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Multiple Opiate Receptors: Different Regional Distribution in the Brain and Differential Binding of Opiates and Opioid Peptides

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SUMMARY

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In rat brain membrane preparations, the parenterally and orally active peptide, [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin, binds to morphine receptor sites ([^{3}H]naloxone or [^{3}H]dihydromorphine binding sites) with an affinity higher than that for enkephalin receptor sites ([^{125}I] [D-Ala^2, D-Leu^5]-enkephalin binding sites). [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin binds to morphine receptor sites stereospecifically, in a saturable manner and with characteristics similar to that of [^{3}H]dihydromorphine; this ligand can be used as an ^{125}I-labeled probe to measure specific binding to morphine receptor sites. Na^+ decreases and Mn^{2+} increases the binding capacity with a concomitant reduction of affinity for [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin. This peptide does not bind to neuroblastoma cells with high affinity. The brain regional distribution of binding of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin or [^{3}H]naloxone and [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin are different. The differential potency of binding of opiate agonists, antagonists, mixed agonist-antagonists, enkephalins and enkephalin analogues is studied by competition of binding of [^{3}H]naloxone or [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin (morphine receptor) and of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin sites (enkephalin receptor). All of these results support the contention that there are multiple opiate receptors with differing characteristics.

INTRODUCTION

H-Tyr-Gly-Gly-Phe-Leu-OH([Leu^5]-enkephalin) and H-Tyr-Gly-Gly-Phe-Met-OH([Met^5]-enkephalin) are two naturally occurring morphine-like substances (1, 2). These two enkephalins, which are widely distributed in the brain and gastrointestinal tract of various species (1-11), inhibit the electrically driven contraction of the isolated guinea pig ileum and the mouse vas deferens (1). Intracisternal injection of enkephalins elicits many morphine-like effects such as analgesia (12, 13), growth hormone and prolactin release (14) and addiction and physical dependence (15, 16). These peptides also bind to opiate receptors with very high affinity (17-21).

Pharmacological studies using a variety of narcotic substances have led Martin and colleagues (22, 23) to suggest the presence in brain of multiple opiate receptors (i.e., putative σ, κ and μ receptors). Recent studies have shown that the potency of the enkephalins in inhibiting the binding of [^{3}H]naloxone to rat brain membrane preparations is less than that exhibited by their inhibition of binding of [^{3}H]enkephalin (21, 24-26) or [^{125}I]enkephalin (26). However, the potency of narcotic drugs in inhibiting the binding of these two labeled ligands is

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reversed (21–26). Lord *et al.* (21) observed a difference in the rank order of potency by opioid peptides and opiates in the mouse vas deferens and guinea pig ileum. Frenk *et al.* (27) reported that different pharmacological responses were obtained by injection of enkephalins and opiate agonists into certain regions of rat brain. Law and Loh (28) observed that the binding of [^{3}H]dihydromorphine and [^{3}H]leu-enkephalin were differentially affected by Na^+, temperature and N-ethylmaleimide and 3,5-diiodo-4-diazosulfonilic acid. These data support the possible existence of multiple opiate receptors.

Using very low concentrations of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin or [^{3}H]narcotics, conditions under which the high affinity binding sites for each labeled ligand are selectively detected (29), reveals that there are at least two distinct opiate receptor populations present in rat brain membrane preparations (29). One of these binds enkephalin better than morphine (enkephalin receptor sites) while another binds morphine better than enkephalin (morphine receptor sites). Sodium ions decrease the binding to both sites. The relative affinities in the absence of Na^+ for these two binding sites are [D-Ala^2, Leu^5]-enkephalin $\geq$ [Leu]-enkephalin or [Met]-enkephalin > naloxone $\geq$ morphine for the enkephalin binding sites and morphine > naloxone > [D-Ala^2, Leu^5]-enkephalin > [Met]-enkephalin > [Leu]-enkephalin for the morphine receptor sites.

Childers *et al.* (30) have compared the potency of a group of opiate drugs in competing for the binding of [^{3}H]opiates and [^{3}H]enkephalin. The differences in these two binding assays seem to depend on the structural difference at the C_6–C_8 region of opiates and Childers *et al.* preferred to explain the results on the basis of a model with several points of attachment to a single opiate receptor (30).

Roemer *et al.* (31) reported that the peptide (Sandoz FK 33-824), Try-D-Ala-Gly-N$^{\alpha}$-MePhe-Met(O)-ol, is orally active and about 30,000- and 1000-times more potent than [Met]-enkephalin and morphine, respectively, after intracerebroventricular administration to mice. In the present paper, we describe our finding that this peptide has a much higher affinity for morphine than for enkephalin receptor sites. The iodinated [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin also binds to morphine receptor sites stereospecifically, saturatably and with characteristics very similar to those of opiate narcotics. The potency of a variety of opiates and enkephalin analogues in inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin, [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin and [^{3}H]naloxone are also compared. In addition, the differential binding of these three labeled ligands to various regions of the rat brain is discussed.

MATERIALS AND METHODS

Enkephalins and their analogues were synthesized by Dr. S. Wilkinson, the Wellcome Research Laboratories, Beckenham, England. [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin and [D-Met^2, Pro^5]-enkephalin were purchased from Peninsula Laboratories, San Carlos, Calif. Morphine sulfate was obtained from Mallinckrodt, naloxone from Endo Laboratory, butorphanol and oxilorphan from Bristol Laboratories and pentazocine from Sterling-Winthrop Research Laboratories. Unlabeled monoiodinated [D-Ala^2, D-Leu^5]- and [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalins were prepared by the method of chloramine T and purified in gel filtration (Bio-Gel P2) and confirmed by the change of OD_{260}/OD_{280} absorption ratio. [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin and [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin were prepared as described previously (26, 29, 32). The specific activity of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin was about 1 to 2 Ci per μmole. [^{3}H]Naloxone (23 Ci per mmole) was purchased from New England Nuclear.

The crude brain membrane preparations were prepared as described previously (29) by the method of differential centrifugation in isotonic sucrose solution. Whole brain (without cerebellum) from Sprague-Dawley rats (150 to 200 g) is homogenized in 10 volumes (v/w) of 0.32 M sucrose with a Polytron PT-20 for 1 minute at a setting of 3. The homogenates are centrifuged at 6,000 $\times$ *g* for 15 minutes to remove the nuclei and

mitochondria. The supernatants obtained from two such centrifugations are combined and centrifuged at 40,000 × *g* for 30 minutes. The pellets are resuspended in 5 volumes (original wet weight) of 5 mM Tris·HCl, pH 7.7, and allowed to swell for 30 minutes. The synaptosomes thus obtained are disrupted with a Polytron homogenizer and centrifuged at 6000 × *g* for 15 minutes. The supernatants are centrifuged at 40,000 × *g* for 30 minutes. 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°.

For studying the brain regional distribution, rat brains were dissected into nine pieces using a modification of the procedure described in Miller *et al.* (5). Each brain is removed and placed with its ventral surface up on an ice-cold petri dish. The brain is initially separated into sections of forebrain, midbrain, and brain stem plus cerebellum by two cuts along the coronal plane, one at the level of the optic chiasm and the other at the level of the mamillary bodies. The "striatums" are removed from the forebrain section and the "limbic system," consisting of olfactory tuberculum, and the septal nuclei are separated from the "frontal cortex" using the lateral margins of the olfactory tract as a guide. Two cuts in the sagittal plane are then made in the midbrain section, extending from the cortical margins of the hypothalamus dorsally to the lateral vertricles. The "hypothalamus" is then separated by a horizontal cut at the level of the anterior commissure from the remaining column of tissue which constitutes the "thalamus." The "hippocampus" and the overlying "sensormotor cortex" are then teased free and separated. The dissection is completed by separating the "cerebellum" from the "brain stem." The brain tissues are homogenized in ice-cold isotonic sucrose (0.32 M) by a Polytron PT-20 (setting at 3.5). The whole membrane particulate samples are centrifuged at 40,000 × *g* for 30 minutes. The synaptosomes are disrupted with a Polytron after suspension (30 minutes) in 5 mM Tris·HCl, pH 7.4. This step is repeated once. The membrane pellets are then incubated with 0.1 M NaCl for 60 minutes to dissociate the endogenous, bound enkephalin as suggested by Simantov *et al.* (33). The final membrane pellets are washed twice with 50 mM Tris·HCl, pH 7.7, and suspended in the same buffer for the binding assays.

Binding assays (24° for 60 minutes) are performed essentially as described previously using a filtration method (26, 29). The protein concentration is about 0.5 to 1 mg/ml. For the competition curves, the concentrations of labeled ligands are 0.05 nM, 0.1 nM and 0.4 nM for [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin, [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-01]-enkephalin and [^{3}H]naloxone, respectively, unless otherwise indicated. The total incubation volumes are 0.25 and 2 ml for ^{125}I- and ^{3}H-labeled ligands, respectively. Non-specific binding is determined in the presence of 0.1 μM [D-Ala^2, D-Leu^5]-enkephalin and 0.1 μM naloxone. The binding reaction is stopped by rapidly filtering through GF/C glass filters and the filters are washed twice with 10 ml of ice-cold Tris·HCl buffer under vacuum. All assays are performed in duplicate and the variability of the duplicates is usually less than 10% of the mean. The protein concentration is determined by the method of Lowry *et al.* (34) using crystalline bovine serum albumin as standard.

RESULTS

1. *Preference of [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin for morphine receptor sites.* We have shown previously (29) that enkephalins and their metabolically stable analogues, [D-Ala^2, Leu^5]-, [D-Ala^2, D-Leu^5]- and [D-Ala^2, Met^5]-enkephalins are more potent in inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin (enkephalin receptor sites) than that of [^{3}H]naloxone and [^{3}H]dihydromorphine (morphine receptor sites). The present study indicates, in contrast, that [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin (Sandoz FK 33-824) is much more potent in inhibiting the binding of [^{3}H]naloxone than [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin in the absence of Na^+ (Fig. 1).

The difference is about 20-fold in the apparent IC_{50} values. Thus [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin serves as an enkephalin analogue with preferential binding to morphine receptor site (29). Consistent with the previous report (31) that Na^+ greatly reduces the potency of this peptide in inhibiting the binding of [^{3}H]naloxone, a nearly 30-fold reduction in affinity is observed (Fig. 1), suggesting that this peptide has agonist properties (35).

2. *Binding properties of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin.* [D-Ala^2, MePhe^4, Met(O)^5-ol] was labeled with ^{125}I and found to be bound to opiate receptor sites with characteristics similar to those expected for morphine receptor sites (29). The binding of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol] is inhibited by levorphanol at nM concentrations and by the biologically much less active stereoisomer, dextrorphan, at much higher concentrations (Fig. 2). Morphine, naloxone and [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin are also very potent in inhibiting this binding. The natural enkephalins and their stable analogues show lower potencies. The order of relative potencies is morphine > naloxone > [D-Ala^2, Leu^5]-enkephalin > [Met^5]-enkephalin ≥ [Leu^5]enkephalin. The same or-

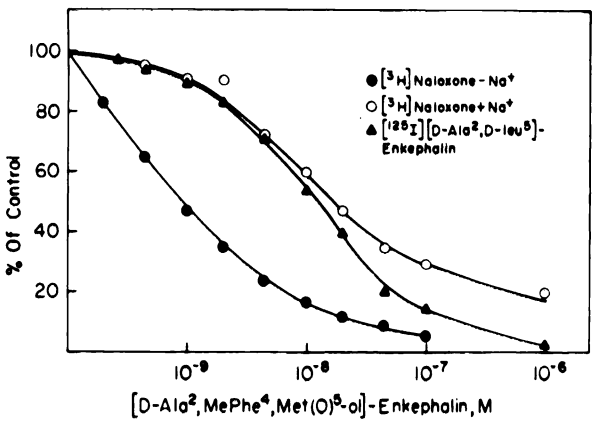

FIG. 1. *Competition curves of [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin in inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin* (▲) *and [^{3}H]naloxone in the absence* (●) *and presence* (○) *of Na^+*

Values represent the mean of duplicate samples (which are ±5% of the mean). The concentrations of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin and [^{3}H]naloxone are 0.05 nM and 0.4 nM, respectively.

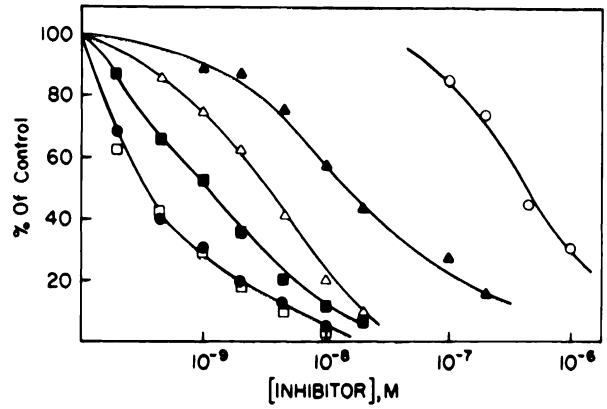

FIG. 2. *Inhibition curves of the binding of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin (0.2 nM) by levorphanol* (●), *dextrorphan* (○), *[Leu]-enkephalin* (▲), *morphine* (□), *naloxone* (■) *and [D-Ala^2, Leu^5]-enkephalin* (△)

Values represent the mean of duplicates, which are ±5% of the mean.

der exists in competing for the binding of [^{3}H]naloxone and [^{3}H]dihydromorphine (29). Furthermore, the binding of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin is reduced in the presence of Na^+ (not shown), and saturation occurs at very low concentrations (Fig. 3) with an apparent dissociation constant (K_d) of 0.8 nM. A linear Scatchard plot is obtained at concentrations below 5 nM. Thus, [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin can be used as a ligand of high specific activity for the detection and study of morphine receptor sites.

3. *Differential effects of Mn^{2+} on the binding of [^{125}I] [D-Ala^2, D-Leu^5]- and [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalins.* Saturation binding curves show that Mn^{2+} does not significantly affect the binding of [^{125}I] [D-Ala^2, MePhe,^{4}, Met(O)^5-ol]-enkephalin at low concentrations (<0.5 nM). However, the binding is increased at concentrations of the peptide higher than 1 nM. The Scatchard plots (Fig. 3) reveal that Mn^{2+} actually increases the binding capacity (about 2-fold) with a concomitant reduction in the affinity (about 2.5-fold).

In contrast, the binding of low concentrations of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin is increased about 2-fold by Mn^{2+} (Fig. 4). The increase in binding is relatively smaller at higher concentrations. The Scatchard plots indicate that both the capacity and affinity for the high affinity sites (enkephalin receptor site) are increased. However, the interaction with the low affinity sites (morphine receptors sites) cannot be assessed clearly because of the high non-specific binding at concentrations greater than 10 nM.

4. *Differential binding in various regions of the brain.* Table 1 shows that marked differences exist in the binding activities of membrane preparations from various regions of the brain. The ratio of binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin to the binding of [^{3}H]naloxone and [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin varies from 5 to 1, suggesting a non-uniform distribution of the two populations of opiate

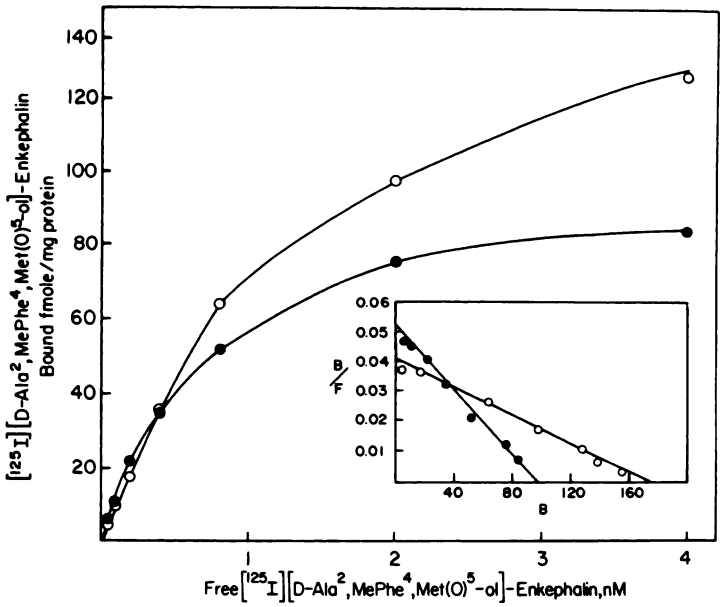

FIG. 3. *Saturation binding isotherms and Scatchard plots (insert) of the binding of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin to brain membranes in the absence (●) and presence (○) of Mn^{2+}*

0.25 ml of brain membrane (0.5 mg per ml protein) is incubated with various concentrations of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin (specific activity, 0.6 Ci per μmole) in the absence and presence of Mn^{2+} at 24° for 60 minutes. Non-specific binding is determined in the presence of 10 μM of [D-Ala^2, Leu^5]-enkephalin. Values represent the means of duplicate determinations, which are 10% of the mean. Note: The apparent dissociation constants are 0.8 and 2.5 nM in the absence and presence of Mn^{2+}. Both are linear under the conditions studied.

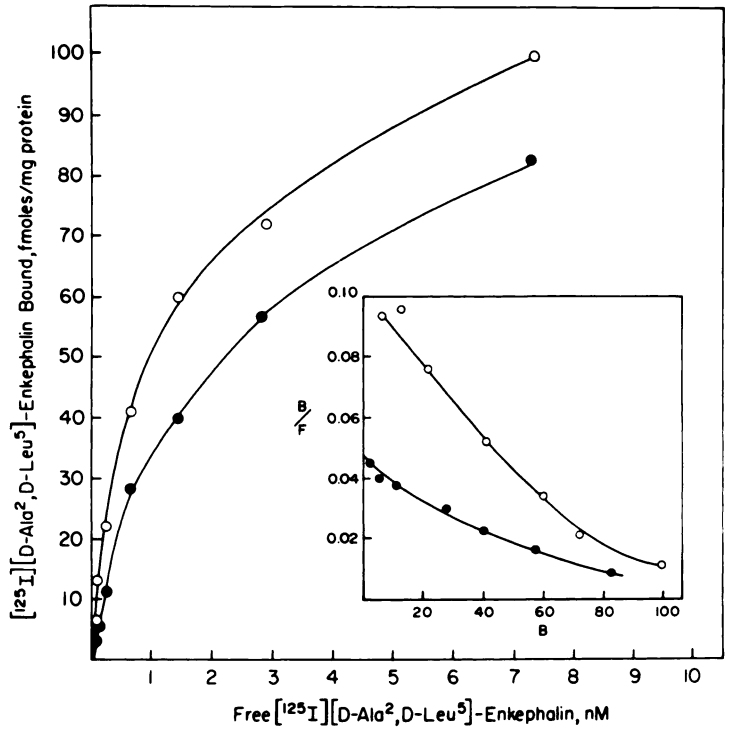

FIG. 4. *Saturation binding isotherms and Scatchard plots for the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin to brain membrane in the absence (●) and presence (○) of Mn^{2+}*

0.1 ml of brain membranes (0.8 mg/ml protein concentration) is incubated with various concentrations of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin (specific activity, 2 Ci per μmole) in the absence and presence of Mn^{2+} at 24° for 60 minutes. Non-specific binding is determined in the presence of 10 μM of [D-Ala^2, Leu^5]-enkephalin.

binding sites. The maximal binding capacities of the two ligands used to assess morphine sites, as measured with [^{3}H]naloxone and [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin, are quite similar to each other in all areas and suggest again that both ligands bind to the same receptor population. Preparations from the cortex, striatum and limbic system bind about equally well, while those from the hippocampus, thalamus and hypothalamus bind somewhat less. The brain stem binds only one-half as much as the frontal cortex. Quite a different pattern of distribution of binding is found for the ligand, [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin. The frontal cortex and striatum bind best, while the sensomotor cortex and limbic system bind about half as much. The hippocampus, brain stem, thalamus and hypothalamus bind only about one-fourth as much as the frontal cortex. These data suggest that the thalamus and hypothalamus contain relatively more morphine binding sites. The differences in the distribution of binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin compared to [^{3}H]naloxone or [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin are not due to differences in the content of endogenous enkephalins since the membrane preparations are preincubated with 0.1 N NaCl for 1 hour and washed before being assayed. Under these conditions most of the bound, endogenous enkephalin is removed (33). The difference in the binding of [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin and [^{3}H]naloxone in the preparations from thalamus and hypothalamus is probably due to the difficulty in the accurate dissection of these two areas since they are obtained from different brains.

Since the difference in the apparent affinity of enkephalin and morphine binding sites for [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin is only about 4-fold (29), morphine recep-

TABLE 1

Comparison of the binding activities of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin, [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin and [^{3}H]naloxone to different brain regions

The binding was performed at concentrations of 0.05, 0.2 and 0.5 nM labeled ligands, respectively, at 24° for 60 minutes. Since the binding of these labeled ligands is characteristic of a bimolecular reaction without cooperative interactions (29), the equation,

$$K_d = \frac{[R_f][H_f]}{[RH]},$$

can be used to calculate the maximum binding capacity (R_f + RH) for each binding site, where K_d is the dissociation constant, $[R_f]$, $[H_f]$ and $[RH]$ are the concentrations of free receptor, free ligand and bound ligand, respectively. The corresponding dissociation constants are 1.5, 0.8 and 0.5 nM, respectively. Values are expressed as fmol per mg of membrane protein ± SE. The numbers in parentheses are the number of separate experiments.

Brain regions	Binding activities,[a] fmol/mg			
	[^{125}I] [D-Ala^2, D-Leu^5]-enkephalin	[^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin	[^{3}H]naloxone	ratio[b]
Frontal cortex	99 ± 17 (7)	108 ± 18 (3)	95 ± 6 (4)	1/1.1/1
Striatum	99 ± 12 (7)	142 ± 25 (3)	91 ± 20 (4)	1/1.4/1
Sensomotor cortex	65 ± 6 (7)	96 ± 15 (3)	71 ± 14 (4)	1/1.5/1.1
Limbic system	48 ± 5 (7)	90 ± 15 (3)	100 ± 16 (4)	1/1.9/1.8
Hippocampus	32 ± 4 (7)	72 ± 8 (3)	65 ± 6 (4)	1/2.3/2.0
Brain stem	20 ± 3 (7)	43 ± 6 (3)	44 ± 6 (4)	1/2.2/2.1
Thalamus	27 ± 5 (7)	120 ± 10 (3)	85 ± 15 (4)	1/4.5/3.2
Hypothalamus	14 ± 3 (7)	40 ± 16 (3)	73 ± 20 (4)	1/2.8/5.2

[a] The concentrations of labeled ligands were selected to ensure that the majority of the binding occurred to high affinity sites of the respective ligands. The IC_{50} values for [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin and naloxone in competing for their respective labeled ligands are the same in every region. However, the IC_{50} values for [D-Ala^2, D-Leu^5]-enkephalin in inhibiting [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin binding vary by a factor of about 2 to 3. IC_{50} values in hypothalamus and thalamus are higher than those of cortex and striatum. This is because of the relatively higher content of morphine receptors in the hypothalamus and thalamus. In these regions, some of the [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin is bound to morphine receptors and thus the content of enkephalin receptor is overestimated.

[b] Ratio of binding capacity of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin/[^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin/[^{3}H]naloxone.

tors will bind some radioactivity and the proportion bound to each site will depend upon the relative content of these two binding sites. Under the present experimental conditions, the competition between [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin (0.25 nM) and naloxone (Fig. 5) or morphine (not shown) for the binding sites in the frontal cortex preparation suggests that the majority (~80%) of the radioactivity is bound to enkephalin receptors having a low affinity for narcotics and a high affinity for enkepalin. In contrast, in preparations from the thalamus the major portion (70%) is bound to morphine receptors, which have a high affinity for narcotics and low affinity for enkephalin (Fig. 5, lower portion).

The apparent affinities of compounds in competing for the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin is also expected to vary for different regions depending upon the relative quantities of these two binding sites. Fig. 5 shows the relative ability of naloxone (upper) to inhibit the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin. The inhibition curves are extended over a span of four log units, and the apparent IC_{50} values for the frontal cortex and thalamus membrane preparations are 20 nM and 1 nM, respectively. Biphasic curves occur in both cases but their sensitivity to naloxone is reversed. The values obtained are very similar to those described previously (29). The apparent dissociation constants of naloxone for the enkephalin and morphine binding sites are 15 and 0.5 nM, respectively (29).

Morphine sulfate behaves very similarly to naloxone. It is much more potent in inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin to membrane preparations from the thalamus than from the cortex (not shown). The apparent IC_{50} value of naloxone for inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin in the preparations from brain stem and hippocampus is about 4 nM while a value of about 10 nM is observed for the limbic system. These values lie between those of cortex and thalamus and are consistent with the data presented in Table 1.

5. *Characteristics of opiate receptors in neuroblastoma cells.* Previously we reported (26) that neuroblastoma cells bear opiate receptors having properties very similar to those of enkephalin receptor sites, and that they bind enkephalins and opiates with high and low affinities, respectively. All attempts to demonstrate the existence of opiate receptor sites having the characteristics of morphine receptors have failed (29), suggesting that neuroblastoma cells possess only enkephalin receptor sites. This is further confirmed by binding studies using [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin. This iodinated peptide does not bind to neuroblastoma cells under conditions which show substantial binding to rat brain membrane, while [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin binds very well to neuroblastoma cells. Furthermore, the peptide competes for the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin in neuroblastoma cells with a low affinity (about 30 nM) in a way similar to that shown for enkephalin receptor sites in brain membrane preparations.

6. *Comparisons of opiates, enkephalins and their analogues.* All compounds, whether agonists, antagonists, mixed ago-

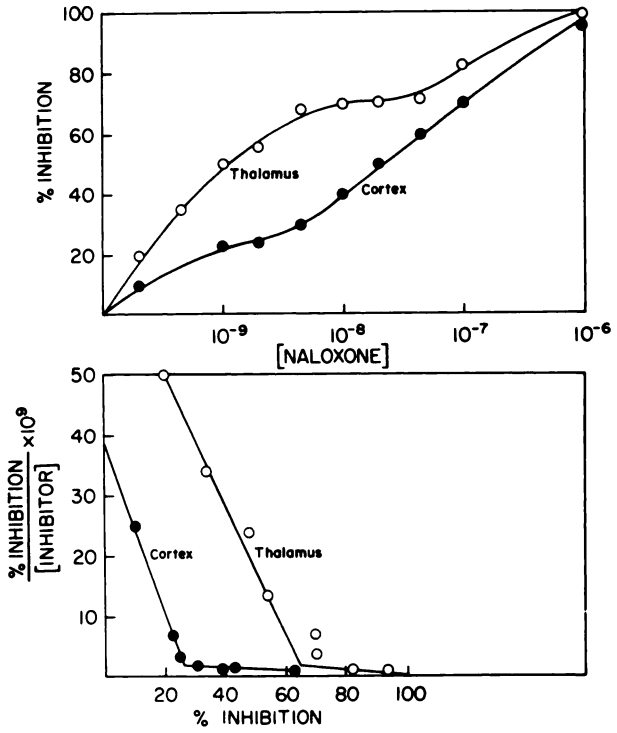

FIG. 5. *Competition curves (upper portion) of naloxone in inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin (0.25 nM) to membrane preparations from the fontal cortex (●) and thalamus (○)*

The lower portion shows the modified Scatchard plots. In competition experiments, when the fraction of total labeled ligand bound to membranes in the absence of competing ligand is less than 5%, the fractional occupancy of the competing ligand is almost equivalent to the percent inhibition of labeled ligand binding and the free component of the competing or radioactive ligand equals approximately the total ligand concentration. The Scatchard analysis can be plotted as % inhibition/ligand concentration versus % inhibition (36).

nist-antagonists or opioid peptides, exhibit similar affinities in inhibiting the binding of [^{3}H]naloxone and [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin (Table 2) in the absence of Na^+. However, considerable differences exist in the inhibition of binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin compared to [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin. Narcotics such as morphine, normorphine, oxymorphone and noroxymorphone are about 30 to 60-times more potent in competing for [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin than for [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin. Naloxone, nalorphine and levorphanol are about 10 to 25-times more potent in inhibiting the binding of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin than that of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin. So far, all mixed agonist-antagonists display less difference in their affinity for these two binding sites, as shown by low IC_{50} ratios (Table 3). In contrast, opiate peptides behave very differently (Table 2). [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin appears very similar to opiate agonists, being more potent in competing for the binding of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin or [^{3}H]-naloxone than of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin. [D-Met^2, Pro^5]-enkephalin displays similar properties but the difference for both binding sites is reduced. However,

TABLE 2

Comparison of the potency of opiates and enkephalin analogues in competing for the [^{125}I] [D-Ala^2, D-Leu^5]enkephalin, [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin and [^{3}H]naloxone binding sites

IC_{50} values (the concentration of competing ligand causing 50% inhibition of specific binding) are estimated from the competition curves using concentrations of 0.05, 0.2 and 0.38 nM for [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin, [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin and [^{3}H]naloxone, respectively. Values are expressed as nM ± SE of 3 to 6 separate experiments.

Compounds	IC_{50}, nM			
	[^{125}I] [D-Ala^2, D-Leu^5]-enkephalin	[^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin	[^{3}H]naloxone	
			$-Na^+$	$+Na^+$
Morphine	35 ± 5	0.4 ± 0.2	0.5	20
Normorphine	52 ± 14	0.8 ± 2	2	50
Oxymorphone	21 ± 6	0.3 ± 0.1	0.8	20
Noroxymorphone	83 ± 20	1.3 ± 0.4	1	55
Levorphanol	4.0 ± 1.0	0.3 ± 0.1	0.3	5
Nalorphine	8.0 ± 1.2	0.3 ± 0.1	1.5	5
Butorphanol	1.7 ± 0.3	0.4 ± 0.2	1.0	4
Oxilorphan	1.0 ± 0.1	0.3 ± 0.1	0.7	2.1
Pentazocine	67 ± 20	9.3 ± 0.7	10	30
Cyclazocine	1.2 ± 0.3	0.3 ± 0.1	0.4	1.3
Naloxone	15 ± 8	1.1 ± 0.2	1.0	1.0
[D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin	14 ± 5	1.2 ± 0.4	0.8	20
[D-Met^2, Pro^5]-enkephalin	4.7 ± 0.3	1.2 ± 0.4	1.5	10
[D-Ala^2, Met^5]-enkephalin	2.1 ± 0.2	5.3 ± 1.3	10	50
[Met^5]-enkephalin	4.4 ± 0.9	8 ± 2	9	50
	(1.6 + 0.4)[a]	(3 ± 1)[a]	(3.5)[a]	
[Leu^5]-enkephalin	3.0 ± 0.3	20 ± 5	28	250
	(1.5 ± 0.8)[a]	(4± 1)[a]	(3.4)[a]	
[D-Ala^2, Leu^5]-enkephalin	1.5 ± 0.1	4 ± 0.2	6	30
[D-Ala^2, D-Leu^5]-enkephalin	1.6 ± 0.2	4 ± 0.2	5	33
Monoiodo [D-Ala^2, D-Leu^5]-enkephalin	1.5 ± 0.2	4.5 ± 0.5	—	—
Monoiodo [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin	20 ± 5	1.4 ± 0.3	—	—

[a] Mn^{2+} largely increases the affinities for receptor sites. The values in the parentheses were obtained in the presence of 1 mM $MnCl_2$.

TABLE 3

Comparison of the relative affinities of binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin and [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin with the sodium ratio of [^{3}H]naloxone binding

Compound	IC_{50} ratio[a]	Sodium ratio[b]
	[^{125}I] [D-Ala^2, D-Leu^5]-enkephalin / [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin	$\frac{+Na^+}{-Na^+}$
Morphine	75	40
Normorphine	65	35
Oxymorphone	70	25
Noroxymorphone	64	55
Levorphanol	13	15
Nalorphine	27	3
Butorphanol	4.3	4
Oxilorphan	3.3	3
Pentazocine	7.2	3
Cyclazocine	4.0	3
Naloxone	15	1
[D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin	12	25
[D-Met^2, Pro^5]-enkephalin	3.9	6
[D-Ala^2, Met^5]-enkephalin	0.4	5
[Met^5]-enkephalin	0.73	6
[Leu^5]-enkephalin	0.16	9
[D-Ala^2, Leu^5]-enkephalin	0.40	5
[D-Ala^2, D-Leu^5]-enkephalin	0.40	7

[a] The ratio of IC_{50} values in inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin and [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin.

[b] The ratio of IC_{50} values in inhibiting the binding of [^{3}H]naloxone in the presence and absence of 0.1 M Na^+.

the natural enkephalins, [D-Ala^2]- and [D-Ala^2, D-Leu^5]-enkephalins are all more potent in inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin than that of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin. Unlabeled monoiodinated [D-Ala^2, D-Leu^5]- and [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin compete for the binding of the ^{125}I-labeled peptides with potencies nearly the same as those of the native peptides. This confirms previous observations (29, 32) and is consistent with the present data on ^{125}I-labeled enkephalin analogues.

Table 2 also shows the potencies for the competition of [^{3}H]naloxone binding in the absence and presence of Na^+. The "sodium ratio" is compared to the "IC_{50} ratio" for inhibition of binding of the [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin to [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin binding sites (Table 3). With the exception of naloxone and nalorphine all agonists and mixed agonist-antagonists show similar ratios. In contrast, all opioid peptides except [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin and [D-Met^2, Pro^5]-enkephalin display opposite values. Despite the reduction by Na^+ of the potency of these peptides in competing for [^{3}H]naloxone binding, they still remain more potent in inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin than of [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin.

DISCUSSION

The following evidence has accumulated from pharmacological and biochemical sources supporting the notion that there are multiple opiate receptors.

1. Lord *et al.* (21) and Beddell *et al.* (37) observed different potencies of opioid peptides and opiates in the mouse vas deferens and guinea pig ileum. Based on studies on chronic spinal dogs, Martin and colleagues (22, 23) observed differential effects of various opiates and proposed three types of opiate receptors.

2. Frenk *et al.* (27) reported different pharmacological responses on administration of enkephalin and opiates into different regions of the brain. The same group also observed (38, 39) that behavioral epileptic seizures were produced by intracerebroventricular injection of low doses of enkephalin and high doses of morphine and that these effects could be blocked by high doses of naloxone. Analgesic effects can be elicited by low doses of morphine and high doses of enkephalins.

3. Different potencies between enkephalins and opiates in their ability to compete for the binding of ^{3}H-labeled narcotics and [^{3}H] or [^{125}I]-labeled enkephalins or their substituted analogues have been reported by Lord *et al.* (21) and confirmed by many other laboratories (24–26, 28). These differential potencies are not due to homogeneous or heterogeneous cooperativities or agonist-antagonist conformations (29).

4. Differential effects of cations (28, 29), temperature, N-ethylmaleimide and 3,5-diiodo-4-diazosulfonilic acid on the binding of [^{3}H]enkephalin or [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin and [^{3}H]dihydromorphine have been observed.

5. In cultured neuroblastoma cells only one type of site (enkephalin receptor sites) is detectable for opiates, enkephalins and their analogues regardless of whether labeled narcotics, enkephalins or enkephalin analogues are used in the binding assays (26, 29).

6. In the present paper, we further describe the different regional distribution in brain of morphine receptors ([^{3}H]naloxone or [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin binding sites) and enkephalin receptors ([^{125}I] [D-Ala^2, D-Leu^5]-enkephalin binding sites).

Childers *et al.* (30) have also described similar results with opiates and enkephalins and their analogues. They proposed a model having several points of attachment to a single opiate receptor (30). Our data show that cultured neuroblastoma cells do not exhibit differential binding for opiates and enkephalins and only enkephalin receptor sites can be demonstrated regardless of the type of labeled ligand used. In addition, there are differences in the regional distribution for morphine and enkephalin receptor sites. It is difficult to explain these results on the basis of the model of multiple points of attachment to a single opiate receptor.

It is interesting to note that Sandoz's peptide (FK 33-824), [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin, differs substantially from other enkephalins including [D-Ala^2, D-Leu^5]-, [D-Ala^2, Leu^5], [D-Ala^2, Met^5]-enkephalins. This peptide binds to morphine receptor sites with an affinity about 20-times higher than that for enkephalin receptors. It has been shown that this peptide is about 30,000-times more active than the naturally occurring enkephalin in producing analgesia when it is injected into the mouse brain, and that it also produces analgesia after oral and parenteral administration. However, it does not produce analgesia in humans even though it promotes the release of prolactin and growth hormone (40). At present, it is not known whether the difference in the relative potencies toward these two opiate receptor sites has any relevance to these *in vivo* results.

The present results confirm previous observations (29) that mixed agonist-antagonists display similar affinities for enkephalin and morphine receptor sites. These drugs also show low sodium ratios in the binding assay of [^{3}H]naloxone (35). All opiate agonists and [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin show similar sodium ratios and IC_{50} ratios of enkephalin receptor sites to morphine receptor sites (Tables 2 and 3). However, these relationships do not hold for the enkephalins and their analogues. All of these peptides have sodium ratios of about 5 to 20. IC_{50} ratios for these two binding sites are about 12 and 4 for [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin and [D-Met^2, Pro^5]-enkephalin, respectively. All other peptides described in Tables 2 and 3 show IC_{50} ratios less than 1. Wei and Loh (15) have reported that enkephalins can cause physical dependence when they are infused continuously into the periaqueductal gray-fourth ventricular spaces of rat. In comparison, [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin is much more potent than [D-Met^2, Pro^5]-enkephalin and [D-Ala^2, D-

Leu^5]-enkephalin in inducing physical dependence. The median effective doses of these peptides in inducing physical dependence are 1.4, 7.0 and 300 nmoles per animal, respectively (41). These relative activities are inversely related to their IC_{50} ratio, but are not correlated to their sodium ratio. The opiate, mixed agonist-antagonists, known to be the least potent in inducing physical dependence, also show small IC_{50} ratios. All of these data imply that the relative activities for these two binding sites may play some role in inducing physical dependence.

The relative distribution of morphine and enkephalin receptor sites in the rat brain can be classified into three categories. (1) Cortex and striatum have about equal quantities of these two receptor sites. (2) The relative ratio of enkephalin to morphine receptor sites in the limbic system, hippocampus and brain stem is about 1 to 2. (3) Thalamus and hypothalamus contain relatively greater quantities of morphine receptor sites (about 4 to 1) than other regions. Therefore, it should be possible to use the hypothalamus plus thalamus as potential sources of relatively enriched morphine receptor sites. Because of the different content and the wide differences in the affinities of opiate agonists to these two opiate sites, the apparent IC_{50} values will vary depending upon the labeled ligand, its concentration and the brain region used. For instance, the apparent IC_{50} values for either morphine or naloxone in inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin are about 20 nM for region 1 (cortex, striatum), 10 to 4 nM for region 2 (limbic system, hippocampus, brain stem) and 1 nM for region 3 (thalamus, hypothalamus). Similar results have recently been reported by Della Bella *et al.* (42), who showed that the potency of morphine for the [^{3}H] [Met^5]-enkephalin binding sites varied among different brain regions, being most potent in brain stem, least potent in cortex and striatum, and intermediate in spinal cord.

Childer *et al.* (30) have compared the potencies of various opiates in competing for the binding of [^{3}H]-enkephalin and [^{3}H]dihydromorphine to rat brain membranes. They concluded that the difference among these opiates was due to their C-ring moieties of the opiate structure. Our results confirm this conclusion. The compounds with relatively less hydrophilic components in their C-ring moieties, such as butorphanol, oxilorphan, pentazocine cyclazocine and levorphanol, show less difference in inhibiting the binding of [^{125}I] [D-Ala^2, D-Leu^5]-enkephalin and [^{125}I] [D-Ala^2, MePhe^4, Met(O)^5-ol]-enkephalin or [^{3}H]-naloxone. In the series of opioid peptides, the natural enkephalins and [D-Ala^2, D-Leu^5]-substituted analogues bind to enkephalin sites better than to morphine receptor sites. Sandoz's peptide with N-methyl-Phe^4 and Met(O)^5-ol-substituted enkephalin show reverse potencies. Thus, it is possible to synthesize opioid peptides with selective potency and pharmacological profiles.

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