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Morphine Allosterically Modulates the Binding of [^{3}H]Leucine Enkephalin to a Particulate Fraction of Rat Brain

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SUMMARY

Equilibrium binding studies have demonstrated that [^{3}H]leucine enkephalin labels a single class of binding sites in a particulate fraction of rat brain with a dissociation constant (K_D) of 3.2 ± 0.1 nM. Methionine enkephalin was a competitive inhibitor of [^{3}H]leucine enkephalin binding, changing the K_D to 14.1 ± 1.5 nM. In contrast, Scatchard analysis of the binding of [^{3}H]leucine enkephalin in the absence and presence of 10, 50, and 100 nM morphine demonstrated that these concentrations of morphine decreased the number of binding sites by 23%, 32%, and 42%, respectively, with no change in the K_D. In contrast, morphine at 500 nM caused a 45% decrease in the number of binding sites and an increase in the K_D. On the basis of these data, the inhibitory dissociation constant (K_I) of morphine was calculated to be 400 ± 17 nM. The noncompetitive inhibition by morphine of [^{3}H]leucine enkephalin binding was shown to be rapidly reversible, ruling out pseudoirreversible binding of morphine to the enkephalin binding site as the underlying mechanism. Computer analysis of the displacement [^{3}H]leucine enkephalin binding by various concentrations of morphine has demonstrated that an allosteric model, not a two-site model, best describes the observed data. We conclude that at low concentration morphine binds to a receptor not labeled by [^{3}H]leucine enkephalin and by doing so allosterically induces an apparent loss of enkephalin receptors.

INTRODUCTION

By using a variety of experimental approaches, the existence of distincts subslasses of opiate receptors has been proposed. On the basis of experiments in chronic spinal dogs, Martin *et al.* (1) and Gilbert and Martin (2) hypothesized three classes of opioid receptors, which they called *mu*, *kappa*, and *sigma* receptors. By using guinea pig ileum and mouse vas deferens bioassays as well as ^{3}H-opioid binding assays, Lord *et al.* (3) obtained evidence for two receptor populations in these preparations as well as in brain. Comparison of the actions of opiates and opioid peptides in more complex test systems *in vivo* also provides data that cannot be readily explained by a single-receptor hypothesis (4–8).

Opioid peptides displace the binding of radiolabeled peptides more potently than do the alkaloids, and the alkaloids displace the binding of a ^{3}H-opiate more potently than do the opioid peptides (3, 9–11). This is consistent with the hypothesis that certain opioid peptides bind to a receptor distinct from that labeled by ^{3}H-opiates such as [^{3}H]dihydromorphine or [^{3}H]naloxone. These results have received strong support from data which suggest that opiates and opioid peptides act on different receptor populations in mouse vas deferens (3) and from the lack of cross-tolerance between morphine-like opiates and certain opioid peptides in this tissue (12).

A major discrepancy between ligand binding studies in brain (3, 9–11) and bioassay data (3) is that the binding data suggest that ME[3] and LE are only 2- to 10-fold selective for the enkephalin receptor, yet in mouse vas deferens they would appear to interact predominantly with the enkephalin receptor. Cox and co-workers (13) have reported that the binding of [^{3}H]dihydromorphine and [^{3}H]D-Ala^2-D-Leu^5-enkephalin to guinea pig ileum and mouse vas deferens is similar to that observed with brain. This finding suggests that both of these peripheral tissues contain morphine and enkephalin receptors and that they have the same characteristics as do the receptors of brain. The fact that the enkephalins bind with apparent high affinity to the morphine receptor of mouse vas deferens yet do not appear to interact physiologically with it remains unexplained.

Additionally, Vaught and Takemori (6) have reported that LE at subanalgesic doses potentiates morphine analgesia. This strongly suggests that LE may act centrally

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[3] The abbreviations used are: ME, Met^5-enkephalin; LE, Leu^5-enkephalin; TLC, thin-layer chromatography.

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at a receptor distinct from that which mediates morphine analgesia. This important observation has been confirmed by two other laboratories (14, 15). Furthermore, receptor autoradiography studies utilizing conditions which allow the selective labeling of morphine and enkephalin receptors demonstrate a differential distribution of these receptors (16–18).

In ligand binding studies the method commonly used to determine the affinity of a drug for a receptor involves displacement of the binding of a fixed concentration of ^{3}H-ligand with increasing concentrations of a test drug. The concentration of drug which inhibits the binding by 50% (IC_{50}) is graphically determined and the inhibitory dissociation constant (K_I) is calculated according to the Cheng-Prusoff equation (19), which assumes competitive inhibition. Morphine has been reported to displace the binding of [^{3}H]ME with a Hill coefficient of 0.59 (11). Because such a low Hill coefficient might not be consistent with competitive inhibition, we decided to determine whether morphine was a competitive inhibitor of [^{3}H]LE binding. We therefore examined in detail the displacement of [^{3}H]LE binding by morphine by determining the effect of fixed concentrations of morphine on the saturation binding of [^{3}H]LE; we observed that morphine causes a dose-dependent masking of [^{3}H]LE binding sites. From computer analysis of these data, we conclude that [^{3}H]LE labels a single class of binding sites, not two as would be expected according to published information (3, 9–11). We additionally conclude that morphine allosterically masks enkephalin receptors as a consequence of binding to a site not labeled by [^{3}H]LE.

MATERIALS AND METHODS

Preparation of a crude particulate fraction (membranes). Male Sprague-Dawley rats weighing 200–300 g (Hilltop Lab Animals, Scottsdale, Pa.) were decapitated and their brains were rapidly removed in a cold room. The cerebellum was removed from each brain and the brain was divided into two halves by cutting the corpus callosum and brain stem with a glass manipulator. One-half of each brain, weighing approximately 700 mg, was homogenized in about 20 ml of ice-cold 50 mM NH_4Ac (pH 7.70 at room temperature) using a Wheaton 30-ml glass homogenizer with a Teflon pestle. The homogenate was diluted with ice-cold 50 mM NH_4Ac to a final volume of 70 ml and centrifuged at 27,000 × *g* at 4° for 15 min. The pellets were resuspended in 70–100 ml of ice-cold 80 mM NH_4Ac (pH 7.70 at 4°) (referred to subsequently as buffer) for the [^{3}H]LE binding assay, and 160–200 ml of buffer for the [^{3}H]etorphine binding assay. The pH of the final membrane suspension was routinely checked and readjusted to 7.70 at 4° if necessary.

Binding assays. The binding of [^{3}H]LE was determined according to the following protocol. Volumes of 500 μl or 1000 μl of ice-cold membranes containing between 400 and 600 μg of protein per milliliter were added to 12 × 75 mm borosilicate glass test tubes which had been immersed in an ice-water bath and prefilled with isotope and drug (delivered in 25 μl or 50 μl of buffer). Following a 2- to 3-hr incubation on ice (equilibrium conditions), 500-μl or 1000-μl aliquots were filtered under reduced pressure over Whatman GF/C filters and washed twice with 5-ml aliquots of ice-cold buffer. All points were determined in triplicate and the nonspecific binding was determined by incubations in the presence of 10 μM naloxone.

Metabolism. The metabolism of [^{3}H]LE was examined by TLC as described by Malfroy *et al.* (20), using the solvent system $CHCl_3$:CH_3OH:AcOH:H_2O (45:30:6:9). A concentration of 6 nM [^{3}H]LE was incubated for 2 hr on ice in the absence of membranes, in the presence of membranes, and with membranes which had been immersed in boiling water bath for 15 min. After acidification with 0.1 N HCl, the membranes were pelleted by centrifugation in a Beckman Microfuge, and 20 μl of the supernatant were spotted on Eastman 6061 silica gel TLC plates. Following development of the plates, each plate was cut into 1-cm fractions and the tritium in each fraction was determined after extraction with 0.5 ml of methanol.

Statistics and calculations. All experiments, except where indicated, were performed three times, each time with the freshly prepared membranes of a different rat brain. The standard error of the mean of the three experiments was less than 10% of the mean.

Data from saturation binding experiments were plotted according to the method of Scatchard (21) and fitted to a straight line by the method of least squares. The lines drawn in graphs of these binding curves are the linear regression lines. The standard errors of the K_D and B_{max} were determined from the parameters of the regression line.

In other experiments, increasing concentrations of an unlabeled drug were used to displace the binding of a fixed concentration of ^{3}H-ligand. These data were plotted according to the Hill equation, fitted to a straight line, and the IC_{50} was determined as the *X*-intercept of the regression line. The correlation coefficients squared (r^2) were greater than 0.98. The standard errors of the IC_{50} values were determined from the parameters of the regression line.

In plotting data according to the Hill equation, the transformation used by Chang and Cuatrecacas (10) was used. According to this transformation the logarithm of the concentration of the drug is plotted versus the logarithm of the percentage inhibition (%*I*) divided by the percentage of control (%*C*). If we let B_o and B_e be the concentration of ligand bound in the absence and presence of the displacing drug, then $\%C = 100 \times (B_e/B_o)$, $\%I = 100 - \%C$, and

$$\frac{\%I}{\%C} = \left(1 - \frac{B_e}{B_o}\right)\frac{B_o}{B_e} = \left(\frac{B_o - B_e}{B_o}\right)\frac{B_o}{B_e} = \frac{B_o - B_e}{B_e}$$

Most workers plot log $B_e/(B_o - B_e)$ on the *Y*-axis. The only difference between this transformation and the one we have used is that the slope of the Hill plot is positive instead of negative.

The K_I of a test drug was calculated from the IC_{50} according to the equation (19) $K_I = IC_{50}/(1 + [\text{ligand}]/K_D)$ when it was demonstrated by Scatchard plots of saturation binding experiments that the displacement was competitive. When the saturation binding experiments were performed in the absence and presence of an unlabeled drug, the K_I was calculated according to the following equation (ref. 22 and Appendix): $K_I = [\text{inhibitor}]/(K_D'/K_D - 1)$, where K_D' is the apparent K_D determined in the presence of the inhibitor.

Linear regression analysis was performed using either a Texas Instruments SR-51-II calculator or a Radio Shack TRS-80 computer. Nonlinear least-squares analysis of data was accomplished by use of the computer program CURFIT as described by Bevington (23) and modified to run on a TRS-80. The goodness of fit was determined by calculating the χ^2 statistic between the observed and predicted data.

Statistical significance between experimental and control conditions was measured by Student's t-test. A value of $p < 0.05$ was considered to be significant and is indicated in the legends to tables and figures by an asterisk.

The protein content of the membrane suspensions were determined by the method of Lowry *et al.* (24).

Chemicals. ^{3}H-LE (specific activity 25, 32.6, and 26.8 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). ME and bacitracin were purchased from Sigma Chemical Company (St. Louis, Mo.). Morphine sulfate and dextrorphan were gifts of Merck (Rahway, N. J.). Diprenorphine was a gift of Dr. Willette, National Institute on Drug Abuse (Bethesda, Md.). Naloxone was donated by Endo Laboratories (Garden City, N. Y.). Ammonium acetate (Malinkrodt) was purchased from Scientific Products (McGraw Park, Ill.).

RESULTS

Ligand Metabolism

The metabolism of [^{3}H]LE was examined by TLC as described under Materials and Methods. Following a 2-hr incubation at 0°, about 15% was degraded by an aminopeptidase, as previously reported (25). No ^{3}H-labeled Tyr-Gly was formed, indicating no detectable dipeptidyl dipeptidase activity. These data agree with the data of Law and Loh (9). Bacitracin has been shown to protect partially the metabolism of [^{3}H]LE at 24° and 37° (9). Bacitracin at 50 μg/ml reduced the metabolism of [^{3}H]LE at 0° to undetectable levels. In another experiment, bacitracin (50 μg/ml) did not enhance the binding of [^{3}H]LE. This and a stable time course suggest that the small metabolism [^{3}H]LE which does occur at 0° did not significantly affect the results reported here.

Nonspecific Binding, Buffer Concentration, and Time to Equilibrium

The concentration of drug used to define the nonspecific binding must be chosen carefully (26). As shown in Fig. 1, naloxone, morphine, and diprenorphine inhibited the total binding of 3 nM [^{3}H]LE in a dose-dependent manner until a maximal displacement to 30% of total binding was attained by all drugs. Similar displacement curves have been run with many other drugs with equivalent results. ME, which displaces 100% of the specific binding at 100 nM and 1000 nM (Fig. 5) does not alter the binding in the presence of 10 μM naloxone. This indicates that with NH_4Ac as the buffer there is no evidence for the presence of a morphine- and naloxone-insensitive peptide binding site which was reported by Audigier *et al.* (27) and Rothman and Westfall (28) using Tris buffer.

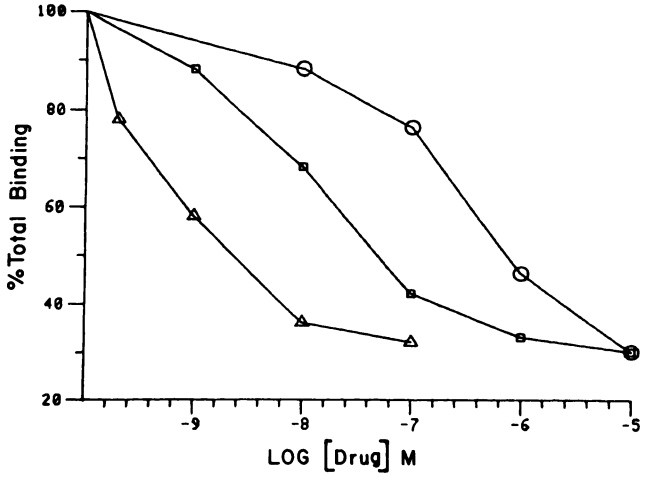

FIG. 1. *Displacement of total [^{3}H]LE binding by morphine, naloxone, and diprenorphine*

Displacement of the total binding of 3 nM [^{3}H]LE by morphine (○), naloxone (□), and diprenorphine (△) was determined as described under Materials and Methods. The total binding was 66 ± 6 fmoles/mg of protein and the binding in the presence of 10 μM naloxone was 20 ± 1 fmoles/mg of protein. All values are means ± standard error of the mean; $n = 3$.

The binding assays described in this paper were run in 80 mM NH_4Ac (pH 7.70 at 4°). The initial reason for using this buffer was that the artifactual binding of [^{3}H]LE to the glass-fiber filters previously described (28) did not occur in the ammonium acetate buffer. The reason for choosing a buffer concentration of 80 mM, as shown in Fig. 2, was that the binding of 3 nM [^{3}H]LE was maximal at this concentration. We have not yet examined in detail the binding of [^{3}H]LE at any other concentration of buffer. The differential effect of the concentration of NH_4Ac on the binding of [^{3}H]LE and [^{3}H]ET suggests that these ligands may be labeling different receptors.

It is critical that an equilibrium ligand binding assay be at equilibrium and maintained at equilibrium during filtration (26). Both 0.5 nM [^{3}H]LE and 0.125 nM [^{3}H]ET (the lowest concentrations generally used) were at equilibrium after 2-hr and 3-hr incubations, respectively, in an ice-water bath. Thus, the data reported in this paper are representative of true equilibrium binding experiments.

Equilibrium Binding Studies

The saturation binding of [^{3}H]LE from 0.5 nM to 16 nM, 17% to 87% saturation, is shown in Fig. 3, which illustrates a typical experiment with the freshly prepared membranes of three rats. The ratio of total to nonspecific binding varied from 3.6 at 0.5 nM to 1.6 at 16 nM. A Scatchard plot of these data was consistent with the hypothesis that [^{3}H]LE labeled a single class of binding

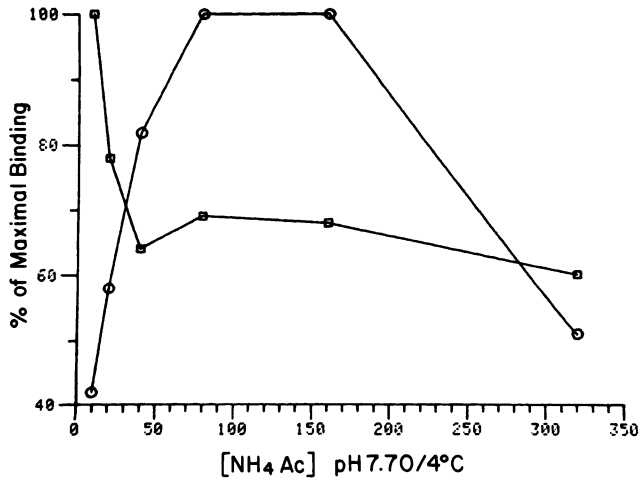

FIG. 2. *Binding of [^{3}H]LE and [^{3}H]etorphine: effect of buffer concentration*

Whole brain was homogenized and centrifuged as described under Materials and Methods. Pellets were resuspended into ice-cold 10, 20, 40, 80, 160, and 320 mM NH_4Ac(pH 7.70 at 4°), and the binding of 3 nM [^{3}H]LE (○) and 0.25 nM [^{3}H]etorphine (□) was assayed as described under Materials and Methods, except that the filters were washed with the same buffer used in the binding assay. The results are expressed as percentages of maximal binding, which were 48 ± 4 fmoles/mg of protein for [^{3}H]etorphine and 40 ± 1 fmoles/mg of protein for [^{3}H]LE. All values are means ± standard error of the mean; $n = 3$.

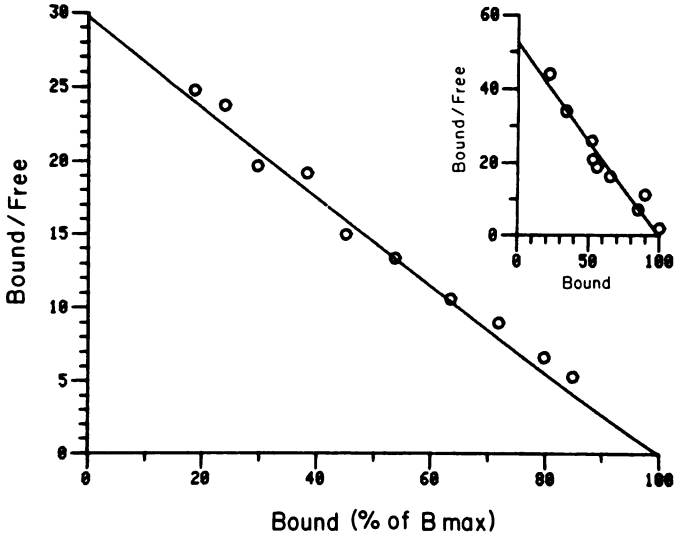

FIG. 4. *Combined Scatchard plots of 30 separate experiments*

The binding of 0.75, 1.5, 3, 6, and 12 nM [^{3}H]LE was determined in 20 separate experiments, and the binding of 1, 2, 4, 8, and 16 nM [^{3}H]LE was determined in 10 other experiments. Each experiment was analyzed by linear regression, and the binding at each concentration of [^{3}H]LE was expressed as a percentage of the B_{max}. The combined data, expressed as a Scatchard plot, were characterized by a K_D of 3.4 nM and an r^2 of 0.98. The mean values ± standard error of the mean of the K_D and B_{max} values calculated from individual experiments were 3.4 ± 0.1 nM and 152 ± 7 fmoles/mg of protein. Shown in the *inset* is a Scatchard plot of [^{3}H]LE binding at nine concentrations between 0.5 and 52 nM. The K_D was 1.9 ± 0.1 nM and the B_{max} was 100 ± 2 fmoles/mg of protein.

sites. Similar linear Scatchard plots have been observed by some investigators (9, 29) but not by others (3).

In order to rule out the possible presence of curvilinearity in Scatchard plots of the binding of [^{3}H]LE, the data from 30 experiments were collated. A Scatchard plot of these data is shown in Fig. 4. Over the concentration range examined, 18%–85% saturation, there is no evidence of a significant deviation from linearity. The K_D of the combined data was 3.4 nM, whereas the K_D of the same experiments calculated separately was 3.4 ± 0.1 nM. In another experiment, performed three times, the binding of [^{3}H]LE over a broader concentration range of 0.24 nM–16 nM was examined and analyzed by linear regression. There was no significant deviation from linearity ($r^2 = 0.97$), and the percentage saturation values at 0.24 nM and 16 nM [^{3}H]LE were 8.4% and 90%, respectively. In another experiment, using isotopic dilution, the binding [^{3}H]LE at nine concentrations between 0.5 and 52 nM was determined. The Scatchard plot (Fig. 4, *inset*) was unambiguously linear with a $K_D = 1.9 \pm 0.1$ nM. On the basis of these data, we believe that analysis of [^{3}H]LE Scatchard plots by linear regression is justified, and that deviation from strict linearity in the data of a single experiment results from experimental variation, i.e., the limited number of animals and points used in a single experiment.

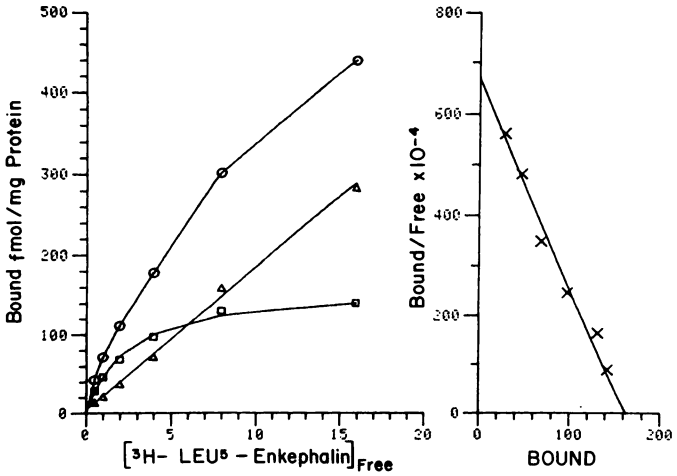

FIG. 3. *Total, nonspecific, and specific binding of [^{3}H]LE*

Total (○), nonspecific (△), and specific (□) binding of 0.5, 1, 2, 4, 8, and 16 nM [^{3}H]LE is plotted as a binding isotherm in the *left* panel, and as a Scatchard plot in the *right* panel. The K_D is 2.4 ± 0.07 nM and the B_{max} is 163 ± 2 fmoles/mg of protein. All values are means ± standard error of the mean; $n = 3$.

The displacement of 3 nM [^{3}H]LE by ME, morphine, and dextrorphan is shown in Fig. 5. Morphine (IC_{50} = 150 nM) was 54 times less potent than ME (IC_{50} = 2.8 nM). The Hill coefficients were 0.63 and 1.3 for morphine and ME, respectively. The 54-fold difference in potency is similar to the 21-fold difference observed by Law and Loh (9). The small inhibition caused by 1 μM dextrorphan demonstrates the stereospecificity of the [^{3}H]LE binding site. Assuming competitive inhibition, the K_I values of ME and morphine, calculated with the Cheng-Prusoff equation (19), were 1.4 nM and 77 nM, respectively.

To validate the assumption of competitive inhibition, we examined the effect of fixed concentrations of ME and morphine on the saturation binding of [^{3}H]LE. These data are shown in Fig. 6. ME was a competitive inhibitor

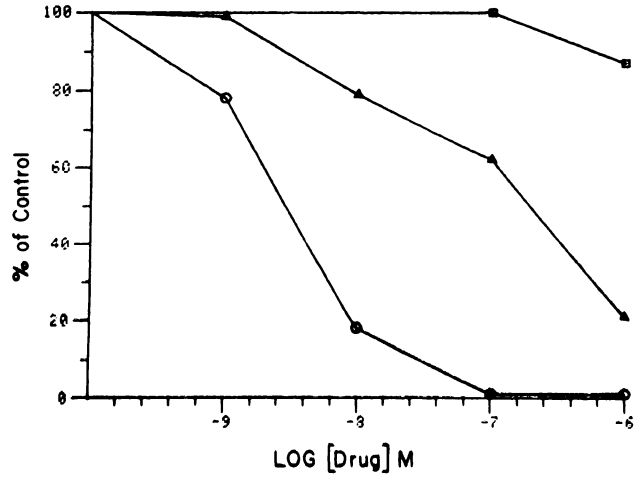

FIG. 5. *Displacement of [^{3}H]LE specific binding by ME, morphine, and dextrorphan*

The specific binding of 3 nM [^{3}H]LE was determined in the absence and presence of increasing concentrations of ME (○), morphine (△), and dextrorphan (□). The IC_{50} of ME was 2.8 nM and that of morphine was estimated to be 150 nM. The control binding was 45 ± 2 fmoles/mg of protein. All values are means ± standard error of the mean; $n = 3$.

of [^{3}H]LE binding. A concentration of 5 nM ME increased the K_D from 3.2 ± 0.1 nM to 14.1 ± 1.5 nM without significantly affecting the B_{max}. Because of the large inhibition produced by ME, it was difficult to obtain clean data at higher concentrations of [^{3}H]LE. The calculated K_I of 1.5 ± 0.2 nM agrees well with that calculated from the displacement curve shown in Fig. 5. In contrast, 10, 50, and 100 nM morphine caused 23 ± 2, 32 ± 2 and 43 ± 2% decreases, respectively, in the B_{max} with no significant alteration in the K_D. On the other hand, 500 nM morphine, in addition to decreasing the B_{max} by 45 ± 2%, also increased the K_D from 3.2 ± 0.1 nM to 7.1 ± 0.3 nM. A K_I of 400 ± 17 nM was calculated as described under Materials and Methods. As shown in Table 1, the effects of 50 and 100 nM morphine have been replicated many times.

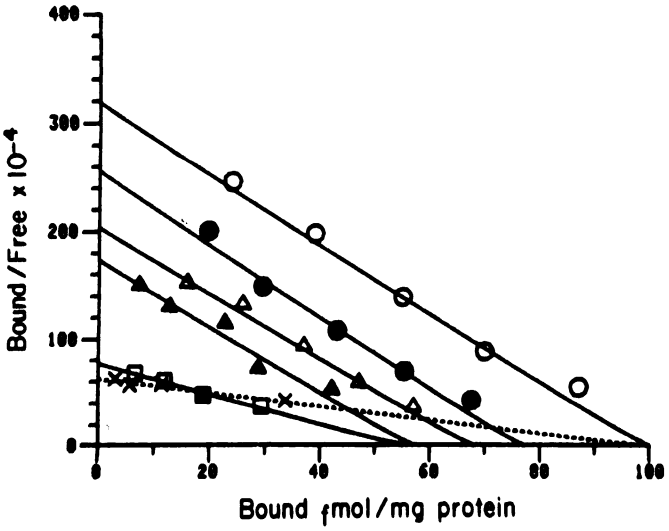

FIG. 6. *Saturation binding of [^{3}H]LE in the absence and presence of ME and morphine*

The saturation binding of [^{3}H]LE at concentrations between 0.5 and 16 nM was examined in the absence (○) and the presence of 5 nM ME (×), 10 nM (●), 50 nM (△), 100 mM (▲), and 500 nM (□) morphine. The B_{max} values were 100 ± 2, 99 ± 8, 77 ± 1*, 68 ± 2*, 57 ± 3*, and 55 ± 2* fmoles/mg of protein, respectively. Only 500 nM morphine and 5 nM ME caused significant increases in the K_D values from 3.2 ± 0.1 nM to 7.4 ± 0.3* nM and 14.1 ± 1.5* nM, respectively. The K_I values of morphine and ME were calculated to be 400 ± 17 nM and 1.5 ± 0.2 nM, respectively. All values are means ± standard error of the mean; $n = 3$. * $p < 0.05$ when compared with control. In another experiment, 650 nM morphine decreased the B_{max} by 52% and increased the K_D 2.5-fold. A K_I of 433 ± 36 nM was calculated.

These data indicate that the displacement of a fixed concentration of [^{3}H]LE by morphine is more complex than is apparent from a simple displacement curve. Clearly, the assumption that morphine competitively displaces [^{3}H]LE from a single site, inherent in the Cheng-Prusoff equation, is not correct. Because of this the K_I of morphine, calculated assuming competitive inhibition (77 nM), is 5.2-fold lower than the actual K_I of approximately 400 nM.

Model Testing

The basic observation documented by the preceding data is that morphine causes a dose-dependent masking of [^{3}H]LE binding sites. In an attempt to understand the mechanism underlying this phenomenon, we have examined three models: a kinetic model, a two-site model, and an allosteric model, which are described in greater detail below.

The kinetic model. The kinetic model postulates that the decrease in [^{3}H]LE binding sites elicited by morphine arises from the persistent occupation of these receptors by morphine. According to this model, morphine binds pseudoirreversibly to the LE receptor. A direct test of this model is to examine whether or not the decrease in the B_{max} caused by morphine is reversible. If the kinetic model is correct, then the masking of receptors should not be rapidly reversible. An excellent example of such a model is depicted in the action of phenyoxybenzamine on opiate receptors (30).

In the experiment shown in Fig. 7, membranes were incubated for 60 min on ice in the absence (control) and in the presence (treated) of 50 nM morphine. The membranes were collected by centrifugation, the supernatants were aspirated, and the pellets were resuspended in ice-cold morphine-free buffer. The binding of [^{3}H]LE was then assayed at concentrations between 0.5 nM and 16 nM. The binding of [^{3}H]LE to control and treated membranes was identical except at 2 nM [^{3}H]LE, where there was a significant 10% reduction. Thus, the morphine-induced reduction of the number of [^{3}H]LE binding sites was rapidly reversible. This did not result from a loss of the ability of the treated membranes to respond to morphine, since treated membranes assayed in the presence of 50 nM morphine showed a 30% reduction in the B_{max} with no alteration in the K_D. This experiment therefore rules out the kinetic model.

The two-site model. The two-site model postulates that [^{3}H]LE labels two distinct receptor populations, morphine (*mu*) and enkephalin (*delta*) receptors, for which [^{3}H]LE has equal affinity and for which morphine has high and low affinity, respectively (3, 9–11). This

TABLE 1

Comparison of data predicted by the allosteric model and the two-site model with the observed data

The K_D and the B_{max} of the Scatchard plots shown in Fig. 9A and B are compared with the observed data. The K_D in the presence of morphine was divided by the K_D in its absence and multiplied by 100 to give the K_D as a percentage of control. The B_{max} values were compared by calculating the percentage decrease in the B_{max} induced by morphine. Each observed value is the mean ± standard error of the mean; the number of rats used are indicated in parentheses. The χ^2 statistic between the observed and predicted data was calculated. The K_D and B_{max} values predicted by the two-site model did not fit the observed data ($p > 0.5$). The K_D and B_{max} values predicted by the allosteric model significantly fit the observed data ($p < 0.05$ and $p < 0.005$, respectively).

	Morphine concentration	K_D			% Decrease in B_{max}		
		Observed	Two-site	Allosteric	Observed	Two-site	Allosteric
	nM	% control					
18	0	100	100	100	0	0	0
	10 (3)	92 ± 4	133	100	23 ± 0.4	3	21
	50 (18)	106 ± 3	157	110	31 ± 2	17	36
	100 (9)	107 ± 1	160	120	40 ± 2	26	40
	500 (6)	214 ± 6	233	207	42 ± 2	39	44

model is consistent with the linear Scatchard plots which we have observed. If the affinities of morphine for the two sites were sufficiently separated, then a high concentration of morphine might be able to prevent the binding of [^{3}H]LE to the morphine receptor without inhibiting the binding of the peptide to the enkephalin receptor. By Scatchard analysis, morphine would appear to decrease the number of [^{3}H]LE binding sites. The maximal possible decrease in binding sites would occur at a concentration of morphine sufficiently high to mask completely all of the morphine receptors. Thus, this model postulates that morphine competitively displaces [^{3}H]LE from two classes of binding sites. A good example of how such a model can result in apparent losses of binding sites is the effect of spiroperidol on the binding of ^{3}H-labeled 5-hydroxytryptamine to cortical membranes (31).

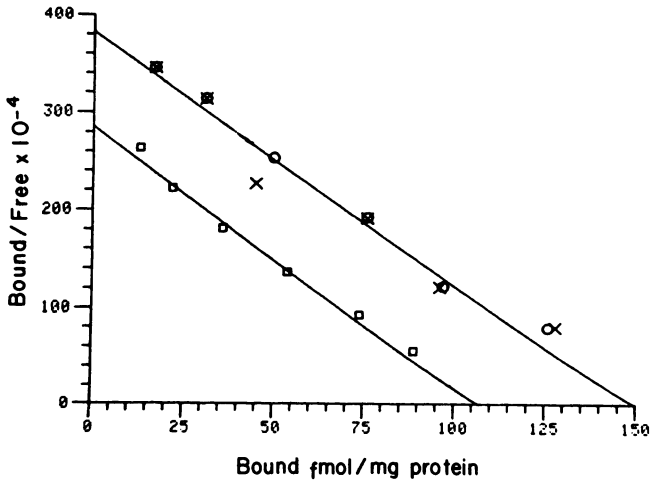

FIG. 7. *Binding of [^{3}H]LE to control and morphine-pretreated membranes*

Membranes were incubated in the absence (○) (control) and the presence (×) (treated) of 50 nM morphine for 60 min on ice. The membranes were collected by centrifugation, and the binding of 0.5, 1, 2, 4, 8, and 16 nM [^{3}H]LE was assayed in the absence of morphine. The binding of [^{3}H]LE to treated membranes was also examined in the presence of 50 nM morphine (□). When analyzed separately by linear regression, the K_D values and B_{max} values of the control and treated conditions were not significantly different. The combined data was therefore analyzed by linear regression which yielded ($r^2 = 0.98$) a K_D = 3.9 ± 0.1 nM and a B_{max} = 150 ± 3 fmoles/mg of protein. The binding of [^{3}H]LE to treated membranes in the presence of morphine was characterized by a K_D of 3.8 ± 0.08 nM and a B_{max} of 107 ± 1* fmoles/mg of protein ($r^2 = 0.99$). All values are means ± standard error of the mean; $n = 6$. * $p < 0.05$ when compared with control.

The allosteric model. The allosteric model postulates that morphine, as a consequence of binding to a receptor not labeled by [^{3}H]LE (the allosteric site), masks [^{3}H]LE binding sites via an allosteric mechanism. This implies that morphine increases the K_D of a fraction of the [^{3}H]LE receptors to such an extent that it is not significantly labeled over the concentration range of [^{3}H]LE examined. This model differs fundamentally from the two-site model. First, the allosteric model postulates that [^{3}H]LE labels a single class of binding sites, the enkephalin receptor, and not two sites. Second, this model postulates that the displacement of a fixed concentration of [^{3}H]LE by morphine occurs in part by a removal of binding sites from the system, rather than from a competitive displacement of [^{3}H]LE from two binding sites.

The interaction of morphine with [^{3}H]LE binding, shown in Fig. 6, can be thought of as a family of displacement curves, since these data examine the effect of several concentrations of morphine on a broad range of [^{3}H]LE concentrations.

In order to distinguish between these models, the displacement of the binding of 3 nM [^{3}H]LE by 11 concentrations of morphine between 2 nM and 2048 nM was analyzed by the nonlinear least-squares curve-fitting program for the parameters of the equations which describe the two-site and allosteric models. The equations obtained were then used to predict what would be the Scatchard plots of the binding of [^{3}H]LE in the absence and presence of 10, 50, 100, 500, and 1500 nM. These data were compared with the observed Scatchard plots. The correct model should be consistent with both sets of data, since parameters derived from one member of the family of displacement curves should predict the entire family of data.

The displacement of [^{3}H]LE by morphine was characterized by a Hill coefficient of 0.54 ± 0.001 nM and an IC_{50} of 156 ± 8 nM, as shown in Fig. 8. Assuming competitive inhibition and a K_D of 3.0 nM, a K_I of 78 ± 4 nM was calculated, which agrees closely with the experiment shown in Fig. 5.

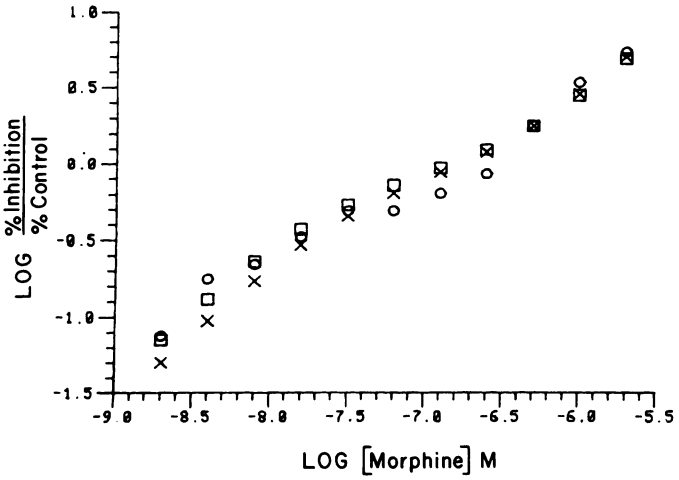

FIG. 8. *Displacement of [^{3}H]LE binding by morphine: comparison with the allosteric and two-site models*

A Hill plot of the displacement of 3 nM [^{3}H]LE by 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, and 2048 nM morphine (○) is shown. Data predicted by the allosteric model (□) and the two-site model (×) are also plotted. The Hill coefficients and IC_{50} values determined by linear regression for the observed data, allosteric model, and the two-site model were 0.54 ± 0.01, 0.55 ± 0.01, and 0.62 ± 0.01, and 156 ± 7 nM, 139 ± 5 nM, and 160 ± 4 nM, respectively. The observed data represent the mean of three separate experiments utilizing quadruplicate points.

The displacement curve was analyzed according to the two-site model as described by the following equation for the K_I of morphine for the morphine receptor, K_{mu}, and the enkephalin receptor, K_{enk}:

$$\text{Bound} = 45 \times \frac{\text{LE}}{\text{LE} + K_D(1 + M/K_{mu})} + 55 \times \frac{\text{LE}}{\text{LE} + K_D(1 + M/K_{enk})}$$

In this analysis, we have fixed two additional parameters. On the basis of the linear nature of [^{3}H]LE Scatchard plots, we assume that the ^{3}H-peptide labels both sites with equal affinity and have fixed the K_D of [^{3}H]LE to 3.0 nM for both sites. We have additionally set the number of high-affinity morphine binding sites, the *mu* receptor, to 45% of the total. This is based upon the fact that Scatchard analysis of the concentration-dependent morphine-induced masking of [^{3}H]LE binding sites indicated a maximal loss of 45% (32) (Fig. 6). We have in essence simplified an equation involving four parameters to two parameters. This has been accomplished by feeding into the equation independently determined experimental information not immediately apparent from the displacement curve shown in Fig. 8, but present in the data of Fig. 6. The importance of independently determining as many parameters as possible has been emphasized (33, 34). In this equation, M equals the concentration of morphine. This equation is derived under Appendix.

The result of this analysis indicated that the observed displacement curve was described quite well ($p < 0.001$) by a two-site model with K_{mu} = 8.6 nM and K_{enk} = 438 nM. The Hill plot of the predicted data is also shown in Fig. 9 and was characterized by a Hill coefficient of 0.62 ± 0.007 and an IC_{50} of 160 ± 4 nM. It is significant to note that Hill plots exaggerate the difference beween points. Thus, a change in the percentage of control from 68% to 70%, a 3% difference, results in a difference of 12% when transformed by the Hill equation, giving the appearance of a poorer fit than is actually the case.

The data of the same displacement curve were analyzed according to the allosteric model as described by the following equation:

$$\text{Bound} = \underbrace{\left(1 - 0.45 \times \frac{M}{M + K_{mu}}\right)}_{\text{Term A}} \times B_{max} \times \underbrace{\frac{\text{LE}}{\text{LE} + K_D(1 + M/K_{enk})}}_{\text{Term B}}$$

According to this equation, morphine acts to decrease the binding of [^{3}H]LE in two ways. By binding to a receptor not labeled by [^{3}H]LE with a dissociation con-

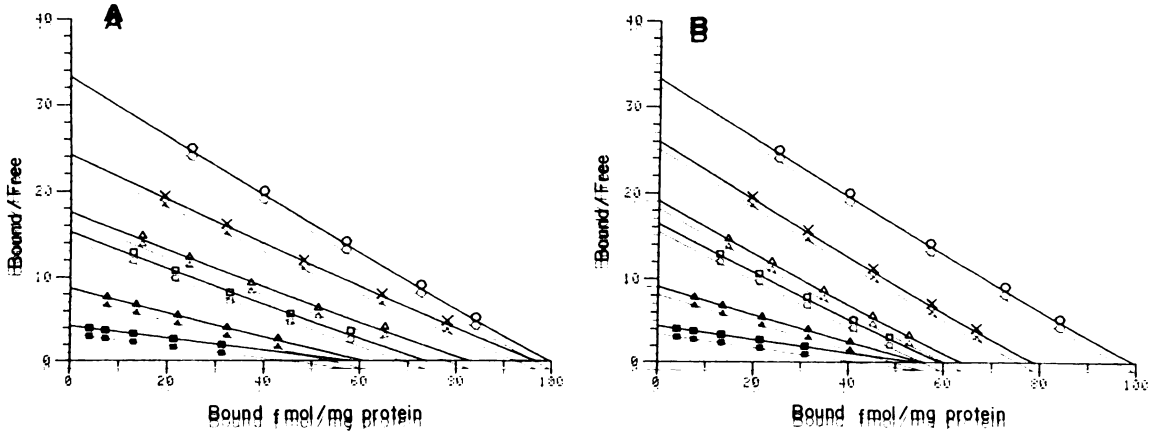

FIG. 9. *Scatchard plots of [^{3}H]LE binding generated by equations describing the two-site model (A) and the allosteric model (B)*

A. The equation describing the two-site model, as described in the text, was used to generate Scatchard plots of the binding of 1, 2, 4, 8, and 16 nM [^{3}H]LE in the absence (○), and presence of 10 nM (×), 50 nM (△), 100 nM (□), 500 nM (▲), and 1500 nM (■) morphine. In this equation, K_{mu} = 8.6 nM and K_{enk} = 438 nM.

B. The equation describing the allosteric model, as described in the text, was used to generate Scatchard plots as described in A. In this equation, K_{mu} = 12 nM and K_{enk} = 467 nM.

stant K_{mu} (Term A), morphine causes a dose-dependent decrease in the B_{max}. By binding to the [^{3}H]LE binding site (Term B) with dissociation constant K_{enk}, morphine causes a dose-dependent increase in the K_D. In this analysis, the maximal decrease in the B_{max} has been set to 45% and the K_D of [^{3}H]LE set to 3.0 nM for the reasons described above. This simplifies an equation involving four parameters to an equation involving two parameters. As with the two-site model, the observed displacement curve was described quite well ($p < 0.001$) by this model, with K_{mu} = 12 nM and K_{enk} = 467 nM. The Hill plot of the predicted data, also shown in Fig. 9, was characterized by a Hill coefficient of 0.56 ± 0.007 and an IC_{50} of 139 ± 5 nM. The derivation of this equation is also shown under Appendix.

Thus, both models accurately predict the same displacement curve and are consistent with this single member of a family of displacement curves. These equations were used to generate Scatchard plots of what would be the binding of 1, 2, 4, 8, and 16 nM [^{3}H]LE in the absence and presence of 10, 50, 100, 500, and 1500 nM morphine if the respective model described the interaction of morphine with the binding site(s) labeled by [^{3}H]LE. These data are shown in Fig. 9A and B.

By careful visual inspection, it is clear that the types of data predicted by each model are quantitatively different. The predicted Scatchard plots were analyzed by linear regression for the K_D and B_{max} values and the results were compared with the observed data as shown in Table 1. The observed and predicted data were statistically compared by calculating the χ^2 statistic. The two-site model did not significantly fit the observed data ($p > 0.50$). In contrast, the allosteric model did significantly fit the observed data ($p < 0.05$).

Of considerable importance is the effect of 10 nM morphine. The allosteric model predicts no alteration in the K_D with a 21% decrease in the B_{max}. The two-site model predicts the opposite: no change in the B_{max} and a 33% increase in the K_D. The effect of 10 nM morphine clearly distinguishes these two models. We conclude therefore that the allosteric model constitutes a working hypothesis as to the mechanism underlying the ability of morphine to cause apparent decreases in the number of [^{3}H]LE receptors.

DISCUSSION

The binding of [^{3}H]LE to brain membranes has been examined by several laboratories (3, 9, 29), and our data do not appear to differ substantially from those reported by others. In our hands, a Scatchard plot of the binding of [^{3}H]LE is linear, confirming the work of Meunier and Moisand (29) and Law and Loh (9). The fact that we find linear Scatchard plots and others (3) find curvilinear Scatchard plots is most likely due to differences in the assay conditions. Furthermore, when the IC_{50} values of LE, etorphine, naloxone, and morphine were determined, the order of potency of these compounds relative to ME were 2.4, 1.9, 5.8, and 58, respectively. This finding compares favorably with the data of Law and Loh (9), who observed an order of potency of 2.0, 0.62, 3.4, and 21 for the same drugs.

In our studies we have examined the displacement of [^{3}H]LE by morphine. Scatchard analysis of the binding of [^{3}H]LE in the absence and presence of morphine was characterized by linear Scatchard plots and indicated that morphine caused a concentration-dependent decrease in the number of [^{3}H]LE binding sites. The rapid reversibility of this phenomenon demonstrated that the pseudoirreversible binding of morphine to the site labeled by [^{3}H]LE could not be the mechanism underlying the masking of receptors.

On the basis of the hypothesis of Chang and Cuatrecacas (19) that LE has similar apparent K_I values for morphine and enkephalin receptors, it was possible that [^{3}H]LE was in fact labeling two classes of binding sites in our assay system. According to this hypothesis morphine would be binding to one class with high affinity (the morphine receptor) and to the other with considerably lower affinity (the enkephalin receptor). If the K_I values of morphine for the two classes of receptors were sufficiently far apart, then a concentration of morphine between the K_I values might prevent the binding of [^{3}H]LE to the morphine receptor while not inhibiting the binding of [^{3}H]LE to the enkephalin receptor. This is illustrated in Fig. 9A.

Alternatively, it was also possible that [^{3}H]LE was labeling a single class of sites. This possibility is well supported by physiological data from mouse vas deferens (3, 12) which suggest that LE interacts predominantly with the enkephalin receptor. In this case, the morphine-induced decrease in the number of [^{3}H]LE binding sites must have arisen as a consequence of the binding of morphine to a receptor not labeled by [^{3}H]LE. This very large increase in the K_D of a fraction of enkephalin receptors represents an allosteric effect.

By analyzing the interaction of a broad range of morphine concentrations with a broad range of [^{3}H]LE concentrations, we in essence generated a family of displacement curves. The correct model should be consistent with the entire family. To that end, a single displacement curve was analyzed according to the equation describing the two-site and allosteric models. As described under Results the allosteric model fit the entire set of data much better than did the two-site model.

Based upon this analysis, we have formulated a working hypothesis which includes two elements: first, that [^{3}H]LE labels a single class of binding sites, the enkephalin receptor; second, that morphine allosterically masks enkephalin receptors by binding to a receptor not labeled by [^{3}H]LE, the morphine receptor. From this working hypothesis, we have formulated two predictions.

The first prediction is that under these conditions the existence of the morphine recepetor should be demonstrable by direct labeling. The K_D of morphine for this site measured directly should equal its dissociation constant measured indirectly as the concentration of morphine which causes a half-maximal decrease in the number of enkephalin receptors, its K_M. In the formalism of the equation used earlier to describe the allosteric model, the K_D of morphine measured by direct binding should equal the K_{mu} determined by monitoring the effect of morphine on the number of enkephalin receptors. Furthermore, from the apparent K_I values of LE for the morphine receptor calculated assuming competitive inhibition, it might be expected that [^{3}H]LE at low con-

centrations would also label the morphine receptor (3, 9–11). Since the working hypothesis states that [^{3}H]LE does not label the morphine receptor, the second prediction is that the true K_D of LE for the morphine receptor must be higher than its apparent K_I would indicate. This implies that the assumption must be incorrect that LE displaces the binding of the ^{3}H-opiate used to label the morphine receptor in a competitive manner. Both of these predictions have been confirmed and are described in another paper (32).

In summary, in this paper we have reported that morphine masks [^{3}H]LE binding sites. The rapid reversibility of this phenomenon has ruled out the pseudoirreversible binding of morphine to the [^{3}H]LE binding site as the underlying mechanism. Computer analysis of the displacement of [^{3}H]LE binding by morphine according to the equations describing a two-site model and an allosteric model has demonstrated that only the allosteric model is consistent with all of the data. From these data we have formulated the working hypothesis that morphine allosterically modulates the enkephalin receptor, which is selectively labeled by [^{3}H]LE.

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APPENDIX

Derivation of two-site model. The equilibrium between a ligand, L, and a receptor, R, is described by the equation

$$L + R = LR \tag{1}$$

As a direct consequence of the law of mass action, an equation describing the concentration of bound ligand as a function of the concentration of free ligand can be derived (35):

$$B = B_{max} \times \frac{[L]}{[L] + K_D} \tag{2}$$

In Eq. 2, B is the concentration of bound ligand, LR, and K_D is the dissociation constant. When $[L] = K_D$, $B = 0.50 \times B_{max}$. When L binds to two distinct noninteracting sites, R and R', the chemical equilibria are described by the following equations:

$$\begin{aligned} L + R &= LR \\ L + R' &= LR' \end{aligned} \tag{3}$$

The total concentration of bound ligand is given by the equation

$$B = [LR] + [LR'] \tag{4}$$

Since R and R' do not interact, the binding of L to each receptor is described by Eq. 2, and the concentration of bound ligand will be given by the following equation:

$$B = B_{max} \frac{[L]}{[L] + K_D} + B'_{max} \frac{[L]}{[L] + K'_D} \tag{5}$$

A competitive inhibitor by definition binds to the same site as does L. An equation describing the concentration of bound ligand as a function of the free concentrations of ligand and inhibitor and the dissociation constant of the inhibitor, K_I, can be derived (35):

$$B = B_{max} \frac{[L]}{[L] + K_D(1 + [I]/K_I)} \tag{6}$$

When both L and I bind to two distinct noninteracting receptors, the concentration of bound ligand will be given by substitution of Eq. 6 into Eq. 4:

$$B = B_{max} \frac{[L]}{[L] + K_D(1 + [I]/K_I)} + B'_{max} \frac{[L]}{[L] + K'_D(1 + [I]/K'_I)} \tag{7}$$

In Eqs. 5 and 7 the total number of receptors is given by

$$B_{total} = B_{max} + B'_{max} \tag{8}$$

The units used in Eq. 8 are arbitrary. For use with computer modeling, it is most convenient to express B_{max} and B'_{max} as either a fraction or as a percentage of B_{total}. Dividing Eq. 7 by B_{total} yields

$$F = A \frac{[L]}{[L] + K_D(1 + [I]/K_I)} + A' \frac{[L]}{[L] + K'_D(1 + [I]/K'_I)} \tag{9}$$

where $A = B_{max}/B_{total}$, $A' = 1 - A$, and F is the fractional saturation, B/B_{total}.

Calculation of K_I values from Scatchard plots. Equation 2 can be rearranged to the form derived by Scatchard (21):

$$\frac{B}{[L]} = \frac{1}{K_D} B_{max} - \frac{B}{K_D} \tag{10}$$

A plot of B versus $B/[L]$ is a straight line whose slope is $1/K_D$ and whose X-intercept is the B_{max}. When the binding of L is determined in the presence of a fixed concentration of inhibitor, I_0, the K_D is multiplied by the constant, as described in Eq. 6, to yield an apparent K_D, K_D^{app}:

$$K_D^{app} = K_D(1 + [I]_0/K_I) \tag{11}$$

Equation 11 can easily be rearranged to yield the K_I as a function of K_D, K_D^{app}, and $[I]$:

$$K_I = [I]_0/(K_D^{app}/K_D - 1) \tag{12}$$

A Scatchard plot of the binding of L in the absence and presence of a fixed concentration of I will yield K_D and K_D^{app} as 1/slope of the respective lines. By using Eq. 12, the K_I can be calculated.

Derivation of the allosteric equation. When it is assumed that a measured biological response is directly proportional to the number of receptors occupied by ligand, then the laws of mass action can be used to derive the following (35):

$$V = V_{max} \times \frac{[L]}{[L] + K_M} \tag{13}$$

In Eq. 13, V represents the measured response and V_{max}

is the maximal possible response. The K_M is the concentration of L which gives a half-maximal response. Since the response is directly proportional to receptor occupancy, the K_M equals the K_D.

Equation 13 is identical in form with Eq. 2, and can similarly be transformed to Eq. 10. A Scatchard plot of Eq. 13 will be linear with the K_M given by 1/slope, and the V_{max} given by the X-intercept. Strictly speaking, this is an Eadie-Hofstie plot.

The allosteric model postulates that [^{3}H]LE labels a single class of sites, the enkephalin receptor. Morphine is postulated to bind not only to the enkephalin receptor, but also to a site not labeled by [^{3}H]LE, the morphine receptor. Occupation of the morphine receptor is hypothesized to cause a decrease in the number of enkephalin receptors via an allosteric mechanism. The binding of [^{3}H]LE is described by Eq. 6:

$$B = B_{max}^{app} \frac{[LE]}{[LE] + K_D(1 + [M]/K_{enk})} \quad (14)$$

where K_{enk} is the dissociation constant of morphine for the enkephalin receptor. The actual value of the: B_{max}^{app} is a function of the fractional occupancy of the morphine receptor:

$$B_{max}^{app} = B_{max}\{1 - V_{max}[M]/([M] + K_{mu})\} \quad (15)$$

In Eq. 15, V_{max} is the maximal fractional decrease possible, and K_{mu} is the dissociation constant of morphine for the morphine receptor. The term $V_{max}[M]/([M] + K_{mu})$ is subtracted from 1 to convert a fractional inhibition to a fraction of control. For example, if $V_{max} = 0.80$, then 100% occupancy of the morphine receptor would result in an 80% decrease in the B_{max}, meaning that $B_{max}^{app} = 0.20 \times B_{max}$. Combining Eqs. 14 and 15 gives the equation used in the text. Rearranging Eq. 15 yields

$$V = \{1 - B_{max}^{app}/B_{max}\} = V_{max}[M]/([M] + K_{mu}) \quad (16)$$

In Eq. 16, V is defined as the fractional decrease in the B_{max} induced by morphine. Equation 16 could be defined equally well in terms of a percentage decrease in the B_{max}, and is identical in form with Eq. 13. A Scatchard plot of the morphine-induced masking of [^{3}H]LE receptors will yield a K_M and V_{max}. As long as the occupancy assumption is valid, the K_M of morphine will equal its K_{mu} determined by direct binding.

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