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Allosteric Coupling between Morphine and Enkephalin Receptors *in Vitro*

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SUMMARY

In a recent paper from our laboratory [*Mol. Pharmacol.* 21:538–547 (1982)] evidence was presented which suggested that [^{3}H]leucine enkephalin labels a single class of binding sites (the enkephalin receptor) and that morphine allosterically induces a masking of enkephalin receptors as a consequence of binding to a receptor (the morphine receptor) not labeled by the ^{3}H-peptide. Evidence is presented in this paper that [^{3}H]etorphine can be used to label selectively the morphine receptor and that the inhibitory dissociation constants (K_I) of morphine, etorphine, and human β-endorphin for the [^{3}H]etorphine binding site closely approximate the concentration of these drugs which produce a half-maximal decrease in the number of enkephalin receptors. Furthermore, an examination of the interaction of leucine enkephalin and methionine enkephalin with the morphine receptor has demonstrated that the pentapeptides are not competitive inhibitors of [^{3}H] etorphine binding, and that they have much lower affinities for the morphine receptor than previously thought. On the basis of these data, a working hypothesis has been formulated which postulates that distinct morphine and enkephalin receptors coexist in an opioid-receptor complex.

INTRODUCTION

A variety of data support the classification of opioid receptors into morphine (*mu*) receptors and enkephalin (*delta*) receptors (1–13). We recently presented data (14) which suggest that [^{3}H]LE[3] labels a single class of binding sites in a crude particulate fraction of rat brain (membranes), the enkephalin receptor. We further showed that morphine as a consequence of binding to a receptor not labeled by [^{3}H]LE, i.e., the morphine receptor, allosterically decreases the number of [^{3}H]LE binding sites. These data suggest that [^{3}H]LE selectively labels the enkephalin receptor, and that occupation of the morphine receptor by morphine results in a masking of enkephalin receptors. On the basis of this working hypothesis, we have formulated two predictions.

First, the inhibitory dissociation constant (K_I) of morphine for the morphine receptor as measured by direct binding should equal its dissociation constant as measured indirectly from its ability to mask enkephalin receptors.

Second, data in the literature suggest that LE has 2 to 10 times lower K_D values for the enkephalin receptor than the morphine receptor (2–5). These apparent K_D values were calculated by assuming competitive inhibition from the concentration of LE which inhibited by 50% (the IC_{50}) the binding of the ^{3}H-ligands used to label morphine and enkephalin receptors. Since our hypothesis states that [^{3}H]LE does not label the morphine receptor, a prediction which follows is that the apparent K_D of LE for the morphine receptor is considerably lower than the actual K_D of the peptide for this binding site.

As a probe for the morphine receptor, we selected [^{3}H] ET because the binding of this ligand was characterized by linear Scatchard plots (15, 16). In contrast, markedly biphasic Scatchard plots have been consistently observed with [^{3}H]dihydromorphine. Data from bioassays suggest that ET is not selective for the morphine receptor(s). The current interpretation of ligand binding data (2–5) supports this notion. The K_D of etorphine for the enkephalin receptor has been calculated by assuming competitive inhibition, but this assumption has not been experimentally validated (2–5). This suggested to us that ET, like morphine, might have a higher K_D for the enkephalin receptor *in vitro* than previously thought. The data presented in this paper demonstrate that, with the proper experimental conditions, [^{3}H]ET can be used to label selectively the morphine receptor. With the use of [^{3}H]ET to label the morphine receptor, the predictions of the allosteric model have been confirmed.

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[3] The abbreviations used are: LE, Leu^5-enkephalin; ME, Met^5-enkephalin; ET, etorphine.

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MATERIALS AND METHODS

The binding of [^{3}H]LE and [^{3}H]ET was determined as previously described (14), with two exceptions. First, β-endorphin was diluted with buffer containing bovine serum albumin (1 mg/ml) and with the use of polystyrene test tubes. The binding assays were conducted in polystyrene test tubes (17). The final concentration of albumin was 45 μg/ml, which had no effect on the binding of either ^{3}H-ligand. Second, an experiment reported here used washed membranes (Table 2). The binding of [^{3}H]ET to washed membranes was equivalent to that observed with unwashed membranes (prepared by centrifugation of the initial homogenate at 27,000 × g for 15 min) except that the reproducibility of the triplicate samples was poorer. It was found that inclusion of 5 mM $MgCl_2$ improved the reproducibility without altering the specific binding of [^{3}H]ET. Thus the binding of [^{3}H]ET to washed membranes was conducted in the presence of 5 mM $MgCl_2$.

Calculations and statistics were carried out as previously described (14). Each experiment was carried out three times with the freshly prepared membranes of separate rats. Except where indicated, the standard error of the mean was less than 10% of the mean. An asterisk indicates $p < 0.05$ when compared with control, as determined with Student's t-test.

[^{3}H]ET (specific activity 41 and 37 Ci/mmole) was purchased from Amersham Corporation (Arlington Heights, Ill.), and [^{3}H]LE (specific activity 31 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). Morphine was a gift of Merck (Rahway, N. J.), and human β-endorphin was kindly donated by Professor C. H. Li, Hormone Research Laboratory, University of California (San Francisco, Calif.). LE and methionine enkephalin were purchased from Sigma Chemical Company (St. Louis, Mo.), and ET was a gift of Dr. James Willette, National Institute on Drug Abuse (Bethesda, Md.). Naloxone HCl was donated by Endo Laboratories (Garden City, N. Y.).

RESULTS

Evidence that [^{3}H]ET labels the morphine receptor. The morphine receptor has previously been detected indirectly by monitoring the morphine-induced loss of [^{3}H]LE binding sites. The first prediction of the allosteric hypothesis states that the morphine receptor should be demonstrable by direct labeling. This prediction additionally states that the K_I of morphine for this site should equal the concentration of morphine which causes a half-maximal decrease in the number of enkephalin receptors, which for the sake of convenience we will call its K_M.

In Fig. 1 is shown a typical saturation binding curve over the range of 0.125–2 nM [^{3}H]ET, which is 29%–85% saturation. The ratio of total to nonspecific binding ranged from 6.1 at 0.125 nM [^{3}H]ET to 3.3 at 2 nM [^{3}H]ET. A Scatchard plot of these data was linear, consistent with the hypothesis that [^{3}H]ET was labeling a single class of binding sites, or two sites with similar K_D values.

Since [^{3}H]ET could be labeling both morphine and enkephalin receptors with similar affinity, consistent with a linear Scatchard plot, the binding of [^{3}H]ET was examined at nine concentrations between 0.0625 nM and 16 nM. These data are shown in Fig. 2. Because of the high nonspecific binding at concentrations about 2 nM, the binding of [^{3}H]ET at 4 nM, 8 nM, and 16 nM was very imprecise. The Scatchard plot deviated from linearity at concentrations above 2 nM. Analysis of the data by a

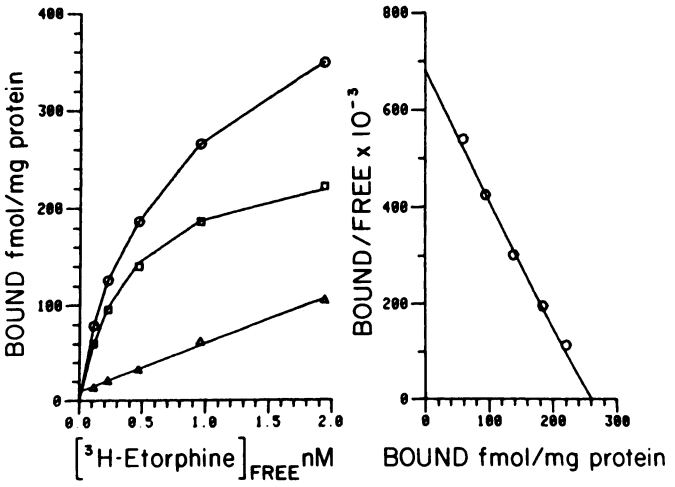

FIG. 1. *Total, specific, and nonsppcific binding of [^{3}H]ET*

Binding of 0.125, 0.25, 0.5, 1, and 2 nM [^{3}H]ET in the absence (○) and presence of 10 μM morphine (△) to a crude particulate fraction of whole rat brain is shown in the *left* panel. The specific binding (□) was calculated as the total binding minus the nonspecific binding. A Scatchard plot of the specific binding, shown in the *right* panel was analyzed by linear regression and was characterized by a K_D of 0.38 ± 0.01 nM and a B_{max} of 262 ± 2.5 fmoles/mg of protein ($r^2 = 0.99$). All values are means ± standard error of the mean; $n = 3$.

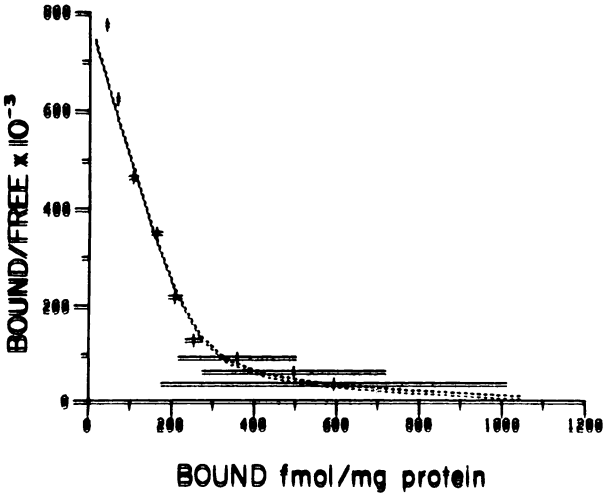

FIG. 2. *Binding of 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 nM [^{3}H] ET as a Scatchard plot*

The standard deviation of the binding observed at each concentration of ligand is shown as a *horizontal solid line*. Linear regression of the first six points (0.0625 nM–2 nM) was consistent with a high-affinity binding component characterised by a K_D of 0.33 ± 0.01 nM and a B_{max} of 279 ± 5.6 fmoles/mg of protein ($r^2 = 0.97$). Nonlinear least-squares analysis of the data for high- and low-affinity K_D and B_{max} values yielded $K_{D_H} = 0.33$ nM, $K_{D_L} = 37$ nM, $B_{max_H} = 250$ fmoles/mg of protein, and $B_{max_L} = 1089$ fmoles/mg of protein, which fit the data well ($p \ll 0.05$). The curve predicted by these parameters is shown (- - -). The *vertical lines* do not indicate error bars. The intersections of these lines with the error bars represent the experimental points. The experimental medium was filtered after a 4-hr incubation on ice. All values are means ± standard error of the mean; $n = 3$.

nonlinear least-squares curve-fitting program according to a two-site model yielded high- and low-affinity K_D values of 0.33 nM and 37 nM, and high- and low-affinity B_{max} values of 250 and 1089 fmoles/mg of protein, respectively, which fit the observed data well ($p < 0.05$). Linear regression of the high-affinity component of the Scatchard plot (the binding at concentrations between 0.0625 nM and 2 nM [^{3}H]ET) yielded a K_D of 0.33 ± 0.01 nM and a B_{max} of 279 ± 6 fmoles/mg of protein. Thus, linear regression of the first six points of the Scatchard plot yielded an excellent estimate of the high-affinity binding component. This is consistent with the 112-fold difference in the K_D values of [^{3}H]ET for the two binding sites. According to these parameters, 80% of [^{3}H]ET binding at 2 nM is to the high-affinity component. These data indicate that the high-affinity binding site can be studied by using concentrations of [^{3}H]ET less than or equal to 2 nM.

In order to explore the hypothesis that the high-affinity binding site of [^{3}H]ET may in fact be a composite of morphine and enkephalin receptors, the displacement of 0.4 nM [^{3}H]ET by 10 concentrations of morphine between 2 nM and 1024 nM was examinned. A Hill plot of these data, shown in Fig. 3, was characterized by a Hill coefficient of 0.72 ± 0.008 and IC $_{50}$ of 37 ± 1 nM. A K_I of 14 ± 0.4 nM was calculated assuming competitive displacement from a single site to which [^{3}H]ET bound with a K_D of 0.25 nM. This K_D value was chosen since a Scatchard plot of the binding of [^{3}H]ET was not determined in the same membranes used for the displacement experiment. The K_D was assumed to be about 0.25 nM, since this value yielded a K_I of morphine very close to what had been observed in two other experiments (K_I = 12.5 ± 0.5 nM and 13 ± 1 nM, respectively) and was within the range of K_D values we typically obtained with [^{3}H]ET.

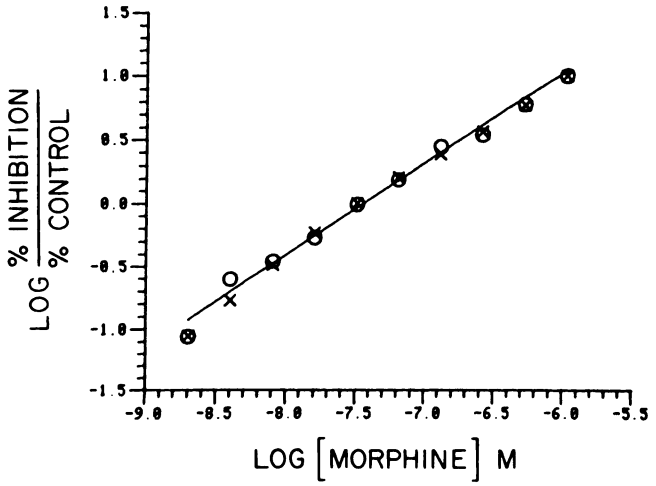

FIG. 3. *Displacement of the binding of 0.4 nM [^{3}H]ET by 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 nM morphine (○) as a Hill plot*

The data predicted by the two-site model, as described in the text, are also shown (×). Linear regression yielded Hill coefficients and IC_{50} values for the observed and predicted data of 0.72 ± 0.008 and 0.74 ± 0.007, and 37 ± 1 nM and 39 ± 0.8 nM, respectively (r^2 = 0.99 for both sets of data). The control binding was 113 ± 5 fmoles/mg of protein. For the observed data, all values are means ± standard error of the mean; n = 3. The observed data fit the predicted data ($p < 0.001$) as calculated using the χ^2 statistic.

(The variation in the K_I of morphine was always less than the variation in the K_D [^{3}H]ET.)

The data of the same displacement curve were analyzed by the nonlinear least-squares curve-fitting program according to a two-site model, as described by the following equation:

$$\text{Bound} = A \times \frac{\text{ET}}{\text{ET} + K_D(1 + M/K_{mu})} + (100 - A) \times \frac{\text{ET}}{\text{ET} + K_D(1 + M/K_{enk})}$$

where ET equals the concentration of [^{3}H]ET (0.4 nM), K_D equals 0.25 nM (assumed to be the same for both morphine and enkephalin receptors), K_{mu} equals the K_D of morphine for the morphine receptor, K_{enk} equals the K_D of morphine for the enkephalin receptor, A equals the percentage of total binding sites that are morphine receptors, and M equals the concentration of morphine. The parameters that yielded the best fit to the observed data were K_{mu} = 6.6 nM, K_{enk} = 172 nM, and A = 74. This solution in fact closely resembles both the selectivity of morphine for enkephalin and morphine receptors, and the relative numbers of these two receptors reported by Chang and Cuatrecacas (2). The calculated data described the observed data quite well ($p < 0.001$). A Hill plot of the displacement curve predicted by the two-site model using the above parameters is shown in Fig. 3. The Hill coefficient and IC_{50} were not significantly different from that of the observed data. (The derivation of this equation is given in ref. 14.)

Quite clearly, then, the displacement of [^{3}H]ET from the high-affinity binding site by morphine is consistent with both a single-site and a two-site model. In order to distinguish between these models, the effect of 10 and 40 nM morphine on the high-affinity binding component of [^{3}H]ET was examined and compared with data predicted by the two-site model.

The two-site model predicts that 10 nM morphine will be a competitive inhibitor of the binding of [^{3}H]ET with a K_I = 10 nM. An additional prediction is that 40 nM morphine would be expected to cause an apparent 19% decrease in the B_{max} with a 2.88-fold increase in the K_D from which a K_I of 21 nM can be calculated. A Scatchard plot of the predicted data is shown in Fig. 4A. On the other hand, the single-site model predicts that both 10 and 40 nM morphine will be competitive inhibitors with K_I values of about 12 nM. The obs ved data are shown in Fig. 4B. As predicted by both models, 10 nM morphine was a competitive inhibitor with a K_I of 12 ± 0.4 nM. The effect of 40 nM morphine was not in accord with the data predicted by the two-site model. This concentration of morphine was a competitive inhibitor and caused a 4.14-fold increase in the K_D from which a K_I of 12.7 ± 1.1 nM was calculated. Thus, the observed data are not in agreement with the data predicted by the two-site model, but are consistent with the one-site model.

Taken together, the preceding data strongly suggest that the high-affinity [^{3}H]ET binding site is the morphine receptor. A Scatchard plot of the binding of [^{3}H]ET over the concentration range of 0.125 nM to 2 nM is linear and provides an accurate estimate of the high-affinity [^{3}H]ET binding site. Additionally, displacement of [^{3}H]ET

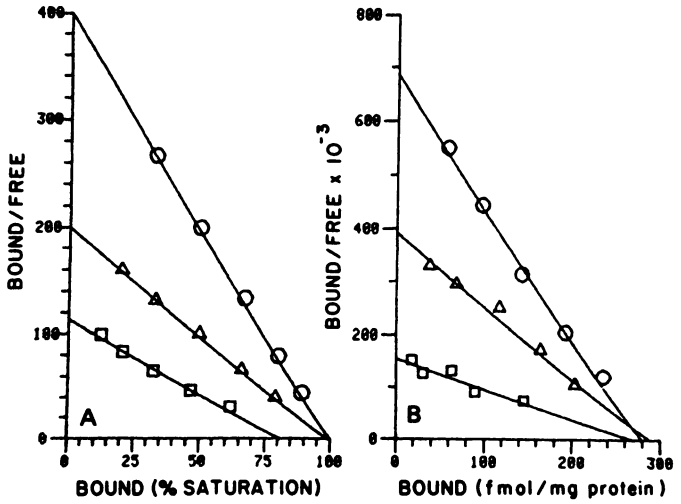

FIG. 4. *Scatchard plots of the binding of 0.125, 0.25, 0.5, 1, and 2 nM [^{3}H]ET in the absence (○) and presence of 10 nM (△) and 40 nM (□) morphine*

A. The binding curves predicted by the two-site model (see text) were characterized by K_D values of 0.25, 0.50, and 0.72 nM and B_{max} values of 100, 99, and 81% saturation, respectively (r^2 = 0.99 for all three curves).

B. Linear regression of the data yielded r^2, K_D, and B_{max} values of 0.99, 0.98, and 0.87; 0.41 ± 0.008 nM, 0.74 ± 0.024* nM, and 1.7 ± 0.15* nM; and 280 ± 3, 289 ± 6, and 270 ± 18 fmoles/mg of protein, respectively. K_I values of 12 ± 0.4 nM and 12.7 ± 1.1 nM, respectively, were calculated. All values are means ± standard error of the mean; n = 3. *$p < 0.05$ when compared with control.

from the high-affinity binding site by morphine is not consistent with the hypothesis that this binding site is a composite of both morphine and enkephalin receptors. Furthermore, linear Scatchard plots of [^{3}H]ET binding have been observed by Dunlap *et al.* (15), Minneman *et al.* (16), Ward and Takemori,[4] and Barrett and Vaught.[5]

In order to determine the concentration of morphine which caused a half-maximal decrease in the number of enkephalin receptors, the percentage decrease in the B_{max} versus the percentage decrease in the B_{max} divided by the concentration of morphine was plotted (a modified Scatchard plot) and analyzed by linear regression. These data were presented in a previous paper (14). The K_M of morphine (1/slope), as shown in Table 1, is 10 ± 1.5 nM, whereas the K_I of morphine for the [^{3}H]ET binding site is 12 ± 0.4 nM (Fig. 4B). The close agreement between the K_I of morphine determined by direct binding and the K_M of morphine determined by measuring an effect of morphine strongly suggest that [^{3}H]ET labels the morphine receptor predicted by the allosteric hypothesis. The extrapolated maximal decrease in the B_{max} of 45%, which we have called the V_{max}, is consistent with the observed data documented in our previous paper (14).

Interaction of ET and β-endorphin with morphine and enkephalin receptors. To provide additional data with which to examine the first prediction of the allosteric hypothesis, the effect of unlabeled ET and β-endorphin on the saturation binding of [^{3}H]LE was examined. As shown in Fig. 5A, 1 nM etorphine caused a 28% decrease in the B_{max}, with no significant alteration in the K_D. A

[4] S. Ward and A. E. Takemori, personal communication.

[5] R. Barrett and J. L. Vaught, personal communication.

TABLE 1

Scatchard analysis of the drug-induced masking of enkephalin receptors

Modified Scatchard plots of the concentration-dependent decrease in the number of [^{3}H]-LE receptors induced by ET, morphine, and β_H-endorphin were analyzed by linear regression and the K_M (1/slope) and the extrapolated maximal percentage decrease (the X-intercept) V_{max} were compared with the dissociation constant (K_I or K_D) of these drugs for the [^{3}H]ET binding site. All values are means ± standard error of the mean; n = 3.The correlation between the K_M and K_I values was characterized by an r^2 of 0.99.

Drug	r^2	K_M	V_{max}	K_I
			% decrease	*nM*
Etorphine	0.97	0.6 ± 0.07	45 ± 1.8	0.47 ± 0.01
Morphine	0.74	10 ± 1.5	45 ± 1.7	12 ± 0.4
β-Endorphin	0.88	5.0 ± 1.1	42 ± 4.7	4.7 ± 0.2

concentration of 5 nM ET elicited a 39% decrease in the B_{max} and a 1.7-fold increase in the K_D, from which a K_I of 7.1 ± 0.2 nM was calculated. In another experiment (data not shown), 0.5 nM ET elicited a significant 19% decrease in the B_{max} without significantly altering the K_D. Scatchard analysis of the dose-dependent decrease of the B_{max}, shown in Table 1, resulted in a K_M of 0.6 ± 0.07 nM, which is in close agreement with the K_D of [^{3}H]ET determined by direct binding. The V_{max} of 45 ± 1.8% also agrees with that determined for morphine. These data provide further evidence that the high-affinity [^{3}H]ET binding site is the morphine receptor predicted by the allosteric working hypothesis.

As shown in Fig. 5B, 5 and 15 nM β-endorphin also

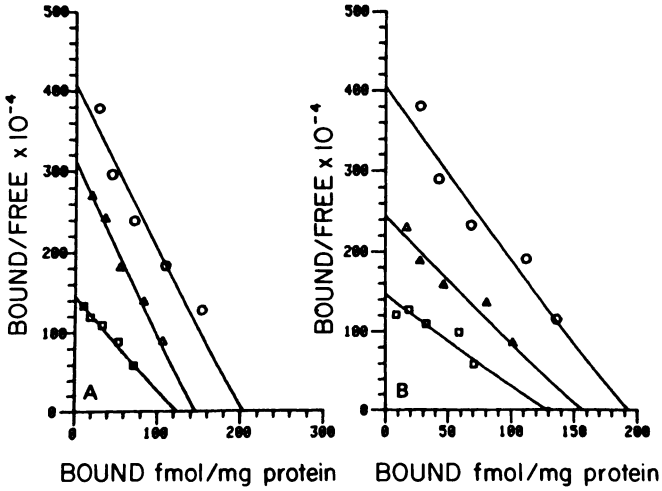

FIG. 5. *Scatchard plots of the binding of [^{3}H]LE in the absence and presence of ET and β-endorphin*

A. The binding of 0.75, 1.5, 3, 6, and 12 nM [^{3}H]LE in the absence (○) and presence of 1 nM (△) and 5 nM (□) etorphine. The r^2, K_D, and B_{max} values were 0.93, 0.98, and 0.98; 5.0 ± 0.3 nM, 4.7 ± 0.1 nM, and 8.5 ± 0.3* nM; and 204 ± 9, 146 ± 3*, and 124 ± 3* fmoles/mg of protein, respectively. All values are means ± standard error of the mean; n = 3. *$p < 0.05$ when compared with control.

B. The binding of 0.75, 1.5, 1.5, 3, 6, and 12 nM [^{3}H]LE in the absence (○) and presence of 5 nM (△) and 15 nM (□) β-endorphin. The r^2, K_D, and B_{max} values were 0.93, 0.94, and 0.81; 4.8 ± 0.3 nM, 6.4 ± 0.4 nM* and 8.8 ± 0.1* nM; and 193 ± 8, 151 ± 7*, and 129 ± 12* fmoles/mg of protein, respectively. All values are means ± standard error of the mean; n = 3. *$p < 0.05$ when compared with control.

caused dose-dependent decreases in the number of [^{3}H] LE binding sites of 19% and 33%, respectively. Both concentrations of β-endorphin increased the K_D, from which K_I values of 15 ± 0.9 and 18 ± 2 nM were calculated. The mean of these two values, 17 ± 2 nM, is therefore a good estimate of the K_I of β-endorphin for the enkephalin receptor. An additional experiment (data not shown) indicated that 2 nM β-endorphin caused a significant 12% decrease in the B_{max} with no significant alteration in the K_D. Scatchard analysis of the dose-dependent decrease in the B_{max}, shown in Table 1, yielded a K_M of 5.0 ± 1.1 nM and a V_{max} of 42 ± 4.7 nM. Scatchard analysis of the binding of [^{3}H]ET in the absence and presence of 4 nM β-endorphin (a concentration well below its K_I for the enkephalin receptor) as shown in Fig. 6 demonstrated competitive inhibition. A K_I of 4.7 ± 0.2 nM was calculated, which closely agrees with the K_M.

The effect of ET and β-endorphin on the saturation binding of [^{3}H]LE is consistent with the same allosteric model hypothesized to describe the interaction of morphine with the enkephalin receptor (14). As a check of the internal consistency of this interpretation, the equations describing the allosteric interactions of these drugs with the enkephalin receptor were used to generate predicted displacement curves of the binding of [^{3}H]LE by ET and β-endorphin, which were then compared with the observed displacement curve. The equation used was

$$\text{Bound} = \left(1 - 0.45 \times \frac{D}{D + K_M}\right) \times 100 \times \frac{2.1}{2.1 + 4.8\,(1 + D/K_I)}$$

where D equals the concentration of drug, K_M is the value given in Table 1, and K_I is the dissociation constant of the drug for the enkephalin receptor. The K_D of [^{3}H] LE was fixed to 4.8, since in the experiments shown in Fig. 5A and B this closely approximated the observed

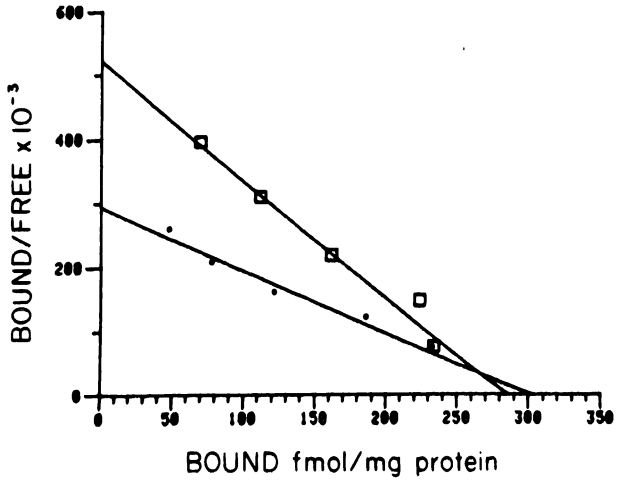

FIG. 6. *Binding of 0.2, 0.4, 0.8, 1.6, and 3.2 nM [^{3}H]ET in the absence (□) and presence of 4 nM β-endorphin (●) as a Scatchard plot*

The r^2, K_D, and B_{max} values were 0.94 and 0.97, 0.54 ± 0.02 nM and 1.0 ± 0.04* nM, and 286 ± 6 and 303 ± 8 fmoles/mg of protein, respectively. A K_I of 4.7 ± 0.2 nM was calculated. All values are means ± standard error of the mean; $n = 3$. *$p < 0.05$ when compared with control.

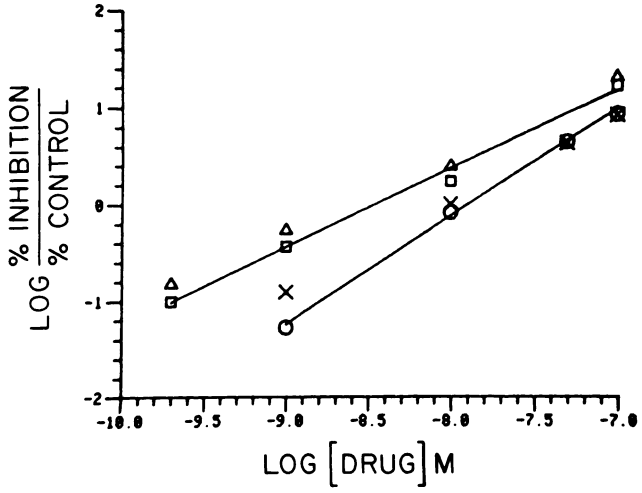

FIG. 7. *Displacement of the binding of 1.5 nM [^{3}H]LE by 1, 10, 50, and 100 nM β-endorphin (○) and 0.2, 1, 10, and 100 nM ET (□) as Hill plots*

The curves were analyzed by linear regression and were characterized by r^2, Hill coefficients, and IC_{50} values of 0.99 and 0.99, 1.1 ± 0.02 and 0.80 ± 0.02, and 13.7 ± 0.5 nM and 3.8 ± 0.25 nM, respectively. Apparent K_I values of 9.6 ± 0.4 nM and 2.7 ± 0.2 nM, respectively, were calculated. All values are means ± standard error of the mean; $n = 3$. Binding in the absence of drug was 38 ± 2 fmoles/mg of protein. The displacement curves predicted by the allosteric model (see text) for β-endorphin (×) and etorphine (△) are also shown and were characterized by Hill coefficient and IC_{50} values of 0.90 ± 0.002 and 0.77 ± 0.02, and 10 ± 1 nM and 2.4 ± 0.13 nM, respectively. The r^2 between the observed and predicted data was 0.99.

K_D. The concentration of [^{3}H]LE was set to 0.44 times the K_D (2.1 nM), since the concentration of [^{3}H]LE used in the observed displacement curves (1.5 nM) was 0.44 times the K_D of [^{3}H]LE in that series of experiments. The maximal percentage decrease in the B_{max} was set to 45%, since this value approximated the V_{max} values observed with morphine, ET, and β-endorphin. (The derivation of this equation is given in ref. 14.)

As shown in Fig. 7, the observed displacement of [^{3}H] LE by β-endorphin was well described by the displacement curve predicted by the allosteric model. Similarly, the observed displacement of [^{3}H]LE by ET was almost identical with the data predicted by the allosteric model. For both drugs, the r^2 between the observed and predicted data was 0.99. Thus, the allosteric model describes well both the effect of fixed concentrations of ET and β-endorphin on the saturation binding of [^{3}H]LE, as well as the displacement of a fixed concentration of [^{3}H]LE by increasing concentrations of these drugs as has been shown to be the case for morphine in our previous paper (14).

Two important points are made by the preceding experiments. As pointed out previously, LE, β-endorphin, and ET are thought to bind to both morphine and enkephalin receptors with similar K_D values. If this were correct, then ET and β-endorphin would be competitive inhibitors of [^{3}H]LE binding. That this is not the case strengthens the hypothesis that [^{3}H]LE does not label the morphine receptor. Additionally, the ability of β-endorphin to mask [^{3}H]LE binding sites and displace [^{3}H]LE binding with a Hill coefficient of about 1.0 is

inconsistent with the two-site model. Such a two-site model is characterized by Hill coefficients less than 1. A good example of such a model is the displacement of [^{3}H] spiroperidol by *N*-propylapomorphine (18).

We believe that the preceding data provide strong confirmation of the first prediction of the allosteric hypothesis. Data have been presented which demonstrate that appropriate concentrations of [^{3}H]ET label a single class of binding sites distinct from that labeled by [^{3}H] LE. The K_I values of morphine, β-endorphin, and etorphine for the [^{3}H]ET binding site agree well with the K_M values determined by Scatchard analysis of the dose-dependent drug-induced masking of enkephalin receptors.

Interaction of LE and ME with morphine and enkephalin receptors. The second prediction of the allosteric model is that the apparent K_I of LE for the morphine receptor is considerably lower than actual K_I of LE for the morphine receptor. These apparent K_I values have been determined from displacement curves and calculated by assuming competitive inhibition. This suggests that LE may not be a competitive inhibitor of [^{3}H] ET binding.

The displacement of 0.4 nM [^{3}H]ET by LE and ME, shown as a Hill plot in Fig. 8, was characterized by Hill coefficients of 0.69 ± 0.02 and 0.50 ± 0.02 and IC_{50} values of 60 ± 4.2 nM and 5.2 ± 0.6 nM, respectively. Apparent K_I values of 35 ± 2.4 nM and 3.0 nM were calculated for LE and ME, respectively.

As previously pointed out, displacement curves characterized by low Hill coefficients are consistent with the

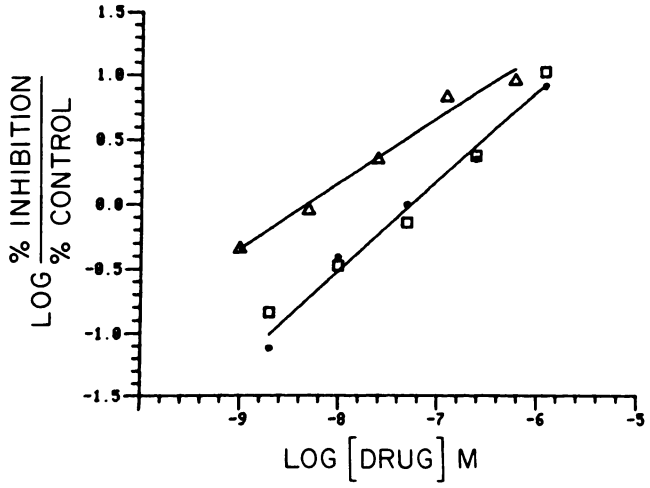

FIG. 8. *Displacement of the binding of 0.4 nM [^{3}H]ET by 2, 10, 50, 250, and 1250 nM LE (●) and 1, 5, 25, 125, and 625 nM ME (△) as Hill plots*

The curves were analyzed by linear regression and were characterized by r^2, Hill coefficient, and IC_{50} values of 0.98 and 0.98, 0.69 ± 0.02 and 0.50 ± 0.02, and 60 ± 4.2 nM and 5.2 ± 0.6 nM, respectively. Binding in the absence of added drug was 149 ± 8 fmoles/mg of protein. Scatchard analysis of the binding of [^{3}H]ET to the same membranes indicated a K_D of 0.53 ± 0.02 nM. With this value, apparent K_I values of 35 ± 2.4 nM and 3.0 ± 0.4 were calculated for LE and ME, respectively. All values are means ± standard error of the mean; $n = 3$. Also shown is the LE displacement curve predicted by the two-site model (□) as described in the text. This curve was characterized by a Hill coefficient of 0.65 ± 0.02 and an IC_{50} of 53 ± 4 nM. The r^2 between the observed and predicted data was 0.99.

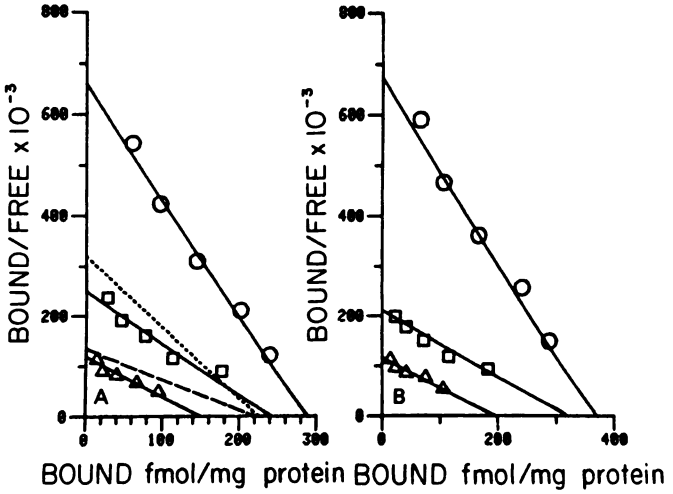

FIG. 9. *Scatchard plots of [^{3}H]ET in the absence and presence of LE and ME*

A. The binding of 0.125, 0.25, 0.5, 1, and 2 nM [^{3}H]ET in the absence (○) and presence of 50 nM (□) and 250 nM (△)LE. The r^2 values were 0.99, 0.91 and 0.93, and the K_D and B_{max} values were 0.44 ± 0.01, 0.97 ± 0.08*, and 1.24 ± 0.1* nM, and 289 ± 4, 242 ± 13*, 150 ± 8*, respectively. Apparent K_I values of 42 ± 3.5 nM and 136 ± 9 nM, respectively, were calculated. The *dotted* and *dashed lines* indicate the Scatchard plots predicted by the two-site model for 50 nM and 250 nM LE, respectively. *$p < 0.05$ when compared with control. All values are means ± standard error of the mean; $n = 3$.

B. The binding of 0.125, 0.25, 0.5, 1, and 2 nM [^{3}H]ET in the absence (○) and presence of 20 nM ME (△) and 50 nM ME (□). The r^2 values were 0.98, 0.96, and 0.93, and the K_D and B_{max} values were 0.53 ± 0.02, 1.44 ± 0.08*, and 1.6 ± 0.2* nM, and 371 ± 7, 305 ± 12*, and 195 ± 10* fmoles/mg of protein, respectively. Apparent K_I values of 12 ± 0.6 nM and 25 ± 3 nM were calculated for 20 nM and 50 nM ME, respectively. All values are means ± standard error of the mean; $n = 3$. *$p < 0.05$ when compared with control.

two-site model. These data could be taken to indicate that [^{3}H]ET labeled both morphine and enkephalin receptors, and that LE displaced the binding of [^{3}H]ET with a Hill coefficient much less than 1 owing to a considerable selectivity for the enkephalin receptor. The displacement [^{3}H]ET binding by LE was analyzed by the nonlinear least-squares curve-fitting program. The two-site model, which was shown to describe quite well the displacement of the binding of [^{3}H]ET by morphine, was used. The result was that K_{mu} = 97.4 nM and K_{enk} = 1.6 nM, with A fixed to 74. The displacement curve predicted by these parameters fit the observed data extremely well (r^2 = 0.99). The Hill plot, shown in Fig. 8, agrees quite well with the observed data.

The same equation was used to predict the effect of 50 and 250 nM LE on the saturation binding of [^{3}H]ET, as shown in Fig. 9A. Both 50 nM and 250 nM LE are predicted to cause about a 22% decrease in the B_{max}. Apparent K_I values of 85 nM and 94 nM, respectively, can be calculated. The observed data are also shown in Fig. 9A. Similar to the predicted data, 50 nM LE caused about a 17% decrease in the B_{max} and increased the K_D from which an apparent K_I of 42 ± 3.5 nM was calculated. The effect of 250 nM LE on the binding of [^{3}H]ET was not in accord with the predicted data. As shown in Fig. 9A, 250 nM LE produced a 48% decrease in the B_{max}. An apparent K_I of 136 ± 9 was calculated. As shown in Fig. 9B, similar

data have been obtained with ME. Thus, both ME and LE produced essentially parallel shifts to the left in the Scatchard plot of the binding of [^{3}H]ET. This is clearly inconsistent with the predictions of the two-site model.

The observations documented in Fig. 9A and B have been replicated. In another experiment, 250 nM LE caused a 49% decrease in the B_{max} with a 1.78-fold increase in the K_D (K_I = 320 nM), and 50 nM ME caused a 39% decrease in the B_{max} with a 2.4-fold increase in the K_D (K_I = 35 nM).

Thus, the interaction of LE and ME with the morphine receptor is complex. Like the interaction of ET, morphine, and β-endorphin with the enkephalin receptor, LE and ME induce apparent decreases in the number of morphine receptors. As shown above, the interaction of LE with the high-affinity [^{3}H]ET binding site is not consistent with a two-site model. This further supports the conclusions reached earlier on the basis of the data described in Figs. 1–5. The ability of LE and ME to mask morphine receptors contrasts sharply with the competitive interaction of morphine and β-endorphin with this binding site. In fact, it is interesting to note that, although morphine and LE displace a fixed concentration of [^{3}H]ET with similar Hill coefficients of 0.72 and 0.69, respectively, they do so via different mechanisms. This illustrates the limited ability of Hill coefficients to distinguish between models.

From these data we conclude that LE and ME do not interact in a competitive manner with the morphine receptor. The fact that the fold increases in the K_D of [^{3}H]ET induced by 50 and 250 nM LE and 20 and 50 nM ME were almost identical suggests that even at these high concentrations the pentapeptides do not bind to the morphine receptor. At a concentration of LE about 6 times its apparent K_I of 40 nM, the K_D of [^{3}H]ET remains essentially identical with that observed in the presence of 50 nM LE, resulting in a much larger apparent K_I of 136 nM. In contrast, morphine at a concentration about 4 times its K_I for the morphine receptor (40 nM) caused a very large increase in the K_D of [^{3}H]ET, as shown in Fig. 4B. Thus, the apparent K_I values of LE and ME for the morphine receptor, calculated from displacement curves (35 nM and 3 nM, respectively), are markedly lower than the actual K_I values of these peptides for the morphine receptor. The decrease in the binding of a fixed concentration of [^{3}H]ET elicited by these drugs arises not from competitive displacement but from a removal of binding sites from the system. This implies that the assumption of competitive inhibition is incorrect and confirms the second prediction of the allosteric hypothesis.

The mechanism underlying the ability of LE and ME to mask morphine receptors has been investigated. As described earlier, a two-site model is inconsistent with the observed data, as well as with the data presented in the first part of Results. The fact that the LE-induced inhibition of the binding of [^{3}H]ET is rapidly reversible, as shown in Table 2, rules out the pseudoirreversible binding of LE to the morphine receptor as a possible mechanism. In this experiment, complete reversal presumably would have been demonstrated if the postcentrifugation preincubation were long enough to allow complete dissociation of LE from the enkephalin receptor.

TABLE 2

Rapid reversibility of the LE-induced masking of morphine receptors

Membranes were incubated for 60 min on ice in the absence (control) and presence (treated) of 250 nM LE. After centrifugation at 27,000 × *g* for 15 min, the membranes were resuspended in ice-cold buffer, and the binding of 1 nM [^{3}H]ET was assayed 1 hr later. All values are means ± standard error of the mean; n = 3.

Assay	Binding of [^{3}H]ET	
	Control	Treated
	fmoles/mg protein	
Without LE	180 ± 8	152 ± 2[a]
With 250 nM LE	61 ± 1[b]	63 ± 5[b]

[a] $p < 0.05$ when compared with control membranes.

[b] $p < 0.05$ when compared with membranes assayed in the absence of LE.

DISCUSSION

In a previous paper (14), we presented evidence that [^{3}H]LE labels a single class of binding sites, the enkephalin receptor, and that morphine allosterically modulates the number of enkephalin receptors by binding to a distinct receptor not labeled by [^{3}H]LE, the morphine receptor. On the basis of this allosteric model two predictions were formulated, and data presented in this paper have confirmed these predictions. These data are discussed in reference to the following equation, which describes the allosteric model:

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

This equation describes the concentration-dependent binding of [^{3}H]LE to a single class of binding sites represented by Term B and characterized by a dissociation constant, K_D, and a B_{max}. Morphine (M) can inhibit the binding of [^{3}H]LE by binding to the enkephalin receptor with a dissociation constant K_{enk}, which results in an increase in the K_D, and also by a mechanism which results in a decrease in the B_{max}. This latter mechanism, represented by Term A, results from the binding of morphine to a site not labeled by [^{3}H]LE, the morphine receptor, where its dissociation constant is K_{mu}. The data presented in our previous paper (14) and in this paper fully support this model.

Before we discuss the ramifications of this model, an important point to be made is that the results we have reported do not represent something unique to our assay system.

In order to compare our binding assay with those used by other investigators, we have determined the IC_{50} values of several drugs by displacement of the binding of [^{3}H]LE and [^{3}H]ET. Apparent K_I values were calculated assuming competitive inhibition. The apparent K_I of a drug for the enkephalin receptor was divided by its apparent K_I for the morphine receptor to yield *delta* to *mu* ratios. These ratios were compared with the ratios determined by Chang *et al.* (19), who used ^{125}I-labeled [D-Ala^2-MePhe^4-Met (O)^5-ol]-enkephalin to label the

TABLE 3

Comparison of K_I values determined from IC_{50} values with literature values

The displacement of the binding of [^{3}H]LE and [^{3}H]ET by nine drugs was examined. The IC_{50} values were determined by linear regression of Hill plots, and the K_I values were calculated assuming competitive inhibition. The K_I for the enkephalin receptor was divided by the K_I for the morphine receptor to yield a *delta* to *mu* ratio which was compared with the ratios determined by four other laboratories.

Drug	*Delta* to *mu* ratio				
	Our laboratory	Ref. 3	Ref. 19	Ref. 20	Ref. 21
ME	0.54	0.17	0.73	0.46	0.10
LE	0.11	0.19	0.16	0.52	0.10
Naloxone	1.7	1.0	15	4.4	6.0
Cyclazocine	6.6	—	4.0	3.0	—
ET	4.5	4.5	—	2.0	2.5
Morphine	8.1	14	75	12	11.5
β-Endorphin	2.0	3.4	—	6.0	—
DALA[a]	0.07	0.27	0.4	—	—
DAMA[b]	0.20	—	—	1.0	1.5

[a] D-Ala^2-D-Leu^5-enkephalin.

[b] D-Ala^2-Met^5-enkephalinamide.

morphine receptor and ^{125}I-labeled D-Ala^2-D-Leu^5-enkephalin to label the enkephalin receptor; with those determined by Law and Loh (3), who labeled morphine and enkephalin receptors with [^{3}H]dihydromorphine and [^{3}H]LE, respectively; with those determined by Childers *et al.* (20), who labeled morphine and enkephalin receptors with [^{3}H]dihydromorphine and [^{3}H]Met-enkephalin, respectively; and with those determined by Simon and co-workers (21), who labeled morphinne and enkephalin receptors with [^{3}H]naloxone and [^{3}H]LE, respectively.

As shown in Table 3, there was good agreement between the five sets of data. The ratios reported by *Chang et al.* (19) for morphine and naloxone are considerably higher than the ratios found by ourselves and other investigators. The probable reason for this discrepancy is that morphine and naloxone displace the binding of ^{125}I-labeled D-Ala^2-D-Leu^5-enkephalin in a biphasic manner (2). The K_I values of morphine and naloxone for the enkephalin receptor were determined from the low-affinity component of the displacement curves, not from the actual IC_{50}. This was based upon the reasonable assumption that the high-affinity component of the displacement curve represented displacement of the radiolabeled peptide from the morphine receptor, and that the low-affinity component represented displacement of the ligand from the enkephalin receptor. Calculation of the *delta* to *mu* ratio of morphine based upon the actual IC_{50} of about 14 nM (2) yields a ratio of 35, which brings the ratio to a value close to that observed by ourselves and the other workers.

Because of the close agreement between the results generated by our binding assay and those used by other investigators, we believe that the data we have presented in this and our previous paper (14) do not represent something unique to our particular set of conditions, but rather reflect the fact that we have used experimental approaches not utilized by other workers. The good agreement between our results and those of other investigators who have used other ligands to label the morphine receptor is further evidence that [^{3}H]ET can be used to label the morphine receptor.

Kosterlitz and Paterson (5) have reported the presence of a putative *kappa* receptor in guinea pig brain homogenates. According to their data, etorphine binds with similar affinity to *mu, delta,* and *kappa* receptors. In contrast, LE and D-Ala^2-D-Leu^5-enkephalin have extremely low affinities for the putative *kappa* receptor. If this receptor were present in our assay system, then the ability of LE to mask [^{3}H]ET binding sites might arise from the displacement of [^{3}H]ET from the *mu* and *delta* receptors. The residual binding sites would then be the *kappa* receptor. The fact that 160 nM D-Ala^2-D-Leu^5-enkephalin is a competitive inhibitor of [^{3}H]ET binding[6] rules out this possibility.

As pointed out under Introduction, the existence of distinct morphine and enkephalin receptors is well supported by a variety of experimental data. On the basis of this information, a reasonable hypothesis is that morphine and enkephalin receptors are physically distinct and that LE and ME are 5.6-fold and 1.9-fold selective for the enkephalin receptor (19).

The data we have presented in this paper and in a previous paper (14) represent an extension and reinterpretation of these findings. The demonstration that a distinct morphine receptor allosterically modulates the number of enkephalin receptors suggests that these two distinct binding sites must be in close physical proximity to each other. Our interpretation is that these two receptors must coexist in an "opioid receptor complex" in rough analogy with the model of allosteric enzymes proposed by Monod *et al.* (22), i.e., morphine and enkephalin receptors may be "subunits" of a larger receptor complex. The ability of morphine to mask enkephalin receptors and the ability of enkephalin to mask morphine receptors would represent allosterically induced increases in the K_D of such magnitude that the binding of drugs to these high K_D conformers would not be detectable. The differential distribution of morphine and enkephalin receptors in brain could therefore represent varying ratios of the two receptors within the receptor complex. In fact, the morphine-induced masking of enkephalin receptors also occurs in striatum, cortex, and pooled nonstriatal-noncortical brain tissue,[6] although the ratios of number of morphine receptors to number of enkephalin receptors in these three regions vary (ratios of 1.13, 1.33, and 1.9, respectively). These findings are not dissimilar to the data reported by Chang *et al.* (19). However, there may certainly be discrete regions of the brain where the allosteric effects do not occur. Because full Scatchard plots are needed to determine whether or not such allosteric effects occur, the limited amount of tissue available precludes the examination of such regions of the brain at the present time.

The second important observation documented in this paper is that both ME and LE have much higher K_D values for the morphine receptor than peviously thought. According to IC_{50} values determined from displacement curves, the pentapeptides appear to bind with high affinity to the morphine receptor. As shown in this paper, the mechanism underlying the displacement of [^{3}H]ET from

[6] R. B. Rothman and T. C. Westfall, in preparation.

the morphine receptor by LE and ME involves a masking of receptors. This effect is rapidly reversible, and does not occur via competitive inhibition. The most likely mechanism is that the pepetides allosterically mask morphine receptors as a consequence of binding to the enkephalin receptor.

At this point it is not possible to determine with precision the K_D values of LE and ME for the morphine receptor. At the highest concentrations tested, 250 nM LE and 50 nM ME, the increase in the K_D of [^{3}H]ET was essentially the same as that observed in the presence of 50 nM LE and 20 nM ME. Thus, as the concentration of the peptides is increased, the calculated K_I is also increased. In principle, at even higher concentrations of peptides, a further increase in the K_D of [^{3}H]ET might occur, but good data at such high concentrations of peptides are difficult to obtain.

Although we cannot state with precision what are the K_D values of LE and ME for the morphine receptor, we believe that the sum of the evidence is that they are much higher than previously thought. Rather than being 2- to 10-fold selective for the enkephalin receptor, they are probably at least 100-fold selective for the enkephalin receptor.

This interpretation resolves two long-standing paradoxes. First, both ME and LE are weakly analgesic. The rapid metabolism of these peptides cannot fully account for their low analgesic potency, as has been elegantly demonstrated by Craves *et al.* (23). An excellent discussion of this issue can be found in their paper. The low affinity of the peptides for the morphine receptor would therefore be consistent with the low analgesic potency of these compounds. Second, in the mouse vas deferens, both pentapeptides appear to interact very selectively with the enkephalin receptor (1, 11). A K_D higher than previously thought for the morphine receptor is therefore consistent with the observations made with this preparation.

ME and LE were isolated and identified as endogenous morphine-like compounds (24). An important implication of the hypothesis that ME and LE have very low affinity for the morphine receptor is that these peptides are not endogenous ligands of the morphine receptor but would appear to be the endogenous ligands for the enkephalin receptor. This raises the question, what is the endogenous ligand for the morphine receptor? As shown in this paper, β-endorphin binds with high affinity to the morphine receptor. Dynorphin, a recently isolated tridecapeptide (25), also binds with high affinity to the morphine receptor.[6] Thus, there exist at least two known endorphins which could function as endogenous ligands of the morphine receptor.

A summary of our current working hypothesis is shown in Fig. 10. Distinct morphine and enkephalin receptors are shown as black boxes since there are many phenomena not yet understood about each individual receptor. *Hatched arrows* connecting the two receptors symbolize the allosteric interactions between the two receptors. These arrows do not indicate thermodynamic equilibrium or interconversion between two receptor conformers. ME and LE are postulated to bind selectively to the enkephalin receptor, and morphine to bind selectively to the morphine receptor. Since the K_I values of β-endorphin for morphine and enkephalin receptors are only about 5-fold apart, we postulate that β-endorphin is not selective for either receptor and can bind to both receptors. This of course is an exaggeration of the real data for the purposes of illustration. Unlike the model proposed by Lee and Smith (26), we do not postulate that β-endorphin must interact with both receptors in order to bind to the receptor complex.

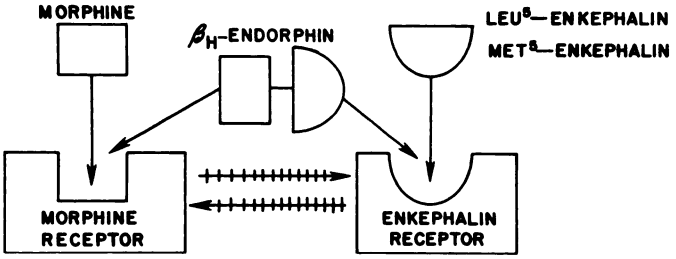

FIG. 10. *Schematic illustration of the allosteric model*
Distinct morphine and enkephalin receptors are proposed to be allosterically coupled (*hatched arrows*) and therefore to coexist in an opioid-receptor complex. The *hatched arrows* do not symbolize thermodynamic equilibrium or interconversion between receptor conformers, but represent the ability of one receptor to influence the other. ME and LE are postulated to bind selectively to the enkephalin receptor and morphine to bind selectively to the morphine receptor. β-Endorphin is postulated not to be selective for either receptor. This model does not postulate that β-endorphin must interact with both receptors in order to bind to the receptor complex, as proposed by Lee and Smith (26).

In conclusion, data have been presented that [^{3}H]ET can be used in our assay system to label selectively the morphine receptor. The dissociation constants of etorphine, morphine, and β-endorphin for the morphine receptor are essentially identical with the dissociation constants predicted by the allosteric model. This confirms a key prediction of the allosteric model. Furthermore, both LE and ME have been shown to have lower affinity for the morphine receptor than previously thought, thereby confirming a second key prediction of the allosteric model. These data therefore support the hypothesis that morphine and enkephalin receptors are allosterically coupled and coexist in an opioid-receptor complex.

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Note added in proof. Based on the data presented in this paper and ref. 14, a model of the opioid analgesic receptor has been proposed (27). Data supporting the predictions of this model have been published (28). During the preparation of the manuscript of this paper, additional data demonstrating the ability of LE to mask morphine receptors was published (29).

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