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Interactions of Leucine Enkephalin and Narcotics with Opioid Receptors

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SUMMARY

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In superfusion experiments *in vitro*, facilitation of naloxone-releasable morphine was observed with slices of corpus striatum which were obtained from mice pretreated *in vivo* with leucine enkephalin (20 mg/kg) i.p. 15 min prior to killing. Preincubation of striatal slices for 15 min with leucine enkephalin *in vitro* at concentrations ranging from 1.0 nM to 0.1 pM also enhanced the morphine-releasing effect of naloxone. Thus, very minute concentrations of leucine enkephalin are required to produce this effect. Methionine enkephalin pretreatment either *in vivo* or *in vitro* did not exhibit any of the above effects. Simultaneous incubation of an equimolar concentration of naloxone blocked this enhancing effect of leucine enkephalin. Prior incubation with leucine enkephalin did not influence naltrexone-releasable [^{3}H]naltrexone but markedly enhanced morphine-releasable [^{3}H]naltrexone and morphine- or levorphanol-releasable [^{3}H]morphine. We suggest that the apparent increase in naloxone potency is mediated by narcotic agonists and that leucine enkephalin potentiates this agonistic effect via a receptor-mediated process. Similar effects of leucine enkephalin on the narcotic-induced enhancement of naloxone potency were observed *in vivo*. These data also indicate a possible modulatory effect of leucine enkephalin on the opioid receptor.

INTRODUCTION

Recently we reported that, although leucine enkephalin and methionine enkephalin may be pharmacologically classified as pure narcotic-like agonists, their interactions with morphine are markedly different (1). We found that prior administration of leucine enkephalin to mice, either peripherally or centrally, markedly enhanced morphine-induced analgesia, acute tolerance, and dependence (2). On the other hand, methionine enkephalin had no effect on any of these pharmacological effects of morphine. Furthermore, this potentiation of narcotic-induced effects by leucine enkephalin was found to be a specific property of pure narcotic analgesics, to occur at spinal as well as supraspinal structures, not to be species- or strain-specific, to require the intact pentapeptide for maximal potency, and to be naloxone-reversible, which, indirectly, suggested a receptor-mediated phenomenon (3, 4).

In order to characterize more directly the mechanism of the leucine enkephalin-morphine interaction, we have utilized in the present study a superfusion technique in which striatal slices from mice were allowed to accumulate radioactive narcotic and subsequently were superfused with solutions containing naloxone (5, 6). We have demonstrated that a small amount of the morphine which has been accumulated by striatal slices is specifically released by naloxone and have proposed that this naloxone-releasable morphine represented receptor-bound drug. Using this system as a model for drug-receptor interactions, we now report that very minute amounts of leucine enkephalin are able to alter the characteristics of binding of narcotic to opioid receptors.

METHODS AND MATERIALS

Striatal Slice Superfusion Assay

Male Swiss-Webster mice (21-24 g) were decapitated, the brains were rapidly removed, and corpus striatal areas were separated as diagrammed by Marcucci *et al.* (7). A slice of corpus striatum was made by hand using a razor blade and a frosted glass slide, weighed, and placed in ice-cold Krebs-Ringer bicarbonate solution. Each slice consistently weighed between 3.1 and 3.5 mg. The slice was then placed in a 20-ml beaker containing 3 ml of incubation medium which consisted of Krebs-Ringer bicarbonate solution, pH 7.4. The medium was

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saturated with a gas mixture of 95% O_2 and 5% CO_2 by bubbling it through the solution for 30 min prior to use. Depending on the protocol, the slice was then either incubated in the medium for 15 min, after which 10 nM ^{3}H-labeled ligand was added, or the ^{3}H-labeled ligand was added immediately. In each instance, after the addition of the radioactive ligand, an additional 30-min incubation period ensued.

Under these conditions and at this concentration of ligand, the accumulation of ligand was saturable (5, 6). After incubation, the slice was removed from the medium, rinsed twice with fresh medium, and placed in a 0.1-ml chamber constructed from the tip portion of a 1-ml plastic syringe (5). Two polyethylene inlet tubings (PE-60) were fitted on the bottom of the chamber and the syringe tip, fitted with a 23-gauge needle, served as the outlet for the superfusion of slices. One inlet tubing was used to introduce fresh Krebs-Ringer bicarbonate solution and the other was used to introduce the solution containing various concentrations of naloxone. The entire chamber was immersed in a tissue bath to maintain the temperature at 37°. Superfusion of the slices was accomplished with a syringe pump at a rate of 0.3 ml/min. Samples were collected into 5-ml counting vials every minute for 15 to 30 min using a modified automatic fraction collector. Aquasol II (4.5 ml) was added to each sample and the samples were shaken for 10 min. Radioactivity was determined in a liquid scintillation spectrometer to <10% error at a counting efficiency of 35–38% after storage for 24 hr in the dark.

Enkephalin treatment. To assess the effects of leucine or methionine enkephalin on the release of a ^{3}H-labeled ligand, two procedures were followed. Fifteen minutes prior to killing the animal, leucine or methionine enkephalin was injected i.p. (20 mg/kg). The slice was then prepared, loaded with ^{3}H-labeled ligand, and superfused as described. The second procedure involved the preparation of the slice as described, followed by a 15-min incubation with varying concentrations of leucine or methionine enkephalin. After this incubation period, the slice was rinsed twice with standard buffer and placed in 3 ml of fresh buffer containing ^{3}H-labeled ligand. Accumulation of the ^{3}H-labeled ligand by the slice and superfusion of the slice was then followed as described. The amount of releasable ^{3}H-labeled ligand was then compared by Student's *t*-test for control and enkephalin-treated slices.

Drugs. The drugs used in this series of experiments were [^{3}H]morphine (22–28 Ci/mmole; Amersham/Searle Corporation, Arlington Heights, Ill., or New England Nuclear Corporation, Boston Mass.), [^{3}H]naltrexone (21 Ci/mmole; National Institute of Drug Abuse, Bethesda, Md.), leucine and methionine enkephalin (CalBiochem, LaJolla, Calif.), morphine sulfate (Merck and Company, Inc., Rahway, N. J.), levorphanol tartrate (Hoffman-LaRoche Inc., Nutley, N. J.), naloxone hydrochloride (Endo Laboratories, Inc., Garden City, N. Y.), and H_2-Gly_2-Phe-Leu-OH (Bachem, Torrance, Calif.).

Analgesic, Tolerance, and Dependence Assays

Animals. Male Swiss-Webster mice (Biolab, White Bear Lake, Minn.) weighing 20–25 g were used in all experiments. All animals were housed for at least 1 day before experimentation and were allowed food and water *ad libitum.*

Analgesia. Analgesia was measured by the tail-flick method of D'Amour and Smith (8) as modified by Tulunay and Takemori (9). A reaction time for drug-treated animals greater than 3 SD from the control reaction time for all of the animals in the group was the criterion for an analgesic response. In experiments in which naloxone was administered, naloxone was given i.p. 10 min following the s.c. injection of morphine.

Acute tolerance and dependence. A single dose of morphine was used to produce acute tolerance and dependence (10). Morphine sulfate (30 mg/kg) was administered to animals s.c., and standard analgesic assays using morphine were performed when reaction times had returned to control values, generally within 3½ hr. The degree of physical dependence on morphine was assessed by estimating the amount of naloxone needed to induce vertical withdrawal jumping (10).

Statistics. The method of Litchfield and Wilcoxon (11) was utilized to calculate analgesic ED_{50} values of morphine sulfate and to compare ED_{50} values and slopes of the dose-response curves in control and treated groups. A minimum of 30 animals was used to determine each dose-response curve.

The ED_{50} of naloxone required to induce jumping was estimated by the method of Dixon (12), using five or six animals per group. The ED_{50} of naloxone was determined five to seven times. Student's *t*-test was used to determine whether there were significant differences between control and treated groups.

RESULTS

Effect of in vivo or in vitro enkephalin treatment on the release of ^{3}H-labeled ligands from striatal slices by opiate-like drugs. It has been demonstrated previously in this laboratory that a small amount of the morphine which had been accumulated by slices of corpus striatum of mice was released specifically by superfusion with solutions containing naloxone (5, 6). Intraperitoneal injection of an animal with leucine enkephalin, 20 mg/kg, 15 min prior to killing resulted in a marked enhancement of this naloxone-releasable morphine from the striatal slices (Fig. 1). Prior treatment of mice with leucine enkephalin was found to shift the naloxone-releasable morphine curve parallel and to the left, which decreased the RC_{50} (concentration of naloxone required to release 50% of the maximal releasable morphine) to approximately one-tenth the control value (Fig. 2). Methionine enkephalin treatment, at the same dose and time interval, had no effect on the amount of morphine released by naloxone. It is important to note that the accumulation of morphine by the slice was not affected by leucine enkephalin pretreatment. The release characteristics and the amount of maximal release also were not significantly different between control and leucine enkephalin pretreated slices.

To determine whether similar effects on naloxone-releasable morphine could be produced *in vitro*, various concentrations of leucine enkephalin were added to the incubation medium and the slice was incubated for 15

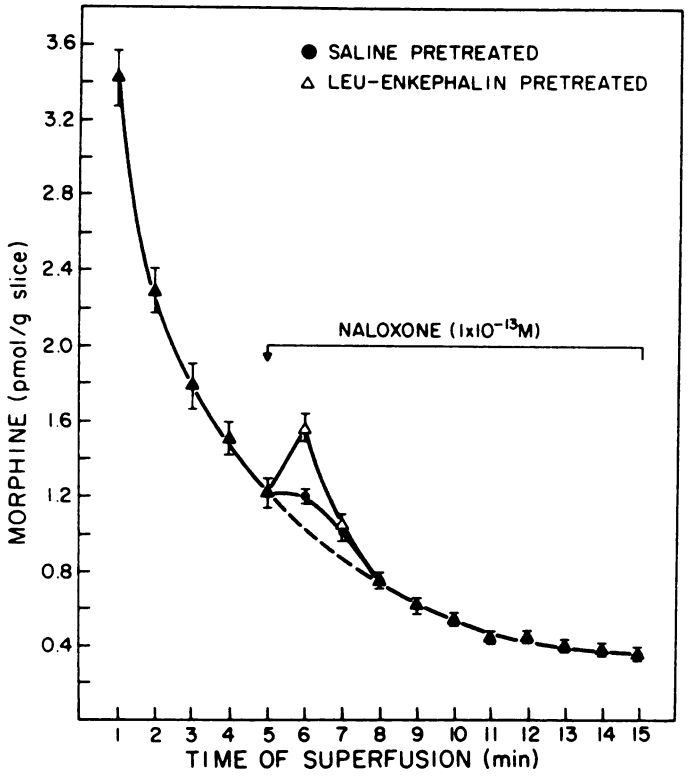

FIG. 1. *Displacement of morphine by naloxone in superfused striatal slices of saline and leucine enkephalin pretreated mice*

The *horizontal line* indicates the beginning and duration of superfusion with solution containing naloxone. Values on the release curve represent means ± standard error from five experiments.

min before the addition of ^{3}H-labeled ligand. This preincubation period without leucine enkephalin was found to have no effect on control naloxone-releasable morphine. Preincubation of the slice with leucine enkephalin at concentrations ranging from 1 nM to 0.1 pM was found to enhance significantly the amount of naloxone-releasable morphine (Table 1). Prior incubation with a 1-nM concentration of the tetrapeptide H_2-Gly_2-Phe-Leu-OH or methionine enkephalin had no significant effect on the amount of morphine released by a given concentration

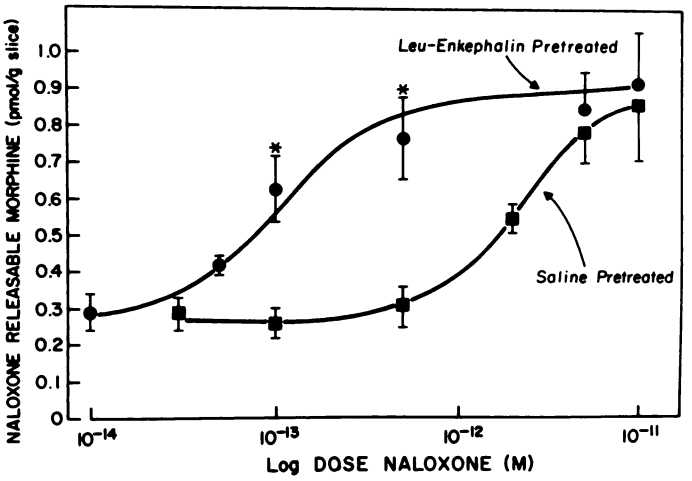

FIG. 2. *Concentration-release curve for naloxone in striatal slices from control and leucine enkephalin-pretreated mice*

Values represent means ± standard error from three to seven experiments.

TABLE 1

Effect of leucine enkephalin in vitro on the amount of morphine released by naloxone in superfused striatal slices

Slices were preincubated with the drugs for 15 min, rinsed, and incubated with [^{3}H]morphine for 30 min in fresh medium before superfusion. Incubated slices were superfused with Krebs-Ringer bicarbonate solution containing 5×10^{-13} M naloxone 5 min after the start of the superfusion.

Preincubated drug	Naloxone-releasable morphine[a]
	pmoles/g slice
Saline	0.38 ± 0.03 (9)
Leucine enkephalin (1×10^{-9} M)	0.78 ± 0.08[b] (3)
Leucine enkephalin (1×10^{-11} M	0.65 ± 0.07[b] (4)
Leucine enkephalin (1×10^{-13} M)	0.54 ± 0.02[b] (3)
Leucine enkephalin (1×10^{-15} M)	0.43 ± 0.15 (6)
H_2-Gly-Gly-Phe-Leu-OH (1×10^{-9} M)	0.48 ± 0.06 (6)
Methionine enkephalin (1×10^{-9} M)	0.43 ± 0.08 (4)
Naloxone (1×10^{-9} M)	0.41 ± 0.08 (5)
Leucine enkephalin (1×10^{-9} M) + Naloxone (1×10^{-9} M)	0.45 ± 0.05 (4)

[a] Values are means ± standard error. The number of experiments is shown in parentheses.

[b] Values significantly different from control values ($p < 0.05$).

of naloxone. Additionally, preincubation of an equimolar concentration of naloxone with leucine enkephalin was found to block the enhancing effects of leucine enkephalin on naloxone-releasable morphine.

Introduction of 1 nM levorphanol or morphine into the superfusing medium 5 min following the start of superfusion similarly resulted in an immediate transient release of [^{3}H]morphine from the slice. As was observed with naloxone-releasable morphine, prior incubation with 1 nM leucine enkephalin resulted in a significant enhancement of levorphanol- or morphine-releasable [^{3}H]-morphine (Fig. 3). Using [^{3}H]naltrexone as the loading ligand, the introduction of 1 nM naltrexone or 10 nM morphine into the superfusing medium resulted in an immediate and transient release of [^{3}H]naltrexone from the slice. However, prior incubation with 1 nM leucine enkephalin had no effect on the amount of naltrexone-releasable [^{3}H]naltrexone but significantly enhanced morphine-releasable [^{3}H]naltrexone. Prior incubation with leucine enkephalin had no effect on the accumulation of the ^{3}H-labeled ligand by the slice and the normal release characteristics of the ^{3}H-labeled ligand from the slice.

Effect of leucine enkephalin on morphine-induced acute tolerance and dependence. On the basis of the above observations, it appeared that leucine enkephalin did not influence the antagonist per se but that an agonist had to be present in the assay system for the effect of leucine enkephalin to be manifested. The postulate that the effects of leucine enkephalin reflected an agonist-mediated event was corroborated by studies *in vivo*. It has been shown that pretreatment of mice with morphine at doses which resulted in no detectable tolerance development markedly enhanced the antagonistic potency of naloxone (9, 13) and that this enhanced antagonistic potency was related to the development of narcotic tolerance (14). Pretreatment of mice with leucine enkepha-

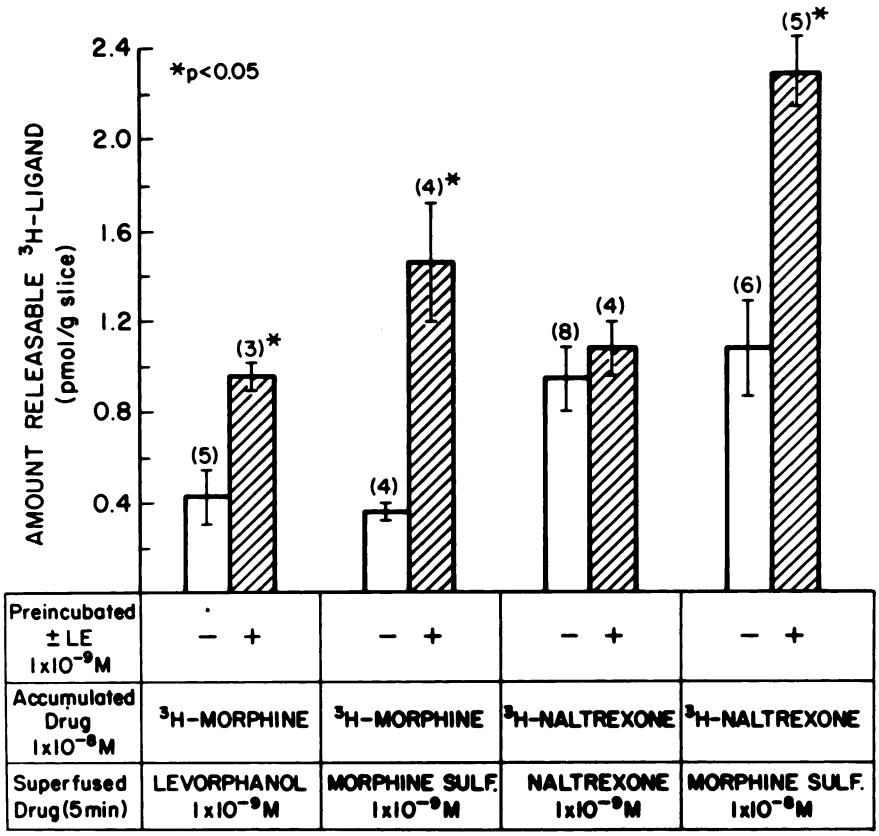

FIG. 3. *The effects of leucine enkephalin (LE) on the release of [³H]naltrexone and [³H]morphine by various agonists and antagonists in superfused striatal slices in mice*

The superfused drug was introduced 5 min after the start of the superfusion. The *bars* and *vertical lines* represent means ± standard error; the number of experiments is indicated in parentheses.

lin significantly enhanced morphine analgesia, as described previously, but the potency of naloxone was unaffected as seen by an equivalent shift of approximately 7-fold in the ED_{50} values of morphine for both control and leucine enkephalin-treated animals (Table 2). In mice pretreated with morphine, 30 mg/kg, the same dose of naloxone shifted the morphine ED_{50} 16.5-fold. In mice pretreated with morphine, 30 mg/kg, and leucine enkephalin, 20 mg/kg, tolerance to the analgesic effects of morphine became evident and naloxone produced an even greater shift of approximately 34-fold in the morphine ED_{50}. Prior treatment with morphine, 15 mg/kg, slightly increased naloxone potency as was evidenced by a 9-fold shift in the ED_{50} of morphine. Pretreatment with morphine, 15 mg/kg, and leucine enkephalin, 20 mg/kg, resulted in an enhanced naloxone potency

TABLE 2

Effect of leucine enkephalin on morphine tolerance and naloxone potency

Morphine sulfate was administered s.c. 3½ hr before ED_{50} determinations. Leucine enkephalin was administered i.p. 15 min prior to morphine sulfate.

Pretreatment	Morphine ED_{50}	Morphine ED_{50} after naloxone, 0.2 mg/kg	Potency ratio
	mg/kg		
Saline	3.61 (1.98–6.50)	25.0 (16.89–37.0)	6.9
Leucine enkephalin (20 mg/kg)	0.64 (0.36–1.12)[a]	4.25 (2.58–7.01)	6.6
Morphine sulfate (30 mg/kg)	4.55 (2.80–7.37)	75.0 (46.58–120.75)[a]	16.5
Leucine enkephalin (20 mg/kg) + morphine sulfate (30 mg/kg)	8.50 (5.21–13.85)[a]	290.0 (134.2–626.4)[b]	34.1
Morphine sulfate (15 mg/kg)	3.98 (2.04–6.99)	35.0 (22.01–55.65)	8.8
Leucine enkephalin (20 mg/kg) + morphine sulfate (15 mg/kg)	4.65 (2.87–7.53)	70.0 (49.64–98.7)[b]	15.1
Methionine enkephalin (20 mg/kg) + morphine sulfate (30 mg/kg)	4.65 (2.84–7.53)	82.5 (56.12–121.27)[a]	17.7

[a] Value is significantly different from that of saline controls.

[b] Value is significantly different from that of saline controls and morphine pretreatment controls.

TABLE 3

The effect of leucine enkephalin on acute dependence and striatal release of morphine

Naloxone (5×10^{-13} M) was introduced into the superfusion medium at 5 min; morphine sulfate was administered s.c. 3½ before testing; leucine enkephalin was administered i.p. 15 min prior to morphine.

Pretreatment	Naloxone ED_{50} for jumping	Naloxone-releasable morphine[a]
	mg/kg	*pmoles/g slice*
Saline	>100	0.26 ± 0.04 (5)
Morphine sulfate (30 mg/kg)	16.63 ± 4.26[a]	0.63 ± 0.07 (5)[a]
Leucine enkephalin (20 mg/kg) + morphine sulfate (30 mg/kg)	5.96 ± 1.70[b]	0.91 ± 0.06 (5)[b]
Morphine sulfate (15 mg/kg)	53.29 ± 12.24[a]	
Leucine enkephalin (20 mg/kg) + morphine sulfate (15 mg/kg)	20.6 ± 5.98[b]	

[a] Value is significantly different from that of saline controls ($p < 0.05$).

[b] Value is significantly different from that of saline controls and morphine pretreatment controls ($p < 0.05$).

with no demonstrable tolerance to the analgesic effects of morphine. Methionine enkephalin pretreatment with the high dose of morphine had no effect on naloxone potency.

The enhancement of the morphine-induced increase in naloxone potency correlated well with the decrease in the ED_{50} of naloxone to produce withdrawal jumping in acutely dependent mice (Table 3). Table 3 also reveals that there was a positive correlation between leucine enkephalin-induced enhancement of naloxone-releasable morphine from superfused striatal slices and the leucine enkephalin-induced decrease in the naloxone ED_{50}.

DISCUSSION

By utilizing the mouse striatal slice superfusion assay, we have demonstrated that minute concentrations of leucine enkephalin *in vivo* and *in vitro* can enhance the amount of naloxone-releasable morphine. The potency of leucine enkephalin *in vitro* is of particular interest. These data demonstrate that subpicomolar concentrations of leucine enkephalin are able to modify the naloxone-induced release of morphine. Thus, even though the pentration of the enkephalins across the blood-brain barrier is minimal[3] and their half-lives are relatively short, only minute concentrations of the pentapeptide appear to be required to modify narcotic ligand-receptor interactions. The inability of H_2-Gly-Gly-Phe-Leu-OH and methionine enkephalin to alter naloxone-releasable morphine closely parallels the results found in assay systems *in vivo* (2, 3) in that methionine and leucine enkephalin have differing effects and the intact pentapeptide of leucine enkephalin is required to enhance naloxone-releasable morphine. Furthermore, since naloxone can block the potentiating effect of leucine enkephalin, these data could be interpreted to mean that the enhancement of narcotic potency by leucine enkephalin is directly associated with a change in the binding characteristics of the narcotic ligand to opioid receptors.

The mechanism by which leucine enkephalin might modify the narcotic ligand-receptor interaction resulting in an enhanced potency of the narcotic could conceivably involve an increase in the available receptor population or an increase in the affinity of the receptor for the narcotic ligand. On the basis of the release curves for naloxone in the presence and absence of leucine enkephalin, the maximal amount of releasable morphine is not altered after treatment with leucine enkephalin, which suggests that the available receptor population has not been affected. However, it is not immediately evident whether or not the effects observed after leucine enkephalin treatment are due to effects on the agonist, antagonist, or both. Incubation of striatal slices with various agonists and antagonists helped to resolve this issue. We were able to demonstrate that, for the effects of leucine enkephalin to be manifested, an agonist had to be present at some time during the experimental protocol. The agonist may be present as the accumulated radiolabeled ligand, the superfused ligand, or both. However, if only antagonists are present throughout the experiment as both the accumulated and superfused ligand, prior leucine enkephalin treatment was without effect on the release of the ligand from striatal slices. Thus the apparent increase in the potency of naloxone to release morphine from striatal slices is postulated to be an agonist-mediated effect at the receptor level.

In vivo, leucine enkephalin did not alter the antagonistic effect of naloxone per se but did enhance the morphine-induced increase in naloxone potency to antagonize analgesia or to induce withdrawal jumping. These increases in naloxone potency *in vivo* (induced by morphine or morphine plus leucine enkephalin) could be directly correlated to the enhanced potency of naloxone to release morphine from striatal slices. We suggest that the apparent increases in naloxone potency both *in vitro* and *in vivo* are mediated through narcotic agonists and that leucine enkephalin potentiates this agonistic effect. The enhancement by leucine enkephalin of morphine-releasable [^{3}H]naltrexone might be interpreted to indicate an enhanced affinity of morphine for the receptor, since naltrexone-releasable [^{3}H]naltrexone was unaffected by prior leucine enkephalin treatment. However, using this procedure, an affinity change in the receptor for morphine could not be clearly evaluated.

We have shown previously that leucine enkephalin, but not methionine enkephalin, potentiated narcotic agonistic effects in guinea pig ileal longitudinal muscle preparations (1), on analgesia using the tail-flick and writhing assays (2, 3), on spinal analgesia (4), on acute tolerance (2), and on acute dependence (2). In yet another test system, in this study we report the potentiation by leucine enkephalin of a narcotic agonistic effect of increasing naloxone potency *in vitro* and *in vivo*. We also suggest that this potentiating effect of leucine enkephalin occurs at the opioid receptor level.

In summary, we have demonstrated that only minute concentrations of leucine enkephalin are required to modify narcotic ligand-receptor interactions. This effect

[3] J. L. Vaught and A. E. Takemori, unpublished observations.

does not appear to involve an increase in the available receptor population. Indirect evidence suggests that leucine enkephalin may act by enhancing the affinity of the receptor for narcotic agonists. Furthermore, coupled with previous observations and the longevity of the effect of leucine enkephalin, these data indicate a possible modulatory role for leucine enkephalin on the interactions of ligands with opioid receptors.

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