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Biochemical Characterization of High-Affinity ^{3}H-Opioid Binding

Further Evidence for Mu_1 Sites

STEPHEN L. NISHIMURA, LAWRENCE D. RECHT, AND GAVRIL W. PASTERNAK[1]

Cotzias Laboratory of Neuro-Oncology, Memorial Sloan-Kettering Cancer Center, and Departments of Neurology and Pharmacology, Cornell University Medical College, New York, New York 10021

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In saturation studies with [^{3}H]dihydromorphine, unlabeled D-Ala^2-D-Leu^5-enkephalin (1 nM) inhibited the high-affinity binding component far more potently than the lower-affinity one. Similarly, morphine (1 nM) inhibited the higher-affinity binding of ^{3}H-D-Ala^2-D-Leu^5-enkephalin to a greater extent than its lower-affinity binding component, consistent with a common high-affinity binding site for opiates and enkephalins. Treatment of tissue with either trypsin (1 μg/ml) or *N*-ethylmaleimide (25 μM) effectively eliminated the high-affinity binding component of a series of ^{3}H-opiates and opioid peptides. Competition studies following both treatments were consistent with a common high-affinity binding site. Both treatments also eliminated the ability of low morphine concentrations (<1 nM) to inhibit ^{3}H-D-Ala^2-D-Leu^5-enkephalin binding and of low D-Ala^2-D-Leu^5-enkephalin concentrations (<1 nM) to inhibit [^{3}H]dihydromorphine binding. Protection experiments examining *N*-ethylmaleimide (25 μM) inhibition of [^{3}H]dihydromorphine binding showed significant protection ($p < 0.002$) by both unlabeled D-Ala^2-D-Leu^5-enkephalin and morphine (both at 1 nM). When studied together, both naloxonazine and *N*-ethylmaleimide inhibited [^{3}H]dihydromorphine binding to a similar extent. Equally important, tissue previously treated with naloxonazine was far less sensitive to *N*-ethylmaleimide than was untreated control tissue, consistent with the possibility that both treatments affected the same site. Together, these results support the concept of a common high-affinity binding site for opiates and opioid peptides.

INTRODUCTION

Peripheral bioassay systems have played an important role in the identification of selective morphine (*mu*) and enkephalin (*delta*) sites. While morphine was more potent than the enkephalin peptides in the guinea pig ileum assay, the reverse selectivity was demonstrated in the mouse vas deferens (1). In addition, little cross-tolerance could be demonstrated between the morphine-like drugs and the enkephalin compounds in these peripheral systems (2, 3). Together, these studies provided strong evidence for receptors selective for either morphine or the enkephalins. Results from the central nervous system were similar. The existence of binding sites which selectively bound either opiates or enkephalins has been clearly shown by a number of laboratories (4–6). In addition to their different pharmacological specificities, these morphine- and enkephalin-selective sites also have different regional localizations, by both homogenate binding techniques (5) and autoradiography (7, 8).

Unlike the results of the peripheral bioassay systems, studies of opiate and opioid peptide analgesia noted a number of similarities between the different types of compounds. For example, the enkephalins, β-endorphin, and the opiates were all potent analgesics given intracerebroventricularly (9–14). Analgesic tolerance following chronic administration was reported for the enkephalins and β-endorphin (12–14). More important, both the enkephalins and β-endorphin showed cross-tolerance to morphine (12–15). Enkephalins also suppressed withdrawal symptoms in morphine-dependent animals (12). The common actions of the opioid peptides and opiates with regard to analgesia raised the possibility of a unique, common receptor for both types of ligands in contradistinction to the receptors observed in the peripheral system, which were selective for only one type.

Recent studies from our laboratory have suggested that the high-affinity opiate binding site, originally described in 1975 (16), might correspond to this common site. The high-affinity binding component for both opiates and enkephalins had a similar sensitivity to the irreversible

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antagonist naloxazone (17–20). These findings, coupled with detailed competition experiments, suggested that both classes of compounds bound with highest affinity to a common site (21). Other evidence also suggested that this high-affinity binding component corresponded to a distinct binding site. The high-affinity binding site had a unique developmental appearance (22, 23), phylogenetic distribution (24, 25), and regional localization (26). *In vivo* studies using both the naloxazone and the developmental models also indicated an association of the high-affinity binding site with opiate and opioid peptide analgesia (17–20, 22, 23, 27). In these studies, the lower-affinity (K_D 1–10 M) binding components selectively bound the various subclasses of opiates and opioid peptides (21, 28). We now present additional biochemical evidence supporting the existence of mu_1 (high-affinity) opioid binding sites.

MATERIALS AND METHODS

Male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). All ^{3}H-labeled ligands and Formula 963 scintillation fluor were obtained from New England Nuclear Corporation (Boston, Mass.). Trypsin (210 units/mg) was purchased from Worthington Biochemicals (Freehold, N. J.), and *N*-ethylmaleimide from Sigma Chemical Company (St. Louis, Mo.). Binding assays for all ^{3}H-labeled ligands were performed as previously described (29). Briefly stated, brain membranes were homogenized with a Brinkmann Polytron, centrifuged (49,000 × *g* for 20 min), resuspended, incubated at 37° for 30 min, recentrifuged, and resuspended. The particulate preparations were then subjected to further treatments or used immediately for assays. Treatments with naloxonazine were performed at 25° for 30 min, followed by two washes. Each wash consisted of resuspension, incubation at 37° for 10 min, and centrifugation. Previous studies have documented that this washing procedure effectively removed reversible opiates originally present at the same concentrations (30). Treatments with *N*-ethylmaleimide (25 μM), or trypsin (1 μg/ml; 210 units/mg) were performed at 25° for 30 min followed by centrifugation and resuspension before assaying. These methods have been utilized previously (29, 31). All binding assays were performed at 25° for 60 min and terminated by rapid filtration over Whatman glass-fiber filters (GF/B). All results are reported as specific binding, the difference between binding in the presence and absence of levallorphan (1 μM). All experiments were replicated a minimum of three times with similar results. Analysis of saturation experiments was performed by computerized nonlinear regression analysis of the saturation data. For the convenience of the reader, the results are presented graphically as Scatchard plots. Statistical analysis was determined, where appropriate, by either Student's *t*-test or analysis of variance.

RESULTS

Competitive interactions between mu and delta ligands. Morphine inhibited the binding of ^{3}H-labeled enkephalin derivatives in a multiphasic manner, with only a small portion of the radiolabeled enkephalin binding easily competed for by low morphine concentrations (<1 nM) (4, 5, 21). Similar multiphasic curves were observed in the competition of [^{3}H]dihydromorphine binding by unlabeled D-Ala^2-D-Leu^5-enkephalin (21). In addition, saturation studies of both [^{3}H]dihydromorphine and ^{3}H-D-Ala^2-D-Leu^5-enkephalin demonstrated high- (K_D < 1 nM) and low- (K_D 2–10 nM) affinity binding components (1, 4–6, 16–23, 27, 28, 30). To determine whether the morphine-sensitive ^{3}H-D-Ala^2-D-Leu^5-enkephalin binding and the enkephalin-sensitive [^{3}H]dihydromorphine

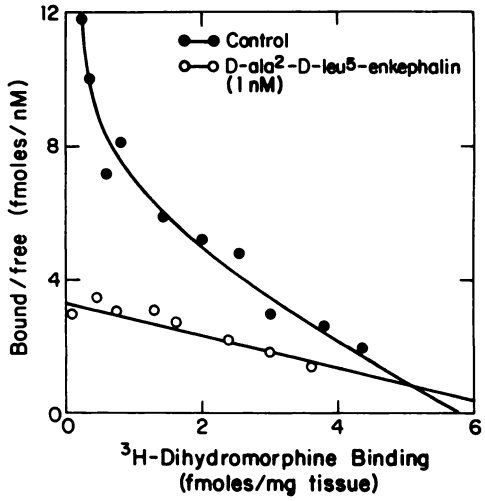

FIG. 1. *Saturation analysis of [^{3}H]dihydromorphine binding in the presence and absence of unlabeled D-Ala^2-D-Leu^5-enkephalin*

Rat brain particulate preparations were prepared and assayed in absence (●) and presence (○) of unlabeled D-Ala^2-D-Leu^5-enkephalin (1 nM) using concentrations of [^{3}H]dihydromorphine ranging from 0.02 to 2.3 nM. Results (specific binding) are from a representative experiment. Binding parameters were determined by computer analysis as described under Materials and Methods. The control binding was best fit with two binding components. The higher-affinity binding component had a K_D of 0.14 nM and a B_{max} of 0.91 fmole/mg of tissue. The lower-affinity component had a K_D of 1.0 nM with a B_{max} of 4.8 fmoles/mg of tissue. The binding data obtained in the presence of unlabeled D-Ala^2-D-Leu^5-enkephalin were best fit as a single component whose K_D was 2.1 nM and whose B_{max} was 6.9 fmoles/mg of tissue. The experiment was replicated three times with similar results.

binding observed in the competition studies corresponded to the high- or low-affinity component of each ^{3}H-ligand seen in the Scatchard plots, we performed saturation studies with [^{3}H]dihydromorphine and ^{3}H-D-Ala^2-D-Leu^5-enkephalin in the presence and absence of low concentrations of unlabeled D-Ala^2-D-Leu^5-enkephalin and morphine, respectively. First we examined the competitive interactions between [^{3}H]dihydromorphine and unlabeled D-Ala^2-D-Leu^5-enkephalin (Fig. 1). The control binding for [^{3}H]dihydromorphine yielded a curvilinear Scatchard which consisted of high- (K_D 0.14 nM) and lower-affinity (K_D 1.0 nM) components as determined by computer analysis. In the presence of unlabeled D-Ala^2-D-Leu^5-enkephalin (1 nM), the shape of the Scatchard plot became linear with an apparent affinity of 2.1 nM and with a B_{max} value (6.9 fmoles/mg of tissue) similar to the sum of the two B_{max} values of both components from the control curves (5.7 fmoles/mg of tissue). This greater effect of D-Ala^2-D-Leu^5-enkephalin at 1 nM upon the high-affinity component of [^{3}H]dihydromorphine compared with its lower-affinity binding component suggested that the "*mu* characteristics" of enkephalins resulted from interactions with the high-affinity [^{3}H]dihydromorphine binding site.

Similar results were seen using ^{3}H-D-Ala^2-D-Leu^5-enkephalin and unlabeled morphine (Fig. 2). Control binding was nonlinear, with a high-affinity (K_D 0.6 nM; B_{max} 1.9 fmoles/mg of tissue) and a lower-affinity component (K_D 3.2 nM; B_{max} 9.2 fmoles/mg of tissue). The presence of unlabeled morphine at 1 nM linearized the plot (K_D 2.5 nM; B_{max} 9.9 fmoles/mg of tissue). Thus morphine

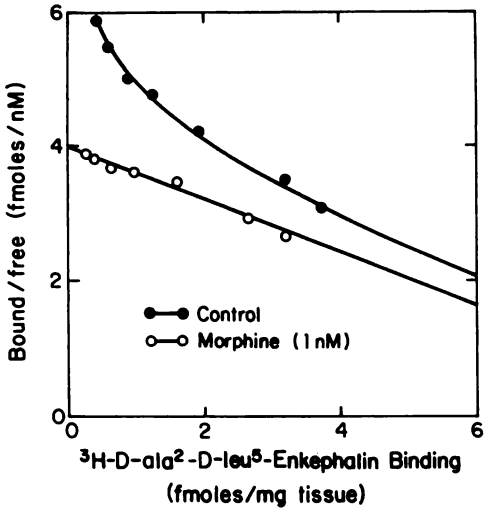

FIG. 2. *Saturation analysis of ^{3}H-D-Ala^2-D-Leu^5-enkephalin binding in the presence and absence of unlabeled morphine*

Rat brain homogenates were prepared and assayed in the absence (●) and presence (○) of unlabeled morphine (1 nM) using concentrations of ^{3}H-D-Ala^2-D-Leu^5-enkephalin ranging from 0.04 to 2.7 nM. Results (specific binding) are from a representative experiment. Binding parameters were determined by computer analysis as described under Materials and Methods. The control binding was best fit with two binding components. The higher-affinity component had a K_D of 0.6 nM and a B_{max} of 1.9 fmoles/mg of tissue. The lower-affinity component had a K_D of 3.2 nM and a B_{max} of 9.2 fmoles/mg of tissue. The binding data obtained in the presence of unlabeled morphine were best fit as a single component whose K_D was 2.5 and whose B_{max} was 9.9 fmoles/mg of tissue. The experiment was replicated three times with similar results.

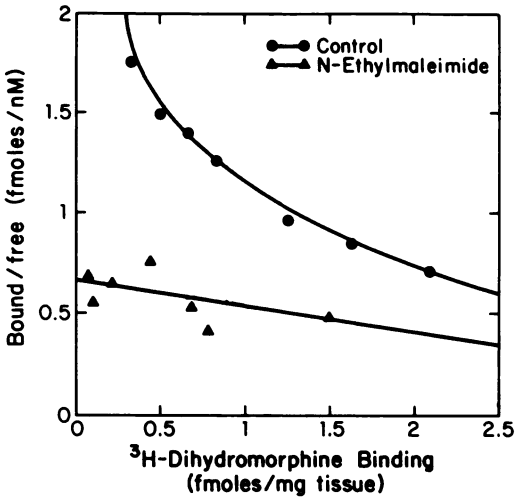

FIG. 3. *Saturation analysis of [^{3}H]dihydromorphine binding in reagent-treated tissue*

Rat brain homogenates were prepared and incubated with nothing or *N*-ethylmaleimide (25 μM; ▲) and washed as described under Materials and Methods. Saturation analysis was performed on tissue from both treatments at the same time in the same experiment using concentrations of [^{3}H]dihydromorphine ranging from 0.1 to 3.0 nM. Only specific binding was reported. Binding parameters were determined by computer analysis of the saturation data as described under Materials and Methods. The control binding was best fit with two binding components. The higher-affinity binding component had a K_D of 0.4 nM and a B_{max} of 0.64 fmole/mg of tissue. The lower-affinity binding component had a K_D of 7.0 nM and a B_{max} of 5.1 fmoles/mg of tissue. The data obtained in tissue treated with *N*-ethylmaleimide were best fit with a single component whose K_D was 8 nM and whose B_{max} was 5.4 fmoles/mg of tissue. The experiment was replicated three times with similar results.

also inhibited with greatest potency of the high-affinity binding component of ^{3}H-D-Ala^2-D-Leu^5-enkephalin.

The question of agonist versus antagonist conformations was addressed by performing saturation studies with the *mu* antagonist [^{3}H]naloxone in the presence and absence of D-Ala^2-D-Leu^5-enkephalin. As with previous results, the enkephalin inhibited the higher-affinity [^{3}H]naloxone binding to a greater degree than did the lower-affinity component.

Effects of N-ethylmaleimide on ^{3}H-opioid binding. Since ^{3}H-opiate binding was also quite sensitive to a variety of protein-modifying reagents, particularly sulfhydryl group reagents (29), we investigated the effects of a low concentration of *N*-ethylmaleimide (25 μM) on saturation studies using [^{3}H]dihydromorphine (Fig. 3) and ^{3}H-D-Ala^2-D-Leu^5-enkephalin (Fig. 4). The high-affinity binding components of both ^{3}H-ligands seen in control tissue were lost following incubations with these low concentrations of *N*-ethylmaleimide (25 μM). Competition experiments (Fig. 5) were also performed. In both the competition for [^{3}H]dihydromorphine binding by D-Ala^2-D-Leu^5-enkephalin and for ^{3}H-D-Ala^2-D-Leu^5-enkephalin binding by morphine, allowing tissue to react with *N*-ethylmaleimide markedly reduced the ability of low concentrations of unlabeled drug to compete for ^{3}H-ligand binding. For example, D-Ala^2-D-Leu^5-enkephalin at 1 nM lowered [^{3}H]dihydromorphine binding about 30% in control tissue. Following treatment of the membranes with *N*-ethylmaleimide, this low enkephalin concentration did not lower [^{3}H]dihydromorphine binding. Similarly, morphine (1 nM) decreased ^{3}H-D-Ala^2-D-Leu^5-en-

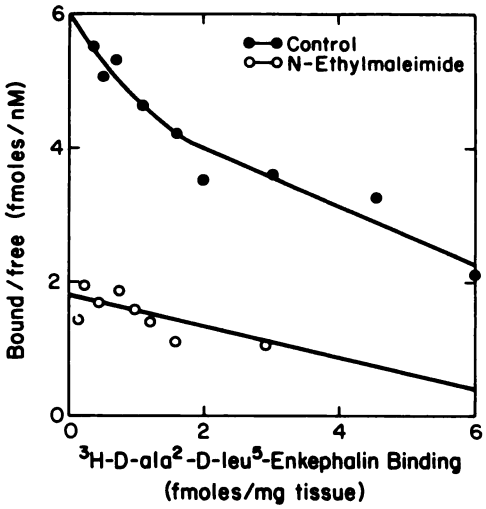

FIG. 4. *Saturation analysis of ^{3}H-D-Ala^2-D-Leu^5-enkephalin binding in reagent-treated tissue*

Rat brain homogenates were prepared, divided, and incubated with nothing (●) or *N*-ethylmaleimide (25 μM; ○) and washed as described under Materials and Methods. Saturation analysis was then performed on both treatments at the same time in the same experiments using a range of ^{3}H-D-Ala^2-D-Leu^5-enkephalin concentrations from 0.07 to 2.7 nM. Control binding was best fit with two binding components. The higher-affinity component had a K_D of 0.5 nM and a B_{max} of 1.4 fmoles/mg of tissue whereas the lower-affinity binding component had a K_D of 4.1 nM and a B_{max} of 12.1 fmoles/mg of tissue. The binding remaining after trypsin treatment was best fit with a single component. After trypsin treatment, the K_D was 4.8 nM and the B_{max} was 8.5 fmoles/mg of tissue. The experiment was repeated three times with similar results.

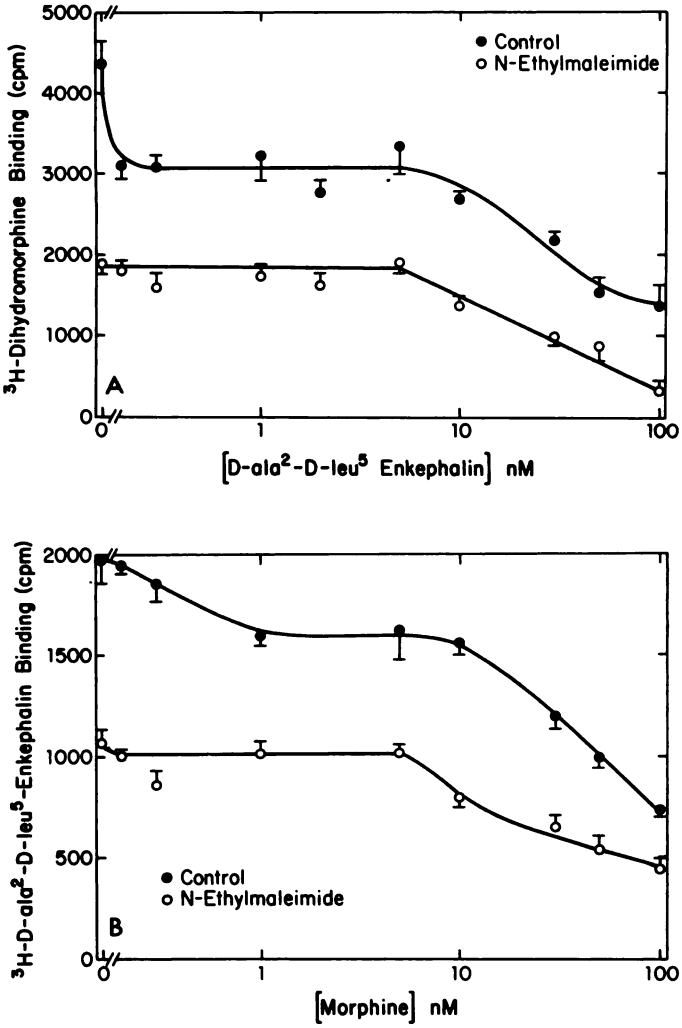

FIG. 5. *Effect of N-ethylmaleimide on inhibition of ^{3}H-opioid binding*
Rat brain particulate preparations were incubated with either nothing (●) or *N*-ethylmaleimide (25 μM; ○), washed, and assayed with (A) [^{3}H]dihydromorphine (2 nM) or (B) ^{3}H-D-Ala^2-D-Leu^5-enkephalin (2 nM) and the designated amount of unlabeled drug. Only specific binding is reported, and each value represents the mean ± standard error of the mean of triplicate samples from an experiment which was replicated three times with similar results.

kephalin binding almost 25% in control tissue, whereas binding in reagent tissue was virtually unaffected by 1 nM morphine. Thus, *N*-ethylmaleimide lowered the high-affinity binding component of *mu* and *delta* ligands observed in saturation studies as well as the morphine-sensitive radiolabeled enkephalin binding and the enkephalin-sensitive [^{3}H]dihydromorphine binding demonstrated in competition experiments.

Protection of [^{3}H]dihydromorphine binding from N-ethylmaleimide by morphine and D-Ala^2-D-Leu^5-enkephalin. Previous reports using a concentration of *N*-ethylmaleimide (500 μM) 20-fold greater than that used in our studies (25 μM) failed to show cross-protection between opiates and enkephalins (32). Using the low concentration of reagent, which was far more selective for the high-affinity binding component, we examined the ability of both morphine and D-Ala^2-D-Leu^5-enkephalin to protect [^{3}H]dihydromorphine binding from *N*-ethylmaleimide (Table 1). Unlike the previous report using 500 μM *N*-

TABLE 1

Protection of [^{3}H]dihydromorphine binding from N-ethylmaleimide by morphine and D-Ala^2-D-Leu^5-enkephalin

Rat brain particulate preparations were preincubated at 25° for 10 min with morphine (1 nM), D-Ala^2-D-Leu^5-enkephalin (1 nM), or nothing and then incubated for an additional 30 min at 25° in the presence and absence of *N*-ethylmaleimide (25 μM). All samples were then centrifuged (49,000 × *g* for 20 min), washed twice, and assayed with [^{3}H]dihydromorphine (1 nM). Each wash consisted of a 10-min incubation at 37°, centrifugation, and resuspension in the original volume of buffer. The results represent the means ± standard error of the mean of three separate experiments. Control binding (mean ± standard error of the mean) was 3257 ± 363 cpm in the three separate experiments. Unlabeled morphine and D-Ala^2-D-Leu^5-enkephalin significantly decreased the inhibition of [^{3}H]dihydromorphine binding by *N*-ethylmaleimide as determined by analyses of variance ($p < 0.002$; $F_{2,6} = 22.9$).

Addition	Inhibition of binding
Nothing	50 ± 6%
Morphine	28 ± 4%
D-Ala^2-D-Leu^5-enkephalin	9 ± 2%

ethylmaleimide, both the *mu* ligand morphine and the *delta* compound D-Ala^2-D-Leu^5-enkephalin protected [^{3}H]dihydromorphine binding from 25 μM *N*-ethylmaleimide ($p < 0.002$). In fact, D-Ala^2-D-Leu^5-enkephalin was more effective than morphine.

Effects of both N-ethylmaleimide and naloxonazine on ^{3}H-opiate binding. Previous work from our laboratory demonstrated a selective, persistent loss of the high-affinity binding component of a number of ^{3}H-labeled opiates and opioid peptides following treatment of tissue with naloxonazine or naloxazone (17–21, 28, 30). To examine whether *N*-ethylmaleimide treatment was inhibiting the same site as that blocked by naloxonazine, we examined the ability of *N*-ethylmaleimide (25 μM) to inhibit [^{3}H]dihydromorphine binding in tissue previously treated with either naloxone or naloxonazine (50 nM) (Fig. 6). Under the conditions used, binding in naloxone-treated tissue was the same as that in untreated tissue, indicating that the wash procedures effectively eliminated all of the naloxone. Both *N*-ethylmaleimide and naloxonazine alone decreased [^{3}H]dihydromorphine binding to a similar extent (48 ± 6% and 52 ± 3%, respectively). *N*-Ethylmaleimide decreased [^{3}H]dihydromorphine binding in tissue initially treated with naloxonazine only an additional 9% (63 ± 2% less than control), implying that the binding lost with the naloxonazine treatment included the binding most sensitive to the reagent.

Effect of trypsin on ^{3}H-opiate binding. Earlier studies examining the actions of proteolytic agents on ^{3}H-opiate binding raised the possibility of binding site heterogeneity (31). Subsequent phylogenetic studies have confirmed differing sensitivities of the various subtypes of binding sites to enzymes (25). In rats, a portion of binding was very sensitive to low trypsin concentrations (<1 μg/ml) whereas the remainder was far more resistant (31). To determine whether the trypsin-sensitive portion of binding corresponded to a particular subtype of binding site, we performed saturation experiments in trypsinized tissue with *mu* and *delta* ^{3}H-ligands. The control binding

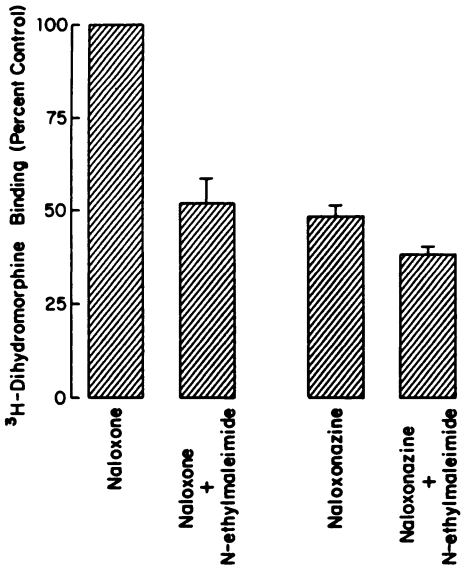

FIG. 6. *Effect of N-ethylmaleimide on [^{3}H]dihydromorphine binding in tissue pretreated with either naloxone or naloxonazine*

Rat brain particulate preparations were incubated with either naloxone or naloxonazine (50 nM), centrifuged, and washed twice by incubating for 10 min at 37° and centrifuging. Homogenate from each treatment was then divided and incubated with either nothing or *N*-ethylmaleimide (25 μM) at 25° for 30 min, centrifuged, resuspended, and assayed with [^{3}H]dihydromorphine (1 nM). Binding in all groups was expressed as the percentage of binding in the tissue treated only with naloxone. Under these wash conditions, naloxone is completely removed. Results represent the mean ± standard error of the mean of three separate experiments. Naloxonazine lowered binding 52 ± 3%. Following naloxone treatment, *N*-ethylmaleimide decreased binding 48 ± 6%. However, *N*-ethylmaleimide lowered binding only an additional 9% (63 ± 2%) in naloxonazine-treated tissue as compared with naloxonazine alone.

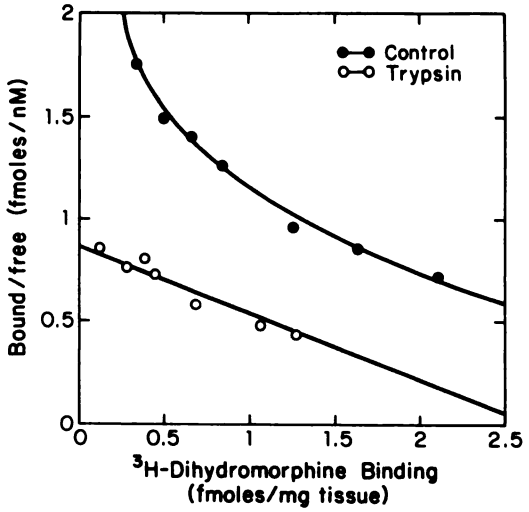

FIG. 7. *Saturation analysis of [^{3}H]dihydromorphine binding in enzyme-treated tissue*

Rat brain particulate preparations were incubated with nothing (●) or trypsin (1 μg/ml; ○) and washed as described under Materials and Methods. Saturation analysis was performed on tissue from both treatments at the same time in the same experiment using concentrations of [^{3}H]dihydromorphine ranging from 0.1 to 3.0 nM. Results (specific binding) are from a representative experiment. Binding parameters were determined by computer analysis as described under Materials and Methods. The control binding was best fit with two binding components. The higher-affinity binding component had a K_D of 0.4 nM and a B_{max} of 0.64 fmole/mg of tissue. The lower-affinity component had a K_D of 7.0 nM and a B_{max} of 5.1 fmoles/mg of tissue. The trypsin data were best fit with a K_D of 3.1 nM and a B_{max} of 2.6 fmoles/mg of tissue. The experiment was replicated three times with similar results.

for both [^{3}H]dihydromorphine (Fig. 7) and ^{3}H-D-Ala^2-D-Leu^5-enkephalin (Fig. 8) yielded curvilinear Scatchard plots which were dissected into high- and lower-affinity binding components by nonlinear regression analysis of the saturation curve. As previously reported (16, 17, 21), the density (B_{max}) of the higher-affinity was much less than the lower-affinity ones. Prior treatment of the tissue with trypsin (1 μg/ml) converted the curvilinear Scatchard plots to linear ones whose affinities (K_D 3 and 4.8 nM, respectively) corresponded more closely to the lower-affinity binding components. In contrast to the apparent complete loss of the high-affinity component, the lower-affinity components for both ligands appeared to be less sensitive to trypsinization, with decreases of only approximately 50% and 35%, respectively. The similar ability of trypsin to decrease preferentially the high-affinity binding component of [^{3}H]naloxone (data not shown) implied that the enzyme was not merely inducing an agonist/antagonist conformation shift. Similar results were seen with [^{3}H]ethylketocyclazocine and ^{3}H-labeled SKF 10,047. Treating the tissue with trypsin (1 μg/ml) virtually eliminated the high-affinity (K_D 0.6 and 0.2 nM, respectively) component of both ^{3}H-ligands while having far less effect on the lower-affinity (K_D 9 and 13 nM, respectively) components.

This decrease in binding by trypsin was due to its proteolytic actions. Prior administration of soybean tryp-

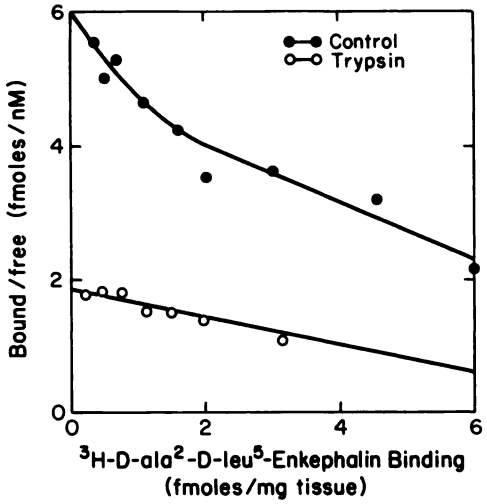

FIG. 8. *Saturation analysis of ^{3}H-D-Ala^2-D-Leu^5-enkephalin binding in enzyme-treated tissue*

Rat brain homogenates were prepared, divided, and incubated with nothing (●) or trypsin (1 μg/ml; ○) and washed as described under Materials and Methods. Saturation analysis was then performed on all three treatments at the same time in the same experiment using a range of ^{3}H-D-Ala^2-D-Leu^5-enkephalin concentrations from 0.07 to 2.7 nM. Control binding was best fit with two binding components. The higher-affinity component had a K_D of 0.5 nM and a B_{max} of 1.4 fmoles/mg of tissue, whereas the lower-affinity component had a K_D of 4.1 nM and a B_{max} of 12.1 fmoles/mg of tissue. The binding remaining after trypsin treatment was best fit with a single component (K_D was 4.8 nM with a B_{max} of 8.5 fmoles/mg of tissue). The experiment was repeated three times with similar results.

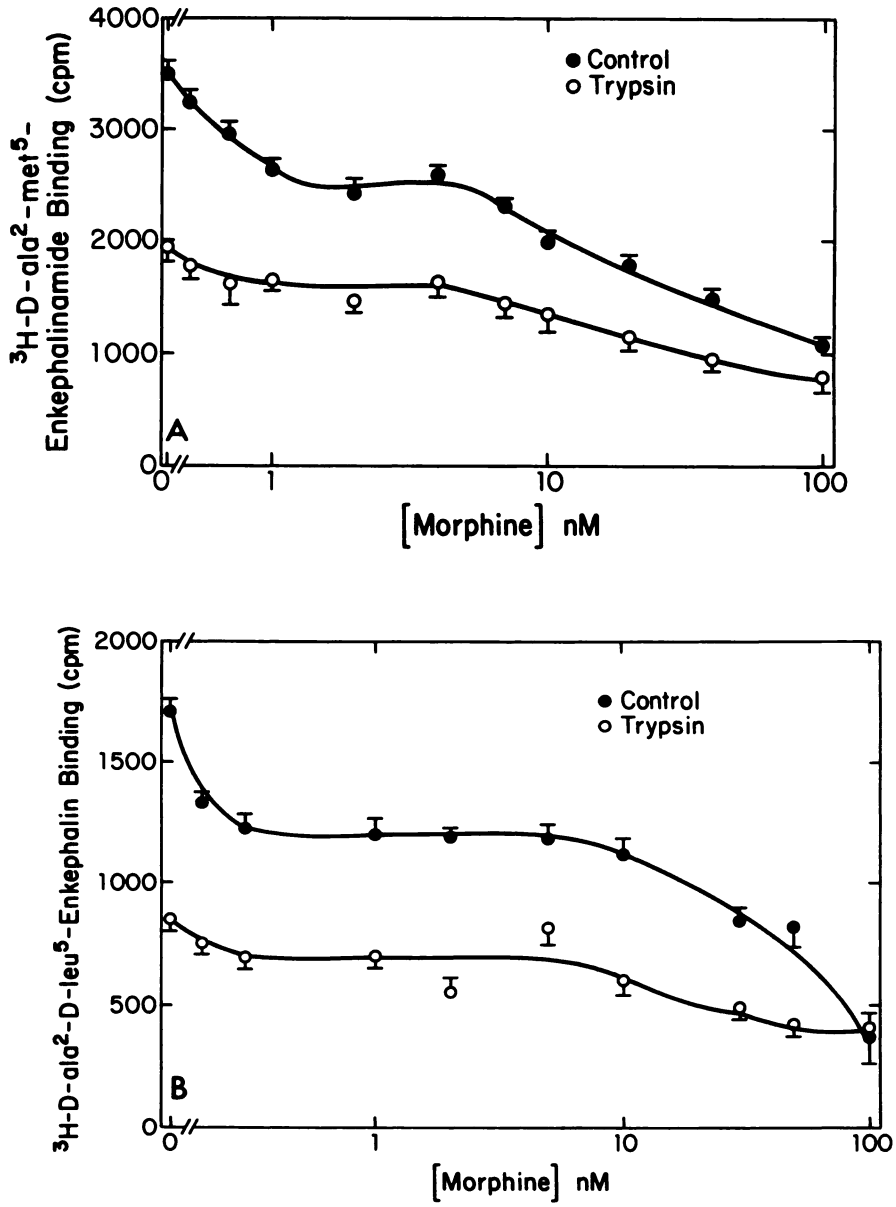

FIG. 9. *Effect of trypsin on the competition for ^{3}H-opioid binding by morphine*

Rat brain particulate preparations were incubated with nothing (●) or trypsin (1 μg/ml; ○) at 25° for 30 min, centrifuged, resuspended, and assayed with (A) ^{3}H-D-Ala^2-D-Leu^5-enkephalinamide (1.7 nM) or (B) ^{3}H-D-Ala^2-D-Leu^5-enkephalin (1 nM). Only specific binding is presented (means ± standard error of the mean) from a representative experiment which was replicated three times with similar results.

sin inhibitor (4 μg/ml of inhibitor with 1 μg/ml of enzyme) prevented any decrease in binding of either [^{3}H] dihydromorphine or ^{3}H-D-Ala^2-D-Leu^5-enkephalin. The actions of the trypsin were also confined to the initial 30-min incubation, since the inhibition of ^{3}H-opioid binding in samples where the soybean trypsin inhibitor was added immediately after the 30-min incubation was the same as that in samples where the trypsin was merely washed out without adding the inhibitor.

To examine further the possibility of a common high-affinity site for these opioids, we next performed detailed morphine competition experiments using control and trypsinized tissue (Fig. 9). A portion of both ^{3}H-D-Ala^2-Met^5-enkephalinamide and ^{3}H-D-Ala^2-D-Leu^5-enkephalin binding was quite sensitive to low morphine concentrations, replicating previous reports (4, 21). This biphasic competition profile suggested that both enkephalin compounds bound to several sites, presumably a *mu* site which bound morphine with high affinity and was therefore competed for by low morphine concentrations and a *delta* site which bound morphine far less potently. Treatment of the tissue with trypsin markedly reduced the ability of low morphine concentrations to decrease the binding of either ^{3}H-enkephalin analogue. At 1 nM, morphine lowered ^{3}H-D-Ala^2-Met^5-enkephalinamide binding approximately 30% in control tissue as compared with only 13% in trypsinized tissue. Morphine also lowered ^{3}H-D-Ala^2-D-Leu^5-enkephalin binding by approximately 30% in control tissue as opposed to only 15% in enzyme-treated tissue. Similar losses in the ability of morphine at low concentrations (<5 nM) to decrease the binding of the putative *kappa* ligand [^{3}H]ethylketocycla-

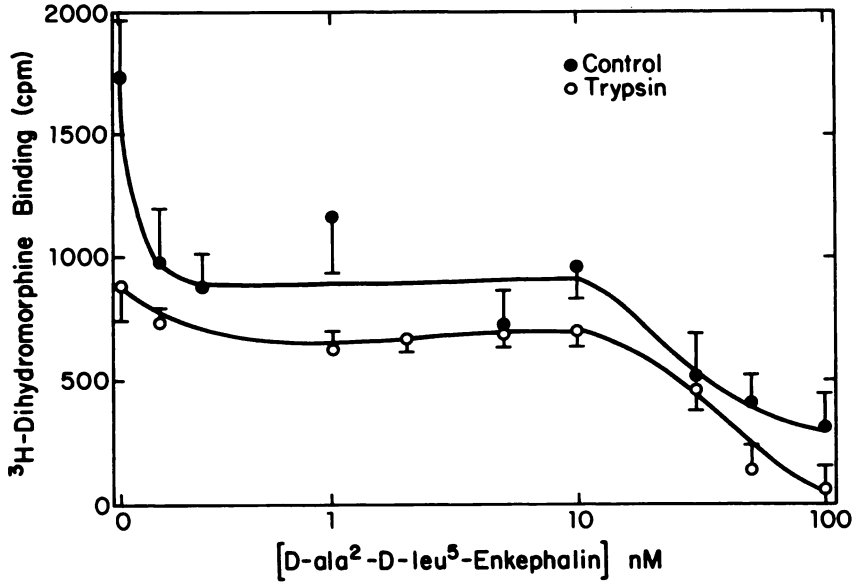

FIG. 10. *Effect of trypsin on the displacement of ^{3}H-opioid binding by D-Ala^2-D-Leu^5-enkephalin*

Rat brain homogenate was prepared with trypsin (1 μg/ml; ○) or nothing (●) for 30 min at 25° and then centrifuged, resuspended, and assayed with [^{3}H]dihydromorphine (1 nM) in the presence of varying concentrations of D-Ala^2-D-Leu^5-enkephalin. Points represent the means ± standard error of the mean of triplicate determinations from an experiment which was replicated three times with similar results.

zocine and the *sigma* ligand ^{3}H-labeled SKF 10,047 were observed following trypsin treatments.

Competition experiments examining the effects of D-Ala^2-D-Leu^5-enkephalin on [^{3}H]dihydromorphine binding in trypsinized tissue yielded similar results (Fig. 10). Trypsin treatment markedly decreased the ability of low concentrations of D-Ala^2-D-Leu^5-enkephalin to inhibit [^{3}H]dihydromorphine binding. For example, D-Ala^2-D-Leu^5-enkephalin (0.3 nM) depressed [^{3}H]dihydromorphine binding approximately 50% in control tissue but only 25% in trypsinized samples. A similar decrease in the sensitivity of ^{3}H-labeled SKF 10,047 and [^{3}H]ethylketocyclazocine binding to D-Ala^2-D-Leu^5-enkephalin (1 nM) in trypsinized tissue was observed.

DISCUSSION

Previous pharmacological studies have established the existence of *mu* (morphine) and *delta* (enkephalin) receptors in peripheral tissue (1). Correlating these pharmacological receptors with radioligand binding sites in the central nervous system has not been easy. Clearly, differences between the binding of radiolabeled opiates and enkephalins exist. Investigators have demonstrated selective sites for either the enkephalins or morphine (4, 6). Furthermore, the regional distribution of radiolabeled enkephalin binding differed from that of morphine-like drugs (5, 7, 8). However, both radiolabeled enkephalins and opiates bound to more than type of site, as indicated by their multiphasic competition profiles and curvilinear Scatchard plots demonstrating high- (K_D < 1 nM) and low- (K_D 1–10 nM) affinity binding components (1, 4–6, 16–28). These results were interpreted by many to mean that the high-affinity binding component [^{3}H]dihydromorphine represented *mu* sites and correlated with the lower-affinity component of radiolabeled enkephalin binding whereas *delta* sites corresponded to the high-affinity [^{3}H]enkephalin and low-affinity [^{3}H]dihydromorphine binding. Previous work from our laboratory raised serious questions about this interpretation (17–20, 22–25, 27), leading us to propose an alternative explanation (21). Our results suggested three classes of morphine and enkephalin binding sites. Both morphine and the enkephalins appeared to bind with highest affinity to a common high-affinity (mu_1) site (K_D < 1 nM). The lower-affinity (mu_2) binding component of [^{3}H]dihydromorphine (K_D approximately 3 nM) preferentially bound morphine far better than enkephalins whereas the lower-affinity binding component of ^{3}H-D-Ala^2-D-Leu^5-enkephalin (K_D approximately 5 nM) selectively bound enkephalins more potently than morphine and corresponded to the previously proposed *delta* receptor (1, 4–6). Our current studies support this concept of three classes of morphine and enkephalin binding sites.

In our current series of experiments, we examined the competitive interactions between enkephalins and morphine. As noted earlier, a portion of radiolabeled enkephalin binding was easily competed for by low morphine concentrations. To determine whether this morphine-sensitive binding corresponded to the high- or low-affinity component of ^{3}H-D-Ala^2-D-Leu^5-enkephalin binding, we performed saturation studies in the presence and absence of a low concentration of morphine (1 nM). Assuming competitive interactions, the unlabeled drug will lower the apparent affinity of the radiolabeled ligand; i.e., higher concentrations of radiolabeled ligand will be needed to saturate the binding sites in the presence of the unlabeled compound. The greater the affinity of an unlabeled compound for a particular binding site, the greater the change in apparent affinity of the radiolabeled ligand. The inclusion of morphine at 1 nM, a concentration sufficient to compete selectively for the morphine-sensitive ^{3}H-enkephalin binding in competition studies, had a far greater effect upon the higher-affinity component of ^{3}H-enkephalin binding than its

lower-affinity component. Similarly, the high-affinity component of [^{3}H]dihydromorphine binding was more sensitive to competition by D-Ala^2-D-Leu^5-enkephalin.

We next examined the sensitivity of the high-affinity binding component of a series of ^{3}H-opioids to a low concentration of *N*-ethylmaleimide. Saturation studies indicated that the high-affinity components of [^{3}H] dihydromorphine, ^{3}H-D-Ala^2-D-Leu^5-enkephalin, [^{3}H] ethylketocyclazocine, ^{3}H-labeled SKF 10,047, and [^{3}H] naloxone were all quite sensitive to this reagent. On the basis of these studies alone, it was not possible to determine whether the loss of the high-affinity component reflected the destruction of the site or its conversion to a lower-affinity one. However, the similar actions of the reagent on several ^{3}H-agonists and the antagonist [^{3}H] naloxone implied that these results could not be simply explained by a conversion between agonist and antagonist conformations. Thus, the similar sensitivities of all of these high-affinity binding components remained consistent with a common site, a possibility further supported by the competition studies. These competition experiments indicated that the loss of the high-affinity binding component on saturation analysis was associated with a marked loss of morphine-sensitive ^{3}H-D-Ala^2-D-Leu^5-enkephalin binding and of enkephalin-sensitive [^{3}H]dihydromorphine binding. Although not shown, a similar decrease in morphine-sensitive and enkephalin-sensitive [^{3}H]ethylketocyclazocine and ^{3}H-labeled SKF 10,047 binding was also observed following *N*-ethylmaleimide treatment of the membranes.

The ability of both morphine and D-Ala^2-D-Leu^5-enkephalin to cross-protect [^{3}H]dihydromorphine binding from *N*-ethylmaleimide also provided strong evidence for a common binding site. Previous attempts to demonstrate cross-protection with *N*-ethylmaleimide were unsuccessful (32). However, the concentration of reagent used in these earlier drugs (0.5 mM) was 20-fold higher than those used in our investigations (25 μM). The differences between these two studies might be explained by the greater selectivity of the high-affinity component for the low *N*-ethylmaleimide concentration.

Much of the previous work suggesting a common high-affinity morphine and enkephalin binding site was based upon the ability of the irreversible antagonists naloxazone and naloxonazine to block selectively the high-affinity binding of a series of ^{3}H-opiates and ^{3}H-opioid peptides. We therefore examined the sensitivity of [^{3}H] dihydromorphine binding to *N*-ethylmaleimide in tissue previously treated with naloxonazine. Treating membranes with *N*-ethylmaleimide alone lowered binding to a similar extent as naloxonazine. However, tissue whose high-affinity sites had been previously blocked with naloxonazine were far less sensitive to the reagent, implying that the naloxonazine had eliminated the reagent-sensitive binding site. The absence of an additive effect between the two treatments suggested that both treatments might involve the same site. It was interesting to note that the small decrease in [^{3}H]dihydromorphine binding produced by *N*-ethylmaleimide in naloxonazine-treated tissue was similar to that previously reported in goldfish brain membranes, a species with little or no high affinity [^{3}H]dihydromorphine binding (25). Therefore, this decrease might reflect the sensitivity of the lower-affinity (mu_2) site to the reagent.

The actions of trypsin were quite similar to those of *N*-ethylmaleimide. Again, the high-affinity binding of [^{3}H]dihydromorphine, ^{3}H-D-Ala^2-D-Leu^5-enkephalin, [^{3}H]ethylketocyclazocine, ^{3}H-labeled SKF 10,047 and [^{3}H]naloxone were quite sensitive to the low concentration of enzyme. In addition, the loss of the high-affinity binding was also associated with the loss of morphine- and enkephalin-sensitive binding of all four ^{3}H-ligands. As with the reagent, it was not clear whether the enzymes actually destroyed the site or merely changed its binding characteristics.

Our previous proposal (21) of three morphine and enkephalin binding sites (mu_1, mu_2, and *delta*) and our observations that *kappa* compounds such as ethylketocyclazocine and the *sigma* drug SKF 10,047 also bound with highest affinity to the mu_1 site (18, 19, 23, 28) were based upon a number of biochemical and pharmacological approaches. These included the effects of naloxazone and naloxonazine, the developmental appearances of these binding sites, their phylogenetic distribution, and their regional localization within the brain. These studies also included *in vivo* correlations of the common, high-affinity (mu_1) site with a number of specific opioid actions, such as analgesia, prolactin release, catalepsy, and acetylcholine turnover, but not other opioid actions (respiratory depression, sedation, growth hormone release, dopamine turnover, bradycardia, and the guinea pig ileum bioassay) (33–35). Our current results supported this concept of three morphine and enkephalin binding sites. In addition, they provided strong evidence against the hypothesis that the high-affinity [^{3}H]dihydromorphine site corresponded to the lower-affinity ^{3}H-D-Ala^2-D-Leu^5-enkephalin site and vice versa. Despite the mounting evidence for a common high-affinity site for opiates and opioid peptides, further work is needed to establish it firmly as a distinct receptor site. However, its proposal does provide an hypothesis which can be tested both *in vivo* and *in vitro*.

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Send reprint requests to: Dr. Gavril W. Pasternak, Department of Neurology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, N. Y. 10021.