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Differential Effects of Protein-Modifying Reagents on Receptor Binding of Opiate Agonists and Antagonists

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SUMMARY

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Opiate receptor binding is enhanced by prior incubation, which removes an endogenous inhibitor of receptor binding. Protein-modifying reagents which affect sulfhydryl groups differentially influence the binding of agonists and antagonists to the opiate receptor. These reagents, including iodoacetamide, *N*-ethylmaleimide, mercuriacetate, mersalyl acid, *p*-aminophenylmercuric acetate, and *p*-chloromercuribenzoate, strongly inhibit [^{3}H]dihydromorphine binding at concentrations which do not affect [^{3}H]naloxone binding. Prior treatment with opiates protects the receptor binding from reagents. The reagents decrease the apparent number of dihydromorphine binding sites without altering their affinity, and also increase the sensitivity of agonist binding to the inhibitory effects of sodium.

INTRODUCTION

Opiate agonists and antagonists have markedly different pharmacological actions despite their considerable structural similarities. Agonists elicit analgesia and euphoria; antagonists block or reverse these effects while having no observable pharmacological effects of their own. Houde and Wallenstein (1, 2) reported that low doses of nalorphine reverse the analgesic effects of morphine in man, while higher doses of nalorphine in combination with the same fixed dose of morphine restore much of the lost analgesia. Based on these and other data not readily explained by simple competitive displacement (3-8), Martin suggested that agonists and antagonists have multiple binding sites (9).

Biochemical identification of opiate receptor binding (10-12) has permitted a direct examination of the receptor interactions of opiates. The subcellular (13) and regional (14, 15) distribution, as well as specificity (16, 17), chemical properties (18, 19), multiple sites (20), presence in tissue culture (21), and influence of addiction (22, 23), have been described. Initial investigations suggested that opiate agonists and antagonists bind competitively to the same sites (16). Subsequently we were able to differentiate clearly between the binding of agonists and antagonists and mixed drugs on the basis of their sensitivity to the effects of sodium (23, 24). Physiological concentrations of sodium markedly decrease agonist binding while increasing

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the binding of antagonists. In this paper we report the effects of protein-modifying reagents on the binding of agonists and antagonists.

MATERIALS AND METHODS

[^{3}H]Naloxone and [^{3}H]dihydromorphine were obtained from New England Nuclear Corporation. [^{3}H]Levorphanol, [^{3}H]levallorphan, [^{3}H]oxymorphone, and [^{3}H]nalorphine were prepared and purified as previously described (23, 24). Tris-HCl buffer, iodoacetamide, *N*-ethylmaleimide, *N*-bromosuccinimide, iodoacetate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, glycine *O*-methyl ester, 2-methoxy-5-nitrobenzyl bromide, and 2-hydroxy-5-nitrobenzyl bromide were purchased from Sigma Chemical Company, and iodine, reduced glutathione, and 2-mercaptoethanol from Baker. *p*-Aminophenylmercuric acetate was obtained from K & K Laboratories, Inc., and mersalyl acid from Nutritional Biochemicals. Naloxone and oxymorphone were generously donated by Endo, and levorphanol, dextrorphan, levallorphan, and (+)-3-hydroxy-*N*-allymorphinan by Roche. We thank Dr. L. Hellerman for gifts of *p*-chloromercuribenzoate and mercuriacetate.

Male Sprague-Dawley rats (ASG Sprague-Dawley, Madison, Wis.) (180–220 g) were decapitated and their brains were rapidly removed. The cerebellum, which contains negligible ^{3}H-opiate binding (10), was excised, and the remainder of the brain was immediately placed in 30 volumes of iced 50 mM Tris-HCl buffer (pH 7.7 at 25°; designated as standard buffer) and homogenized with a Brinkmann Polytron. The homogenate was centrifuged at 49,000 × *g* for 15 min, and the pellet was resuspended in standard buffer with the Brinkmann Polytron. The resuspension was then incubated at 37° for 30 min and recentrifuged. This pellet was then resuspended in 100 volumes of standard buffer and allowed to react with the appropriate reagent for 20 min at 25°. After the reaction, the homogenate was recentrifuged and the pellet was resuspended in the original volume of standard buffer. Assays were performed at 25° for 30 min with 2-ml aliquots of the tissue homogenate and appropriate amounts of ^{3}H-opiate. All determinations were performed in triplicate with and without 1 μM levallorphan. The incubation was terminated by filtration under vacuum over Whatman glass fiber filters (GF-B). The filters were then washed with two 5-ml volumes of cold standard buffer and placed in 12 ml of Hydromix scintillation fluor (Yorktown Research). All values are expressed as specific ^{3}H-opiate binding, i.e., the binding of triplicate samples in the absence of 1 μM levallorphan minus the binding of triplicate samples in its presence. The triplicate determinations always varied by less than 10%.

RESULTS

Influence of prior incubation on [^{3}H]naloxone binding. Previous studies in our laboratory measured opiate receptor binding without any prior incubation (10, 13, 14, 16, 18, 20). In the present study we routinely first incubated preparations at 37° for 30 min because the procedure enhances [^{3}H]naloxone and [^{3}H]dihydromorphine binding. Preliminary incubation at 37° for as little as 5 min increases [^{3}H]naloxone binding about 30%, assayed in the presence or absence of sodium (Table 1). Maximal increases occur at 30 min, with a 50% augmentation of

TABLE 1

Effect of prior incubation on [^{3}H]naloxone binding

Rat brains, minus cerebella, were homogenized and centrifuged, and the pellets were resuspended in 100 volumes of standard buffer as described in MATERIALS AND METHODS. Aliquots were incubated for the appropriate period of time at 37° and assayed in the presence and absence of 100 mM NaCl with [^{3}H]naloxone (1 nM) as described in MATERIALS AND METHODS. Data are the means of triplicate determinations, which varied less than 10%. The experiment was replicated twice.

Prior incubation time	Specific [^{3}H]naloxone binding	
	Assayed with NaCl	Assayed without NaCl
min	*cpm*	*cpm*
0	2207	1533
5	2811	1887
15	3442	
30	3350	3520
45		3413
60	3319	3380

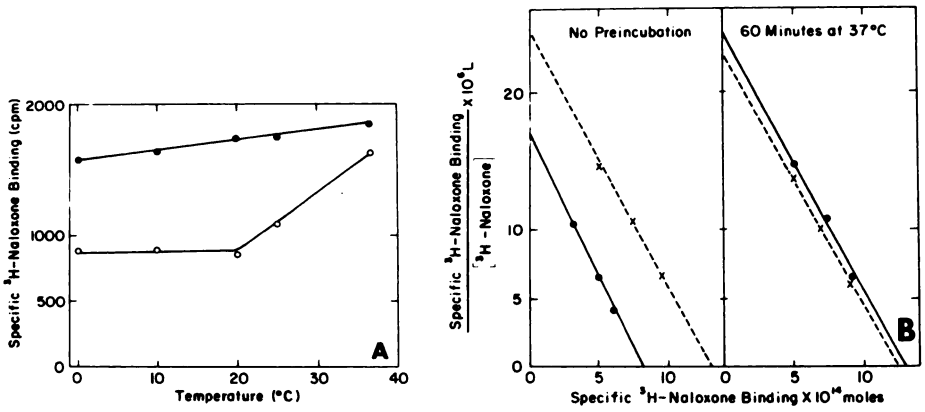

FIG. 1.

A. Temperature dependence of enhancement of binding induced by prior incubation. Rat brains minus cerebella were homogenized and centrifuged, and the pellets were resuspended in 100 volumes of standard buffer as described in MATERIALS AND METHODS. Aliquots were then incubated at the stated temperature for 30 min and centrifuged at 49,000 × *g* for 15 min, and the pellets were resuspended in the original volume of standard buffer. The aliquots were then assayed in the presence (●) or absence (○) of 100 mM NaCl with [³H]naloxone (1 nM) as described in MATERIALS AND METHODS. Points represent specific [³H]naloxone binding and are the means of triplicate determinations.

B. Scatchard analysis of the influence of prior incubation on [³H]naloxone binding. Rat brains minus cerebella were homogenized and centrifuged, and the pellets were resuspended in 100 volumes of buffer. The samples receiving no preliminary incubation were immediately assayed with the appropriate amount of [³H]naloxone. The remaining homogenate was incubated at 37° for 60 min and then assayed as described in MATERIALS AND METHODS. Values represent specific [³H]naloxone binding and are the means of triplicate determinations. ●, assayed without NaCl; ×, assayed with 100 mM NaCl. The experiment was replicated twice.

[³H]naloxone binding assayed with sodium and a doubling of [³H]naloxone binding assayed in the absence of sodium. Interestingly, a 30-min prior incubation virtually abolishes the ability of sodium to further enhance [³H]naloxone binding. This incubation increases the binding of [³H]dihydromorphine to a similar extent. The resultant [³H]dihydromorphine binding is still as sensitive to the inhibitory effects of sodium as before preliminary incubation.

The influence of prior incubation on binding is temperature-dependent (Fig. 1A). Incubation at temperatures under 25° for 30 min does not significantly alter [³H]naloxone binding, and a 37° incubation is more effective than a 25° one. Scatchard analysis indicates that the increase in binding after incubation for 60 min at 37° results from an increase in the number of [³H]naloxone binding sites with no alteration in their affinity (Fig. 1B).

The mechanism whereby prior incubation increases [³H]naloxone binding is unclear. However, preliminary experiments suggest that it may result from the removal of an endogenous inhibitor of binding (Table 2). When homogenates are first

TABLE 2

Release of factor inhibiting opiate receptor binding by incubation of brain tissue at various temperatures

Rat brains were homogenized in 5 volumes of standard buffer and centrifuged for 15 min at 49,000 × *g*, and the pellets were resuspended in the original volumes. Aliquots were then incubated at 0°, 25°, or 37° for the stated times. After centrifugation (15 min at 49,000 × *g*), supernatants (1.5 ml) were added to homogenates (0.5 ml of one brain in 30 ml) prepared as described in MATERIALS AND METHODS and assayed in the presence of 100 mM NaCl with 1 nM [³H]naloxone. Values are defined as specific opiate binding and are the means of triplicate determinations. The experiment was replicated twice.

Addition	Specific [³H]naloxone binding	Decrease
	cpm	%
None	4142	
0° supernatant, 40 min	3154	24
25° supernatant, 40 min	1905	54
37° supernatant, 40 min	1493	64
37° supernatant, 120 min	1166	72

incubated at 37°, then centrifuged, and the supernatant fluid is added to a standard opiate receptor assay, [^{3}H]naloxone binding is diminished. The release of the inhibitory factor into the supernatant fluid shows a temperature dependence similar to the increase in [^{3}H]naloxone binding induced by prior incubation. A 40-min preliminary incubation at 0° decreases binding only 24%, while the 25° incubation inhibits by 54%, and the 37° one by 64%. Extending the 37° incubation to 2 hr results in only a slight increase of the inhibition to 72%. The rate of dissociation of this endogenous inhibitor is increased several fold in the presence of sodium chloride, resembling the behavior of opiate agonists.[3]

*Influence of protein-modifying reagents on binding of [^{3}H]naloxone and [^{3}H]*dihydromorphine. Several protein-modifying reagents affect the specific binding of [^{3}H]naloxone and [^{3}H]dihydromorphine differentially (Fig. 2; see Table 4). When assayed in the presence of sodium, 0.1 mM iodoacetamide decreases [^{3}H]dihydromorphine binding 25%, and 5 mM iodoacetamide lowers binding 90% (Fig. 2A), while [^{3}H]naloxone binding is affected very little. Without NaCl in the assay, [^{3}H]dihydromorphine binding is decreased somewhat less, while [^{3}H]naloxone binding is only slightly reduced. The greater sensitivity of [^{3}H]dihydromorphine binding to iodoacetamide when assayed in the presence of sodium does not simply reflect the reduction of opiate agonist binding by sodium. All values for [^{3}H]dihydromorphine binding by iodoacetamide-treated tissue assayed in the presence of sodium were compared with untreated tissues which were also assayed with sodium.

Previously we showed that iodoacetamide and other protein-modifying reagents differentiate agonist and antagonist receptor binding for [^{3}H]oxymorphone, [^{3}H]levorphanol, and [^{3}H]levallorphan (19). Dithionitrobenzoic acid distinguishes among a variety of ^{3}H-agonists and ^{3}H-antagonists (Table 3). Thus the discriminatory effects of these reagents depend on the agonist and antagonist properties of opiates and are not restricted to naloxone and dihydromorphine.

The effects of iodoacetamide and *N*-ethylmaleimide on [^{3}H]dihydromorphine binding do not involve a reaction with the opiate itself, since all experiments were performed with membrane preparations extensively washed free of the reagents (19). Moreover, after five ^{3}H-opiates—[^{3}H]levorphanol, [^{3}H]levallorphan, [^{3}H]oxymorphone, [^{3}H]naloxone, and [^{3}H]dihydromorphine—were incubated with 100 mM iodoacetamide for 20 min at 25°, thin-layer chromatograms in three solvent systems showed no detectable changes in the relative fronts of any of the ^{3}H-drugs (19).

Similar effects on receptor binding have been obtained with other protein reagents. The differential influence of *N*-ethylmaleimide on ^{3}H-opiate agonist and antagonist binding is evident over a wide range of concentrations. As little as 10 μM *N*-ethylmaleimide reduces [^{3}H]dihydromorphine binding by 65% when assayed with sodium and by 40% when assayed without sodium, whereas the same concentration of reagent lowers [^{3}H]naloxone binding 20% when

TABLE 3

Effect of dithionitrobenzoic acid on binding of opiate ^{3}H-agonists and ^{3}H-antagonists

Rat brain membrane preparations were allowed to react with dithionitrobenzoic acid (0.5 mM) and assayed as described in MATERIALS AND METHODS. The following amounts of ^{3}H-opiates were used: [^{3}H]naloxone, 60,000 cpm; [^{3}H]dihydromorphine, 90,000 cpm; [^{3}H]levorphanol, 54,000 cpm; [^{3}H]levallorphan, 90,000 cpm; [^{3}H]nalorphine, 35,000 cpm; [^{3}H]oxymorphone, 48,000 cpm. Results are the means of quadriplicate determinations, which varied less than 10%.

^{3}H-Opiate	Binding	
	Assayed with 100 mM NaCl	Assayed without NaCl
	% control	
Antagonists		
[^{3}H]Naloxone	87	83
[^{3}H]Levallorphan	91	92
[^{3}H]Nalorphine	66	46
Agonists		
[^{3}H]Dihydromorphine	0	29
[^{3}H]Levorphanol	48	108
[^{3}H]Oxymorphone	9	35

[3] G. W. Pasternak and S. H. Snyder, manuscript in preparation.

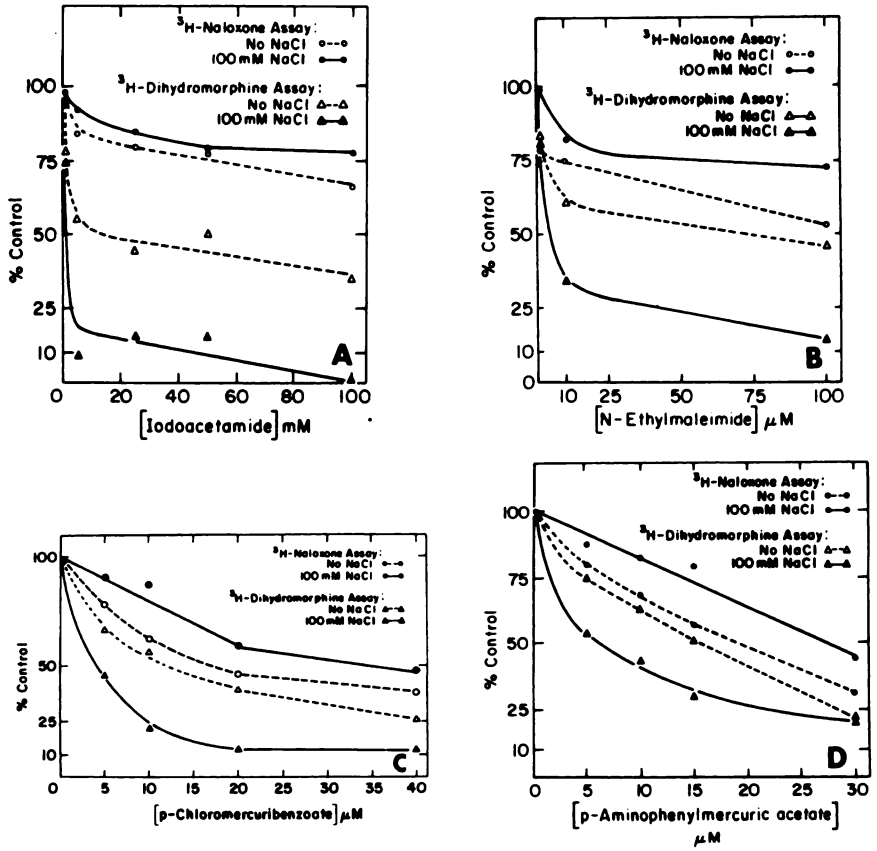

FIG. 2. *Effects of iodoacetamide, N-ethylmaleimide, p-chloromercuribenzoate, and p-aminophenylmercuric acetate on [³H]naloxone and [³H]dihydromorphine binding*

Rat brain homogenate was prepared, allowed to react with the appropriate concentration of the stated reagent, and centrifuged, and the pellets were resuspended and assayed as described in MATERIALS AND METHODS. Assays were performed with [³H]naloxone (1 nM) in the presence (●) and absence (○) of 100 mM NaCl, as well as with [³H]dihydromorphine (0.5 nM) in the presence (▲) and absence (△) of 100 mM NaCl. Values were determined by comparing specific binding in reacted homogenates with specific binding in unreacted homogenates. Reacted samples assayed in the presence of 100 mM NaCl were compared with unreacted samples also assayed in the presence of 100 mM NaCl. All reagents were dissolved in water immediately before use except for *p*-aminophenylmercuric acetate and *p*-chloromercuribenzoate, which were dissolved in dimethylsulfoxide. Dimethylsulfoxide has no effect on binding at these concentrations. The experiment was replicated three times.

assayed with sodium and 25% when assayed without sodium. Even a concentration of the reagent as high as 1 mM reduces [³H]naloxone binding only 35% when assayed with sodium, while [³H]dihydromorphine binding is virtually abolished.

The sulfhydryl reagents *p*-chloromercuribenzoate and *p*-aminophenylmercuric acetate also differentiate agonist from antagonist binding. Both these reagents, at concentrations under 10 μM, exert markedly different effects on [³H]naloxone and [³H]dihydromorphine binding (Fig. 2C and D). As with the other reagents, the differential effect is most pronounced when the tissue is assayed in the presence of sodium chloride. The effects of *p*-aminophenylmercuric acetate can be totally prevented if excess 2-mercaptoethanol (20–100 mM) or dithiothreitol (20–50 mM) is added prior to the reagent.

These results have been extended to an array of protein-modifying reagents which vary in their ability to differentiate agonist and antagonist binding (Table 4). 5,5'-Dithiobis(2-nitrobenzoic acid, one of the most specific sulfhydryl reagents, is also extremely effective. When assayed in the

TABLE 4

Effects of various reagents on [^3H]naloxone and [^3H]dihydromorphine binding

Brain homogenates were prepared, allowed to react with the appropriate reagent at the indicated concentrations, and assayed in the presence and absence of 100 mM NaCl as described in MATERIALS AND METHODS. Values were determined by comparing specific binding in reacted homogenates with specific binding in unreacted homogenates. Reacted samples assayed in the presence of 100 mM NaCl were compared with unreacted samples also assayed in the presence of 100 mM NaCl. Assays were performed with 1 nM [^{3}H]naloxone or 0.6 mM [^{3}H]dihydromorphine. All reagents were dissolved in water immediately before use except for mercuriacetate, which was dissolved in dimethylsulfoxide. Dimethylsulfoxide has no effect on binding at these concentrations. Values are the means of triplicate determinations. The experiment was replicated three times.

Reagent	Assayed with 100 mM NaCl		Assayed without NaCl		Reagent	Assayed with 100 mM NaCl		Assayed without NaCl	
	[^{3}H]-Naloxone	[^{3}H]Dihydromorphine	[^{3}H]-Naloxone	[^{3}H]Dihydromorphine		[^{3}H]-Naloxone	[^{3}H]Dihydromorphine	[^{3}H]-Naloxone	[^{3}H]Dihydromorphine
	% control		*% control*			*% control*		*% control*	
N-Ethylmaleimide									
0.1 mM	85	7	75	53	5 μM	96	78	83	114
0.3 mM	71	9	54	46	10 μM	110	18	71	80
1.0 mM	65	6	42	21	30 μM	61	3	36	35
3.0 mM	40	3	25	11	75 μM	10	7	8	2
Iodoacetamide					Iodine				
1 μM	88	90	86	85	10 μM	90	67	91	81
10 μM	109	107	98	102	20 μM	73	52	75	72
100 μM	98	75	96	78	50 μM	34	18	34	17
5,5'-Dithiobis-(2-nitrobenzoic acid)					100 μM	17	11	14	8
					2-Methoxy-5-nitrobenzyl bromide				
0.1 mM	100	21	100	84	10 μM	102	101	95	92
0.5 mM	100	14	91	75	30 μM	89	103	112	92
1.0 mM	100	26	97	87	100 μM	71	65	57	58
2.0 mM	100	18	100	75	1 mM	65	50	55	53
5.0 mM	86	8	68	13	10 mM	35	37	34	28
Mercuriacetate					2-Hydroxy-5-nitrobenzyl bromide				
1 μM	80	43	82	90					
3 μM	70	41	58	58					
10 μM	18	0	9	10	0.1 mM	94	94	80	92
Mersalyl acid					0.3 mM	83	80	83	83
1 μM	77	88	98	99	1.0 mM	86	91	85	88
3 μM	89	88	89	84	10 mM	72	47	58	44
4 μM	83	32	86	90	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide				
10 μM	76	23	59	35					
30 μM	39	0	26	28					
Glutathione									
1 mM	89	89	87	101					
10 mM	77	68	90	82	1 μM	112	84	128	114
25 mM	66	35	51	50	10 μM	96	99	119	105
50 mM	52	0	132	6	30 μM	99	95	118	101
100 mM	80	0	169	4	100 μM	101	73	113	81
N-Bromosuccinimide					1 mM	81	30	89	52
					10 mM	53	26	50	41
1 μM	109	83	104	107					

presence of sodium chloride, [^{3}H]naloxone binding is not affected while [^{3}H]dihydromorphine binding is lowered 80% by only 0.1 mM reagent. As with other reagents, this effect is most pronounced in the presence of sodium chloride. Mercuriacetate affects [^{3}H]naloxone and [^{3}H]dihydromorphine binding differentially, but not as strikingly as does *N*-ethylmaleimide. Mersalyl acid, which is fairly selective for sulfhydryl groups, discriminates between [^{3}H]naloxone and [^{3}H] dihydromorphine binding effectively, reducing agonist binding almost 80% in concentrations which lower antagonist binding only 25% when assayed in the presence of sodium. Glutathione is also effective and, in higher concentrations, even appears to increase [^{3}H]naloxone binding, producing 30% and 70% increases at 50 mM and 100 mM concentrations when assayed without sodium. At these concentrations glutathione completely abolishes [^{3}H]dihydromorphine binding assayed with or without sodium. Since in the same concentrations dithiothreitol and 2-mercaptoethanol do not affect receptor binding so drastically, it is conceivable that the effect of glutathione is not due solely to its sulfhydryl character.

N-Bromosuccinimide, which interacts with a variety of protein residues besides sulfhydryl groups, is effective in discriminating between agonist and antagonist binding. Iodine exerts less differential effects, with only a slightly enhanced reduction of [^{3}H]dihydromorphine binding compared to [^{3}H]naloxone binding at several concentrations. The reagents 2-hydroxy-5-nitrobenzyl bromide and 2-methoxy-5-nitrobenzyl bromide, which primarily modify tryptophan residues, have only a limited ability to distinguish between agonist and antagonist binding. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, a water-soluble compound which is fairly selective for carboxyl groups, can differentiate [^{3}H]naloxone and [^{3}H]dihydromorphine binding to a limited extent at 1 and 10 mM concentrations. The effects of this reagent are completely prevented by the prior addition of 40 mM glycine *O*-methyl ester.

With iodoacetamide, *N*-ethylmaleimide, *p*-chloromercuribenzoate, *p*-aminophenylmercuric acetate, and *N*-bromosuccinimide, [^{3}H]naloxone binding is consistently more resistant to inhibition by reagents when assayed in the presence than in the absence of sodium. This contrasts strikingly with the greater sensitivity of [^{3}H]dihydromorphine binding in the presence of sodium.

We performed saturation studies of [^{3}H]dihydromorphine binding in the presence of iodoacetamide and *N*-ethylmaleimide. Scatchard analysis of these experiments indicates that both reagents lower the number of binding sites with no alteration in receptor affinity for opiates (Fig. 3).

Reversal of p-aminophenylmercuric acetate inhibition. The reagents which best differentiate the binding of agonists and antagonists primarily affect sulfhydryl groups, but can also interact with other residues (25–31). If the influence of the mercurial reagents on opiate receptor binding involves sulfhydryl groups, these effects should be at least partially reversed by sulfhydryl-containing compounds. Accordingly we endeavored to reverse the effects of *p*-aminophenylmercuric acetate with dithiothreitol and 2-mercaptoethanol (Table 5). After incubation with *p*-aminophenylmercuric acetate (30 or 100 μM), either dithiothreitol (10, 20, or 50 mM) or 2-mercaptoethanol (20 or 50 mM) was

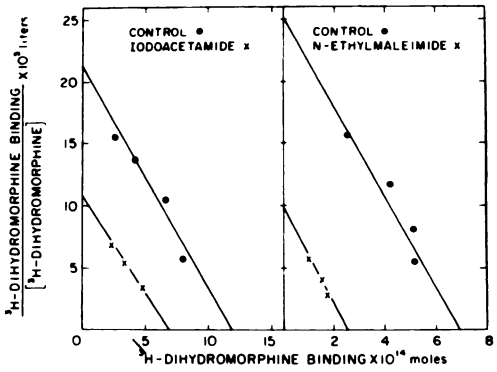

FIG. 3. *Scatchard analysis of [^{3}H]dihydromorphine binding in tissues treated with iodoacetamide and N-ethylmaleimide*

Rat brain homogenate was prepared and allowed to react with 10 mM iodoacetamide, 0.1 mM *N*-ethylmaleimide, or buffer, and assayed in triplicate with varying concentrations of [^{3}H]dihydromorphine as described in MATERIALS AND METHODS. The experiment was repeated three times.

TABLE 5

Reversal of effects of p-aminophenylmercuric acetate by 2-mercaptoethanol and dithiothreitol

Rat brains minus cerebella were homogenized in standard buffer and centrifuged at 49,000 × *g* for 10 min. The pellet was resuspended in standard buffer, incubated for 30 min at 37°, and recentrifuged at 49,000 × *g* for 10 min, and the resulting pellets were resuspended in 100 volumes of standard buffer. The homogenates were incubated with *p*-aminophenylmercuric acetate for 15 min at 25°; then either dithiothreitol or 2-mercaptoethanol was added, and the incubation was continued at 25° for 15 min. The homogenates were then centrifuged at 49,000 × *g* for 15 min, and the pellets were resuspended in the original volume of standard buffer and assayed with either [^{3}H]naloxone (1 nM) or [^{3}H]dihydromorphine (0.6 nM) in the presence and absence of 1 μM levallorphan. Values were determined by comparing specific binding in samples treated with the indicated drugs with specific binding in samples treated with all indicated drugs except *p*-aminophenylmercuric acetate. Values are the means of triplicate determinations. The experiment was replicated three times.

Additions	[^{3}H]Naloxone binding	[^{3}H]Dihydromorphine binding
	% control	
p-Aminophenylmercuric acetate, 30 μM	16	6
+Dithiothreitol, 10 mM	30	20
+Dithiothreitol, 20 mM	78	37
+Dithiothreitol, 50 mM	73	63
+2-Mercaptoethanol, 20 mM	56	55
+2-Mercaptoethanol, 50 mM	73	49
p-Aminophenylmercuric acetate, 100 μM	0	0
+2-Mercaptoethanol, 10 mM	65	51

added, and the incubation was continued for another 15 min before centrifuging the homogenate, resuspending it, and assaying opiate binding in pelleted membranes. In samples not exposed to 2-mercaptoethanol or dithiothreitol, the mercurial reagent virtually abolishes the binding of both [^{3}H]dihydromorphine and [^{3}H]naloxone. The binding of [^{3}H]naloxone is affected because of the high concentrations of *p*-aminophenylmercuric acetate employed, considerably greater than concentrations which differentiate between agonist and antagonist binding. Both dithiothreitol and 2-mercaptoethanol reverse the effects of *p*-aminophenylmercuric acetate, suggesting that this reagent acts on sulfhydryl groups.

Protection from iodoacetamide inhibition by opiates. To determine whether the reagents react at or near the opiate binding site itself, we attempted to protect the receptor by prior incubation with unlabeled naloxone, oxymorphone, morphine, nalorphine, levorphanol, and dextrorphan (Table 6). Samples treated with iodoacetamide alone or with the inactive opiate, dextrorphan, show a 50% reduction in [^{3}H]dihydromorphine binding. In samples to which an opiate was added prior to iodoacetamide, the decrease in receptor bind-

TABLE 6

Protection of [^{3}H]dihydromorphine binding from iodoacetamide inhibition by opiates

Rat brains were homogenized, centrifuged, resuspended, incubated at 37°, centrifuged, and resuspended as described in MATERIALS AND METHODS. The homogenates were then incubated with the appropriate opiate for 15 min at 25°, after which they were allowed to react with 10 mM iodoacetamide for 2 min at 25°. The reaction was terminated by the addition of 20 mM 2-mercaptoethanol. The homogenates were then centrifuged at 49,000 × *g* for 15 min. The pellets were resuspended in the original volume of standard buffer, incubated at 37° for 15 min, and recentrifuged. The incubation and centrifugation were repeated twice. The pellets were resuspended in their original volume and assayed as described in MATERIALS AND METHODS. Values were determined by comparing specific binding of the iodoacetamide-treated samples with the specific binding of unreacted tissue treated with the same opiate. Assays were performed with 0.6 nM [^{3}H]dihydromorphine. Values are the means of triplicate determinations. The experiment was replicated four times.

Addition	Binding
	% control
None	49
Naloxone, 0.2 μM	83
Oxymorphone, 0.2 μM	85
Morphine, 0.2 μM	95
Nalorphine, 0.2 μM	87
Levorphanol, 0.1 μM	93
Dextrorphan, 0.1 μM	50

ing is considerably reduced, suggesting that iodoacetamide acts at or very near the opiate binding site. Because very high concentrations of opiate were used in an attempt to saturate the receptor sites totally, and since the wash procedure in some cases did not remove all the unlabeled opiates, control homogenates also contained the appropriate amounts of opiates.

Interactions of protein-modifying reagents and sodium on receptor binding. Sodium markedly enhances the ability of reagents to differentiate agonist and antagonist binding to the opiate receptor. To explain the role of sodium, we examined the effects of several reagents on [^{3}H]dihydromorphine binding over a wide range of sodium concentrations (Fig. 4). Log probit plots show parallel lines, which demonstrate that the concentration of sodium chloride needed to reduce [^{3}H]dihydromorphine binding 50% is significantly lower in homogenates treated with the reagents. Without the reagent treatment the ED_{50} value for sodium chloride is 40–50 mM. The ED_{50} value for sodium chloride is reduced by 63% for *p*-chloromercuribenzoate, by 80% for iodoacetamide, by 84% for *N*-ethylmaleimide, and by 63% for *p*-aminophenylmercuric aceate. If the reagents act by altering sensitivity to sodium, there should be a correlation between the inhibition of [^{3}H]dihydromophine binding by the reagent and the sensitivity of [^{3}H]-dihydromorphine binding to sodium, measured as the inhibition of [^{3}H]dihydromorphine binding by 100 mM sodium chloride. The greater the inhibition by the reagent, the more sensitive the binding of [^{3}H]dihydromorphine should be to sodium. Using the data from Fig. 2 and Table 4, we attempted to demonstrate this correlation (Fig. 5). The increased sensitivity of the binding of [^{3}H]dihydromorphine to sodium appears to be related to the degree of inhibition of

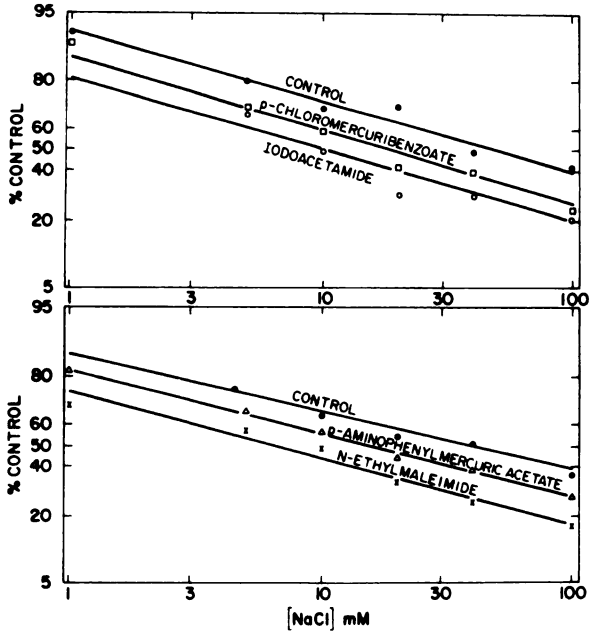

FIG. 4. *Effects of various protein reagents on sodium sensitivity of [^{3}H]dihydromorphine binding*

Rat brain homogenates were prepared and allowed to react with 5 mM iodoacetamide, 10 μM *p*-chloromercuribenzoate, 10 μM *p*-aminophenylmercuric acetate, 50 μM *N*-ethylmaleimide, or buffer as described in MATERIALS AND METHODS. Samples were then assayed with 0.6 nM [^{3}H]hydromorphine in the presence of varying concentrations of sodium. Values represent the percentage of specific binding remaining in the presence of NaCl compared to the specific binding for the same reacted tissue in the absence of NaCl. Values thus indicate the decrease of binding due to the presence of sodium for homogenates reacted with different drugs. All values were determined from the means of triplicate samples. The experiment was repeated twice.

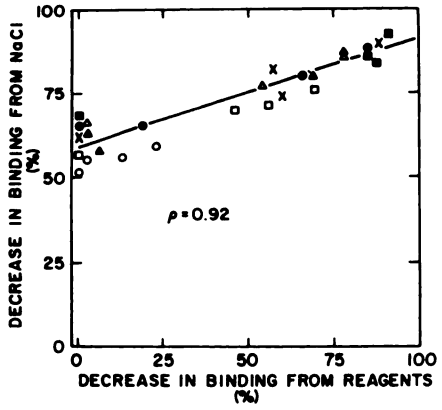

FIG. 5. *Correlation of sodium sensitivity of binding of [³H]dihydromorphine with degree of inhibition of binding of [³H]dihydromorphine by various reagents*

The specific binding of [³H]dihydromorphine remaining after reaction with a variety of reagents at several concentrations was compared with the ability of 100 mM NaCl to decrease the binding of [³H]dihydromorphine in these reacted homogenates. Decrease in binding from reagents is defined as the specific binding of [³H]dihydromorphine in homogenates reacted with reagents and assayed in the presence of 100 mM NaCl divided by the specific binding of [³H]dihydromorphine in unreacted homogenates also assayed with 100 mM NaCl. Decrease in binding from NaCl is defined as the specific binding of reacted homogenates assayed with 100 mM NaCl divided by the specific binding of identically reacted homogenates assayed in the absence of NaCl. ●, *N*-ethylmaleimide; ○, glutathione; ▲, mersalyl acid; △, *p*-chloromercuribenzoate; ■, iodoacetamide; □, *p*-aminophenylmercuric acetate; ×, mercuriacetate.

[³H]dihydromorphine binding, measured in the presence of 100 mM NaCl, in a dose-related manner. When analyzed by least-squares linear regression, a straight line ($y = 0.32$, $x = +59$) can be drawn with a correlation coefficient of 0.92. This indicates that a major action of the reagents is to increase the sensitivity of binding of [³H]dihydromorphine to sodium in a dose-dependent manner.

DISCUSSION

In contrast to previous studies in our laboratory (10, 13, 14, 16, 18–20, 23, 24), experiments in the present investigation employed previously incubated membrane preparations. This treatment enhances receptor binding by dissociating from the receptor an endogenous inhibitor of binding. Recently we have partially characterized this substance (32), which appears to be a naturally occurring ligand of the opiate receptor resembling that reported by Hughes and Kosterlitz (33). In our binding assays the endogenous ligand behaves as an agonist, since its ability to inhibit [³H]naloxone binding is reduced by sodium. Because its effects on [³H]naloxone binding are abolished by carboxypeptidase A and chymotrypsin but not by trypsin, the endogenous ligand described here, like that reported by Hughes and Kosterlitz (33), may be a polypeptide. The regional distribution in rat brain of the endogenous ligand resembles that of the opiate receptor, with the highest concentration in the corpus striatum, the lowest levels in the cerebellum, and intermediate values in other regions (32, 33).

A major finding of this study is that a variety of protein-modifying reagents differentially influence interactions of agonists and antagonists with the opiate receptor. The most effective differentiation is seen with reagents which usually react preferentially with sulfhydryl groups. Similar effects are exerted by a wide selection of sulfhydryl reagents which differ markedly in their structures and chemical mechanism of sulfhydryl inactivation, strongly suggesting the presence of a reactive sulfhydryl group associated with the opiate receptor which is much more important for agonist than antagonist binding. The location of the sulfhydryl group in relation to the binding site is difficult to establish. Protection experiments with high concentrations of opiates suggest that this group may be at or near the binding site. However, it is possible that opiates protect receptor binding by conformational changes induced by binding or by allosteric interactions.

The mechanism by which these sulfhydryl reagents act is still not known. They appear to decrease the number of agonist binding sites. They also increase the sensitivity of [³H]dihydromorphine binding to sodium ion. Although it is not possible to establish a causal relationship between the increase in sodium sensitivity and reagent

actions, it is striking that these effects are closely correlated.

The possibility that sulfhydryl reagents react with other residues cannot be excluded. Reagents used primarily to modify other residues show varying degrees of differentiation between agonist and antagonist binding. Some, like *N*-bromosuccinimide and the carbodiimide, are effective, while others, like the substituted nitrobenzyl bromides and iodine, are not. *N*-Bromosuccinimide reacts most rapidly with tryptophan, followed by tyrosine and histidine, and carbodiimides react primarily with carboxyl groups (26, 27). Thus it appears that other residues, in addition to sulfhydryl groups, are more important for the binding of agonists than antagonists.

The ability of sodium to increase antagonist and decrease agonist binding suggests that the opiate receptor exists in an equilibrium between conformations which preferentially bind agonists or antagonists (20, 24). Sodium shifts the equilibrium to favor the "antagonist" conformation of the receptor. According to this model the selective decrease in agonist binding produced by protein-modifying reagents could be attributed to effects on sites which are involved in the interconversion of receptor conformation. This explanation is consistent with evidence that the reagents act by enhancing sensitivity of the receptor to sodium. It also accords with the ability of reagents that act on different chemical groups to differentially alter agonist and antagonist binding, since one would expect numerous chemical groups to be involved in the transition between conformational states of the receptor. Alternatively, it is conceivable that the receptor contains distinct binding sites for agonists and antagonists.

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