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Morphine-like Peptides, Leucine Enkephalin and Methionine Enkephalin: Interactions with the Opiate Receptor

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SUMMARY

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The morphine-like peptides leucine enkephalin and methionine enkephalin compete for opiate receptor binding with affinities resembling that of morphine. In the absence of added sodium, methionine enkephalin is about twice as potent as leucine enkephalin in reducing [^{3}H]naloxone binding, whereas binding of the agonist [^{3}H]dihydromorphine is reduced equally by the two enkephalins. Sodium decreases competition by both enkephalins for [^{3}H]naloxone, but with twice as great an effect on leucine enkephalin as on methionine enkephalin. In contrast, competition by the enkephalins for [^{3}H]dihydromorphine binding is enhanced by sodium. Manganese increases the apparent affinities of both leucine and methionine enkephalins for the opiate receptor. Incubations exceeding 20 min at 25° and 5 min at 37° result in a marked apparent degradation of both leucine and methionine enkephalins, which can be prevented by bacitracin.

INTRODUCTION

The drug specificity (1-3) and subcellular (4), regional (5, 6), and cellular localization (7) of opiate receptor binding suggest that the opiate receptor may interact with an endogenous opiate-like substance. Hughes (8) elegantly demonstrated a peptide in brain extracts with morphine-like activity on smooth muscle, while Terenius and Wahlstrom (9, 10) and ourselves (11, 12) observed that a closely similar substance in brain extracts competes for opiate receptor binding. Other peptides, extracted from pituitary (13) or hypothalamic-pituitary mixtures (14), with morphine-like effects on nerve plexus of smooth muscle appear to represent different chemical entities (13, 14). Hughes *et al.* (15) identified the opioid activity of pig brain as a mixture of H-Tyr-Gly-Gly-Phe-Met-OH (methionine enkephalin) and H-Tyr-Gly-Gly-Phe-Leu-OH (leucine enkephalin) in a 4:1 ratio. By assaying competition for opiate receptor binding, we isolated enkephalin activity from calf brain and showed it to consist of the same two peptides, but with 4 times more leucine enkephalin than methionine enkephalin (16, 17). The two enkephalins compete for opiate receptor binding with potencies similar to morphine, while effects of sodium on competition for receptor

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binding suggest that methionine enkephalin behaves more like mixed agonists-antagonists than does leucine enkephalin (16, 17). Here we report detailed studies of enkephalin influences on opiate receptor binding.

METHODS

Opiate receptor binding assay. Enkephalin activity was assayed as the ability to inhibit the binding of radiolabeled opiates to brain membranes. Membranes from Sprague-Dawley rat brains minus cerebella were prepared as previously described (18). The binding assay was performed in 2 ml of Tris-HCl buffer (pH 7.7 at 0°) containing 1.4 nM [^{3}H]naloxone (20 Ci/mmole) or 1.2 nM [^{3}H]dihydromorphine (45 Ci/mmole). Ions, enkephalin, or nonlabeled opiates were added in the appropriate concentrations as indicated in each experiment. The reaction mixture was incubated for 2 hr at 0° (ice-water mixture) unless otherwise stated, and then terminated by filtration on glass fiber filters (Whatman GF/B) and counted in a liquid scintillation spectrometer with 42% efficiency. Specific opiate binding was defined as the difference in binding in the presence and absence of 1 μM levallorphan. For calculation of enkephalin activity, 1 unit of enkephalin is defined as that amount of enkephalin which yields 50% receptor occupancy in the standard binding assay and determined according to Colquhoun (19), assuming classical binding interactions.

[^{3}H]Naloxone (20 Ci/mmole), [^{3}H]dihydromorphine (45 Ci/mmole), [^{3}H]levallorphan (7.5 Ci/mmole), [^{3}H]levorphanol (5.4 Ci/mmole), and [^{3}H]morphine (8.5 Ci/mmole) were purchased from New England Nuclear Corporation. The unlabeled opiates were donated by the following companies: Ciba-Geigy [(−)-1,2,3,4,5,6-hexahydro-11β-methyl-6-phenyl-3-propargyl-2,6-methano-3-benazaocin-8-ol methanesulfonate (GPA 2163)], American Cyanamid (etorphine, diprenorphine), Endo (naloxone, oxymorphone), Winthrop (pentazocine), and Roche (levallorphan, levorphanol). Nalorphine was purchased from Merck. Synthetic methionine enkephalin and leucine enkephalin were generously donated by Drs. D. Hauser and F. Cardinaux, Sandoz, Basel.

The pentapeptides were built up by fragment condensation methods (20).

RESULTS

Changes in enkephalin influences on opiate receptor binding at elevated temperature. To develop optimal conditions for studying interactions of enkephalin with the opiate receptor, we compared effects of leucine enkephalin and methionine enkephalin with morphine and naloxone on receptor binding of [^{3}H]naloxone and [^{3}H]dihydromorphine at different temperatures and with varying durations of incubation (Fig. 1). At 0° and 10°, inhibition of [^{3}H]naloxone and [^{3}H]dihydromorphine binding by the two peptides and by morphine and naloxone is approximately the same with incubations ranging from 15 to 180 min. With 25° incubations conducted for only 10 min, inhibition of [^{3}H]naloxone and [^{3}H]dihydromorphine binding is the same as at 0° and 10°. During progressively longer incubations at 25°, leucine and methionine enkephalins lose their inhibitory potency while inhibition by morphine and naloxone is constant. There appears to be a greater decrease in inhibition by leucine enkephalin than by methionine enkephalin with longer incubations. Inhibition of [^{3}H]naloxone and [^{3}H]dihydromorphine binding by leucine enkephalin falls from about 75% with 10-min incubations to about 25% with 60-min incubations, while, over the same interval, inhibition by methionine enkephalin falls from 75% to about 40%. At 37° there is yet a more marked and rapid loss in effects of the two enkephalins. With 5-min incubations at 37°, methionine and leucine enkephalins reduce [^{3}H]naloxone and [^{3}H]-dihydromorphine binding by about 60%, while during 40- and 60-min incubations the two enkephalins do not lower binding. Over the same time interval inhibition by morphine and naloxone is essentially unaltered.

One possible explanation for the loss of enkephalin potency during prolonged incubations relates to kinetics of receptor interaction. Conceivably, at shorter inter-

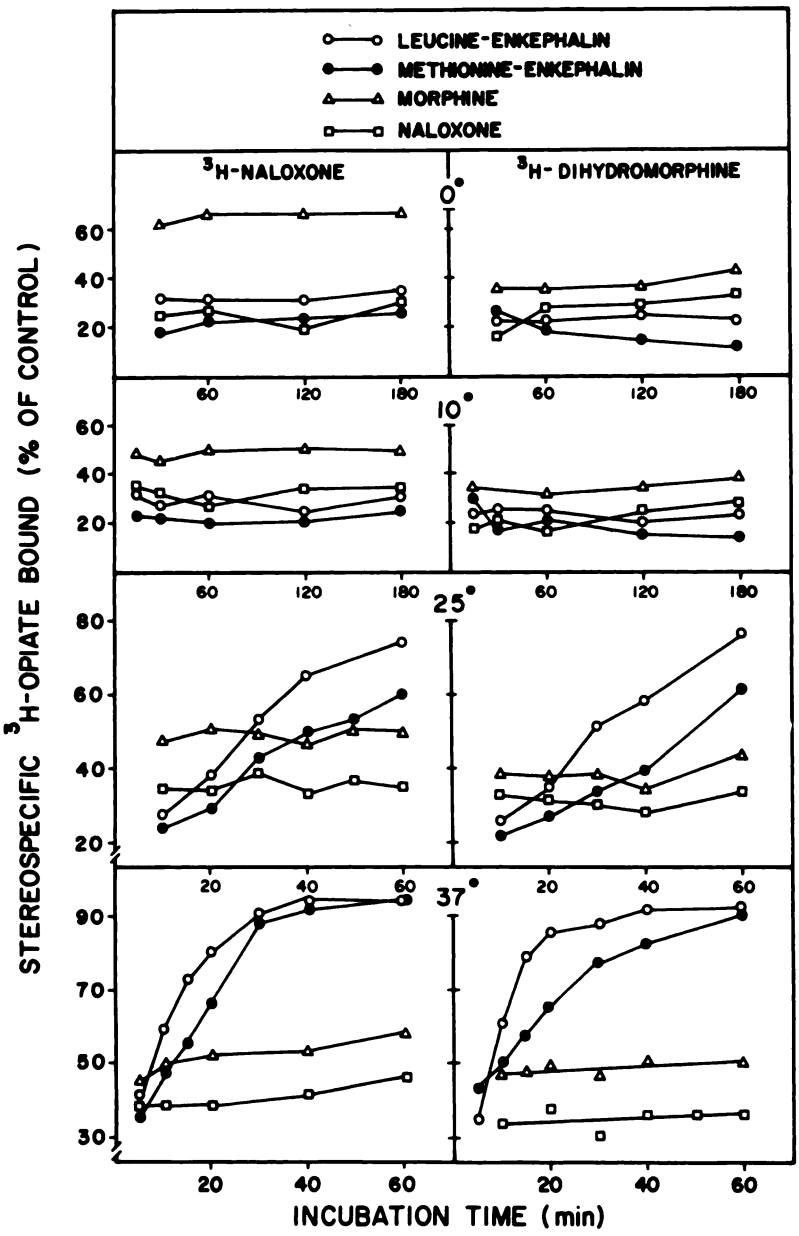

FIG. 1. *Enkephalin influences on opiate receptor binding at different temperatures*

Binding of 1.4 nM [^{3}H]naloxone or 1.2 nM [^{3}H]dihydromorphine was tested at the indicated temperatures for different times in the absence and presence of methionine enkephalin (100 nM), leucine enkephalin (100 nM), naloxone (10 nM), or morphine sulfate (10 nM). All additions were mixed together, after which the incubation was started by adding the rat brain membrane. No ions were added. The experiment was replicated twice.

vals [^{3}H]naloxone and [^{3}H]dihydromorphine binding have not attained equilibrium; as they approach equilibrium with longer incubations, the ^{3}H-opiate might displace enkephalin from binding sites. Accordingly, we examined the time course of [^{3}H]naloxone and [^{3}H]dihydromorphine binding at various temperatures (Fig. 2).

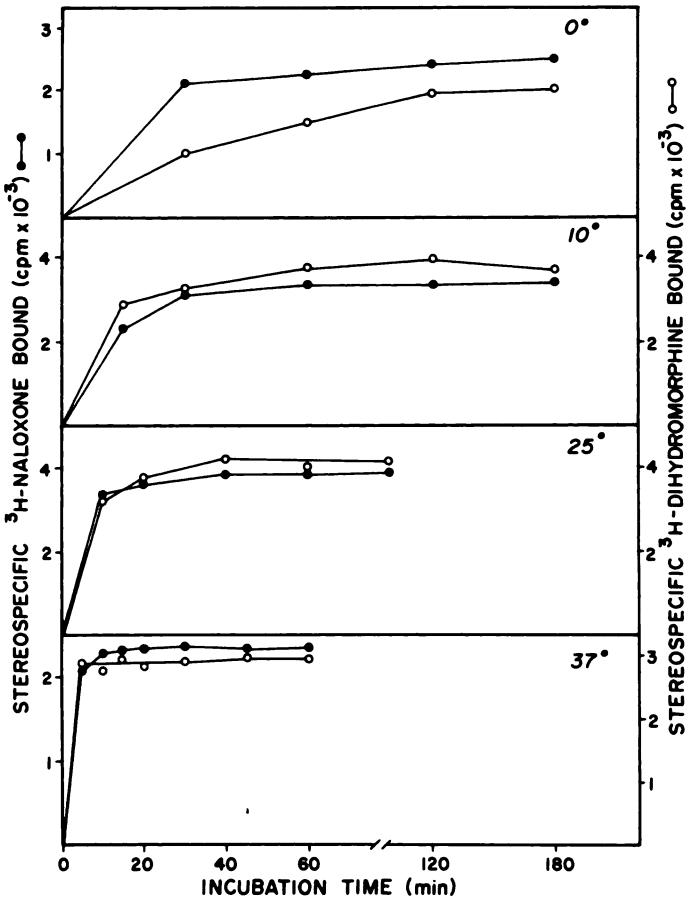

FIG. 2. *Kinetics of [^{3}H]naloxone and [^{3}H]dihydromorphine binding to opiate receptor at different temperatures*

[^{3}H]Naloxone (1.4 nM) or [^{3}H]dihydromorphine (1.2 nM) was incubated in the standard binding assay at the indicated temperatures with no ions added. The experiment was replicated twice.

At 0° [^{3}H]dihydromorphine and [^{3}H]naloxone binding do not attain equilibrium until about 120 min, while equilibrium for the two opiates is reached in about 40–60 min at 10°. At 25° binding of the two ^{3}H-opiates attains equilibrium in about 10 min, and at 37° equilibrium appears complete at about 5 min. Since the loss in inhibitory capacity by the enkephalins takes place after ^{3}H-opiate binding has attained equilibrium, differential kinetics of receptor interaction for the enkephalins and the ^{3}H-opiates cannot readily explain the decreased enkephalin effects with longer incubations. A more likely explanation is a progressive degradation of the enkephalins by enzymes in brain membranes.

Incubations with 1% bovine serum albumin, 0.1% glucagon, or a 0.1 mM concentration of the tripeptide Tyr-Gly-Gly fail to protect enkephalins during incubations of 40–60 min at 25° or 37°. However, bacitracin at 50 μg/ml appears to provide almost complete protection of methionine enkephalin for up to 60 min of incubation at 25° (Fig. 3). At 10 μg/ml, bacitracin affords full protection at 25° for 20 min, though by 60 min considerable destruction of methionine enkephalin can be detected. With 37° incubations, bacitracin (10 or 50 μg/ml) protects methionine enkephalin for only 5 min, with up to 40% destruction apparent by 40 min. The extent of protection of leucine enkephalin by bacitracin at various times and temperatures resembles

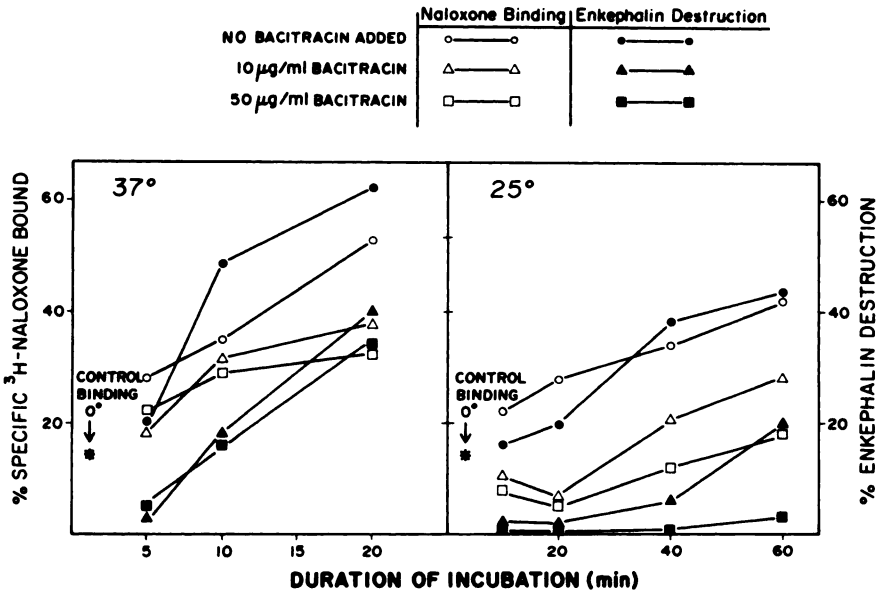

FIG. 3. *Protection of enkephalin by bacitracin*

Binding of [³H]naloxone was assayed in the presence of 100 nM methionine enkephalin for different periods. Bacitracin (10 or 50 μg/ml) was added before the incubation was started by adding rat brain membranes. Data about enkephalin destruction were calculated from a standard curve, assuming classical binding interactions according to Colquhoun (19). The experiment was replicated twice. Values for [³H]naloxone binding in the absence of enkephalin are indicated by asterisks.

results with methionine enkephalin. Bacitracin itself does not inhibit [³H]naloxone binding at 10 or 50 μg/ml, although with 100 μg/ml, 15–20% reduction of binding is detectable (Table 1). At 100 mg/ml, bovine serum albumin, frequently employed to protect peptides from proteolytic degradation, reduces [³H]naloxone binding 15–20%.

Assuming that decreased competition for binding by enkephalins is attributable to degradation, calculation of the number of enkephalin units as defined in METHODS suggests that without bacitracin as little as 40 min of incubation at 25° result in the destruction of about 80% of leucine enkephalin and 73% of methionine enkephalin, while 20 min of incubation at 37° reduce the concentrations of leucine enkephalin and methionine enkephalin by 85% and 65%, respectively. Since enkephalin inhibition of receptor binding at 0° appears constant for 180 min, we conducted routine assays of enkephalin influences on opiate receptor binding at 0° for 2 hr.

Inhibition of opiate receptor binding by enkephalins in the absence of added ions. When opiate receptor binding is assayed in the absence of added ions, methionine enkephalin appears 2.5 times more potent than leucine enkephalin in reducing [³H]naloxone binding, although they have about the same potencies in inhibiting

TABLE 1

Effects of bacitracin and bovine serum albumin on [³H]naloxone binding

Binding with 1.4 nM [³H]naloxone was assayed at 25° for 40 min or at 37° for 20 min. Data are means ± standard errors of four determinations of specific opiate receptor binding as defined in METHODS.

Additions		Specific [³H]naloxone bound	
Bacitracin	Albumin	25°	37°
μg/ml	mg/ml	cpm	cpm
0	0	3670 ± 295	2675 ± 180
10	0	3480 ± 340	2522 ± 310
50	0	3435 ± 385	2500 ± 295
100	0	2920 ± 327[a]	2290 ± 285[a]
0	100	2945 ± 220[a]	2250 ± 180[a]

[a] Significantly lower than values for control binding without additions ($p < 0.05$).

[^{3}H]dihydromorphine binding (Fig. 4). Leucine enkephalin appears more potent in inhibiting [^{3}H]dihydromorphine binding (IC_{50} = 12.5 nM) than [^{3}H]naloxone binding (IC_{50} = 20 nM), whereas methionine enkephalin is more potent in inhibiting [^{3}H]naloxone binding (IC_{50} = 8 nM) than [^{3}H]dihydromorphine binding (IC_{50} = 12.5 nM).

Ionic influences on enkephalin interactions with opiate receptor. In assays conducted at 25°, sodium selectively enhances ^{3}H-antagonist binding and diminishes ^{3}H-agonist binding. Certain properties of opiate receptor binding change at reduced temperature (21). To evaluate ionic influences on enkephalin, we first sought to ascertain whether the effects of monovalent cations on opiate receptor binding at 0° would resemble their effects at 25° (Fig. 5). With 0° incubations, low concentrations of sodium do selectively decrease receptor binding of [^{3}H]dihydromorphine, while potassium, cesium, and rubidium exert virtually no effect. As little as 1 mM sodium markedly enhances the binding of [^{3}H]naloxone. Lithium produces similar effects, while potassium, rubidium, and cesium have negligible influences on [^{3}H]naloxone binding. Thus the previously reported differential actions of sodium on agonist and antagonist binding can be demonstrated with the present incubation conditions.

In preliminary experiments we reported that sodium reduces competition for opiate receptor binding by leucine enkephalin to a greater extent than by methionine enkephalin (16, 17). In the present study we have evaluated numerous concentrations of five monovalent and three divalent cations on inhibition of [^{3}H]naloxone binding by methionine and leucine enkephalins (Fig. 6). Increasing concentrations of sodium reduce inhibition of [^{3}H]naloxone binding by the two enkephalins, with maximal effects apparent at 50 mM sodium. This action shows a selectivity similar to that of sodium on ^{3}H-opiate binding, since lithium is almost as active as sodium while potassium, cesium, and rubidium have negligible effects.

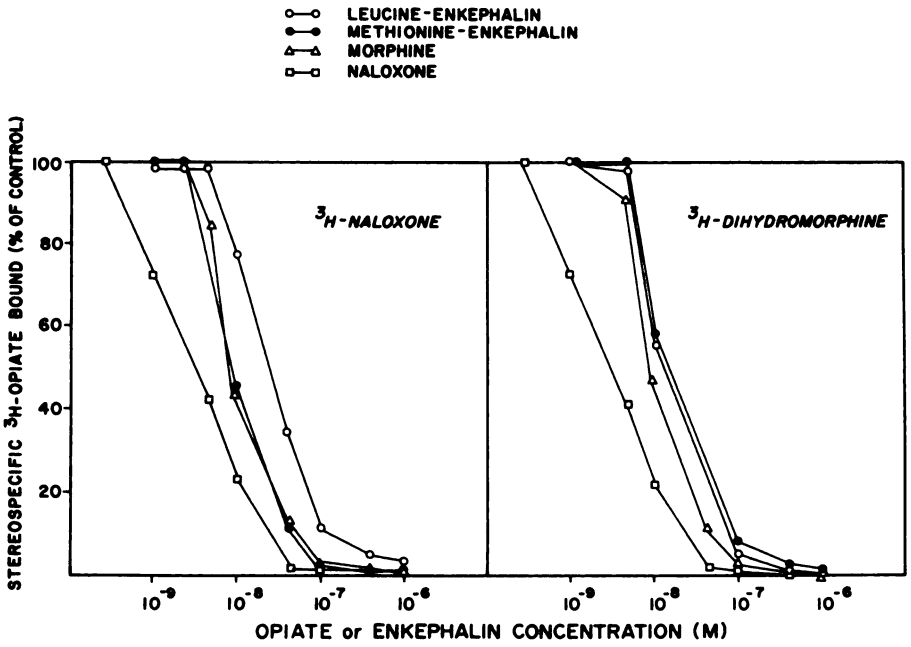

FIG. 4. *Concentration dependence for inhibition of [^{3}H]naloxone and [^{3}H]dihydromorphine binding by enkephalins and other opiates*

Incubations were performed at 0° for 2 hr in the standard binding assay containing 1.4 nM [^{3}H]naloxone or 1.2 nM [^{3}H]dihydromorphine. The experiment was replicated three times.

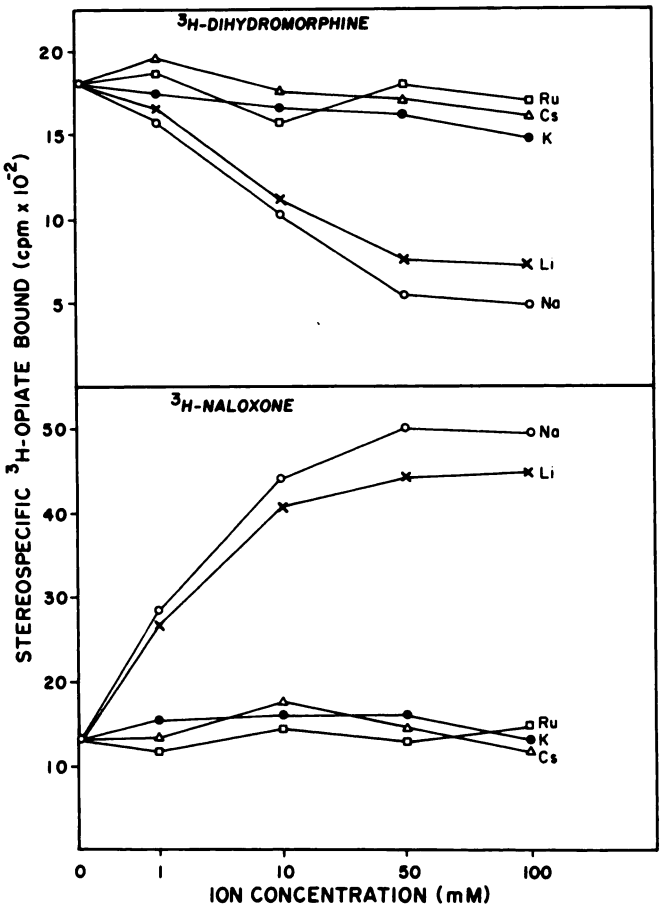

FIG. 5. *Effects of monovalent ions on binding of [^{3}H]naloxone and [^{3}H]dihydromorphine at 0°*
Incubations with brain membranes were conducted at 0° for 2 hr in the standard binding assay with different concentrations of monovalent ions and 1.4 nM [^{3}H]naloxone or 1.2 nM [^{3}H]dihydromorphine. The experiment was replicated three times.

When opiate receptor binding is assayed at 25°, manganese enhances receptor binding of ^{3}H-agonists (22). This effect is selective, since it is exerted by manganese and to a lesser extent by magnesium, and is not apparent with calcium (22). By contrast, at 0° manganese but not calcium depresses the binding of both ^{3}H-agonists and ^{3}H-antagonists.[3] In the present studies, using 0° incubations, as little as 0.25 mM manganese greatly enhances inhibition by methionine and leucine enkephalins of [^{3}H]naloxone binding. This effect is displayed selectively by manganese, with lesser effects elicited by magnesium and calcium. In experiments not depicted, manganese but not calcium enhances the potencies of leucine and methionine enkephalins in competing for [^{3}H]dihydromorphine.

To evaluate ionic influences on interactions of enkephalin with the opiate receptor in greater detail, we assessed effects of sodium and manganese on competition for receptor binding by a wide range of enkephalin concentrations (Table 2). Sodium decreases competition by both leucine and methionine enkephalins for [^{3}H]naloxone binding. As reported previously (16, 17), the sodium-elicited decline in potency is greater for leucine enkephalin (20-fold)

[3] R. Simantov and S. H. Snyder, manuscript in preparation.

than for methionine enkephalin (12.5-fold). Manganese increases the potencies of both enkephalins about 2-fold.

Whereas sodium decreases enkephalin inhibition of [^{3}H]naloxone, sodium increases enkephalin potency in inhibiting [^{3}H]dihydromorphine binding (Table 2), with 20% and 250% enhancement of potency, respectively, for leucine and methionine enkephalins. Manganese increases the respective potencies of leucine and methionine enkephalins 1.6- and 1.25-fold. Thus, whereas sodium increases the potency in competing for [^{3}H]dihydromorphine binding more for methionine enkephalin than for leucine enkephalin, the reverse holds for manganese.

Because the slopes for displacement of [^{3}H]naloxone binding by enkephalins appear steeper in the absence than in the presence of sodium, we examined Hill plots for inhibition of [^{3}H]naloxone binding by leucine and methionine enkephalins as well as by morphine and naloxone in the presence and absence of sodium (Fig. 7). Sodium significantly reduces the Hill coefficient for displacement of [^{3}H]-naloxone binding by leucine and methionine enkephalins ($p < 0.01$). The Hill coefficient for morphine influences on [^{3}H]naloxone binding is reduced to about the same extent, while the Hill coefficient for naloxone itself is unaffected by sodium. In the absence of sodium the Hill coefficient for leucine enkephalin is significantly smaller than for methionine enkephalin ($p < 0.005$). The Hill coefficients for morphine in the presence and absence of sodium are essentially the same as for leucine enkephalin, whereas the Hill coefficient for naloxone is significantly greater than for morphine or either of the enkephalins in both the presence and absence of sodium ($p < 0.005$).

Comparisons of sodium effects on competition by enkephalins and opiates for [^{3}H]naloxone binding. In assays conducted at 25°, the extent to which sodium alters the ability of drugs to compete for [^{3}H]naloxone binding predicts whether

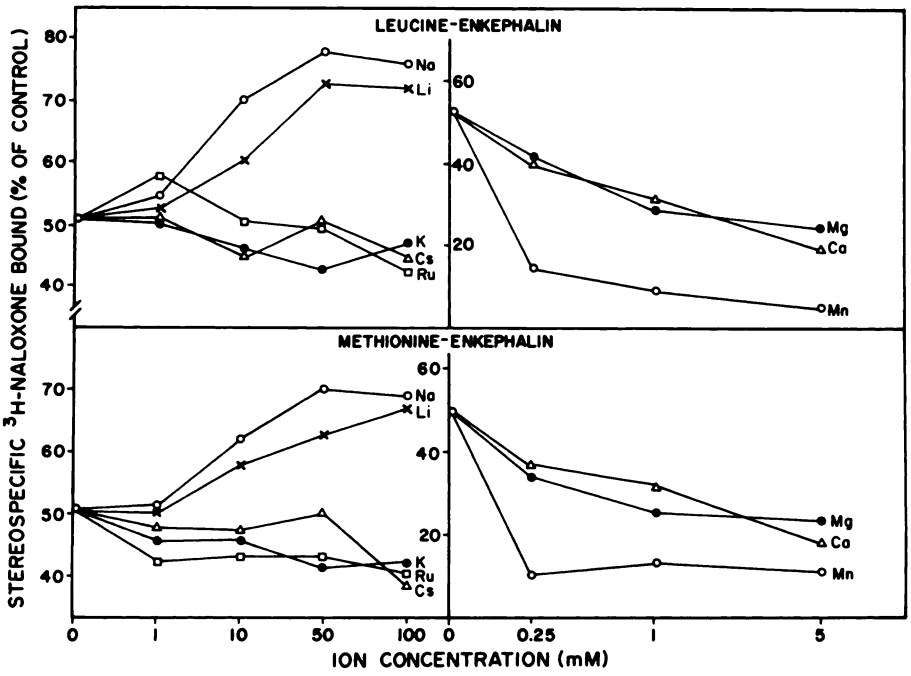

FIG. 6. *Influence of monovalent and divalent ions on inhibition of [^{3}H]naloxone binding by enkephalins*
Binding was performed as indicated in Fig. 5, but in the presence of 20 nM leucine enkephalin or 8 nM methionine enkephalin. Data are presented as percentage of controls in the absence of enkephalin. The experiment was replicated twice.

TABLE 2

Effects of ions on inhibition of specific [^{3}H]naloxone and [^{3}H]dihydromorphine binding by enkephalins

The binding assay was performed at 0° for 2 hr in the absence and presence of 1 nM–1 μM enkephalins. IC_{50} is the concentration of enkephalin required to inhibit specific binding of 1.4 nM [^{3}H]naloxone or 1.2 nM [^{3}H]dihydromorphine by 50%. Data are means ± standard errors of four different experiments.

Ion added	[^{3}H]Naloxone binding			[^{3}H]Dihydromorphine binding		
	IC_{50}	Na shift (+Na/−Na)	Mn shift (+Mn/−Mn)	IC_{50}	Na shift (+Na/−Na)	Mn shift (+Mn/−Mn)
	nM					
Leucine enkephalin						
None	20 ± 2			12.5 ± 1		
NaCl (100 mM)	400 ± 50	20		10 ± 1	0.80	
$MnCl_2$ (1 mM)	10 ± 1		0.5	8 ± 1		0.64
Methionine enkephalin						
None	8 ± 1			12.5 ± 1.5		
NaCl (100 mM)	100 ± 15	12.5		5 ± 0.5	0.4	
$MnCl_2$ (1 mM)	5 ± 0.7		0.6	10 ± 0.8		0.8

drugs have pharmacological properties of pure agonists, pure antagonists, or mixed agonists-antagonists (23, 24). Using assays at 0°, we have compared sodium influences on competition for [^{3}H]naloxone binding by enkephalins and several other opiates (Table 3). Relative potencies of drugs and influences of sodium are similar to those reported earlier at higher incubation temperatures (23), except that agonists tend to be weaker with lower incubation temperatures, as previously noted (21). The potencies of the fairly pure antagonists naloxone and diprenorphine are not altered by sodium, while the pure antagonist GPA 2163 becomes 5 times more potent in the presence of sodium. Levallorphan and nalorphine, antagonists with some agonist activity, exhibit "sodium shifts" of 2.0 and 2.7, respectively, while the potency of the mixed agonist-antagonist pentazocine declines 3.5-fold in the presence of sodium. Potencies of conventional opiate agonists such as etorphine, levorphanol, oxymorphone, morphine, and dihydromorphine fall 10–50-fold in the presence of sodium. The sodium shift for methionine enkephalin is 12.5-fold, while leucine enkephalin has a sodium shift of about 20. Thus both leucine enkephalin and methionine enkephalin are affected by sodium like opiate agonists.

DISCUSSION

Opiate receptor binding assays in many laboratories are routinely conducted at 25° or 37° for periods of 20 min or longer. Although brain membranes used in the present study were washed twice, methionine and leucine enkephalins were degraded 65–85% at 25° and 37° with incubations longer than 10 min. Studies of enkephalin interactions with opiate receptor binding should probably be conducted at 0° or in the presence of bacitracin to minimize degradation.

Both leucine and methionine enkephalins inhibit opiate receptor binding with affinities comparable to that of morphine. Reduced potency in competing for [^{3}H]naloxone binding in the presence of sodium suggests that both leucine and methionine enkephalins have properties of opiate agonists, which coincides with direct evidence in smooth muscle (8, 15). Sodium appears to act both by facilitating binding of [^{3}H]naloxone to opiate receptors and by reducing the affinities of agonists

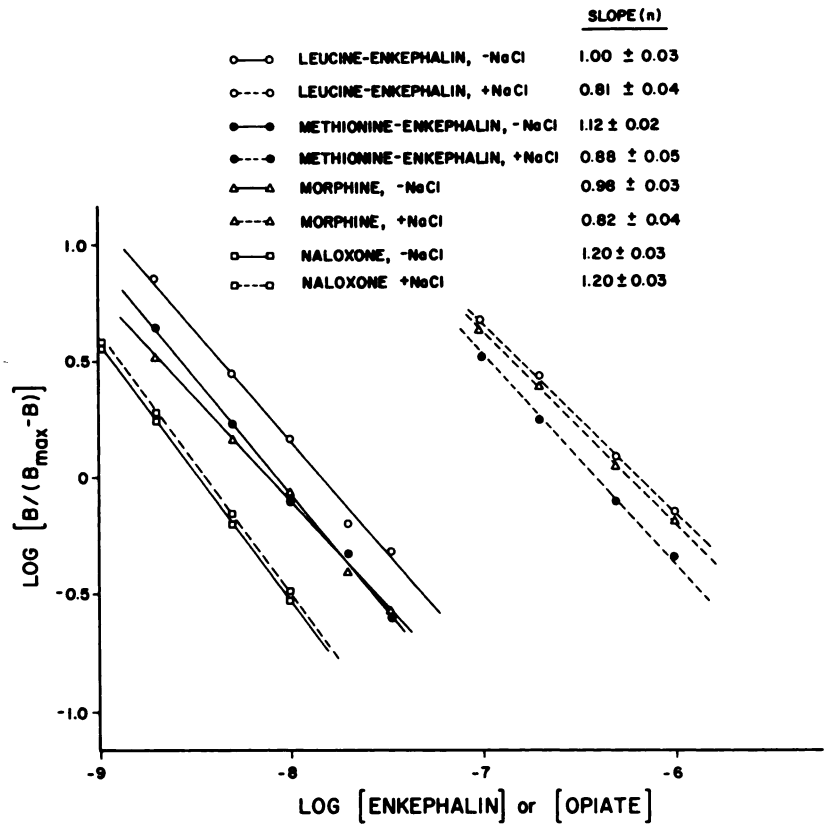

FIG. 7. *Hill plot analysis of inhibition by enkephalins and opiates of [³H]naloxone binding, and effect of sodium*

The standard binding assay was performed as described in Fig. 5, with 1.4 nM [³H]naloxone and 1 nM–1 μM enkephalin, morphine, or naloxone. Data from three experiments were plotted according to the Hill equation, and each point is the mean of triplicate assays, which varied less than 15%. B refers to counts per minute of specifically bound [³H]naloxone in the presence of nonradioactive drugs or enkephalins. B_{max} refers to [³H]naloxone specifically bound in the absence of added unlabeled drugs or enkephalin. Values for the slopes are means ± standard errors from four separate determinations. The statistical significance of difference between mean slopes was determined by Student's t-test and is discussed in the text.

for the receptor. In terms of the extent of the sodium-induced loss of potency, methionine enkephalin behaves more like mixed agonists-antagonists than does leucine enkephalin. This is also suggested by the finding that leucine enkephalin appears somewhat more potent in reducing [³H]dihydromorphine than [³H]naloxone binding, whereas the reverse is true with methionine enkephalin. Because sodium enhances potencies of both enkephalins in competing for [³H]dihydromorphine binding, dihydromorphine behaves as a "purer" agonist than the enkephalins. This is also apparent in the "sodium shifts," which are smaller for enkephalins than for dihydromorphine. The pharmacological significance of different sodium shifts for agonists in competing for [³H]naloxone binding is unclear. Thus etorphine displays a sodium shift of only 10 while the shift for dihydromorphine is about 50; yet both drugs are pure agonists with no obvious differences in their opiate agonist properties.

In the absence of sodium, Hill coefficients for displacement of [³H]naloxone by leucine and methionine enkephalins as well as morphine are close to 1. Sodium reduces these Hill coefficients to values significantly lower than 1.0. According to a two-state model of the opiate receptor

TABLE 3

Comparison of enkephalin and opiate influences on binding of [^{3}H]naloxone

Data are averages of three experiments, which varied less than 15%. IC_{50} is defined in the legend to Table 2.

Nonradioactive opiate	IC_{50}		Na shift (+NaCl/−NaCl)	Pharmacological properties[a]	
	No ions added	+100 mM NaCl		Agonist	Antagonist
GPA 2163	100	20	0.2		1[b, c]
Naloxone	1.5	1.5	1.0		3[b–d]
Diprenorphine	0.5	0.5	1.0	3[c]	3[b, d]
Levallorphan	2.0	4	2.0	2[e]	3[b, d]
Nalorphine	1.5	4.0	2.7	1,[f] 2[e, g]	2[b, d]
Pentazocine	20	70	3.5	1,[f] 2[e, g]	1[b, d]
Etorphine	0.5	5.0	10	3[e, f]	
Methionine enkephalin	8.0	100	12.5		
Levorphanol	4.0	60	15	3[c, e, f]	
Leucine enkephalin	20	400	20		
Oxymorphone	10	350	35	3[c, e, f]	
Morphine	7.0	300	43	2[c, e, f]	
Dihydromorphine	6.0	300	50	2[c, e, f]	

[a] The pharmacological properties of each opiate are designated as follows: 1, weak; 2, intermediate; 3, strong.

[b] Monkey abstinence precipitation (25, 26).

[c] Guinea pig intestine (29).

[d] Pierson-Harris tail flick (28, 29).

[e] Human analgesia (30).

[f] Eddy-Leimbach mouse hot plate (31).

[g] Nielsen tail shock (32, 33).

(24), sodium increases the proportion of "antagonist" receptors, which would resist displacement of the antagonist [^{3}H]naloxone by enkephalin. As predicted by this model, sodium produces no change in the Hill coefficient for reduction of [^{3}H]-naloxone binding by nonradioactive naloxone. Similar data indicating apparent negative cooperativity in displacing ^{3}H-antagonists by unlabeled agonists, and vice versa, exist for dopamine (34, 35) and serotonin (36) receptor binding in brain tissue.

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