solution. Whole brain without cerebellum from Sprague-Dawley rats (150 to 200 g) is homogenized with a Polytron PT-20 for 1 min at a setting of 3 in 10 volumes (v/w) of 0.32 M sucrose. The homogenates are centrifuged at 6,000 × g for 15 min to remove the nuclei and mitochondria. The supernatants obtained from two such centrifugations are combined and centrifuged at 40,000 × g for 30 min. The pellets are resuspended in 5 volumes (original wet weight) of 5 mM Tris.HCl, pH 7.7, and allowed to swell for 30 min. The synaptic vesicles thus obtained are disrupted with a Polytron homogenizer and centrifuged at 6,000 × g for 90 min. The supernatants are centrifuged at 40,000 × g for 30 min. The tight, brownish pellets (which contain most of the mitochondria) are discarded while the top, loose pellets are separated and resuspended in 5 mM Tris.HCl. These steps of swelling, disruption, and centrifugation are repeated and the final loose membrane pellet is suspended in 2 volumes (original wet weight) of 50 mM Tris.HCl and stored at −20°C.

**RESULTS**

**Direct Evidence for Two Opiate Binding Sites with Differing Specificity**—The potency of an inhibitor in competing for binding of a radioactive ligand is dependent upon the concentration of the labeled ligand and receptor concentration used in the assay (28, 29). When the concentrations of receptor and labeled ligand are lower than one-tenth the dissociation constant for labeled ligand, IC$\text{50}$ value (the concentration which can inhibit 50% of the labeled ligand binding) is nearly equal to its dissociation constant (28, 29). In a multiple receptor system, selection of the labeled ligand concentration becomes an especially important factor. A high affinity site can be measured without significant interference from a low affinity site provided certain conditions are met. The comparison of the potency is always done in the same membrane preparation, the same conditions, and at the same time. The results are qualitatively reproducible, and the key experiments are repeated at least three times. The variation between individual experiments gives no more than 2-fold difference in IC$\text{50}$ value.

![Fig. 1](http://www.jbc.org/)  
**Fig. 1.** Upper panel, comparison of the potency of [D-Ala$^2$,L-Leu$^5$]enkephalin in competing for the binding of $^{125}$I-[D-Ala$^2$,D-Leu$^5$]enkephalin (O), $^{[3}H]$Naloxone (●), and $^{[3}H]$Dihydromorphine (Δ). The total specific radioactivity bound was 1180 cpm (2.4% of the total added), 670 cpm (4.6%), and 2430 cpm (8%) for $^{125}$I-[D-Ala$^2$,D-Leu$^5$]enkephalin (0.25 nM), $^{[3}H]$Naloxone (0.38 nM), and $^{[3}H]$Dihydromorphine (0.2 nM), respectively. Values represent the mean of duplicate samples which were ±5% of the mean. **Lower panel**, Hill plot. Hill coefficient, n = 1, 1, and 0.8 are found for $^{125}$I-labeled enkephalin, $^{[3}H]$Dihydromorphine, and $^{[3}H]$Naloxone, respectively.
shown in Table I. The Hill coefficient (n) is 0.8 for both competition curve of naloxone and morphine sulfate against $^{125}$I-\[n-fiaZ,n-Leu5\]enkephalin and $^{3}$H[naloxone in the absence and presence of Na$^+$ or Mn$^{2+}$ ions.

The IC$\text{so}$ values are estimated from competition curves (Figs. 1 and 2) using 0.2 nM of $[^{3}\text{H}]$dihydromorphine, 0.38 nM of $[^{3}\text{H}]$naloxone, and 0.25 nM of $^{[25]}$I-\[d-Ala,d-Leu5\]enkephalin. The concentration of Na$^+$ and Mn$^{2+}$ ions is 0.1 mM and 1 mM, respectively. The two portions of the competition curve of naloxone and morphine sulfate against $^{[25]}$I-\[d-Ala,d-Leu5\]enkephalin are separately estimated. Binding is carried out at 24°C. Values are expressed as mean $\pm$ S.E. The number in parentheses is the number of separate experiments.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$[^{3}\text{H}]$dihydromorphine</th>
<th>$[^{25}]$I-[d-Ala,d-Leu5]enkephalin</th>
<th>$[^{3}\text{H}]$Naloxone</th>
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<tr>
<td>[d-Ala,Leu]enkephalin</td>
<td>6.0 ± 0.5 (3)</td>
<td>1.3 ± 0.1 (3)</td>
<td>7.25 ± 0.6 (4)</td>
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<tr>
<td>[Leu]enkephalin</td>
<td>23 ± 6 (3)</td>
<td>3.1 ± 0.5 (3)</td>
<td>28.3 ± 10.4 (3)</td>
</tr>
<tr>
<td>[Met]enkephalin</td>
<td>8.2 ± 1.8 (3)</td>
<td>4.4 ± 0.9 (4)</td>
<td>9.5 ± 1.2 (2)</td>
</tr>
<tr>
<td>Morphine</td>
<td>0.4 ± 0.1 (3)</td>
<td>2.11 ± 0.14 (4)</td>
<td>0.26 ± 0.29 (3)</td>
</tr>
<tr>
<td>Naloxone</td>
<td>1.4 ± 0.6 (3)</td>
<td>0.43 ± 0.08 (4)</td>
<td>1.06 ± 0.51 (3)</td>
</tr>
</tbody>
</table>

When narcotics are used to compete with the binding of low concentrations of $^{3}$H-labeled narcotics, a linear Hill plot of competition curve of morphine sulfate is found for narcotics in competing with $^{25}$I-\[d-Ala,d-Leu5\]enkephalin. The approximate IC$\text{so}$ values for these two portions of the competition curve are estimated to be about 0.5 nM and 40 nM, respectively (Table I). Naloxone competes with the binding of $^{[25]}$I-\[d-Ala,d-Leu5\]enkephalin in a fashion very similar to morphine (data not shown); the IC$\text{so}$ values are shown in Table I. The Hill coefficient (n) is 0.8 for both enkephalin and morphine when measured against $[^{3}\text{H}]$naloxone binding (Fig. 2). This may be due to the slightly different binding of agonist and antagonist such as suggested by Feinberg et al. (30).

When narcotics are used to compete with the binding of low concentrations of $^{3}$H-labeled narcotics, a linear Hill plot with a Hill coefficient (n) of about 1 is obtained (Fig. 2B). This difference is smaller for $[^{3}\text{H}]$enkephalins (Table I). Although they are less potent than $[^{3}\text{H}]$enkephalin and narcotics, the difference in these two binding assays is still apparent.

This difference is smaller for $[^{3}\text{H}]$enkephalin (Table I). Although they are less potent than $[^{3}\text{H}]$enkephalin and narcotics, the difference in these two binding assays is still apparent. However, the potency relationship is reversed when morphine is used to compete for the binding of $^{125}$I-\[d-Ala,d-Leu5\]enkephalin with an affinity similar to morphine (data not shown); the IC$\text{so}$ values are estimated to be 85 and 83 nM for $[^{3}\text{H}]$naloxone and $[^{3}\text{H}]$dihydromorphine, respectively. Values represent the mean of duplicate samples that are 5% of the mean.

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When narcotics are used to compete with the binding of low concentrations of $^{3}$H-labeled narcotics, a linear Hill plot with a Hill coefficient (n) of about 1 is obtained (Fig. 2B). This evidence suggests that the $^{3}$H-labeled narcotics bind primarily to a homogeneous receptor population under these conditions.

Thus, the bimolecular mass action equation

$$Kd = \frac{[R][H]}{[RH]}$$

is applied to estimate the kinetic parameters, where $Kd$ is the apparent dissociation constant, [R] and [H] are the concentrations of unbound receptor and ligand, and [RH] is the receptor- ligand complex. The total concentration of receptor sites (R$+RH$) are estimated to be 85 and 83 nM for $[^{3}\text{H}]$naloxone and $[^{3}\text{H}]$dihydromorphine, respectively. This nearly equal content of both $^{3}$H-labeled narcotics is consistent with their binding to the same receptor population under the conditions described. This conclusion is further supported by their nearly identical IC$\text{so}$ values regardless of whether $[^{3}\text{H}]$naloxone or $[^{3}\text{H}]$dihydromorphine is used (Table I). If the same receptor population were responsible for the majority of the binding of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin with an affinity of 0.8 nM (22), 6 to 8% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound. However, only about 2.5% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound. However, only about 2.5% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound. However, only about 2.5% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound. However, only about 2.5% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound. However, only about 2.5% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound. However, only about 2.5% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound. However, only about 2.5% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound. However, only about 2.5% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound. However, only about 2.5% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound. However, only about 2.5% of $^{25}$I-\[d-Ala,d-Leu5\]enkephalin should have been bound.
Leu]enkephalin is bound, making it very unlikely that the binding site with high affinity for narcotics can also bind enkephalin with an affinity of 0.8 nM.

Competition curves of morphine sulfate and naloxone against [125I]-(D-Ala2,D-Leu5]enkephalin show biphasic curves and suggest that [125I]-(D-Ala2,D-Leu5]enkephalin at 0.25 nM is bound to two receptor populations at a ratio of about 3:7 (Fig. 2). Thirty percent of the total bound [125I]-(D-Ala2,D-Leu5]enkephalin can be inhibited by very low concentrations of morphine and naloxone, and the remaining 70% can only be inhibited by high concentrations of narcotics. Direct saturation curves of [125I]-(D-Ala2,D-Leu5]enkephalin (Fig. 3) show nonlinear Scatchard plots with parameters of 0.8 nM and 6 nM for the apparent KD, and 35 pm (or 44 fmol per mg of membrane protein) and 70 pm (or 88 fmol per mg of membrane protein) for receptor concentration. The receptor concentration calculated for the latter site is very similar to that which binds [3H]naloxone and [3H]dihydromorphine, as described above. Na+ decreases the binding of both the high and the low affinity sites (Fig. 3). Furthermore, a biphasic rate of dissociation with half-times (t1/2) of 5 and 22 min is observed for the [125I]-(D-Ala2,D-Leu5]enkephalin-receptor complexes (Fig. 4) which is consistent with the hypothesis of two opiate receptors of differing affinities.

The data described above clearly indicate that [125I]-(D-Ala2,D-Leu5]enkephalin binds to two separate receptor sites which can bind narcotics with a 20- to 100-fold, and enkephalin with a 3- to 5-fold difference in affinity (Table I). The use of low concentrations of H-labeled narcotics as reported here should allow detection of only the high affinity sites for narcotics. Indeed, morphine and naloxone compete with the binding of H-labeled narcotics with affinities close to that of the high affinity site (0.5 nM and 0.8 nM). [D-Ala2,D-Leu5]enkephalin competes with [3H]naloxone and [3H]dihydromorphine with an IC50 of about 6 nM. Therefore, this site is called the "morphine receptor" since it binds morphine more strongly than enkephalin. The other site, which binds enkephalin better than morphine and naloxone, is referred to as the "enkephalin receptor." These two sites are not the same as those described by Pasternak and Snyder (31) in their "two state conformation" hypothesis since in our studies the behavior of the agonist, dihydromorphine, is very similar to that of the antagonist, naloxone. In the model of "agonist-antagonist two state conformation" (31), agonists should have less potency in inhibiting the binding of [3H]naloxone than that of [3H]dihydromorphine, and vice versa for antagonist. In the present studies the potency of naloxone and morphine and enkephalin does not vary significantly whether [3H]-naloxone or [3H]dihydromorphine is used.

Low concentrations of labeled enkephalin and narcotics are used subsequently in the evaluation of potency of narcotics and enkephalin on the two binding sites since low concentrations of labeled narcotics bind preferentially to the morphine-binding sites. However, since the affinities of enkephalin for both sites are quite similar (4-fold difference), and since the quaternary structure of the receptor is much higher in the brain preparation, [125I]-(D-Ala2,D-Leu5]enkephalin at a concentration of 0.25 nM will bind to both sites with a ratio (morphine site to enkephalin site) of 3:7, which is readily detected by binding competition curves of naloxone and morphine. This may also explain the slight deviation of the Hill plot from a linear slope (Fig. 1). Since most of the [125I]-(D-Ala2,D-Leu5]enkephalin is bound to the enkephalin site, the apparent IC50 value should be closer to the KD for the enkephalin site. Thus, [125I]-(D-Ala2,D-Leu5]enkephalin at concentrations below 0.25 nM can be used as a marker for the enkephalin receptors.

Absence of Negative Cooperativity. The difference in potency of enkephalin and narcotics in competing for the binding sites of labeled enkephalin and narcotics could also be due to heterogenous, negatively cooperative interactions between enkephalin and morphine receptor sites. To test this possibility, the rate of dissociation of the enkephalin-receptor complex was measured by diluting membranes previously labeled with [125I]-(D-Ala2,D-Leu5]enkephalin in the absence and presence of excess, unlabeled naloxone, morphine, and enkephalin (Fig. 4). The rate of dissociation of [125I]-(D-Ala2,D-Leu5]enkephalin is not significantly affected by any of these conditions. This indicates that negative, heterogenous, or homogenous cooperativity does not exist. This conclusion is further supported...
by the data described in Figs. 1 and 2, where the Hill coefficient ($n$) is very close to 1 when $[\text{D-Ala}^2,\text{L-Leu}^5]$- and $[\text{Met}^5]$enkephalin compete with the binding of any of the labeled ligands and when morphine and naloxone compete with the binding of $^3$H-labeled naloxone and dihydromorphine. Linear plots with two different slopes are obtained when naloxone and morphine compete with the binding of $^{125}$I-$[\text{D-Ala}^2,\text{D-Leu}^5]$enkephalin. These data are explained simply by two independent binding sites with widely different affinities for narcotics.

**Differential Effects of Cations on Enkephalin and Morphine-binding Sites**—In the absence and presence of Na" or Mn"$^{2+}$, the $IC_{50}$ value for $[\text{D-Ala}^2,\text{D-Leu}^5]$enkephalin is unchanged (Fig. 5, inset). But Mn"$^{2+}$ increases significantly the potency of $[\text{Leu}^5]$- and $[\text{Met}^5]$enkephalin in inhibiting the binding (Table I). When morphine (Fig. 5, lower panel) or naloxone (not shown) are used to compete for binding, the portion inhibited by low concentrations of narcotics (morphine-binding sites) disappears completely in the presence of Na". However, in the presence of 1 mM MnCl$_2$, a large increase is noted (about 2-fold) in the portion (the enkephalin-binding site) sensitive to high concentrations of the narcotic.

When $[^3$H]naloxone (0.38 nM) is used as the labeled marker, the potency of enkephalin and morphine is reduced in the presence of 0.1 M NaCl. A 40-fold and a 5- to 10-fold reduction in affinity is observed or morphine and enkephalin analogues, respectively, while the affinity for naloxone is not changed significantly (Table I). This reduction suggests that the morphine-binding sites may be converted by Na" to sites which bind morphine and enkephalin only with low affinity but which retain the high affinity for naloxone (31).

**Neuroblastoma Cells Exhibit Only Enkephalin-binding Sites**—The potency of a series of narcotic agonists and antagonists in competing with the binding of $^{125}$I-$[\text{D-Ala}^2,\text{D-Leu}^5]$enkephalin to intact cultured cells is similar to that seen with rat brain membranes (24). This result suggests that the opiate receptor in neuroblastoma cells is similar to the enkephalin receptor in brain membranes. To examine the possible effects of homogenization procedures, cultured cells were homogenized by the same methods used for the preparation of brain membranes, and the membranes were then analyzed for their affinity for enkephalin and narcotics. Naloxone and morphine compete with $^{125}$I-$[\text{D-Ala}^2,\text{D-Leu}^5]$enkephalin in a manner very similar to that seen in intact cells. Notably, neuroblastoma cells or cell membranes bind very little $[^3$H]naloxone or dihydromorphine (<200 cpm) even though very substantial binding (several thousand counts per min) of $[^3$H]naloxone is noted (about 2-fold) in the portion (the enkephalin-binding site) sensitive to high concentrations of the narcotic.
competing with the binding of labeled enkephalin or naloxone. It can, therefore, be anticipated that \([^{3}H]\)etorphine should bind to both sites equally well. Since the affinities of enkephalin and naloxone for both sites are different, the competition curve of enkephalin and naloxone against \([^{3}H]\)etorphine should behave heterogeneously, and the apparent \(IC_{50}\) value for enkephalin and naloxone should be greater than those values against \([^{3}H]-[\text{D}-\text{Ala}^{2},\text{D}-\text{Leu}^{5}]\)enkephalin and \([^{3}H]\)naloxone, respectively. That is indeed the case is shown in Fig. 7. The apparent \(IC_{50}\) values are \(4\) nm and \(10\) nm for naloxone and \([\text{D}-\text{Ala}^{2},\text{D}-\text{Leu}^{5}]\)enkephalin, respectively, and the Hill plots are not linear.

Effect of Enkephalin Analogs and Various Narcotics on the Binding of Enkephalin and Naloxone—The apparent \(IC_{50}\) values of various narcotic agonists, antagonists, and partial agonists in competing for the binding of the two labeled ligands are given in Table II. The ratios of the \(IC_{50}\) values for both receptor sites range from \(70\) to \(1.4\). Meperidine, a narcotic with a structure quite different from morphine, binds better to the morphine receptor than to the enkephalin receptor (ratio of \(10\)). The mixed agonist-antagonists, pentazocine, butorphanol, and oxilorphan have a similar affinity for both systems. \(N\)-Cyclopropylmethylnorephorine, another mixed agonist-antagonist which is 100 to 300 times more potent than morphine and has antagonist activity about one-fifth that of nalorphine (32, 33), binds to both receptor sites with even more nearly equal affinity. These drugs have been reported to contain less potential for inducing physical dependence. The "sodium shift" is smaller for pentazocine, butorphanol, and oxilorphan than for morphine (Table II). Sodium ions do not affect the potency of \(N\)-cyclopropylmethylnorephorine (Fig. 8) in competing for the binding of \([^{3}H]\)naloxone (Table II). In these terms, \(N\)-cyclopropylmethylnorephorine behaves as a pure antagonist in the \([^{3}H]\)naloxone-binding assay.

The increase in affinity for the enkephalin receptor and the decrease in affinity for the morphine receptor resulting from introduction of a hydrophobic group at C 19 of oripavine suggest that a hydrophobic group in this position is more important for the enkephalin receptor. This hydrophobic area could in principle be occupied by the benzene ring of the phenylalanine residue of enkephalin. This possibility is supported by the fact that the 5-fold difference in enkephalin binding to its receptor compared to the morphine receptor is maintained even with the simple analog, Tyr-Gly-Gly-Phe (Table II, lower part). This difference disappears with Tyr-ethyl ester (Table II, lower part). These drugs have been reported to contain less potential for inducing physical dependence. The "sodium shift" is smaller for pentazocine, butorphanol, and oxilorphan than for morphine (Table II). Sodium ions do not affect the potency of \(N\)-cyclopropylmethylnorephorine (Fig. 8) in competing for the binding of \([^{3}H]\)naloxone (Table II). In these terms, \(N\)-cyclopropylmethylnorephorine behaves as a pure antagonist in the \([^{3}H]\)naloxone-binding assay.

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Table II also shows that the \(IC_{50}\) ratios of the enkephalins and their analogs, determined from inhibiting the binding of the \([^{3}H]\)naloxone in the presence and absence of Na\(^{+}\), range between 3 to 10.

![Fig. 7. Upper panel, competition curve of \([\text{D}-\text{Ala}^{2},\text{L}-\text{Leu}^{5}]\)enkephalin and naloxone against \([^{3}H]\)etorphine binding. 0.13 nm of etorphine (8600 cpn) is incubated with 2 ml of brain membrane (0.27 mg/ml of membrane protein) in the absence and presence of various concentrations of \([\text{D}-\text{Ala}^{2},\text{L}-\text{Leu}^{5}]\)enkephalin (□) or naloxone (○) for 60 min at room temperature. Lower panel, Hill plot of the binding of enkephalin and naloxone against \([^{3}H]etorphine. Note the nonlinear biphasic curve for both ligands. Each point is the mean of duplicate determination.](http://www.jbc.org/)

![Fig. 8. Structure of \(N\)-cyclopropylmethylnorephorine.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>(IC_{50}) ((\text{nm}))</th>
<th>(IC_{50}) ((\text{nm}))</th>
<th>(IC_{50}) ((\text{nm}))</th>
<th>(IC_{50}) ((\text{nm}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>([^{3}H]-[\text{D}-\text{Ala}^{2},\text{D}-\text{Leu}^{5}])enkephalin</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>([^{3}H])naloxone</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>([^{3}H]-[\text{D}-\text{Ala}^{2},\text{D}-\text{Leu}^{5}])enkephalin</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>([^{3}H])naloxone</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\(\text{IC}_{50}\) \text{ ratios and IC}_{50} \text{ ratios using these two labels are shown in the third column. The last column shows the IC}_{50} \text{ ratios in inhibiting the binding of \([^{3}H]\)naloxone in the presence and absence of sodium ion (0.1 M). The concentrations are 0.25 nm and 0.38 nm for \([^{3}H]-[\text{D}-\text{Ala}^{2},\text{D}-\text{Leu}^{5}]\)enkephalin and \([^{3}H]\)naloxone, respectively. The assays are carried at 24°C for 60 min.}
DISCUSSION

Morphine inhibits the binding of $^3$H]dihydromorphine with a $IC_{50}$ value of about 0.5 nM. However, its potency against the binding of $^{125}$I-[d-Ala$^2$,D-Leu$^5$]enkephalin is about 50 times lower, with a $IC_{50}$ value of about 35 nM (Table II). Creese et al. (34) shows that the binding of $^3$H-labeled narcotic agonist and antagonist to brain membrane preparation was discriminatingly affected by temperature. At 25°C, sodium ions increase the number of binding sites for opiate antagonist and decrease the number of agonist with no change in affinity. However, at 0°C, sodium ions increase the binding of opiate antagonist by enhancing its affinity for binding site without changing the number of binding sites. These effects are about 2- to 3-fold. The differences of morphine and naloxone in inhibiting the binding of $^3$H-labeled narcotics and $^{125}$I-[d-Ala$^2$,D-Leu$^5$]enkephalin are about 50- to 100-fold, and thus these differences could not be accounted for by the temperature effect. In addition, the comparison of the potency are carefully carried out at the same time and with the same temperature and the same membrane preparations. Similar to the binding of $^3$H-labeled narcotic agonists (31, 35, 36, 38), the binding of $^{125}$I-[d-Ala$^2$,D-Leu$^5$]enkephalin is decreased by Na$^+$ (Fig. 3) and increased by Mn$^{2+}$ (Fig. 5), and also extremely sensitive to the enzymatic digestion (24). $^{125}$I-[d-Ala$^2$,D-Leu$^5$]enkephalin probably binds to agonistic conformation as postulated by Pasternak and Snyder (31). Under the present experimental condition (low concentration). Because of the wide difference of narcotics and enkephalin in inhibiting the binding of $[^3$H]dihydromorphine and $^{125}$I-[d-Ala$^2$,D-Leu$^5$]enkephalin, and these differences are not due to the cooperativity and agonist-antagonist conformation (31), it has to be concluded that at least two opiate receptors exist in rat brain.

By using the radioactive ligands, $^{125}$I-[d-Ala$^2$,D-Leu$^5$]enkephalin, $[^3$H]naloxone, $[^3$H]dihydromorphine, and $[^3$H]etorphine, it is possible to demonstrate that at least two types of opiate binding sites (receptors) exist in rat brain membrane preparations. Table III summarizes the basic features and parameters of these two binding sites. Enkephalin binds to both sites with affinities ($Kd$, apparent dissociation constant) of about 2 nM and 10 nM. However, narcotic agonists such as morphine, and antagonists such as naloxone, bind to these two affinities which differ by as much as 20- to 100-fold. Both naloxone and morphine bind to the enkephalin sites with affinities of about 30 nM, while their affinities for the morphine sites are about 0.4 nM. In conclusion, the relative order of potency for the enkephalin receptor in the absence of sodium ions is [d-Ala$^2$,L-Leu$^5$]enkephalin > [Leu$^5$]enkephalin or [Met$^5$]enkephalin $\geq$ N-cyclopropylmethyl-noretorphine > naloxone $> \xi$ morphine. Thus, the enkephalin sites seem to resemble the pharmacologic receptor of the mouse vas deferens, and the morphine receptor resembles the receptor in guinea pig ileum. In the guinea pig ileum, enkephalin presumably interacts mainly with the putative $\delta$ receptors which mediate the action of classical morphine-like compounds. In the mouse vas deferens, enkephalin interacts mainly with the putative $\kappa$ receptor (19). Martin et al. (25) previously proposed the existence of multiple opiate receptor, $\sigma$, $\mu$, and $\kappa$ receptors.

The monovalent ion, Na$^+$, and the divalent cation, Mn$^{2+}$, affect the binding to morphine and enkephalin receptors differentially. Na$^+$ increases the binding of naloxone without any significant effect on its affinity. In contrast, the affinity for enkephalins is reduced 5- to 10-fold and that for morphine 40-fold. These results are consistent with previous reports by Pert and Snyder (38) and Miller et al. (22). Mn$^{2+}$ increases slightly the affinity of enkephalin and morphine for the morphine-specific sites. In contrast to the effects on the binding of $[^3$H]naloxone, Na$^+$ completely inhibits the binding of $^{125}$I-[d-Ala$^2$,L-Leu$^5$]enkephalin to the morphine sites but only partially inhibits its binding to the enkephalin sites. Saturation binding isotherms and Scatchard plots indicate that Na$^+$ decreases the binding of enkephalin to both sites, by decreasing the amount of binding without altering the affinity. Mn$^{2+}$ increases the binding of the enkephalin receptor without significantly affecting the affinity of morphine, naloxone, or [d-Ala$^2$,L-Leu$^5$]enkephalin (Table I). The differential effect of cations described here further supports the existence of two opiate receptors in rat brain tissues. Since Mn$^{2+}$ increases the binding capacity of enkephalin receptors nearly 2-fold (Fig. 5), the relative binding of $^{125}$I-[d-Ala$^2$,D-Leu$^5$]enkephalin to enkephalin sites is increased in the presence of 1 mM MnCl$_2$ (Fig. 5).
Thus, it is possible to measure more accurately the affinity of compounds for enkephalin receptors in the presence of 1 mM MnCl₂.

The ratio of the IC₅₀ value of a drug in the presence of sodium ion to that in its absence is known as the "sodium shift" (38), which has been used as an indication of the agonist, antagonist, and partial agonist properties of compounds. The partial agonists, pentazocine, butorphanol, and oxilorphan which have low "sodium shift" ratios also bind to both receptor sites with similar affinity. The most interesting compound is N-cyclopropylmethyl-norleu-enkephalin. This compound shows no "sodium shift" in the [²⁴Na]naloxone-binding assay, and it binds to the enkephalin sites with an affinity higher than morphine and naloxone and binds to morphine sites with an affinity lower than that of morphine. At present, it is not clear what these data mean. However, partial agonists or mixed agonist-antagonists are also known to have less potential in inducing physical dependence. It is conceivable that the relative affinity to these two opiate receptor sites may play some role in the ability of a compound to cause physical dependence or addiction. The affinity of opioid peptides to induce physical dependence has recently been demonstrated by Wei and Loh (39) with constant infusion into the peri-aque ductal gray fourth ventricular space of rat brain. Since the relative affinities of opiates and opioid peptides for these two opiate receptor sites vary, a careful comparison of the analgesic potency and the relative ability to cause physical dependence may provide new insights. Recently, Frenk et al. (40) and Urca et al. (41) showed that intracerebroventricular injection of low doses of [Met] and [Leu]enkephalins or of high dose of morphine caused epileptic seizures and that all such seizures could be blocked by high doses of naloxone. However, morphine at low dose or enkephalin at high dose induced analgesia. Our data show that enkephalin receptors bind enkephalins with an affinity higher than that of opiates. In contrast, morphine receptors bind enkephalins and opiates with the reverse affinity. Thus, the data suggest that morphine receptors may play a role in analgesic effects, and enkephalin receptors mediate behavioral epileptic seizures.

Comparisons of the affinities of enkephalin and its analogs for the enkephalin and morphine sites suggest that the hydrophobic component of phenylalanine may be the structural component which results in the differential recognition of these two receptor sites. N-Cyclopropylmethyl-norleu-enkephalin, which contains a cyclopropylmethyl group at the N position (Fig. 8) and a hydrophobic group at the C-19 position of oripavine, binds to the enkephalin receptor better than naloxone.

We have screened many cultured neuroblastoma cells for opiate receptor binding. Although many of these bear enkephalin receptors, no cells have yet been found to have the morphine receptor sites. Since opiate receptors in situ are continually exposed to enkephalins, it is theoretically possible that exposure of receptors to enkephalins causes some kind of transition which leads to new binding characteristics. This possibility was tested by treating neuroblastoma cells with morphine (10⁻⁷ M) for 24 h; however, the binding properties did not change with this treatment.

It is not possible at this time to determine whether or not both receptors normally interact with [Met]enkephalin and [Leu]enkephalin or related endorphins. It is quite possible that other, yet to be discovered opiate-like substances, such as that described recently by Spector and his colleagues (42, 43), may exist. These may serve as neurotransmitter or modulator substances for one of these two opiate receptors, especially for the morphine receptor sites which on the basis of the present studies are apparently less likely to interact normally with the enkephalin peptides. Our data suggest that the endogenous substances which normally interact with the morphine receptors may not yet be known.

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Multiple Opiate Receptors

Multiple opiate receptors. Enkephalins and morphine bind to receptors of different specificity.
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