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Temperature and Ionic Influences on Opiate Receptor Binding

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SUMMARY

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Treatment of brain membranes by preliminary incubation with sodium or at elevated temperature increases opiate receptor binding of both the agonist [^{3}H]dihydromorphine and the antagonist [^{3}H]naloxone about 2-fold. Enhancement of receptor binding is elicited by sodium but not potassium and cesium, which coincides with the ability of sodium selectively to accelerate dissociation of opiate agonists from receptor sites. Augmented receptor binding associated with elevated preliminary incubation temperature is antagonized markedly by manganese, to a lesser extent by magnesium, and not by calcium, which coincides with the selectivity of divalent cations in increasing opiate agonist binding. Elevated receptor binding produced by these treatments is associated with an increase in the number of binding sites, with minimal changes in affinity. The "new receptors" unmasked by these preliminary incubation conditions have an augmented sensitivity to degradation by protein-modifying reagents. The characteristics of preliminary incubation conditions that increase opiate receptor binding suggest that enhancement of binding is related to dissociation from the receptor of an endogenous inhibitor of binding with the characteristics of an opiate agonist. Regardless of preliminary incubation conditions, manganese reduces both [^{3}H]naloxone and [^{3}H]dihydromorphine binding at 0°, indicating a direct effect on the opiate receptor. The present observations emphasize the importance of defining experimental conditions for opiate receptor binding.

INTRODUCTION

Opiate receptor binding (1-3) is modified markedly by protein-modifying reagents (4), enzymes (5, 6), ionic influences (7-9), and temperature (10). Low concentrations of sodium selectively enhance the binding of opiate antagonists and reduce the binding of agonists in a fashion suggesting that the receptor exists in two states, with selective high affinities for antagonists and agonists, respectively (7, 11). Certain divalent cations, especially manganese and magnesium, selectively enhance the binding of opiate agonists (9). To explore mechanisms related to these influences on opiate receptor binding, we have studied in detail effects of ions, protein-modifying reagents, and different temperatures upon opiate receptor binding.

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METHODS

Male Sprague-Dawley rats (175-220 g)

were decapitated, and their brains minus cerebella were immediately placed in 50 mM Tris-HCl buffer (pH 7.7 at 25°) and homogenized with a Brinkmann Polytron. The homogenate was centrifuged at 49,000 × *g* for 15 min, and the pellet was resuspended in 50 mM Tris-HCl buffer with or without ions and incubated for 60 min at 0° or at 37° as indicated in each experiment. This preliminary incubation was terminated by centrifugation at 49,000 × *g* for 15 min at 0°, and the pellet was resuspended in Tris-HCl buffer and assayed in the standard opiate receptor binding assay (4). The assay, in 2 ml of 50 mM Tris-HCl buffer, contained 20 mg of brain tissue, and ions and [^{3}H]naloxone or [^{3}H]dihydromorphine as indicated for each experiment. Unless otherwise stated, receptor binding was assayed at 0° (ice-water mixture) and terminated after 3 hr by filtration on Whatman glass fiber filters (GF/B). The filters were washed with two 5-ml volumes of cold Tris-HCl buffer, placed in 12 ml of Hydromix scintillation fluor (Yorktown Research), and counted in a liquid scintillation spectrometer with 42% efficiency. Specific opiate binding was defined as the difference between binding in the presence and absence of 1 μM levallorphan.

[^{3}H]Naloxone (49 or 20 Ci/mmole) and [^{3}H]dihydromorphine (46 Ci/mmole) were obtained from New England Nuclear Corporation. Tris-HCl buffer, iodoacetamide, 2-methoxy-5-nitrobenzyl bromide, 5,5′-dithiobis(2-nitrobenzoic acid), and 2-hydroxy-5-nitrobenzyl bromide were obtained from Sigma Chemical Company; *p*-aminophenylmercuric acetate, from K & K Laboratories; and mersalyl acid, from Nutritional Biochemicals.

RESULTS

Previously we reported that incubating brain membranes at 37° for 30 min prior to the addition of ^{3}H-opiates enhances subsequently assayed receptor binding of the agonist [^{3}H]dihydromorphine and the antagonist [^{3}H]naloxone (4, 9). The increased receptor binding elicited by the preliminary incubation is associated with the release into the supernatant fluid of an inhibitor of opiate receptor binding which may be identical with the morphine-like peptide enkephalin (12–17). To explore mechanisms responsible for the preliminary incubation-induced enhancement of receptor binding, we compared the effects of prior incubation at 0° and 37° in the absence and presence of sodium and manganese (Table 1). Membranes incubated in the absence of ions at 37° display about twice as much [^{3}H]naloxone and [^{3}H]dihydromorphine binding as those incubated without ions at 0°. Addition of sodium to the 0° preliminary incubation medium increases naloxone and dihydromorphine binding to levels observed after a 37° sodium-free incubation. Membranes incubated at 37° show the same enhancement of agonist and antagonist binding whether or not the preliminary incubation medium

TABLE 1

Influences of preliminary incubation on opiate receptor binding

Brain membranes were first incubated at 0° or 37° in the presence or absence of NaCl or $MnCl_2$. The opiate binding assay was performed at 0° for 3 hr with 1.4 nM [^{3}H]naloxone or 1.2 nM [^{3}H]dihydromorphine in ion-free buffer. Data are means ± standard errrors of five experiments.

Preliminary incubation conditions		Stereospecific [^{3}H]naloxone bound	Stereospecific [^{3}H]dihydromorphine bound
Temperature	Ion		
		cpm	*cpm*
0°	None	1219 ± 64	757 ± 60
0°	Na^+, 100 mM	2183 ± 153	1599 ± 75
0°	Mn^{++}, 1 mM	1264 ± 85	747 ± 80
37°	None	2239 ± 210	1629 ± 152
37°	Na^+, 100 mM	2697 ± 205	1920 ± 180
37°	Mn^{++}, 1 mM	1158 ± 113	836 ± 75

contained sodium. The finding that sodium- and temperature-induced enhancements of receptor binding are not additive suggests that they act by similar mechanisms. Manganese has no influence on the receptor binding of membranes incubated at 0°, but prevents the enhanced naloxone and dihydromorphine binding associated with 37° incubation.

After incubation with sodium or manganese, opiate receptor binding was assayed in the absence of ions. Conceivably, the ion-induced changes of binding might result from ions remaining bound to membranes. Accordingly, in some experiments, membranes were washed an additional two times following preliminary incubation and prior to the opiate binding assay. Binding results were unaffected by the additional washing procedure. Furthermore, sodium remaining after the washing procedure would be expected to reduce [^{3}H]-dihydromorphine binding, which, instead, was enhanced.

The augmentation of receptor binding following incubation without ions at 25° is less than at 37°, whereas incubations at 10° and 0° fail to increase binding (Fig. 1).

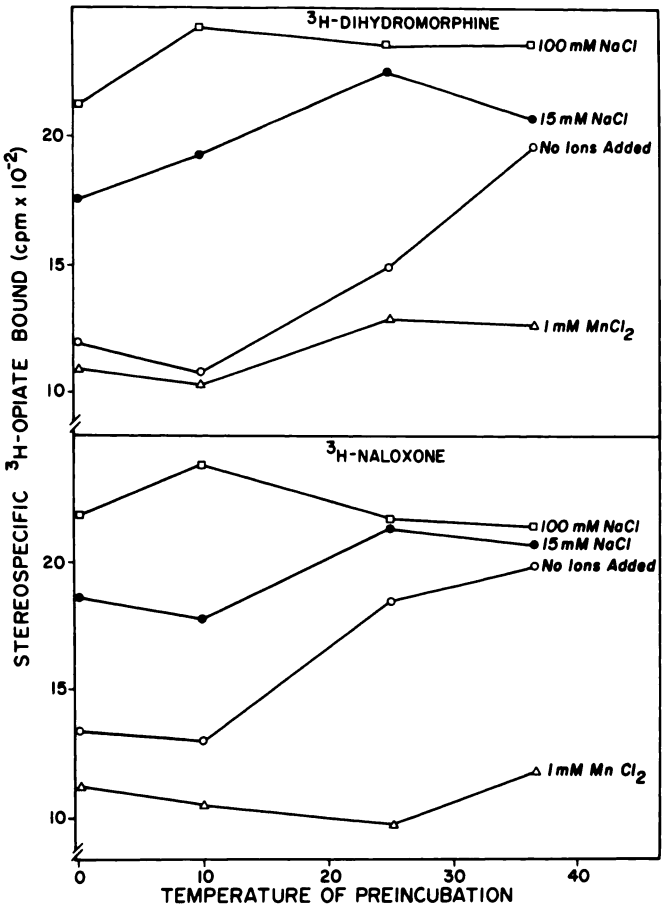

FIG. 1. *Effect of preliminary incubation with NaCl or $MnCl_2$ at different temperatures on opiate receptor binding*

Brain membranes were first incubated in 50 mM Tris-HCl buffer for 60 min at the indicated temperatures, with or without NaCl or $MnCl_2$. The suspension was centrifuged at 49,000 × *g* for 15 min, and the pellet was resuspended in Tris-HCl buffer and assayed for opiate binding. The assay was performed at 0° for 3 hr with no ions added and with 1.4 nM [^{3}H]naloxone or 1.2 nM [^{3}H]dihydromorphine. The experiment was replicated three times.

Enhancement of both [^{3}H]naloxone and [^{3}H]dihydromorphine binding by preliminary incubation with 15 mM NaCl is almost as effective as incubation with 100 mM NaCl.

To determine whether enhanced receptor binding following incubation with sodium is related to the previously reported ability of sodium to influence agonist and antagonist binding differentially (7), we compared sodium with lithium, cesium, and potassium (Fig. 2). Enhancement of antagonist and depression of agonist binding has been demonstrated most strikingly by sodium, somewhat less so by lithium, and hardly at all by cesium and potassium (7). Similarly, incubations with sodium and lithium elevate [^{3}H]naloxone and [^{3}H]dihydromorphine binding to similar extents, while potassium and cesium are much less effective (Fig. 2).

Earlier reported stimulation of agonist binding by divalent cations (9) is most manifest with manganese, less apparent with magnesium, and hardly evident at all with calcium. To determine whether the ability of manganese to prevent the 37° prior incubation enhancement of receptor binding is related to previously reported influences of divalent cations, we compared various concentrations of calcium, manganese, and magnesium (Fig. 3). As little as 0.5 mM manganese prevents the preliminary incubation-induced enhance-

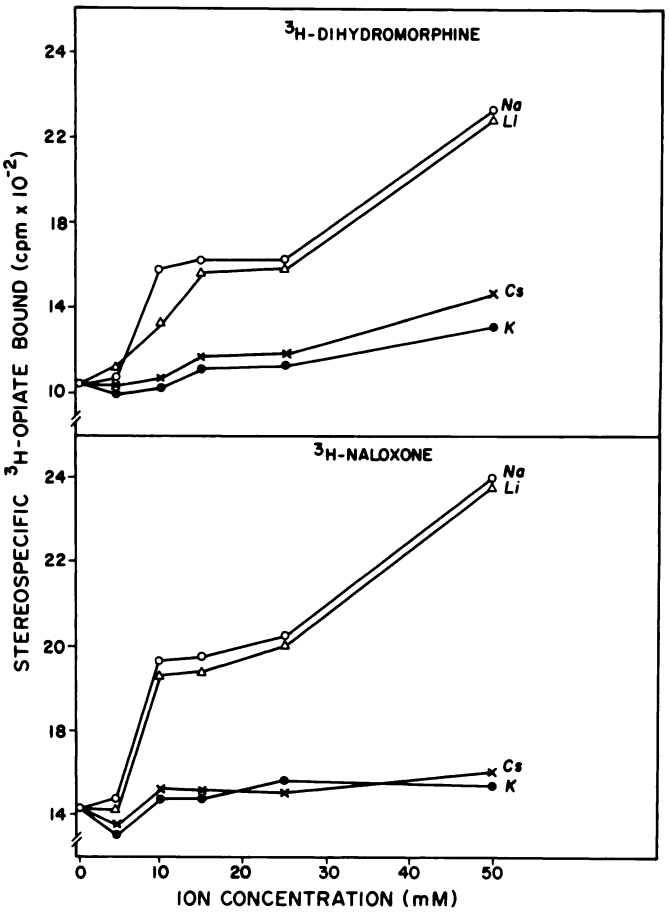

FIG. 2. *Specificity of monovalent ion effects during preliminary incubation on opiate receptor binding*
Brain membranes were first incubated at 0° for 60 min in the absence or presence of different concentrations of the indicated ions. The membranes were then assayed as described in Fig. 1. The experiment was replicated twice.

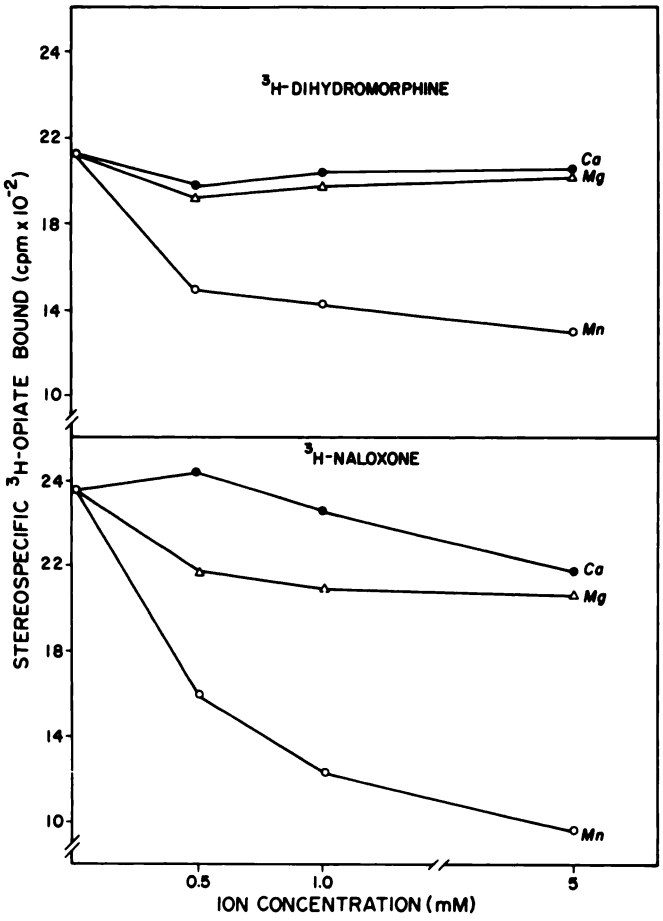

FIG. 3. *Specificity of divalent ion effects during preliminary incubation on opiate receptor binding*
Brain membranes were first incubated at 37° for 60 min in the absence or presence of different concentrations of the indicated ions. The membranes were then assayed as described in Fig. 1. The experiment was replicated twice.

ment of both [^{3}H]naloxone and [^{3}H]dihydromorphine binding, with maximal inhibition at 1.0 mM manganese. Magnesium has a lesser effect on [^{3}H]naloxone binding and almost no detectable influence on [^{3}H]dihydromorphine, while calcium does not alter either naloxone or dihydromorphine binding.

To ascertain whether the temperature and ionic influences on receptor binding are related to alterations in affinity or in the number of binding sites, we conducted incubations at a variety of [^{3}H]naloxone and [^{3}H]dihydromorphine concentrations (Figs. 4 and 5). The changes in binding after preliminary incubation seem related primarily to an alteration in the number of binding sites. Incubation at 37° in the absence of ions or at 0° in the presence of sodium increases the number of binding sites. Similarly, the manganese-induced reduction of binding in tissue incubated at 37° is associated with a decline in the number of binding sites, with only a small change in affinity. The increase in the number of [^{3}H]dihydromorphine binding sites after incubation at 37° or at 0° in the presence of sodium is associated with some decrease in affinity, with a 50% increase in the K_D in three repeated experiments.

Regardless of the conditions of preliminary incubation, all the opiate receptor binding assays described above were conducted in the absence of added ions. Subse-

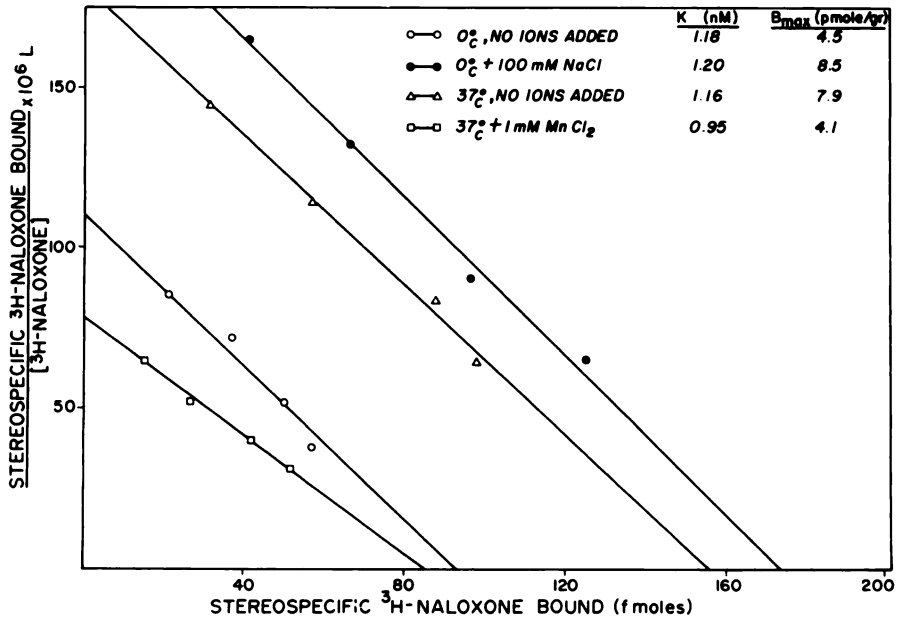

FIG. 4. *Scatchard analysis of [³H]naloxone binding to brain membranes previously incubated under different conditions*

Brain membranes were first incubated at 0° or 37° with or without ions, as indicated. The homogenate was then centrifuged at 49,000 × *g* for 15 min and the pellet was resuspended in Tris-HCl buffer and assayed at 0° for 3 hr with different concentrations of [³H]naloxone (0.25–3.0 nM) with no ions added. The experiment was replicated twice.

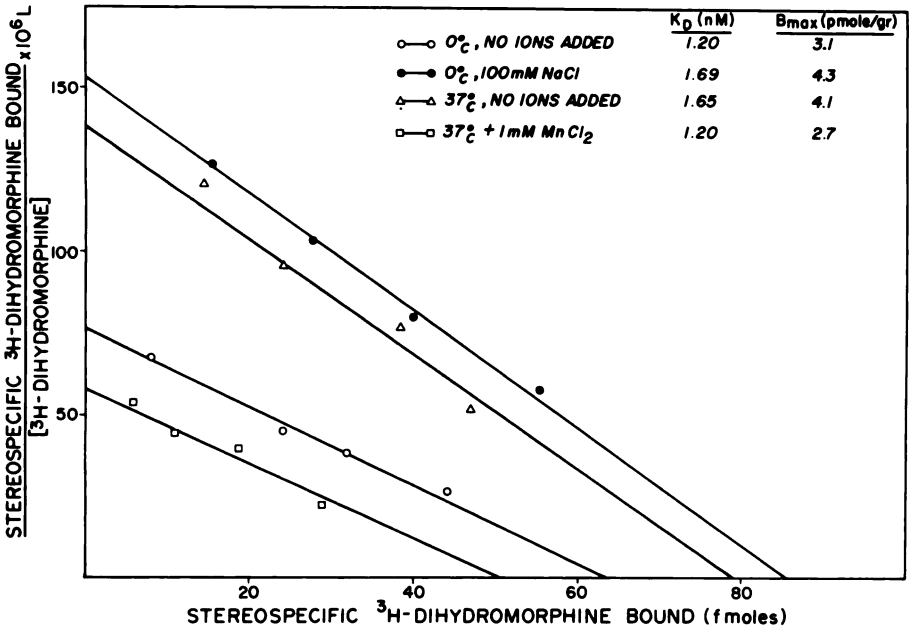

FIG. 5. *Scatchard analysis of [³H]dihydromorphine binding to brain membranes incubated under different conditions*

The experiment was performed as described in Fig. 4, except that [³H]dihydromorphine was used (0.12–1.5 nM). The experiment was replicated twice.

quently we evaluated ion effects on receptor binding in tissue subjected to a variety of preliminary incubation conditions (Table 2). For tissue incubated at 0° with no ions, sodium produces the same marked enhancement of [^{3}H]naloxone and depression of [^{3}H]dihydromorphine binding observed previously in tissue that had not been incubated beforehand (7). By contrast, after incubation at 0° without ions, manganese reduces the binding of both [^{3}H]naloxone and [^{3}H]dihydromorphine, wheras in earlier reports manganese enhanced [^{3}H]dihydromorphine binding (9). In the previous studies opiate receptor binding was assayed at 25° while in the present study assays were conducted at 0°. For membranes incubated with sodium at 0°, opiate receptor binding is enhanced whether receptor binding is assayed with or without sodium or manganese. The relative effects of sodium and manganese in the receptor assay are the same whether tissues have been incubated at 0° or 37°, with or without sodium. Prior incubation at 37° in the absence or presence of sodium enhances naloxone and dihydromorphine binding to the same extent as incubation with sodium at 0°. Previous incubation with manganese at 0° and 37° has the same effect on all aspects of receptor binding as incubation at 0° in the absence of ions. Thus manganese prevents the influence of preliminary incubation at 37° upon receptor binding.

To compare the properties of the "new receptors" unmasked by incubation at 37° or at 0° with sodium, we evaluated effects of several protein-modifying reagents (Table 3). At the concentrations employed, none of the reagents affects [^{3}H]naloxone binding of tissue incubated without ions at 0°. Thus this "baseline" receptor binding is stable to reagent treatment. Opiate binding by membranes incubated with manganese, which prevents preliminary incubation-induced elevations of binding, is also resistant to effects of reagents.

TABLE 2

Interactions of preliminary incubation manipulations and ionic effects on opiate receptor binding

Brain membranes were first incubated at 0° or 37° in the absence or presence of ions (100 mM NaCl or 1 mM $MnCl_2$). The opiate binding assay was performed at 0° for 3 hr in the absence or presence of 100 mM NaCl or 1 mM $MnCl_2$ as indicated, with 1.4 nM [^{3}H]naloxone or 1.2 nM [^{3}H]dihydromorphine. Data are means ± standard errors of five experiments.

Preliminary incubation conditions		Ions added in assay	Stereospecific opiate bound	
Temperature	Ion added		[^{3}H]Naloxone	[^{3}H]Dihydromorphine
			cpm	*cpm*
0°	None	None	1321 ± 110	1128 ± 77
0°	None	Na^+	4785 ± 315	338 ± 50
0°	None	Mn^{++}	705 ± 65	680 ± 63
0°	Na^+	None	2320 ± 172	2080 ± 125
0°	Na^+	Na^+	5812 ± 375	1330 ± 110
0°	Na^+	Mn^{++}	1277 ± 90	1215 ± 127
0°	Mn^{++}	None	1230 ± 105	1090 ± 70
0°	Mn^{++}	Na^+	4750 ± 325	505 ± 60
0°	Mn^{++}	Mn^{++}	673 ± 85	640 ± 64
37°	None	None	2347 ± 193	2106 ± 180
37°	None	Na^+	5270 ± 276	885 ± 45
37°	None	Mn^{++}	1025 ± 65	1124 ± 70
37°	Na^+	None	2870 ± 187	2305 ± 165
37°	Na^+	Na^+	5397 ± 420	970 ± 90
37°	Na^+	Mn^{++}	1248 ± 125	1015 ± 93
37°	Mn^{++}	None	1140 ± 80	1060 ± 40
37°	Mn^{++}	Na^+	6208 ± 415	430 ± 43
37°	Mn^{++}	Mn^{++}	530 ± 68	590 ± 58

TABLE 3

Effects of protein-modifying reagents on preliminary incubation-induced increase in [^{3}H]naloxone binding

Brain membranes were first incubated at 0° or 37° in the absence or presence of 100 mM NaCl or 1 mM $MnCl_2$, after which the homogenate was centrifuged at 49,000 × *g* for 15 min. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.7 at 25°) containing the indicated protein-modifying reagent and incubated at 0° for 60 min. The suspension was recentrifuged at 49,000 × *g* for 15 min, and the membranes were assayed for [^{3}H]naloxone binding at 0° for 3 hr with no ions added and with 1.4 nM [^{3}H]naloxone. Data are means ± standard errors of two to four experiments.

Reagent added	Stereospecific [^{3}H]naloxone bound after various preliminary incubation conditions			
	Temperature	No ions added	+100 mM NaCl	+1 mM $MnCl_2$
	cpm	*cpm*	*cpm*	*cpm*
None	0°	1263 ± 105	1890 ± 132	1175 ± 65
	37°	2075 ± 167	2280 ± 210	1365 ± 80
Iodoacetamide, 50 μM	0°	1060 ± 85	1151 ± 110	1123 ± 110
	37°	1112 ± 80	1270 ± 60	1040 ± 122
Mersalyl acid, 10 μM	0°	1101 ± 107	1185 ± 85	1060 ± 73
	37°	1420 ± 105	1620 ± 135	850 ± 53
5,5′-Dithiobis(2-nitrobenzoic acid), 1 mM	0°	1108 ± 80	1231 ± 140	1020 ± 82
	37°	1112 ± 65	1123 ± 110	920 ± 66
Reduced glutathione, 1 mM	0°	1180 ± 92	1450 ± 120	1008 ± 78
	37°	1375 ± 106	1460 ± 160	1133 ± 100
p-Chloromercuribenzoate, 3 μM	0°	1130 ± 90	1295 ± 112	1080 ± 135
	37°	1344 ± 68	1480 ± 110	1323 ± 120
2-Hydroxy-5-nitrobenzyl bromide, 1 mM	0°	1231 ± 107	1435 ± 145	1080 ± 83
	37°	1382 ± 105	1470 ± 170	1104 ± 72
2-Methoxy-5-nitrobenzyl bromide, 100 μM	0°	1093 ± 67	1374 ± 103	1044 ± 132
	37°	1340 ± 103	1407 ± 140	1127 ± 120
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, 1 mM	0°	1147 ± 88	1375 ± 105	1105 ± 61
	37°	1422 ± 155	1460 ± 115	1062 ± 70
Iodine, 10 μM	0°	1075 ± 110	1140 ± 107	1040 ± 73
	37°	1260 ± 130	1310 ± 123	1166 ± 93

By contrast, all the reagents markedly reduce or abolish the enhancement of receptor binding elicited by prior incubation at 37° with or without sodium, or at 0° in the presence of sodium. Enhanced binding is abolished by iodoacetamide, 5,5′-dithiobis(2-nitrobenzoic acid), and iodine, while partial reduction occurs with mersalyl acid, reduced glutathione, *p*-chloromercuribenzoate, 2-hydroxy-5-nitrobenzyl bromide, and 2-methoxy-5-nitrobenzyl bromide and carbodiimide.

DISCUSSION

The most striking finding of the present study is the alteration of opiate receptor binding produced by various manipulations of temperature and ionic environment. Earlier we described enhanced receptor binding for tissues previously incubated at 37° associated with the release of an endogenous inhibitor of binding (4). This inhibitor might be identical with the endogenous morphine-like peptide enkephalin (12–17). In the present study we have observed that preliminary incubation with sodium at 0° doubles binding of both the agonist dihydromorphine and the antagonist naloxone. The enhanced binding produced by prior 37° incubation is not further augmented by sodium, suggesting that sodium and elevated temperature act by similar mechanisms. If increased binding arises from removal of enkephalin or

some other endogenous ligand, dissociation of enkephalin at 0° may be accelerated by sodium. Such a suggestion is consistent with the agonist properties of enkephalin (12–17) and the accelerated dissociation of opiate agonists from receptor sites produced by sodium (7). Dissociation of an endogenous ligand from receptors during preliminary incubation would also explain the increase in number of binding sites for both agonists and antagonists. This effect is apparent for both the agonist dihydromorphine and the antagonist naloxone. Moreover, it is elicited with an ionic specificity similar to the capacity of manganese and certain other divalent cations to increase agonist binding selectively (9). We suggest that manganese may act by enhancing the binding of enkephalin to the opiate receptor, thus retarding enkephalin dissociation from receptor sites.

When manganese is added to opiate receptor binding assays performed at 0°, it reduces the binding of both [^{3}H]naloxone and [^{3}H]dihydromorphine regardless of the conditions of preliminary incubation. Since maximal dissociation of the endogenous ligand has presumably taken place during the preliminary incubation, it is possible that the decrease in binding induced by manganese under these conditions represents a direct effect of manganese upon the opiate receptor. Alternatively, enhanced affinity of enkephalin binding elicited by manganese (18) could reduce dihydromorphine and naloxone binding. In previous studies in which manganese enhanced agonist but not antagonist binding (9), incubations were performed at 25°, while in the present study opiate receptor binding assays were conducted at 0° for 3 hr. We have confirmed the previous observations that manganese increases agonist but not antagonist binding when receptor binding is assayed at 25°. Thus manganese affects the opiate receptor differently, depending on the incubation temperature.

Earlier studies also showed marked differences in the behavior of the opiate receptor at lower temperatures (10). Thus, whereas incubations at 25° reveal an augmentation by sodium of the number of [^{3}H]naloxone binding sites with no change in their affinity, at 0° sodium enhances affinity without markedly altering the number of binding sites. The sodium-elicited increase in [^{3}H]naloxone binding observed in opiate receptor assays conducted at 25° is attributable to the capacity of sodium to cause a dissociation of enkephalin or some other endogenous ligand from the receptor, since the enhancement is no longer observed in tissue incubated at 37° and assayed at 25° (4, 10). By contrast, sodium can still elevate [^{3}H]naloxone binding in membranes previously incubated to remove endogenous ligands if binding is assayed at 0°, so that at 0° sodium presumably increases naloxone binding by a direct influence on the opiate receptor.

The enhanced receptor binding "unmasked" by preliminary incubation appear to be more susceptible to inhibitory effects of protein-modifying reagents than "basal" receptors. It is unclear whether the protein-modifying reagents decrease the number of binding sites or reduce their affinity. The greater lability of the enhanced binding is supported by preliminary experiments showing that the increased binding is also more sensitive than basal levels to degradation by proteolytic enzymes.[3]

Earlier we observed that low concentrations of protein-modifying reagents reduce dihydromorphine binding with minimal effects of naloxone binding (4). The concentrations of the reagents employed in the present study were high enough under the experimental conditions to produce some reduction in naloxone binding.

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