

## Kinetics of Opiate Receptor Inactivation by Sulfhydryl Reagents: Evidence for Conformational Change in Presence of Sodium Ions

(naltrexone/etorphine/stereospecific binding/antagonist)

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**ABSTRACT** The role of SH groups in opiate-receptor interactions has been further examined. Inactivation by *N*-ethylmaleimide of stereospecific opiate binding by rat brain membrane fractions follows pseudo-first order kinetics and exhibits strong temperature dependence. The kinetics indicate that alkylation of a single SH group suffices to block opiate binding. Considerable protection from SH group inactivation is observed when treatment with *N*-ethylmaleimide is carried out in the presence of an opiate or an antagonist, suggesting close proximity of the SH group to the opiate binding site. The rate of inactivation of receptor binding by *N*-ethylmaleimide is markedly slower in buffers containing 100 mM NaCl ( $t_{1/2} = 30 \pm 1.4$  min) than in sodium-free buffers ( $t_{1/2} = 10 \pm 1.0$  min). Since the rate of alkylation of model SH compounds is unaffected by sodium ions, this protection seems best explained by a conformational change in the receptors that renders the SH groups less accessible to alkylation. The rate of inactivation is not affected by  $K^+$ ,  $Rb^+$ , or  $Cs^+$  and only slightly by  $Li^+$ . This cation specificity as well as the concentration-response to  $Na^+$  are remarkably similar to those previously shown to lead to increased antagonist and decreased agonist binding. We suggest that the same conformational change is involved in the two phenomena.

The existence of stereospecific binding sites for opiates and opiate antagonists in the brain of animals was discovered independently in three laboratories, including our own (1-3), by a modification of an assay first suggested by Goldstein *et al.* (4). Confirmation in many laboratories followed and much evidence has accumulated suggesting that these sites represent pharmacological receptors. Opiate receptors have also been observed in human brain obtained at autopsy (5).

Because we noted (3) that addition of salt decreases binding of the potent agonist etorphine, and Pert and Snyder (2) found no effect of salt on the binding of the antagonist naloxone, we suggested that the differential effect of salt may reflect a general difference in the manner in which agonists and antagonists bind to the receptor (3). This generalization was confirmed by the studies of Pert *et al.* (6), who further showed that discrimination between agonists and antagonists was a specific property of sodium salts, the binding of antagonists being stimulated in the presence of sodium ions. Our recent studies on the mechanism of the sodium effect (7) suggest that sodium acts as an allosteric effector causing a conformational change in the receptor molecule. The resulting "sodium-dependent" form has a higher affinity for antagonists and a lower affinity for agonists than the "sodium-free" form, but the total number of receptor sites does not change.

The inactivation of stereospecific etorphine binding in rat brain homogenate by several sulfhydryl reagents was re-

ported earlier by us (3). We now present kinetic studies of the inactivation of receptor binding by the SH-alkylating agent, *N*-ethylmaleimide (NEM). These experiments were stimulated by the elegant studies of Kennedy and collaborators (8) on the M protein of the *lac* operon of *Escherichia coli*. Our results provide evidence that an SH group essential for binding can be protected from inactivation by opiates. Independent evidence for the conformational change in receptor sites caused by sodium ions is also provided.

### MATERIALS AND METHODS

**Materials.** [ $15,16^3H$ ]Naltrexone (specific activity = 15.3 Ci/mmol) and [ $^3H$ ]etorphine (specific activity = 20.7 Ci/mmol) were generously supplied by Dr. Robert Willette, National Institute on Drug Abuse, Rockville Center, Md., levorphanol tartrate and dextrorphan tartrate by Dr. W. E. Scott, Hoffman-LaRoche, Inc. Tris-(2-carboxyethyl)-phosphine·HCl was obtained from Dr. Donald Kirschenbaum, Dept. of Biochemistry, Downstate Medical Center, to whom it was made available through the courtesy of Dr. Martin Grayson, American Cyanamid Co.

NEM was purchased from General Biochemicals; glutathione, *p*-hydroxymercuribenzoate, and iodoacetamide from Calbiochem; dithiothreitol from Sigma Chemical Co.; and tritium-labeled NEM from New England Nuclear Corp.

**Methods.** The crude mitochondrial-synaptosomal fraction ( $P_2$ ) was prepared as described previously (7) from brains of Sprague-Dawley rats after removal of the cerebellum. The  $P_2$  fraction was hypotonically lysed by 10-fold dilution from 0.32 M sucrose into 0.05 M Tris·HCl buffer, pH 7.4. Stereospecific binding of tritiated naltrexone or etorphine was carried out as described earlier (7).

For inactivation studies, 2 ml of  $P_2$  (1:60, i.e.,  $P_2$  from 1 g wet weight of brain in 60 ml) were incubated in 0.05 M Tris, pH 7.4, with NEM and appropriate additions. Concentrations of salts and reagents, temperature and length of incubation are indicated in legends to table and figures. In all kinetic experiments the reaction was stopped by addition of an excess of a reagent that rapidly inactivates excess NEM. We used dithiothreitol or odorless tris-(2-carboxyethyl)-phosphine, which reduces disulfide bonds efficiently (9) and which reacts rapidly with NEM, as indicated by the disappearance of its light absorption at 302 nm.

Protection against the effect of SH-reagents by drugs was studied by preincubating membranes with an appropriate concentration of a drug for 10 min followed by exposure to NEM (0.5 mM) for 20 min. Unbound NEM and drug were removed by sedimentation and washing at  $20,000 \times g$ .

Abbreviation: NEM, *N*-ethylmaleimide.

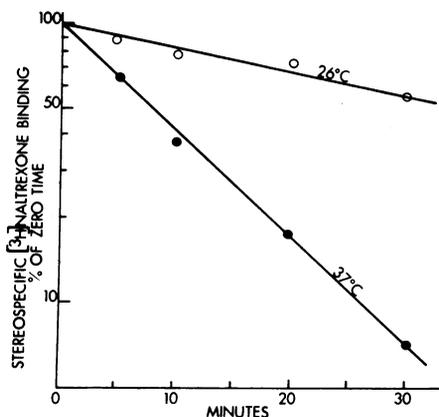


FIG. 1. Effect of temperature on the rate of inactivation of stereospecific binding of [ $^3\text{H}$ ]naltrexone by rat brain  $\text{P}_2$  fraction. Rat brain  $\text{P}_2$  fraction was preincubated with 0.5 mM NEM for various intervals at 26° and 37°. The reaction was stopped by addition of tris-(2-carboxyethyl)-phosphine (2.5 mM). Stereospecific binding of [ $^3\text{H}$ ]naltrexone (specific activity 15.3 Ci/mmol, concentration 1 nM) was assayed. Results are expressed as percent of activity remaining as compared to a sample treated with NEM and phosphine immediately (zero time).

Residual stereospecific binding of [ $^3\text{H}$ ]naltrexone was then assayed. In kinetic studies the concentration of protecting drug used was 3 nM.

Protein was estimated by the method of Lowry *et al.* (10). A  $\text{P}_2$  membrane fraction derived from a 1:60 homogenate had 0.9–1.2 mg of protein per ml.

## RESULTS

**Kinetics of Inactivation and Protection by Opiates.** When rat brain  $\text{P}_2$  membranes are incubated with NEM and the reaction is stopped by destruction of excess NEM, there is a progressive decrease in their capacity to bind [ $^3\text{H}$ ]naltrexone stereospecifically. As shown in Fig. 1, the rate of inactivation follows pseudo-first order kinetics, as would be expected at high NEM concentration (NEM concentration essentially constant) if reaction occurs with a single SH group on the receptor. It also exhibits a strong dependence on temperature, the half-time of inactivation being increased from 9 min to 35 min when the temperature is decreased from 37° to 26°. Inactivation at 0° is exceedingly slow. When the binding of the potent narcotic analgesic [ $^3\text{H}$ ]etorphine is assayed, both the rate and extent of inactivation by NEM are the same as for naltrexone. Similar first-order kinetics have also been obtained for inactivation of binding by *p*-hydroxymercuribenzoate and iodoacetamide.

When preincubation with NEM is performed in the presence of an opiate or opiate antagonist there is considerable protection. Fig. 2 shows the rate of inactivation by NEM in the presence and absence of 3 nM naltrexone. The half-time of inactivation is increased from 9 to 25 min. Similar protection has been observed with levorphanol. Larger concentrations of drug can be used if they are subsequently removed by careful washing before binding assays are performed, although protection was generally not complete even with levels of opiate up to 0.1 or 1  $\mu\text{M}$ .

**Specific Protection from NEM Inactivation by Sodium Ions.** When preincubation of  $\text{P}_2$  membranes with NEM is carried

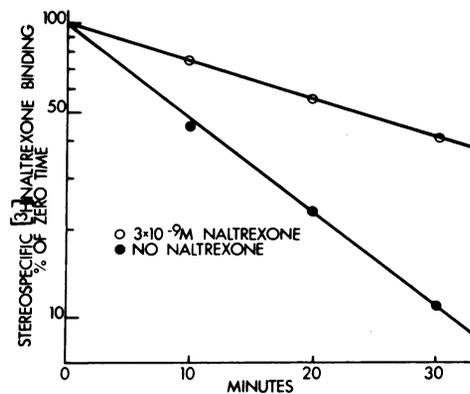


FIG. 2. Kinetics of inactivation of stereospecific [ $^3\text{H}$ ]naltrexone binding by NEM and protection by pretreatment with naltrexone. Inactivation was carried out as in legend to Fig. 1 at 37°.  $\text{P}_2$  fraction was preincubated for 5 min with or without unlabeled naltrexone (3 nM) before addition of NEM. For the binding assay [ $^3\text{H}$ ]naltrexone was added to final concentration of 2 nM (total concentration of naltrexone 5 nM).

out in 100 mM NaCl, the rate of inactivation is markedly decreased (Fig. 3). In six experiments the average half-time of inactivation was  $10 \pm 1.0$  min in the absence of NaCl and  $30 \pm 1.4$  min in its presence. This is not due to retardation of alkylation of SH groups, since we found spectrophotometrically that reaction of NEM with model SH-compounds, such as glutathione, occurs equally rapidly in the presence and absence of 100 mM NaCl. Other investigators have carried out such reactions routinely in 100 mM sodium acetate or sodium phosphate buffer (11). We postulate that the effect of sodium is the result of a conformational change in the receptors which renders SH groups less accessible to alkylation by NEM.

Our evidence suggests, further, that the alteration reflected in the protection of SH groups by  $\text{Na}^+$  may be identical to that which results in enhanced antagonist (and decreased agonist) binding. Table 1 shows the effect of different alkali metal salts on the rate of inactivation. In addition to  $\text{Na}^+$  only  $\text{Li}^+$  has a slight protective effect, whereas  $\text{K}^+$ ,  $\text{Rb}^+$ , and  $\text{Cs}^+$  are totally inactive. This cation specificity is the same as that previously demonstrated for enhancement of antagonist binding (6).

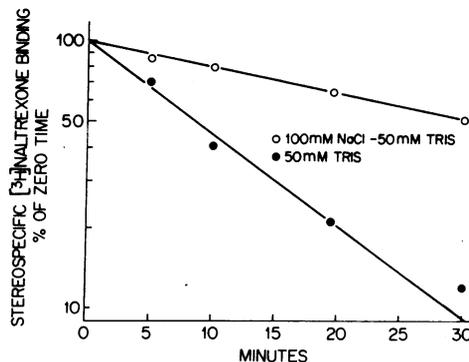


FIG. 3. Kinetics of inactivation of stereospecific binding by NEM in the presence and absence of NaCl. Preincubation with NEM was carried out in the presence and absence of 100 mM NaCl. Reaction was stopped and assay for stereospecific naltrexone binding was done as described in legend to Fig. 1.

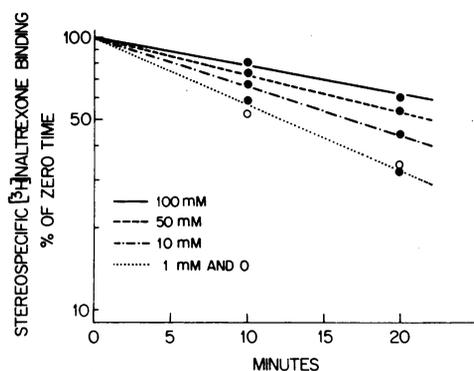


FIG. 4. Kinetics of inactivation of stereospecific binding of [ $^3\text{H}$ ]naltrexone by NEM in the presence of various concentrations of NaCl. Incubations with NEM were carried out in the concentrations of NaCl shown.

Fig. 4 shows the protective effects of various concentrations of sodium. Protection increases with concentrations of NaCl from essentially none at 1 mM to maximal protection at 100 mM, remaining the same or declining slightly at 200 mM NaCl. This concentration-response is identical to that which we previously reported for enhancement of antagonist binding at different NaCl concentrations (7).

Protection by sodium has also been observed when stereospecific binding is assayed with the potent agonist [ $^3\text{H}$ ]etorphine. Inactivation by iodoacetamide is similarly retarded by the presence of sodium salts.

Saturation experiments with and without  $\text{Na}^+$  were carried out with  $\text{P}_2$  membranes in which binding capacity had been reduced 80% by treatment with NEM. The residual binding sites were found to be identical to those in untreated membranes with respect to the affinity of naltrexone in the absence ( $K_D = 1.1 \text{ nM}$ ) and presence ( $K_D = 0.5 \text{ nM}$ ) of NaCl, indicating that the uninhibited binding sites retain their original properties.

**Incorporation of [ $^3\text{H}$ ]NEM.** To determine whether or not NaCl produces a change in the rate of alkylation of all or most of the SH groups present in  $\text{P}_2$  membranes of rat brain, we studied the rate of incorporation of [ $^3\text{H}$ ]NEM into the membranes (Fig. 5). The rates and extents of incorporation are identical in KCl and NaCl. The plateau of incorporation

TABLE 1. Effect of alkali metal cations on inactivation of receptor binding capacity by NEM

Time of preincubation (min)	Binding of [ $^3\text{H}$ ]naltrexone (% of zero time)					
	Tris only	KCl	RbCl	CsCl	LiCl	NaCl
5	73	72	75	—	81	85
10	45	51	51	50	65	78
20	22	24	27	24	42	65
30	12	11	17	12	32	48

All preincubations were carried out in the presence of 0.5 mM NEM in 50 mM Tris with the appropriate salt added to final concentration of 100 mM. The reaction was stopped after the appropriate interval and stereospecific binding of [ $^3\text{H}$ ]naltrexone was measured. Results are expressed as percent of zero time preincubation in the appropriate salt. All results are means of three closely similar experiments.

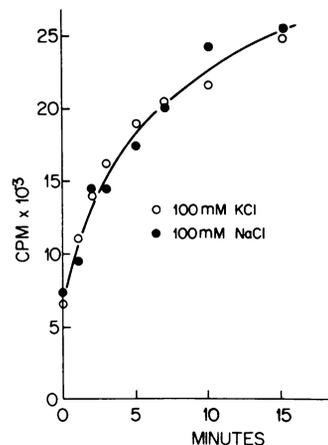


FIG. 5. Rate of incorporation of [ $^3\text{H}$ ]NEM into rat brain  $\text{P}_2$  fraction. Incubation was carried out in the presence of [ $^3\text{H}$ ]NEM (0.5 mM, specific activity 1 Ci/mol) for the intervals indicated. Incorporation was assayed by the same filtration and washing procedure used for the opiate binding assay.

also allows a rough calculation of the number of SH groups, namely about 20 nmol/mg of brain protein or about 100,000 other SH groups for each SH group on an opiate receptor (assuming one SH group per receptor).

## DISCUSSION

Stereospecific binding of narcotic analgesics and their antagonists is effectively inhibited by sulfhydryl reagents (3). The fact that several of these reagents act by quite different mechanisms (alkylation, oxidation, or complexing of mercury) indicates that the reduction in binding is brought about by inactivation of an essential SH group. The presence of an opiate or antagonist in the reaction mixture protects against this inactivation, suggesting that the SH group is located near the opiate binding site of the receptor. Alternatively, the possibility cannot be ruled out that binding of an opiate alters the conformation of the receptor, rendering an SH group located at some distance from the binding site less accessible to alkylation. Inactivation of a single SH group suffices to block binding as indicated by the pseudo-first order kinetics of the inactivation.

Saturation studies on the binding activity remaining after treatment of membranes with NEM have yielded no evidence for active binding sites with altered properties. Our findings, therefore, indicate that alkylation of an essential SH group results in the inactivation of that binding site. Those sites that retain their binding activity are indistinguishable from sites in untreated membranes with respect to their affinity for naltrexone as well as their ability to undergo conformational transformation when exposed to sodium ions, presumably because their SH groups have escaped alkylation.

The present results also provide independent evidence for a conformational transformation of receptors when membranes are transferred from a sodium-free to a sodium-containing buffer (or *vice versa*). This model was previously suggested by us (7, 12) on the basis of the sodium-induced alteration in opposite directions of binding affinities of agonists and antagonists. Since the rate of reaction between NEM and mdoel SH compounds is unaffected by the presence of NaCl, the marked slowing down in the rate of receptor inactivation by NEM is most simply explained by a conformational change in

the receptor caused by NaCl, which renders a vital SH group less accessible to inactivation. The hypothesis that a conformational change occurs and that it may be the same as that previously postulated for changes in binding affinities is supported by the finding that the protection from inactivation by NEM exhibits the same cation specificity and concentration-response relationship to NaCl as is observed for enhancement of antagonist binding.

The observation that the rate of incorporation of [<sup>3</sup>H]NEM into P<sub>2</sub> membranes is identical in KCl and NaCl provides evidence that the change that occurs in the presence of sodium does not affect all or most of the SH groups present in the membranes. Since there are, however, approximately 10<sup>5</sup> SH groups for every one that is involved in opiate binding, it is clearly not possible to conclude that sodium affects only receptor SH groups. One can conclude that decreased accessibility is exhibited by only a fraction of total membrane SH groups, and it is attractive to speculate that the change may occur only in proteins whose structures are appropriately altered by bound sodium ions.

Evidence accumulated to date is consistent with competition of opiate agonists and antagonists for the same binding site. This hypothesis is strengthened by the present results which show protection from inactivation of SH groups by both agonists and antagonists. Moreover, protection of receptor

sites by sodium ions is observed regardless of whether an antagonist (naltrexone) or an agonist (etorphine) is used in the binding assay.

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