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A Quantitative Study of [^{3}H]D-Ala^2-D-Leu^5-Enkephalin Binding to Rat Brain Membranes

Evidence That Oxymorphone Is a Noncompetitive Inhibitor of the Lower Affinity δ-Binding Site

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SUMMARY

The mechanism by which μ ligands inhibit the binding of prototypic δ agonists to preparations of brain membranes is controversial. Most investigators assume competitive inhibition. In this study, we examine the interaction of the μ agonist oxymorphone and δ agonist DSTLE (D-Ser^2-Thr^6-Leu-enkephalin) with [^{3}H]D-Ala^2-D-Leu^5-enkephalin (DADL) binding to membranes of rat brain. According to the two-site competitive model, μ ligands are competitive inhibitors at both sites. The two-site allosteric model supposes that μ ligands are competitive inhibitors at one binding site, and noncompetitive inhibitors at the other binding site. Quantitative analysis of DSTLE and oxymorphone binding demonstrated that the two-site allosteric model fit the data significantly better than did the two-site competitive model, and that oxymorphone is a noncompetitive inhibitor of the lower affinity [^{3}H]DADL-binding site. Autoradiographic studies demonstrated that the lower affinity [^{3}H]DADL-binding site (μ-noncompetitive binding site) had an anatomical distribution apparently indistinguishable from that obtained with [^{3}H]oxymorphone (type I pattern), supporting the hypothesis that the lower affinity δ-binding site is the δ-binding site of an opiate receptor complex consisting of interacting μ- and δ-binding sites.

INTRODUCTION

The classification of opiate receptors into μ, δ, and κ receptors is widely accepted and well supported by experimental data (1), although not proven at the molecular level. Recently, three novel models of the opiate receptors were proposed which share a common theme (2–4): a population of receptors which are composed of interacting μ- and δ-binding sites. Based upon ligand-binding studies, Rothman and coworkers (4–7) suggested the existence of a receptor complex which consisted of physically associated μ- and δ-binding sites. Lee and Smith, integrating physical and pharmacological data, proposed a protein-lipid model of the opiate receptor (3), and subsequently named this receptor the "beta-endorphin receptor" (8). In an elegant autoradiographic study, Bowen *et al.* (2) presented evidence that [^{3}H]DADL[1] labeled two anatomically and biochemically distinct δ-binding sites in rat caudate. The type II receptor, diffusely localized throughout the caudate, was not affected by alterations in the composition of the assay medium. In contrast, the type I receptor localized to striatal patches and the subcallosal streak was affected. Based upon condition-dependent alterations in the ligand selectivity pattern, it was suggested (2) that the type I receptor in the patches could interconvert between μ and δ conformations.

According to a one-site allosteric model, [^{3}H]LE labels a single class of δ receptors at which morphine is a mixed competitive-noncompetitive inhibitor (4–7). In view of

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[1] The abbreviations used are: DADL, D-Ala^2-D-Leu^5-enkephalin; DSTLE, D-Ser^2-Thr^6-Leu-enkephalin; OXY, oxymorphone; BIT, 2-(*p*-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanatobenzimidizole; FIT, *N*-phenyl-*N*-[1-(2-(*p*-isothiocyanato)phenylethyl)-4-piperidinyl] propanamide; MOPS, 3-[*N*-morpholino]propanesulfonic acid; BSA, bovine serum albumin; LE, Leu-enkephalin.

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the data briefly summarized above (2), Rothman and Westfall (7) postulated that [^{3}H]LE labeled two classes of binding sites *in vitro*, distinguished by the manner in which morphine inhibited [^{3}H]LE binding. According to this hypothesis, morphine is a pure competitive inhibitor at one class of sites, and a pure noncompetitive inhibitor at the other class of sites.

In this study, we examine this hypothesis by studying the interaction of μ agonist oxymorphone and δ agonist DSTLE with [^{3}H]DADL binding. We consider two models: the widely accepted two-site competitive model (1, 9) and a two-site allosteric model. The former model hypothesizes that [^{3}H]DADL labels two binding sites, and that μ ligands are competitive inhibitors at both sites. The latter model postulates that μ ligands inhibit [^{3}H]DADL binding competitively at one site, and noncompetitively at the other site.

In recent papers Rothman *et al.* (10, 11) reported that brain membranes pretreated with the δ-selective irreversible ligand FIT (12) were devoid of detectable higher affinity [^{3}H]DADL-binding sites. Using these membranes, μ agonists were apparent noncompetitive inhibitors of δ binding to the residual lower affinity binding sites (10, 11).

An important issue not addressed in those studies was if the noncompetitive interactions resulted from treatment of the membranes with the site-directed alkylating agent. To rule out this possibility requires that the noncompetitive interactions be demonstrated in membranes not treated with alkylating agent. The experiments in this paper address this issue. Using recently described methods of experimental design and analysis (13, 14) and membranes not treated with an alkylating agent, we show that the two-site allosteric model fits the data significantly better than does a two-site competitive model.

MATERIALS AND METHODS

Experimental design. The question addressed in this study requires distinguishing between similar models. One approach is an analysis of binding surfaces (13, 14). The essential concept of this approach is that the observed binding, B, is a function of two independent variables, D, the radiolabeled ligand, and I, the inhibitor: $B = F(D,I)$. Plotting F requires three axes for the concentration of D, the concentration of I, and the observed binding. Thus, the binding function describes a three-dimensional surface. When thought of in this manner, it is clear that a single displacement isotherm and saturation isotherm describe only a small portion of the binding surface, thus providing minimal information about F.

The goal of this quantitative study is to determine the functional form of F, i.e., determine the model which best fits the data, and to determine the parameters of the model as accurately as possible.

Equations describing the models. The two-site competitive model is described by the following equation (6):

$$B = B_H \frac{D}{D + K_{DH}\left(1 + \frac{I}{K_{IH}}\right)} + B_L \frac{D}{D + K_{DL}\left(1 + \frac{I}{K_{IL}}\right)} \quad (1)$$

This equation describes the binding of a radiolabeled drug, D, to two binding sites. The number of binding sites is given by B_H and B_L, respectively. The dissociation constants (K_D) of the drug and the inhibitor, I, for the high and low affinity binding sites are K_{DH} and K_{DL}, and K_{IH} and K_{IL}, respectively.

The two-site allosteric model is described by the following equation:

$$B = B_H \frac{D}{D + K_{DH}\left(1 + \frac{I}{K_{IH}}\right)} + \left(1 - \frac{I}{I + K_\mu}\right) B_L \frac{D}{D + K_{DL}\left(1 + \frac{I}{K_{IL}}\right)} \quad (2)$$

According to this model, D labels two classes of binding sites. The inhibitor I is a competitive inhibitor at the high affinity binding site, and a mixed competitive-noncompetitive inhibitor at the low affinity binding site. K_μ is the dissociation constant of the μ ligand for the μ-binding site of the opiate receptor complex, which is the site through which noncompetitive inhibition of [^{3}H]DADL binding is effected. As formulated above, the model has seven parameters, whereas the two-site competitive model (Eq. 1) has six parameters. When fitting data to Eq. 2, the value of K_{IL} was invariably in excess of 1 M. Since the concentration of I did not exceed 10^{-5} M, this indicates that, within the limitations of the techniques used in this paper, I was a pure noncompetitive inhibitor of [^{3}H]DADL binding to the low affinity binding site. For this reason, the value of K_{IL} is not reported.

Curve-fitting procedure. All curve fitting was done with MLAB (15), a curve-fitting language which utilizes a nonlinear least squares algorithm. Displacement curves were expressed as B/B_0, where B_0 is the specific binding in the absence of inhibitor, and B is the specific binding in the presence of inhibitor. Saturation binding curves were expressed as B/B_0, where B_0 was the initial estimated B_{max}, and B the specific binding. Thus, both displacement curve data and saturation binding data were expressed in units ranging from 0 to 1.0. In all cases, the predicted data points (which changed, of course, with each iteration), were weighted in the following manner: for displacement curve data, $1/(0.0001 + 0.0003y)$, and for saturation curve data, $1/(0.0001y^2)$, where y is the data point predicted by the current parameter values (16).[2]

Statistics. The F-test was used to determine if fits obtained with different models were statistically different (17):

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/df_2}$$

SS_1 and SS_2 are the sum of squares of the residuals of models 1 and 2, respectively, while df_1 and df_2 are the degrees of freedom of models 1 and 2, respectively. Statistical significance between models can be assessed only if the degrees of freedom differ. As defined by Eqs. 1 and 2, the two-site competitive and two-site allosteric models differ by one degree of freedom.

Unfortunately, there is no widely accepted statistical test to determine if a model fits the data significantly. Rothman *et al.* (13) suggested that comparing observed and predicted "secondary characteristics" of curves might accomplish the goal (for instance, comparing observed and predicted IC_{50} values). We have not utilized this approach here (with the exception of Table 6), since the predicted curves clearly fit the data remarkably well.

Preparation of membranes. Membranes were prepared as previously described (18). Briefly, a lysed P2 fraction was prepared from 20 rat brains, minus cerebellum (Sprague-Dawley males, 150–250 g). The membrane suspension was aliquoted to 1.5-ml Microfuge tubes and centrifuged. The supernatant was aspirated and the pellets were stored at −70°. Thalamic/hypothalamic regions were dissected out and membranes were prepared as described above.

Membrane-binding assay. The binding of [^{3}H]DADL and [^{3}H]OXY to brain membranes was determined as previously described (18). Briefly, to 12 × 75 mm polystyrene test tubes were added 100 μl of isotope in protease cocktail (10 mM Tris-HCl, pH 7.4, 40 μg/ml leupeptin, 20 μg/ml chymostatin, 100 μg/ml bestatin, 1000 μg/ml bacitracin), 100 μl of a "salt solution" containing drugs, if indicated, and 800 μl of

[2] D. Rodbard, personal communication.

membranes in buffer. For assay in the Na/Mn/GTP condition, the salt solution contained 10 mM Tris-HCl, pH 7.4, 1 M NaCl, 24 mM $MnCl_2$, and 20 μM GTP. For assay with Mn only, the salt solution contained 24 mM $MnCl_2$. Samples were filtered under reduced pressure over Whatman GF/B glass fiber filters and washed twice with 5 ml of ice-cold 10 mM Tris-HCl, pH 7.4. The filter-bound tritium was determined by liquid scintillation spectrophotometry after an overnight extraction in 10 ml of Aquassure. Nonspecific binding was assessed by incubations in the presence of 20 μM levallorphan.

The frozen membrane pellets were diluted such that 800 μl delivered 400–600 μg of protein. Each point was determined in triplicate with less than 10% variation. Experiments were repeated two to four times with less than 15% variation between experiments. Protein was measured by the method of Lowry *et al.* (19).

The concentration of free ^{3}H-labeled ligand was calculated by subtracting the specifically bound ligand from the total ligand added. In control experiments, the concentration of free ligand was directly determined by pelleting the membranes and counting the supernatant. Less than 5% of added ligand was bound and the calculated concentration of free ligand was essentially identical to the directly determined concentration of free ligand.

Receptor autoradiography. Slide-mounted coronal sections (20 μm thick) of rat brain at the level of the caudate nucleus were prepared as described by Herkenham and Pert (20), except that the sections were stored under vacuum in a desiccated container overnight in a refrigerator. This modification was essential to ensure that the sections did not fall off the slides during the long protocols to be described below. Sections were stored at −70° prior to use.

For treatment with the site-directed alkylating agents BIT and FIT (10–12), slides were removed from slide boxes and placed into stainless steel slide racks (25 slides/rack). Following a 30-min preincubation at 25° in 500 ml of MOPS/sucrose (10 mM MOPS, 100 mM sucrose, pH 7.4), the slides were placed into plastic cytomailers containing 10 ml of MOPS/sucrose with 2.4 mM $MnCl_2$ and alkylating agent at a concentration of 1 μM. Control sections were treated identically, but were not incubated with an alkylating agent. Following a 60-min incubation at 25°, the slides were placed in stainless steel slide racks and washed by three sequential 10-min immersions in 500 ml of 10 mM Tris-HCl, 100 mM sucrose, 0.1 mg/ml BSA, pH 7.4, at 25°. Sections were then assayed for [^{3}H]DADL and [^{3}H]OXY binding as described below.

For assay with [^{3}H]DADL, slides were placed into cytomailers containing 10 ml of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.4 mM $MnCl_2$, 2 μM GTP, 1 mg/ml BSA, 0.1 mg/ml bacitracin, 0.004 mg/ml leupeptin, and 0.004 mg/ml chymostatin. For assay with [^{3}H]OXY, slides were placed into cytomailers containing 10 ml of 50 mM Tris-HCl, pH 7.4, 2.4 mM $MnCl_2$, and 1 mg/ml BSA. Following a 90-min incubation at 25°, the sections were washed by five sequential 40-sec immersions in ice-cold 10 mM Tris-HCl, pH 7.4, and dried with a stream of cool air. After overnight desiccation under vacuum, the slides were placed into a Wolf X-ray cassette and placed against LKB tritium-sensitive film. The film was developed as previously described (20) after a 46-day exposure.

Chemicals and supplies. [^{3}H]DADL (specific activity, 43.6 Ci/mmol) and Aquassure were purchased from New England Nuclear Corp. Peptidase inhibitors leupeptin, chymostatin, bestatin, bacitracin, and GTP (as the lithium salt) were purchased from Sigma Chemical Company. [^{3}H]OXY (specific activity, 41 Ci/mmol) was purchased from Amersham Ltd. Synthetic peptides were purchased from Peninsula Laboratories.

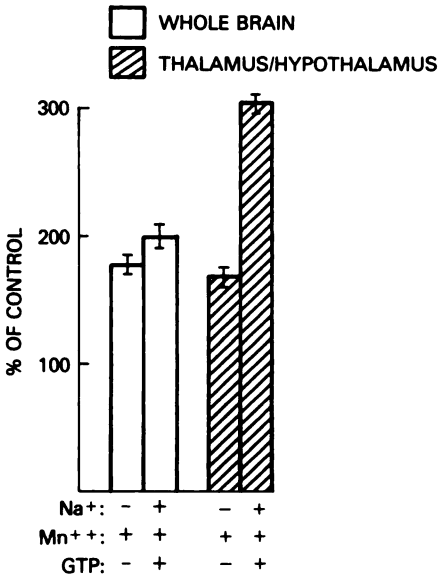

FIG. 1. *Effect of NaCl, $MnCl_2$, and GTP on [^{3}H]DADL binding*

The binding of 3.0 nM [^{3}H]DADL to membranes derived from whole brain and thalamus/hypothalamus was determined as described in Materials and Methods using three different conditions. In the absence of effectors (control), the specific binding was 37.7 (80% specific binding) and 10.2 fmol/ml (57% specific binding) using whole brain and thalamus/hypothalamus, respectively. The concentrations of effectors were: 100 mM NaCl, 2.4 mM $MnCl_2$, and 2 μM GTP. In the presence of Na/Mn/GTP, the per cent specific binding was 87 and 78.5 using membranes derived from whole brain and thalamus/hypothalamus, respectively. Each point is the mean ± standard deviation of two experiments, each determined in triplicate.

RESULTS

Selection of Assay Conditions.

Ionic conditions. The binding of opiate agonists is affected by ionic conditions (21). Bowen *et al.* (2) reported that [^{3}H]DADL binding to slide-mounted sections of rat caudate was substantially augmented when assayed in the presence of 100 mM NaCl, 3 mM MnAc, and 2 μM GTP. They also showed that, using this condition, [^{3}H]DADL labeled two anatomically distinct binding sites in the rat caudate nucleus. In addition, the binding of [^{3}H]DADL to solubilized opiate receptors requires the Na/Mn/GTP condition (22, 23).

The experiments reported in Fig. 1 examine the effect of ionic conditions on [^{3}H]DADL binding to whole brain and thalamic/hypothalamic membranes. As expected, Mn stimulated [^{3}H]DADL binding to both membrane preparations. The Na/Mn/GTP condition similarly stimulated [^{3}H]DADL binding. Thalamic/hypothalamic membranes were especially sensitive to Na/Mn/GTP, in that [^{3}H]DADL binding increased 300% over the binding observed in the absence of ions. All other experiments reported in this paper utilize the Na/Mn/GTP condition, since not only does this condition yield the greatest specific binding, but, using this condition, [^{3}H]DADL labels two anatomically distinct binding sites (2).

Time of incubation. Association and dissociation experiments are shown in Fig. 2. Surprisingly, it took 4 hr at 25° to achieve steady state. Similar results were obtained with freshly prepared, unfrozen membranes. The dissociation half-life was approximately 1 hr. Thus, the typical assay was incubated for four to five half-lives. We have not attempted a more sophisticated analysis of the kinetic data, since, as the following experiments indicate, [^{3}H]DADL must be associating and dissociating to and from two independent binding sites. Also, as

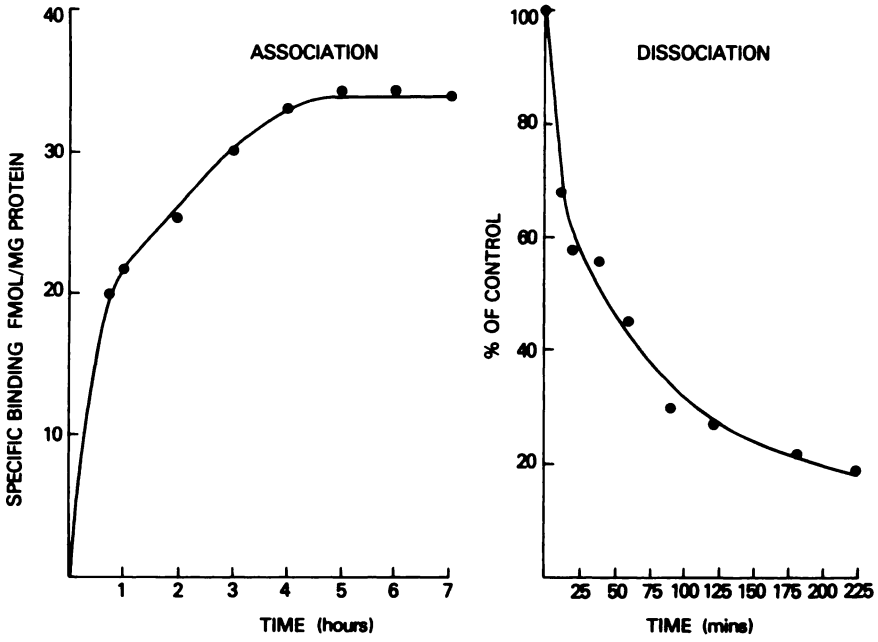

FIG. 2. *Association and dissociation experiments*

Left panel: total and nonspecific binding of 1.3 nM [^{3}H]DADL to whole brain membranes at 25° was determined in the presence of 100 mM NaCl, 2.4 mM $MnCl_2$, and 2 μM GTP. The time course of specific binding is presented. Similar results were obtained in two additional experiments, one using frozen membranes, the other using freshly prepared membranes. Each point is the mean of triplicate determinations, which differed by less than 10%. Right panel: to follow the dissociation of prebound [^{3}H]DADL, 3.2 nM [^{3}H]DADL was incubated with whole brain membranes in the presence of 3 nM DSTLE, in the absence and presence of 20 μM levallorphan. DSTLE was included in the incubation to place a higher proportion of [^{3}H]DADL on the lower affinity binding site. After a 4-hr incubation at 25°, OXY and DADL were added to both the total and nonspecific binding conditions to give final concentrations of 100 nM. Triplicate samples were filtered at the indicated times from both total and nonspecific binding conditions. The specific binding, which was a percentage of the specific binding at steady state, is plotted as a function of time. The nonspecific binding did not change as a function of time. Similar results were obtained in two other experiments.

TABLE 1

Time course of [^{3}H]oxymorphone binding to rat brain membranes in the presence of 100 mM NaCl, 2.4 mM $MnCl_2$, and 2 μM GTP

The specific binding of 2 nM [^{3}H]OXY was determined at the indicated time points. Each point is the mean ± standard deviation of two separate experiments, each determined in triplicate.

Time	Specific binding
hr	*fmol/ml*
0.17	37 ± 2
0.50	52 ± 3
1.00	61 ± 5
2.00	63 ± 3
3.00	70 ± 4
4.00	72 ± 2
6.00	72 ± 3
7.50	72 ± 2

TABLE 2

Effect of protease inhibitors on [^{3}H]DADL and [^{3}H]OXY binding

The specific binding of [^{3}H]DADL and [^{3}H]OXY were determined as described in Materials and Methods in the absence of protease inhibitors (control), in the presence of protease cocktail, and in the presence of 0.1 mg/ml bacitracin. The control specific binding of 2.35 nM [^{3}H]DADL and 2.07 nM [^{3}H]OXY were 74 ± 4 and 73 ± 3 fmol/ml. Each value is the mean ± standard deviation of two experiments, each determined in triplicate.

Condition	Specific binding	
	[^{3}H]DADL	[^{3}H]OXY
	% control	
Control	100	100
Protease cocktail	74 ± 3	72 ± 4
Bacitracin (0.1 mg/ml)	76 ± 2	76 ± 3

reported in Table 1, [^{3}H]OXY binding was at steady state by 4 hr. Additional control experiments indicated that [^{3}H]DSTLE binding was at steady state within 4 hr (data not shown).

Protease inhibitors. In control experiments, the effect of the protease inhibitor cocktail and bacitracin alone on [^{3}H]DADL and [^{3}H]OXY binding was ascertained using the Na/Mn/GTP condition. As shown in Table 2, relative to no protease inhibitors, the cocktail decreased the binding of both ligands by about 25%. The fact that bacitracin alone decreased the binding by about the same extent suggests that bacitracin is responsible for the slight inhibition of binding. Given the long period of incubation required to achieve equilibrium, we opted to maximize protease inhibition at the expense of a slight decrease in specific binding.

Examination of DSTLE-Binding Surfaces

To determine accurately the K_D values of [^{3}H]DADL for the two binding sites, as well as the number of binding sites, we chose to study the interaction of DSTLE with [^{3}H]DADL binding for several reasons. Based upon pharmacological studies (24), DSTLE is very δ selective. Since it is a δ-selective peptide, we expected it to inhibit [^{3}H]

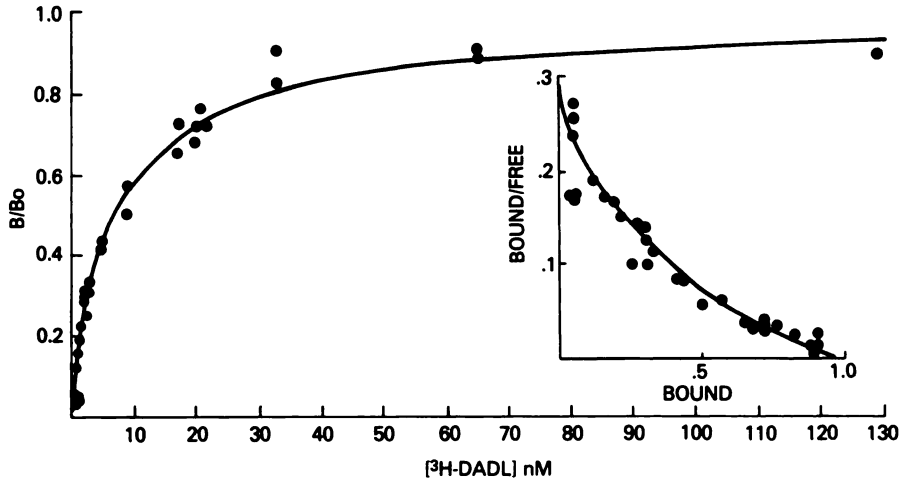

FIG. 3. *Saturation binding of [^{3}H]DADL to whole brain membranes*

The specific binding of [^{3}H]DADL to whole brain membranes was determined at concentrations between 0.17 and 129 nM. The data are presented as a binding isotherm. The concentration of bound ligand is expressed as the ratio of observed binding to an estimated B_{max}, B_0, which in this case was 375 fmol/mg protein. A Scatchard plot of the same data is shown in the inset. Each point is the mean of two experiments, each determined in triplicate. The solid lines are generated by the best fit two-site competitive model (Table 3).

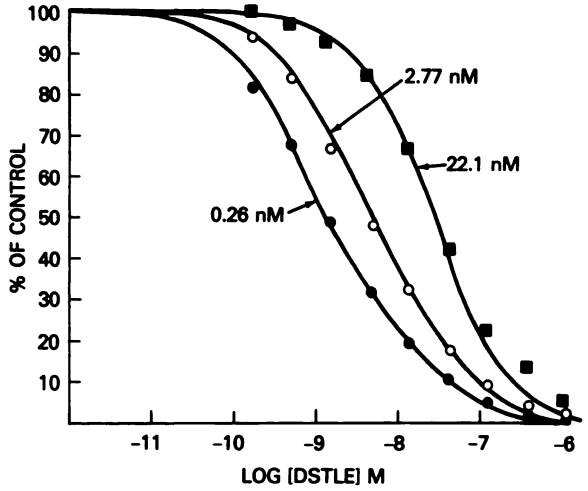

FIG. 4. *DSTLE-binding surface using whole brain membranes*

DSTLE displacement curves were determined using three widely spaced concentrations of [^{3}H]DADL. The concentrations of free [^{3}H]DADL were 0.26, 2.77, and 22.1 nM, respectively. Less than 10% of [^{3}H]DADL was bound. Each point is the mean of four separate experiments, each determined in triplicate. The solid lines are those generated by the test fit two-site competitive model (Table 3).

DADL binding at both sites in a competitive manner. Additionally, the lower affinity DSTLE site could be tentatively identified, according to the two-site competitive model, as the μ receptor.

To generate DSTLE-binding surfaces, [^{3}H]DADL saturation binding curves and DSTLE displacement curves were determined using membranes derived from whole brain as well as from thalamus/hypothalamus. The use of two membrane sources allowed the relative number of binding sites to vary, leading to a more constrained fit and greater precision in the estimates of the parameter values. [^{3}H]DADL saturation curves were calculated from DADL-binding surfaces. The data of Figs. 3 and 4 describe the DSTLE-binding surface obtained with whole brain membranes. Figs. 5 and 6 describe the surface obtained using thalamic/hypothalamic membranes. The experimental details are provided in the figure legends.

The two DSTLE-binding surfaces were simultaneously analyzed according to four models. As expected, the two-site competitive model fit the data much better than a one-site competitive model. More importantly, the two-site competitive model fit the data better than the two-site allosteric model ($p < 0.001$). The parameters of the two-site competitive model are reported in Table 3A. Analysis of the data according to a three-site competitive model resulted in a statistically significant 6% decrease in the sum of squares. But as reported in Table 3B, this is a curve-fitting artifact, since certain B_{max} values were iterated to zero.

Examination of an Oxymorphone-Binding Surface

Having accurately determined the K_D values of [^{3}H]DADL for the two binding sites, as well as the number of binding sites, using a peptide which proved to interact competitively at both binding sites, we next examined the interaction of OXY with [^{3}H]DADL binding. According to the two-site analysis of the DSTLE-binding surfaces, the lower affinity [^{3}H]DADL-binding site is the μ receptor and OXY should be a competitive inhibitor of [^{3}H]DADL binding to this site.

To test this hypothesis, we examined regions of the OXY-binding surface which computer simulation studies showed best distinguished the two-site competitive model from the two-site allosteric model. We used thalamic/hypothalamic membranes, since 90% of the sites are of the lower affinity variety. As shown in Fig. 7, OXY inhibited [^{3}H]DADL binding in an apparently noncompetitive manner. The surface was fit to both models using the parameters listed in Table 3A. As reported in Table 4A, the two-site allosteric model fit the data significantly better than did the two-site competitive model ($p <$

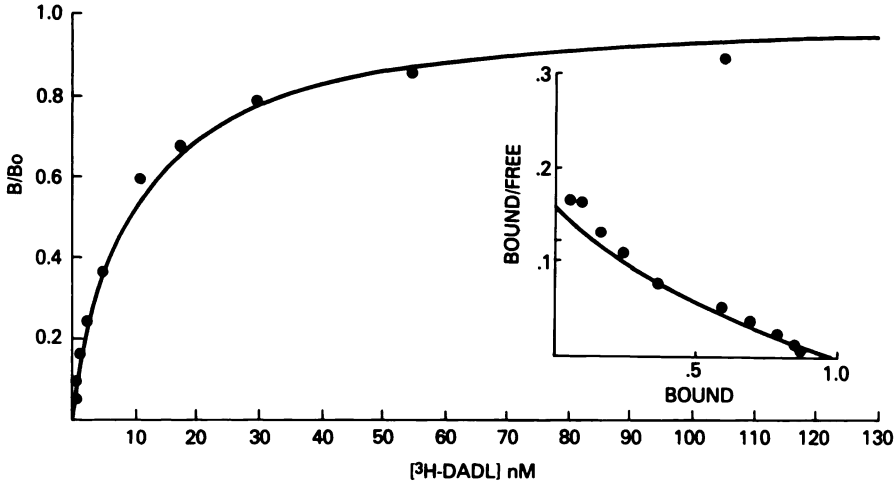

FIG. 5. *Saturation binding of [^{3}H]DADL to thalamic/hypothalamic membranes*

The specific binding of [^{3}H]DADL to thalamic/hypothalamic membranes was determined at concentrations between 0.33 and 105 nM. The estimated B_{max} (B_0) was 243 fmol/mg protein. A Scatchard plot of the same data is shown in the inset. Each point is the mean of two experiments, each determined in triplicate. The solid lines are those generated by the best fit two-site competitive model (Table 3).

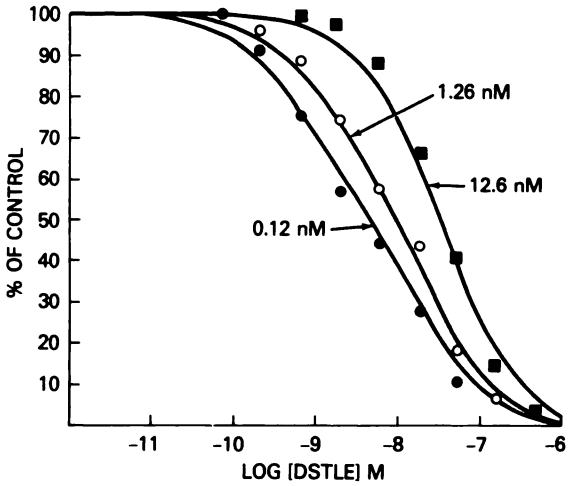

FIG. 6. *DSTLE-binding surface using thalamic/hypothalamic membranes*

DSTLE displacement curves were determined using three widely spaced concentrations of [^{3}H]DADL. The concentrations of free [^{3}H]DADL were 0.12, 1.26, and 12.6 nM, respectively. Less than 10% of [^{3}H]DADL was bound. Each point is the mean of two experiments, each determined in triplicate. The solid lines are generated by the best fit two-site competitive model (Table 3).

0.001). These parameters were used to generate the solid lines in Fig. 7, illustrating the goodness of fit.

A Prediction Experiment

Rothman *et al.* (13) emphasized the importance of "prediction" experiments, which are designed to test for differences between models. To distinguish further between the competitive and allosteric models, we examined the displacement of [^{3}H]DADL binding (at 0.1 nM) to whole brain membranes by OXY. These conditions place about 70% of bound [^{3}H]DADL on the high affinity binding site. Since the two-site competitive model and two-site allosteric model predict the K_D of OXY at the high affinity site to be 675 and 2.3 nM, respectively, this is a discriminating experiment. The results shown in Table 4B indicate that the two-site allosteric model predicted the data better than did the two-site competitive model.

TABLE 3

Parameters of the DSTLE-binding surfaces according to the two-site competitive model (A) and the three-site competitive model (B)

A: the 89-point DSTLE-binding surface defined by the data shown in Figs. 3–6 was analyzed for the parameters of the two-site competitive model. The *SS* (sum of squares) was 0.0784 with 81 degrees of freedom. This was a significantly better fit than that obtained with the one-site competitive model ($SS = 0.517, p < 0.001$) or with the two-site allosteric model ($SS = 0.114, p < 0.001$). B: the 89-point DSTLE-binding surface defined by the data of Figs. 3–6 was fit to the three-site competitive model. The *SS* was 0.068 with 77 degrees of freedom. The 6% decrease in the *SS* relative to the two-site competitive model is a significant decrease ($p < 0.01$), but is a curve-fitting artifact since the best fit parameters describe the same two-site model described above. All values are mean ± SD.

A.	High affinity binding site	Low affinity binding site
K_D (nM)		
DADL	1.42 ± 0.18	10.6 ± 0.9
DSTLE	0.52 ± 0.08	19.2 ± 2.3
B_{max}		
Whole brain	0.28 ± 0.03	0.70 ± 0.04
Thalamus/ hypothalamus	0.10 ± 0.02	0.92 ± 0.03

B.	Site 1	Site 2	Site 3
K_D (nM)			
DADL	1.39 ± 0.19	8.33 ± 7.20	13.1 ± 9.8
DSTLE	0.57 ± 0.09	18.1 ± 6.30	21.2 ± 6.2
B_{max}			
Whole brain	0.29 ± 0.04	0.00 ± 1.1	0.74 ± 1.0
Thalamus/hypothalamus	0.11 ± 0.02	0.85 ± 1.8	0.00 ± 1.8

Autoradiographic Visualization of the Higher and Lower Affinity [^{3}H]DADL-Binding Sites

Studies published elsewhere (10, 11, 25) have demonstrated that the site-directed alkylating agents BIT and

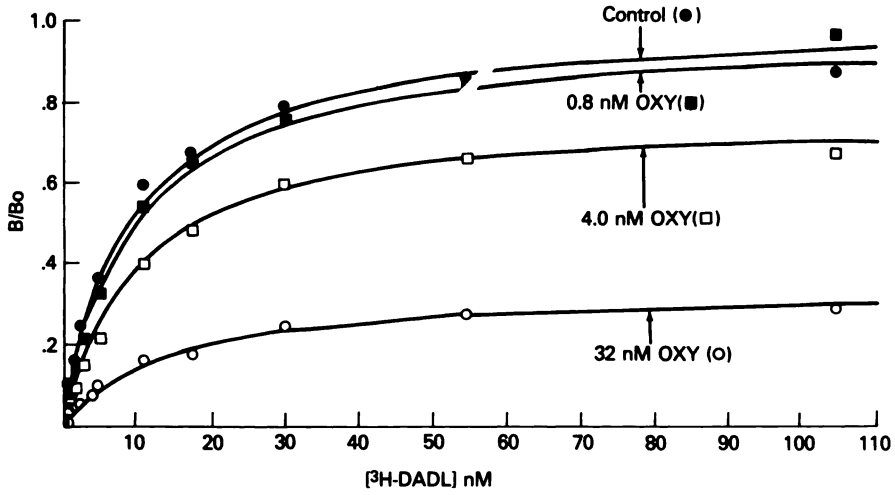

FIG. 7. *Saturation binding of [^{3}H]DADL to thalamic/hypothalamic membranes in the absence and presence of OXY*

The specific binding of [^{3}H]DADL was determined at concentrations between 0.33 and 105 nM, in the absence and presence of 0.8, 4.0, and 32 nM OXY. The data are presented as a binding isotherm. The estimated B_{max} (B_0) was 243 fmol/mg protein. The solid lines are those generated by the best fit two-site allosteric model (Table 4A). Each point is the mean of two experiments, each determined in triplicate.

TABLE 4

Parameters of the oxymorphone-binding surface according to the two-site allosteric model (A) and a prediction experiment (B)

A: the 40-point oxymorphone-binding surface (Fig. 7) was analyzed for the parameters of the two-site allosteric model. The B_{max} and K_D values of DADL were fixed to those obtained by analysis of the DSTLE-binding surfaces (Table 3A). The *SS* was 0.0199. When fit to the two-site competitive model, $SS = 0.0741$ was obtained, with K_D values at the high and low affinity binding sites of 675 ± 2878 and 1.9 ± 0.2 nM, respectively. Application of the *F* test showed that the two-site allosteric model fit significantly better than the two-site competitive model ($p < 0.001$). B: in a competition experiment using whole brain membranes, 0.14 nM [^{3}H]DADL was displaced by 8 concentrations of OXY between 0.274 and 600 nM. Each point was the mean of four separate experiments, each determined in triplicate. The displacement curves were analyzed by MLAB for the IC_{50} using a two-parameter logistic equation. Using the parameters in A, predicted displacement curves were generated and analyzed for predicted IC_{50} values.

A.	High affinity binding site	Low affinity binding site
K_D (nM)	2.3 ± 1.3	10.9 ± 0.9
B.	**IC_{50}**	
	nM	
1. Two-site	211 ± 49[a]	
2. Allosteric	3.6 ± 0.03[b]	
3. Observed data	2.0 ± 0.1	

[a] Significantly different from the observed IC_{50} ($p < 0.001$, Student's *t* test).

[b] Not significantly different from the observed IC_{50} ($p < 0.001$, Student's *t* test).

FIT, first described by Rice *et al.* (12), can be used to prepare membranes highly enriched with either the higher affinity binding site (BIT-treated) or lower affinity binding site (FIT-treated). Similar results are obtained when slide-mounted sections of unfixed rat brain are treated with these alkylating agents (10).

The selectivity of the δ-selective peptide DSTLE for the higher affinity binding site suggests that the lower affinity and higher affinity [^{3}H]DADL-binding sites might correspond to the μ receptor and δ receptor, respectively. To examine this hypothesis, slide-mounted sections of rat brain were treated with either 1 μM BIT or 1 μM FIT, as described in Materials and Methods.

Consistent with the reported selectivity of BIT and FIT for the μ- and δ-binding sites (10–12), respectively, treatment of sections with BIT reduced [^{3}H]OXY binding to the level of nonspecific binding, while treatment of sections with FIT had no detectable affect on the level of [^{3}H]OXY binding or its typically μ-like anatomical distribution (Fig. 8).

Strikingly different results were obtained when BIT- and FIT-treated sections were labeled with [^{3}H]DADL. Higher affinity binding sites (BIT-treated sections) had the distribution characteristic of type II (2) or δ-binding sites, while the lower affinity [^{3}H]DADL-binding site (FIT-treated sections) had an anatomical distribution almost indistinguishable from the μ-binding sites labeled by [^{3}H]OXY (2, 20).

DISCUSSION

The mechanism by which μ ligands inhibit the binding of prototypic δ receptor ligands such as [^{3}H]leucine enkephalin and [^{3}H]DADL is controversial. Rothman and Westfall (26) were the first to report the apparent noncompetitive interaction of μ ligands with δ receptor binding. The major focus of their subsequent work was to distinguish between the widely accepted two-site competitive model and an allosteric model. The former model hypothesizes that ligands such as DADL and leucine enkephalin, which in physiological systems are "prototypic" δ agonists (27), bind equally well to μ and δ receptors *in vitro* (9). Computer simulations (13, 28) and the examination of actual data clearly show that this model can result in the appearance of apparent noncompetitive inhibition.

The alternative model first considered by Rothman and Westfall is best described as the one-site allosteric model. This model supposes that δ ligands label a single class of sites (the δ receptor), and that μ ligands are

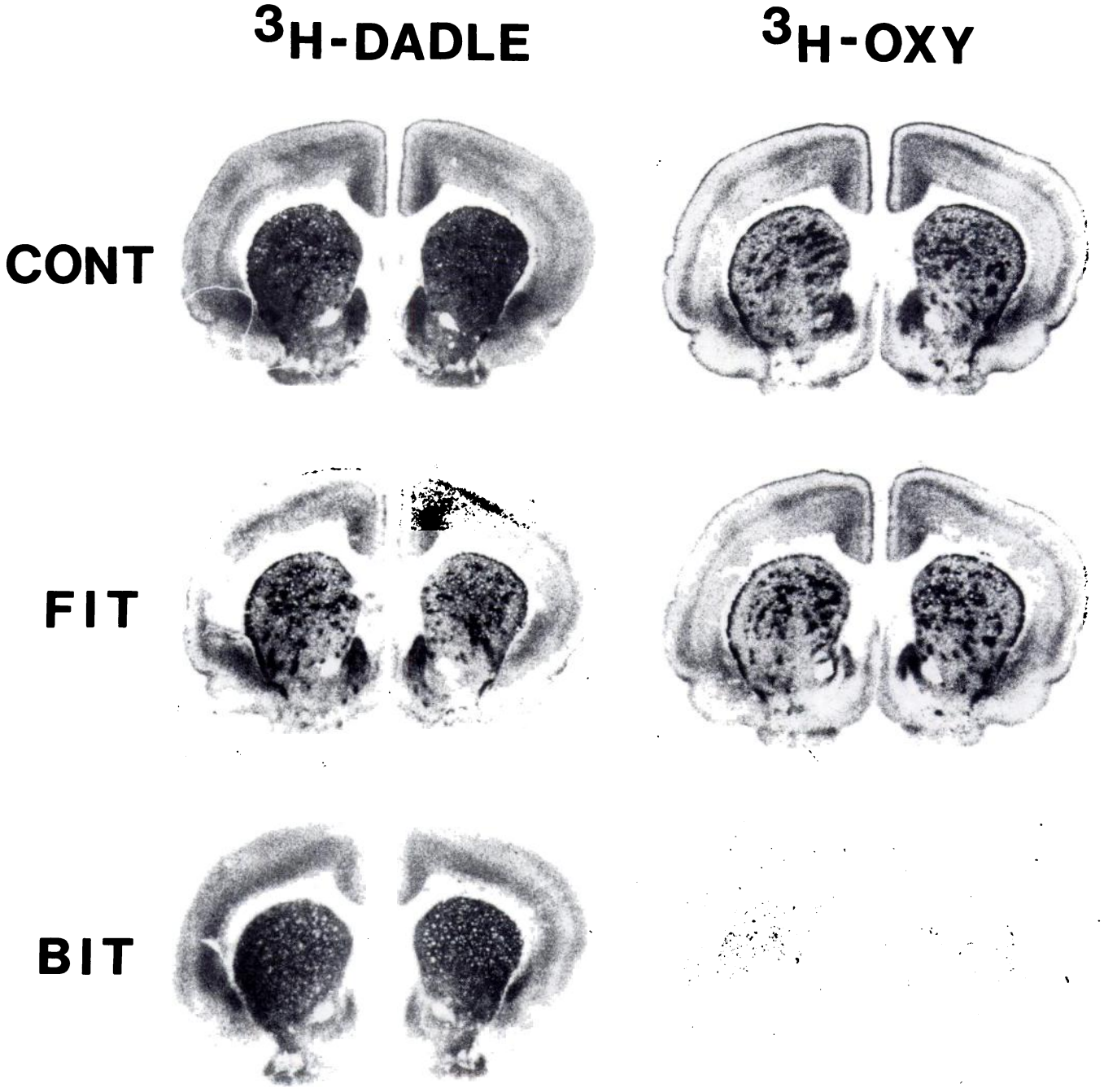

FIG. 8. *Autoradiographic visualization of the higher and lower affinity [^{3}H]DADL-binding sites*

Slide-mounted coronal sections of rat brain at the level of the caudate nucleus were treated with either BIT or FIT as described in Materials and Methods and the anatomical distribution of [^{3}H]DADL- and [^{3}H]OXY-binding sites was autoradiographically visualized. The upper two panels depict the results obtained with sections not treated with alkylating agent, the middle panels show when sections were treated with FIT, while the lower two panels depict the results obtained when sections were treated with BIT. Control binding studies utilizing slide-mounted sections of rat caudate indicated that [^{3}H]DADL labeled two binding sites with K_D values of 1.1 and 7.3 nM, respectively, and that OXY was a competitive inhibitor at the higher affinity binding site (K_D = 22.0 nM) and an apparent noncompetitive inhibitor at the lower affinity binding site (K_μ = 2.0 nM).

mixed competitive-noncompetitive inhibitors of δ receptor binding. Using a variety of approaches, they presented evidence that the one-site allosteric model fit their data better than did a two-site competitive model. To explain the finding of noncompetitive inhibition, they proposed that μ and δ receptors coexisted in an "opioid receptor complex." In their autoradiographic study, Bowen *et al.* (2) demonstrated that, using the Na/Mn/GTP condition, [^{3}H]DADL labeled two anatomically and biochemically distinct populations of binding sites in sections of unfixed caudate. Recognizing this, Rothman and Westfall (7) noted that their data were consistent

TABLE 5

$\mu \rightarrow \delta$ noncompetitive interactions: a summary

A summary of the various *in vitro* conditions in which a $\mu \rightarrow \delta$ noncompetitive interaction has been observed. The last condition is from unpublished data.

Ligand	Assay temp.	Protease inhibitors	Preparation	Assay condition	Ref.
	°C				
[^{3}H]LE	0	No	Membranes	80 mM NH_4Ac buffer; no ions	6
[^{3}H]ME	0	No	Membranes	80 mM NH_4Ac buffer; no ions	7
[^{3}H]LE	0	Yes	Membranes	80 mM NH_4Ac buffer; no ions	11
[^{3}H]DADL	25	Yes	Slide-mounted sections	Na/Mn/GTP	10
[^{3}H]DADL	25	No	Membranes	50 mM Tris-HCl ± $CaCl_2$	33
[^{3}H]LE	25	Yes	Membranes	80 mM NH_4Ac buffer +3 mM $MnCl_2$	

with the possibility that δ ligands label two populations of δ receptors. According to this hypothesis, μ ligands are pure competitive inhibitors at one class and pure noncompetitive inhibitors at the other class of binding sites. This results in the appearance of mixed competitive-noncompetitive inhibition if the K_D of the δ ligand is similar at both sites. This model implies the existence of a receptor complex consisting of interacting μ- and δ-binding sites and, in addition, a pure δ receptor at which μ drugs interact competitively. We call this the two-site allosteric model.

We show in this study that [^{3}H]DADL labels two binding sites at which DSTLE interacts competitively. The experimental design used (i.e., analysis of binding surfaces) led to very precise parameter estimates. An appropriate portion of the OXY-binding surface generated using a source of membranes highly enriched in the lower affinity binding site demonstrated apparent noncompetitive inhibition. Analysis of this surface showed that the two-site allosteric model fit the data significantly better than did the two-site competitive model. Thus, the extensive set of data presented here is best described by a model which hypothesizes that [^{3}H]DADL labels two binding sites, and that OXY is a competitive inhibitor at the higher affinity binding site, and a noncompetitive inhibitor at the lower affinity binding site.

An interpretation consistent with the finding of noncompetitive inhibition is that the lower affinity [^{3}H]DADL-binding site is the δ-binding site of an opiate receptor complex which consists of interacting and presumably physically associated μ- and δ-binding sites. The autoradiographic study reported in Fig. 8 supports this hypothesis, in that the lower affinity [^{3}H]DADL-binding site has the same anatomical distribution as the μ-binding site labeled by [^{3}H]OXY. This is consistent with the findings of Bowen *et al.* (29) that 6-hydroxydopamine lesions of the substantia nigra caused equal decreases of μ and δ binding to the same receptor patches in the ipsilateral striatum. This was taken as evidence that μ- and δ-binding sites are localized to the same striatal dopaminergic nerve terminals. However, since apparently identical anatomical distributions of two binding sites at the light microscopic level does not prove identity of the two binding sites at the molecular level, we interpret the autoradiographic experiments in the context of the membrane-binding studies.

As we document in Fig. 1, the Na/Mn/GTP condition maximizes [^{3}H]DADL binding. Other studies[3] suggest that the enhancement of [^{3}H]DADL binding results primarily from a decrease in the K_D of [^{3}H]DADL for the lower affinity site. Given that the focus of this study is the interaction of μ ligands with the lower affinity [^{3}H]DADL-binding site, the Na/Mn/GTP condition was a logical choice. We have not yet studied in detail interesting, but unrelated, questions about the mechanisms underlying the effect of Na/Mn/GTP (30), although involvement of receptor thiol groups may be indicated (31).

The ionic conditions utilized in this study as well as the fact that the protease inhibitor cocktail inhibited [^{3}H]DADL binding by 25% raises the possibility that the observed noncompetitive interactions might be condition-dependent. However, the ability of μ ligands to inhibit δ agonist binding noncompetitively has been observed using a variety of different conditions. These are reported in Table 5. These data suggest that the noncompetitive interactions reported here are not a consequence of any particular set of assay conditions, but reflect an underlying property of the lower affinity [^{3}H]DADL-binding site.

The finding that μ ligands noncompetitively inhibit [^{3}H]DADL binding to its lower affinity binding site led us to use a terminology which emphasizes the finding that [^{3}H]DADL labels two δ-binding sites (10). The type II δ-binding site (μ-competitive, higher affinity binding site) is analogous to the diffusely distributed type II opiate receptor (2). The type I δ-binding site (μ-noncompetitive, lower affinity binding site) is the δ-binding site of the opiate receptor complex. μ ligands inhibit [^{3}H]DADL binding to this site noncompetitively via an interaction at a physically associated μ-binding site. An interesting question is which of these receptors is analogous to the adenylate cyclase-coupled receptor of the

[3] R. B. Rothman *et al.*, in preparation.

neuroblastoma-glioma hybrid cell (32). Studies in this regard are now underway.

A question of obvious importance is the relationship of the two-site allosteric model to other models of the opiate receptors. The autoradiographic studies reported in Fig. 8 suggest that the μ-competitive (higher affinity) and μ-noncompetitive (lower affinity) [^{3}H]DADL-binding sites are synonymous with the type II and type I opiate receptors (2). Our data support the protein-lipid model of the opiate receptor proposed by Lee and Smith (3), with the exception that the type II δ-binding site is seemingly inconsistent with the unitary concept of the opiate receptor that they recently proposed (8). The data of Pfeiffer and Herz (33) fully support the two-site allosteric model. Most investigators identify the lower affinity [^{3}H]DADL-binding site as the μ receptor (1, 9). Our model extends this widely accepted notion, in that it allows for the finding that μ ligands *noncompetitively* displace [^{3}H]DADL binding from this site.

The relationship of our model to that proposed by Pasternak (34) is not clear. This investigator has proposed a three-site binding model which supposes that μ and δ ligands share a single common high affinity binding site. Analysis of the DSTLE-binding surfaces according to the three-site competitive model provided no compelling evidence that [^{3}H]DADL labels three binding sites. It is possible that our inability to detect three binding sites is condition-dependent. However, in that Pfeiffer and Herz (35) and Barrett and Vaught (28), who used similar quantitative techniques, also failed to find ligand-binding evidence supporting the Pasternak hypothesis suggests that this is not the case. Examination of the effect of naloxonazine in our assay system should help to clarify the relationship between the Pasternak model and that which is considered in this study.

In summary, quantitative analysis of the interaction of OXY and DSTLE with [^{3}H]DADL binding to rat brain membranes has demonstrated that a two-site allosteric model fits the data significantly better than does the widely accepted two-site competitive model. Thus, the noncompetitive interactions observed using membranes treated with the site-directed alkylating agent FIT (10, 11) are not created by the alkylating agent. Additionally, these findings suggest that prototypic δ agonists like [^{3}H]DADL label two δ-binding sites *in vitro* distinguished by the inhibitory mechanism of μ ligands. Our finding of apparent μ-δ-binding site interactions *in vitro* suggests that apparent μ-δ interactions observed *in vivo* may reflect the physiological functions of the opiate receptor complex (36, 37).

REFERENCES

1. Robson, L. E., S. J. Paterson, and H. W. Kosterlitz. Opiate receptors. Handbook *Psychopharmacol.* **17**:13–80 (1983).
2. Bowen, W. D., S. Gentlemen, M. Herkenham, and C. B. Pert. Interconverting mu and delta forms of the opiate receptors in rat striatal patches. *Proc. Natl. Acad. Sci. USA* **78**:4818–4822 (1981).
3. Lee, N. M., and A. P. Smith. A protein-lipid model of the opiate receptor. *Life Sci.* **26**:1459–1464 (1980).
4. Rothman, R. B., and T. C. Westfall. Allosteric coupling between morphine and enkephalin receptors *in vitro*. *Mol. Pharmacol.* **21**:548–557 (1982).
5. Rothman, R. B., and T. C. Westfall. Interaction of leucine enkephalin with [^{3}H]naloxone binding in rat brain: evidence for an opioid receptor complex. *Neurochem. Res.* **8**:913–931 (1983).
6. Rothman, R. B., and T. C. Westfall. Morphine allosterically modulates the binding of [^{3}H]leucine enkephalin to a particulate fraction of rat brain. *Mol. Pharmacol.* **21**:538–547 (1982).
7. Rothman, R. B., and T. C. Westfall. Further evidence for an opioid receptor complex. *J. Neurobiol.* **14**:341–351 (1983).
8. Smith, A. P., N. N. Lee, and H. H. Loh. The multiple site beta-endorphin receptor. *Trends Pharm. Sci.* **4**:163–164 (1983).
9. Chang, K. J., and P. Cuatrecasas. Multiple opiate receptors: enkephalins and morphine bind to receptors of different specificity. *J. Biol. Chem.* **254**:2610–2618 (1979).
10. Rothman, R. B., W. D. Bowen, V. Bykov, U. K. Schumacher, C. B. Pert, A. E. Jacobson, T. R. Burke, Jr., and K. C. Rice. Preparation of rat brain membranes greatly enriched with either type-I-delta or type-II-delta opiate binding sites using site directed alkylating agents: evidence for a two-site allosteric model. *Neuropeptides* **4**:201–215 (1984).
11. Rothman, R. B., C. B. Pert, A. E. Jacobson, T. R. Burke, Jr., and K. C. Rice. Morphine noncompetitively inhibits the binding of [^{3}H]leucine enkephalin to a preparation of rat brain membranes lacking type-II delta receptors. *Neuropeptides* **4**:257–260 (1984).
12. Rice, K. C., A. E. Jacobson, T. R. Burke, Jr., B. S. Bajwa, R. A. Streaty, and W. A. Klee. Irreversible ligands with high selectivity toward mu or delta opiate receptors. *Science* **220**:314–316 (1983).
13. Rothman, R. B., R. W. Barrett, and J. L. Vaught. Multidimensional analysis of ligand binding data: application to opioid receptors. *Neuropeptides* **3**:367–377 (1983).
14. Rothman, R. B. Analysis of binding surfaces: a methodology appropriate for the investigation of complex receptor mechanisms and multiple neurotransmitter receptors. *Neuropeptides* **4**:41–44 (1983).
15. Knott, G. G., and D. K. Reece. MLAB: a civilized curve fitting system, in *Proceedings of the Online 1972 International Conference*, Vol. 1, 497–526 (1972).
16. Rodbard, D., R. H. Lenox, L. Wray, and D. Ramseth. Statistical characterization of the random errors in the radioimmunoassay dose-response variable. *Clin. Chem.* **22**:350–358 (1976).
17. DeLean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose response curves. *Am. J. Physiol.* **235**:E97–E102 (1978).
18. Rothman, R. B., U. K. Schumacher, and C. B. Pert. Binding of radiolabeled opiates to slide mounted sections of molded minced rat brain: a novel method for conducting radioreceptor assays. *Neuropeptides* **3**:493–499 (1983).
19. Lowry, O. H., N. H. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
20. Herkenham, M., and C. B. Pert. Light microscopic localization of brain opiate receptors: a general autoradiographic method which preserves tissue quality. *J. Neurosci.* **2**:1129–1149 (1982).
21. Pasternak, G. W., A. M. Snowman, and S. H. Snyder. Selective enhancement of ^{3}H-opiate agonist binding by divalent cations. *Mol. Pharmacol.* **11**:735–744 (1975).
22. Bowen, W. D., U. K. Schumacher, and C. B. Pert. Characterization of solubilized rat brain opiate receptors with mu, delta, and kappa ligand selectivity. *Fed. Proc.* **42**:1876 (1983).
23. Chow, T., and R. S. Zukin. Solubilization and preliminary characterization of mu and kappa opiate receptor subtypes from rat brain. *Mol. Pharmacol.* **24**:203–212 (1983).
24. Gacel, G., M. C. Fournie-Zaluski, E. Fellion, and B. P. Roques. Evidence for preferential involvement of mu-receptors in analgesia using enkephalins highly selective for peripheral mu and delta receptors. *J. Med. Chem.* **24**:1119–1124 (1981).
25. Rothman, R. B., J. D. Danks, A. E. Jacobson, T. R. Burke, Jr., K. C. Rice, and C. B. Pert. Tritiated-6-beta-fluro-6-desoxyoxymorphone: a highly selective ligand for the opiate mu receptor whose binding is characterized by low nonspecific binding. *Neuropeptides* **4**:311–317 (1984).
26. Rothman, R. B., and T. C. Westfall. Noncompetitive inhibition by morphine of the binding of ^{3}H-leucine enkephalin to crude membranes prepared from rat brain. *Fed. Proc.* **39**:385 (1980).
27. Lord, J. A. H., A. Waterfield, J. Hughes, and H. W. Kosterlitz. Endogenous opioid peptides: multiple agonists and receptors. *Nature* **267**:495–499 (1977).
28. Barrett, R. W., and J. L. Vaught. Evaluation of the interactions of mu and delta selective ligands with [^{3}H]D-Ala^2-D-Leu^5-enkephalin binding to mouse brain membranes. *Life Sci.* **33**:2439–2448 (1983).
29. Bowen, W. D., C. B. Pert, and A. Pert. Nigral 6-hydroxydopamine lesions equally decrease mu and delta opiate binding to striatal patches: further evidence for a conformationally malleable type I opiate receptor. *Life Sci.* **31**:1679–1682 (1982).
30. Chang, K-J., S. G. Blanchard, and P. Cuatrecacas. Unmasking of magnesium-dependent high-affinity binding sites for [DAla^2,DLeu^5]enkephalin after pretreatment of brain membranes with guanine nucleotides. *Proc. Natl. Acad. Sci. USA* **80**:940–944 (1983).
31. Bowen W. D., and C. B. Pert. Conformational malleability of opiate receptors: sulfhydryl modification alters ion-induced mu/delta ligand selectivity shifts in rat striatal sections. *Cell. Mol. Neurobiol.* **2**:115–128 (1982).
32. Sharma, S. K., W. A. Klee, and M. Nirenberg. Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. Natl. Acad. Sci. USA* **72**:3092–3096 (1975).

33. Pfeiffer, A., and A. Herz. Enhancement of delta- but not mu-opiate agonist binding by calcium. *Nauuyn-Schmiedeberg's Arch. Pharmacol.* **319**:147–153 (1982).
34. Pasternak, G. W. High and low affinity opioid binding sites: relationship to mu and delta sites. *Life Sci.* **31**:1303–1306 (1982).
35. Pfeiffer, A., and A. Herz. Discrimination of three opiate receptor binding sites with the use of a computerized curve fitting technique. *Mol. Pharmacol.* **21**:266–271 (1982).
36. Vaught, J. L., R. B. Rothman, and T. C. Westfall. Mu and delta receptors: their role in analgesia and in the differential effects of opioid peptides on analgesia. *Life Sci.* **30**:1443–1455 (1982).
37. D'Amato, R., and J. W. Holaday. Multiple opiate receptors in endotoxic shock: evidence for delta involvement and mu-delta interactions *in vivo*. *Proc. Natl. Acad. Sci. USA* **81**:2898–2901 (1984).

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