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Opiate Receptor Binding: Enzymatic Treatments That Discriminate between Agonist and Antagonist Interactions

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SUMMARY

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Several enzymatic treatments differentially influence receptor binding of opiate agonists and antagonists. Low concentrations of trypsin (EC 3.4.4.4), chymotrypsin (EC 3.4.4.5), and phospholipase A (EC 3.1.1.4) reduce receptor binding of agonists more than that of antagonists, while phospholipases C (EC 3.1.4.3) and D (EC 3.1.4.4) and neuraminidase (EC 3.2.1.18) have negligible influence on the binding of agonists or antagonists. Binding of the opiate agonist [^{3}H]dihydromorphine is more sensitive to inhibition by enzymatic treatments when assays are conducted in the presence than in the absence of sodium. Moreover, enzymatic treatments markedly reduce the concentrations of sodium required to inhibit [^{3}H]dihydromorphine binding. The extent of reduction of [^{3}H]dihydromorphine binding by enzymatic treatment correlates closely with the sensitivity of [^{3}H]dihydromorphine to sodium. These observations suggest that a major action of enzymatic treatments is to enhance the sensitivity of opiate agonist binding to sodium.

INTRODUCTION

Biochemical identification of opiate receptor binding (1-3) has permitted the characterization of many properties of the pharmacologically relevant opiate receptor, including regional variations in the brain (4, 5), occurrence in specific clones of neuroblastoma cultures (6), phylogenetic variations (7), localization to synaptic membranes (8), and the influence of enzymatic treatment (9). The ability of physiological concentrations of sodium to enhance antagonist binding and reduce agonist binding proportionately (10, 11) has suggested a model to explain the pharmacological actions of agonists and antagonists (11, 12). The opiate receptor is postulated to exist in two interconvertible forms, one in the presence of sodium, which binds antagonists preferentially, and one in the absence of sodium, which has preferential affinity for agonists. Interference with conformational changes of the receptor would be expected to affect agonist and antagonist binding differentially, as has been demonstrated for several protein-modifying reagents (13, 14). In the present study we examined the influence of enzymatic

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treatments on binding of opiate agonists and antagonists.

MATERIALS AND METHODS

Trypsin (205 units/mg), α-chymotrypsin (45 units/mg), neuraminidase (0.5 unit/mg), and phospholipase C (from *Clostridium welchii*, 1.5 units/mg) were purchased from Worthington Biochemical Corporation. Phospholipase A (from *Vipera russelli*, 14 units/mg), phospholipase C (from cabbage, 19 units/mg), and soybean trypsin inhibitor were obtained from Sigma Chemical Company. [^{3}H]Naloxone (23.6 Ci/mmole) and [^{3}H]dihydromorphine (46 Ci/mmole) were purchased from New England Nuclear Corporation, and Hydromix scintillation fluor, from Yorktown. [^{3}H]Levorphanol (5.4 Ci/mmole) and [^{3}H]levallorphan (7.6 Ci/mmole) were tritiated and purified as previously described (10). [^{3}H]Diprenorphine (13 Ci/mmole) was purchased from Amersham/Searle Corporation. Levallorphan was the generous gift of the Roche Company.

Brain homogenates, prepared as previously described (13), were incubated with the appropriate amount of appropriated enzyme for 30 min at 25° and centrifuged at 49,000 × *g* for 20 min. The pellets were then resuspended and assayed with both [^{3}H]naloxone and [^{3}H]dihydromorphine in the presence and absence of 100 mM NaCl, according to a slight modification (13) of described methods (9), and counted at 45% efficiency. Unless otherwise indicated, the concentrations of [^{3}H]naloxone and [^{3}H]dihydromorphine were 1 and 0.8 nM, respectively. Solutions of phospholipase A were immersed in a boiling water bath for 7 min to destroy contaminating proteolytic enzymes (15). When soybean trypsin inhibitor was used, it was added prior to the trypsin at a ratio of 4 parts inhibitor to 1 part trypsin, by weight. All values were determined from triplicate samples assayed in the presence and absence of 1 μM levallorphan, and specific binding was defined as the difference between them. All triplicates varied by less than 10%, and all experiments were replicated at least three times.

RESULTS

The influence of several enzymes was examined upon opiate receptor binding of [^{3}H]naloxone and [^{3}H]dihydromorphine assayed in both the presence and absence os sodium (Table 1; Figs. 1-3). Low concentrations of trypsin markedly decrease [^{3}H]dihydromorphine binding assayed in the presence of sodium, with much less effect on the binding of [^{3}H]naloxone. As little as 0.1 μg/ml of trypsin reduces [^{3}H]dihydromorphine binding in the presence of sodium by 50%, while more than 30 times as much trypsin lowers [^{3}H]naloxone binding only by 25%. This trypsin effect is due to enzymatic activity, because soybean trypsin inhibitor abolishes the degradation of opiate receptor binding. Log probit analysis of trypsin effects on [^{3}H]naloxone and [^{3}H]dihydromorphine binding shows a 6-fold steeper slope for the inactivation of [^{3}H]dihydromorphine than of [^{3}H]naloxone, which might indicate different modes of action on agonist and antagonist binding. The influence of trypsin on [^{3}H]naloxone and [^{3}H]dihydromorphine binding de-

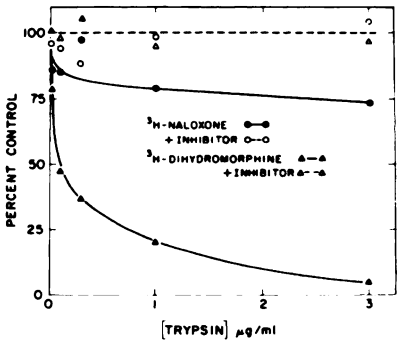

FIG. 1. *Effect of trypsin on binding of [^{3}H]naloxone and [^{3}H]dihydromorphine*

Tissue homogenate was prepared, allowed to react with trypsin, trypsin and soybean inhibitor, or no enzyme, and then assayed with 100 mM NaCl as described in MATERIALS AND METHODS. The specific binding of the treated homogenates was compared with the specific binding of the control, all assayed in the presence of sodium chloride. Trypsinized tissue was assayed with [^{3}H]naloxone (●) and [^{3}H]dihydromorphine (▲). Homogenates trypsinized with soybean inhibitor were also assayed with [^{3}H]naloxone (○) and [^{3}H]dihydromorphine (△). All values are based on the means of triplicate samples and represent specific binding.

pends on sodium. [^{3}H]Dihydromorphine binding is much more sensitive to degradation by trypsin when assays are conducted in the presence than in the absence of sodium. By contrast, [^{3}H]naloxone binding is lowered somewhat more by trypsin when assayed in the absence than in the presence of sodium (Table 1).

Chymotrypsin also clearly differentiates between [^{3}H]naloxone and [^{3}H]dihydromorphine binding (Fig. 2 and Table 1). It is considerably less potent than trypsin, since about 50 times as much chymotrypsin as trypsin is required to reduce [^{3}H]dihydromorphine binding by 50% when assayed in the presence of sodium. When the effects of chymotrypsin digestion assayed in the presence of 100 mM NaCl are analyzed by the log probit technique, the slope of [^{3}H]dihydromorphine inhibition is 1.8 times that of [^{3}H]naloxone inhibition. This suggests that chymotrypsin, like trypsin, inhibits the binding of agonists and antagonists in a different manner. As was observed with trypsin, [^{3}H]dihydromorphine binding is more sensitive to chymotrypsin when the assays are conducted in the presence than in the absence of sodium, while the reverse situation occurs with [^{3}H]naloxone binding.

Phospholipase A is extremely potent in reducing [^{3}H]dihydromorphine binding, lowering it by 85% at only 50 ng/ml (Fig. 3). The influence of phospholipase A cannot be attributed to proteolytic enzymes which often contaminate commercial preparations, because the action on opiate receptor binding requires the presence of calcium ions, is abolished by 1 mM ethylene glycol bis (β-aminoethyl ether)-*N*,*N*'-tetraacetic acid, and is retained after boiling the enzyme preparation. As with trypsin and chymotrypsin, phospholipase A inhibits receptor binding of [^{3}H]dihydromorphine more than that of [^{3}H]naloxone when assayed in the presence of sodium chloride. Moreover, [^{3}H]dihydromorphine binding is reduced more and [^{3}H]naloxone binding reduced less when assays are conducted in the presence rather than in the absence of sodium.

In contrast to the very potent effects of trypsin, chymotrypsin, and phospholipase A, other enzymes, including phospholipases C and D and neuraminidase, have negligible effects on receptor binding of either [^{3}H]naloxone or [^{3}H]dihydromorphine whether assays are conducted in the presence or absence of sodium.

To examine whether the effects of these enzymes are applicable to all agonists and antagonists or are confined to [^{3}H]naloxone and [^{3}H]dihydromorphine, we evaluated the influence of trypsin and chymotrypsin on receptor binding of the additional agonist [^{3}H]levorphanol and the antagonists [^{3}H]levallorphan and [^{3}H]diprenorphine. (Table 2) As was observed with [^{3}H]naloxone and [^{3}H]dihydromorphine, both enzymes reduce the receptor binding of the agonist more than the binding of the antagonist. Moreover, binding of [^{3}H]levorphanol is more sensitive to enzymatic degradation when assays are conducted in the presence than in the absence of sodium, and the reverse situation is true for receptor binding of [^{3}H]levallorphan and [^{3}H]diprenorphine. The effects of enzymatic treatments on the binding of ^{3}H-opiates cannot be due to a direct effect of the enzymes upon the opiates themselves, because tissue preparations were washed free of the enzymes prior to receptor assay with the ^{3}H-opiate. Also, [^{3}H]naloxone and [^{3}H]dihydromorphine, incubated with 1 μg/ml of each enzyme for 30 min at 25°, followed by inactivation of the enzyme, bind to the receptor to the same extent as untreated [^{3}H]naloxone and [^{3}H]dihydromorphine.

Previously we reported that the selective degradation of receptor binding of opiate agonists but not of antagonists by protein-modifying reagents was related to an increase in the sensitivity of agonist binding to inhibition by sodium ion (13, 14). The observation that enzymatic treatments reduced [^{3}H]dihydromorphine binding more when assayed in the presence than in the absence of sodium suggests that these enzymes might also increase the sodium sensitivity of agonist binding. To examine this question directly, we treated rat brain membrane preparations with trypsin (0.1 μg/ml), chymotrypsin (7.5 μg/ml), or no enzyme as described in MATERIALS AND

TABLE 1

Effect of enzymes on binding of [^{3}H]naloxone and [^{3}H]dihydromorphine in the presence and absence of sodium ion

Brain homogenate was prepared and assayed as described in MATERIALS AND METHODS. Values were determined by dividing the specific binding in treated homogenates by the specific binding in control homogenates assayed under identical conditions.

Enzyme	Concentration	Binding			
		Assayed with 100 mM NaCl		Assayed without NaCl	
		[^{3}H]Naloxone	[^{3}H]Dihydromorphine	[^{3}H]Naloxone	[^{3}H]Dihydromorphine
		% control		% control	
Trypsin	30 ng/ml	86	79	104	75
	100 ng/ml	85	48	75	74
	300 ng/ml	98	37	58	52
	1 μg/ml	79	20	65	37
	3 μg/ml	74	5	38	45
Trypsin + soybean inhibitor	30 ng/ml	95	101	96	104
	100 ng/ml	107	98	94	68
	300 ng/ml	88	106	119	92
	1 μg/ml	99	95	121	96
	3 μg/ml	105	97	114	100
Chymotrypsin	1 μg/ml	88	79	86	90
	5 μg/ml	79	53	61	63
	10 μg/ml	71	45	62	54
	25 μg/ml	71	45	52	34
	50 μg/ml	61	15	54	21
Phospholipase A + 5 mM $CaCl_2$	10 ng/ml	97	77	77	85
	50 ng/ml	50	15	19	23
	100 ng/ml	41	0	8	6
	200 ng/ml	8	0	5	0
Phospholipase A + 1 mM EGTA[a]	10 ng/ml	94	89	98	105
	25 ng/ml	110	112	104	106
	100 ng/ml	93	73	85	93
Phospholipase D	10 μg/ml	88	84	91	85
	50 μg/ml	83	78	99	89
	200 μg/ml	90	83	97	84
	400 μg/ml	84	89	84	78
Phospholipase C	10 μg/ml	83	86	92	92
	25 μg/ml	93	65	86	105
	75 μg/ml	83	85	87	84
	100 μg/ml	80	95	82	106
	150 μg/ml	94	95	75	91
	250 μg/ml	102	93	98	92
Neuraminidase	5 μg/ml	91	100	85	91
	10 μg/ml	90	109	94	103
	20 μg/ml	95	108	94	92
	50 μg/ml	98	94	87	100

[a] EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

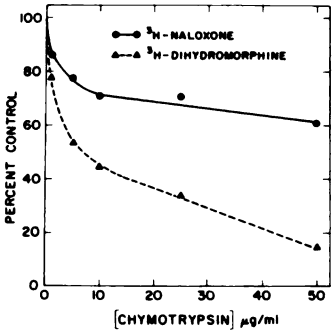

FIG. 2. *Effect of chymotrypsin on binding of [³H]naloxone and [³H]dihydromorphine*

Tissue homogenate was prepared, allowed to react with chymotrypsin or no enzyme, and then assayed with 100 mM NaCl as described in MATERIALS AND METHODS. The specific binding of the treated homogenates was then compared with the specific binding of the control, both assayed in the presence of sodium chloride. The homogenates were assayed with both [³H]naloxone (●) and [³H]dihydromorphine (▲). All values are based on the means of triplicate samples and represent specific binding.

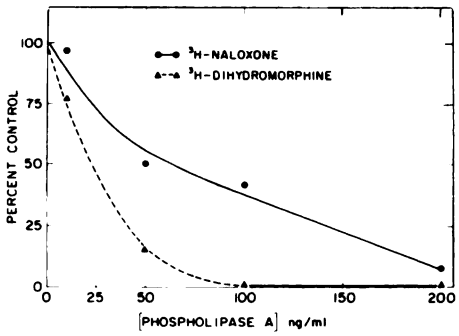

FIG. 3. *Effect of phospholipase A on binding of [³H]naloxone and [³H]dihydromorphine*

Tissue homogenate was prepared, allowed to react with boiled phospholipase A and 5 mM $CaCl_2$ or no enzyme, and then assayed with 100 mM NaCl as described in MATERIALS AND METHODS. The specific binding of the treated homogenates was then compared with the specific binding of the control, all assayed in the presence of sodium chloride. The homogenates were assayed with both [³H]naloxone (●) and [³H]dihydromorphine (▲) in the presence of 100 mM NaCl. All values are based on the means of triplicate samples and represent only specific binding.

METHODS and measured [³H]dihydromorphine binding in the absence of sodium and in the presence of 1, 10, 20, 50, 75, and 100 mM sodium. When assayed in the presence of 100 mM NaCl, trypsin and chymotrypsin reduce [³H]dihydromorphine binding by 52% and 40%, respectively. The concentration of NaCl for 50% inhibition of [³H]dihydromorphine binding without enzymatic treatment is 40 mM, whereas after trypsin treatment the ID_{50} is reduced to 3 mM, and after chymotrypsin digestion, to 14 mM. Thus both trypsin and chymotrypsin increase the sensitivity of [³H]dihydromorphine binding to sodium.

If the enzymatic treatments act by altering sensitivity to sodium, one might expect a close correlation between the reduction in [³H]dihydromorphine binding by enzymatic treatment and the sensitivity of the remaining [³H]dihydromorphine binding to sodium. Greater inhibition of binding by enzymatic treatment should be associated with greater sensitivity of agonist binding to sodium. Previously we demonstrated such a correlation for the influence of protein-modifying reagents upon [³H]dihydromorphine receptor binding (13). Using the experimental results which contributed to the data of Table 1, we have

TABLE 2

Effect of trypsin and chymotrypsin on opiate receptor binding of ³H-agonists and ³H-antagonists

Rat brain homogenates were prepared, allowed to react with 1 µg/ml of chymotrypsin or 1 µg/ml of trypsin, and assayed with the appropriate ³H-opiate in the presence of 100 mM NaCl, as described in MATERIALS AND METHODS. Values are expressed as percentage of control, calculated by dividing the specific binding in the enzyme-treated homogenates by the specific binding in the unreacted homogenates. The following amounts of ³H-opiate per sample were used: [³H]naloxone, 90,000 cpm; [³H]diprenorphine, 4500 cpm; [³H]levallorphan, 26,000 cpm; [³H]dihydromorphine, 76,000 cpm; [³H]levorphanol, 26,000 cpm.

³H-Opiate	Binding	
	Trypsin	Chymotrypsin
	% control	
Antagonists		
Naloxone	71	95
Diprenorphine	81	90
Levallorphan	76	93
Agonists		
Dihydromorphine	16	58
Levorphanol	15	69

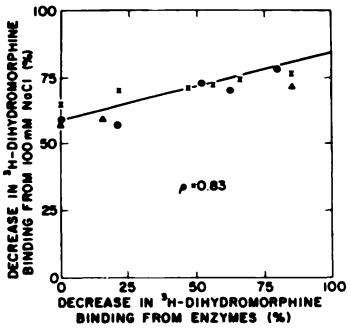

FIG. 4. *Correlation of sodium sensitivity of binding of [^{3}H]dihydromorphine with degree of inhibition of binding of [^{3}H]dihydromorphine by enzymes*

The specific binding of [^{3}H]dihydromorphine remaining after reaction with the enzymes at several concentrations was compared with the ability of 100 mM NaCl to decrease the binding of [^{3}H]dihydromorphine in these reacted homogenates. Decrease in binding from enzymes is defined as the specific binding of [^{3}H]dihydromorphine in homogenates after reaction with enzymes and assayed in the presence of 100 mM NaCl, divided by the specific binding of [^{3}H]dihydromorphine binding 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 treated homogenates assayed in the absence of NaCl. ×, chymotrypsin; O, trypsin; Δ, phospholipase A. The zero-order correlation coefficient (ρ) was calculated on an Olivetti Programma 101 computer and was significant ($p < 0.01$).

demonstrated such a correlation (Fig. 4). In these experiments the decrease of [^{3}H]dihydromorphine binding due to sodium is compared with the decrease to the enzymes. This correlation further supports the concept that one effect of the enzymatic treatments is to enhance the sensitivity of [^{3}H]dihydromorphine binding to sodium.

DISCUSSION

These studies show that opiate agonist binding is more sensitive to the actions of some enzymes than antagonist binding, in a manner resembling the effects of protein-modifying reagents (12, 13). While this differential sensitivity might suggest totally different receptors for agonists and antagonists, agonists and antagonists do show competitive inhibition, which implies an interdependent manner of binding.

The influence of enzymatic treatments on agonist and antagonist binding to the opiate receptor is consistent with a model for opiate receptor binding previously postulated on the basis of differential influences of sodium (10, 11) and protein-modifying reagents (13, 14) on agonist-antagonist binding. According to this allosteric model (12), the opiate receptor can exist in interconvertible "sodium-antagonist" and "non-sodium-agonist" forms. Pharmacological effects of agonists, such as analgesia, occur only when drugs bind to the agonist form of the receptor, and antagonists act by maintaining the receptor in the antagonist form, thus decreasing the number of available agonist-binding forms. Antagonists are postulated to have a high affinity for the antagonist conformation and low affinity for the agonist conformation of the receptor, while the reverse situation occurs for opiate agonists. Mixed agonist-antagonists bind significantly to both forms of the receptor. Protein-modifying reagents and enzymatic treatments are postulated to modify the interconversion of the two forms of the receptor, increasing the ability of sodium to convert the receptor to the antagonist form and thus decreasing the binding of opiate agonists selectively.

This model might also help to explain the greater sensitivity of [^{3}H]naloxone binding to trypsin and chymotrypsin in a previous report (9) than in our present study. [^{3}H]Dihydromorphine binding in the present investigation has about the same sensitivity to trypsin and chymotrypsin as [^{3}H]naloxone binding in the earlier study. In the previous report (9) the low specific activity of [^{3}H]naloxone required us to use relatively high concentrations (about 15 nM), which label both high- and low-affinity binding sites (12). In contrast, the present study involved low concentrations of [^{3}H]naloxone and [^{3}H]dihydromorphine (about 0.8 nM), which bind predominantly to the high-affinity sites (12). The apparent differences between the present and previous studies may be explained if the high-affinity binding site for [^{3}H]dihy-

dromorphine in this study is the same as the low-affinity [^{3}H]naloxone binding site examined in the previous study. Other evidence (12) suggests that the high-affinity binding site for opiate antagonists is equivalent to the low-affinity site for agonists.

An alternative model also consistent with our data involves two allosterically coupled binding sites (one for agonists and one for antagonists) (16). If binding to one site prevents binding to the other by inducing a conformational change, inhibition of agonist and antagonist binding would resemble classical competitive antagonism.

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