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Two Different Modifications of the Neuroblastoma × Glioma Hybrid Opiate Receptors Induced by *N*-Ethylmaleimide

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SUMMARY

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Modification of membrane groups by *N*-ethylmaleimide (NEM) differentially inactivates the binding of the [^{3}H]-labeled opioid peptide agonist [D-Ala^2-Met^5]-enkephalinamide (Dala^{2}met^{5}amide) and the opiate antagonists [^{3}H]naltrexone and [^{3}H]naloxone to NG108-15 opiate receptors. There appear to be at least two different NEM-sensitive groups involved. Alteration of one group by NEM prevents equally the binding of opiate agonists and antagonists and is seen as a reduction in the number of opiate binding sites. Furthermore, this process is first-order, and alteration of one of these groups is therefore sufficient to inactivate the receptor completely. The second group affected by NEM is at least 4-fold more sensitive to NEM, and in its altered state causes a selective reduction in the affinity of opiate agonists for these receptors. The affinity of at least one of the two antagonists tested (i.e., naloxone) actually is increased by 50% after modification of this group. With the opioid agonist Dala^{2}met^{5}amide these losses in affinity have been confirmed by both ^{3}H-labeled peptide saturation studies and by studying the peptide competition against the [^{3}H]naloxone binding. Both analyses indicate a 7-fold loss in affinity accompanying the modification of this group by NEM. All of the opiate agonists, including the enkephalins, β-endorphin, oxymorphone, normorphine, and etorphine, exhibit losses in affinity. The magnitude of these losses is not constant, but a property of the particular agonist, and is found with even the mixed agonist-antagonist pentazocine. Therefore these losses are proposed to be a general property of opiate agonists in this system; pure opiate antagonists along with the mixed antagonist-agonist cyclazocine do not show this loss. Modification of this second site is accompanied by (a) an increased sensitivity to the selective effects of cations (rank order $Na^+ > Li^+ > K^+$ = choline) to decrease preferentially the agonist affinity and (b) a loss in sensitivity of the affinity of Dala^{2}met^{5}amide to GTP. The second NEM-sensitive site is therefore distal to the ligand binding domain and is proposed to be involved selectively in the formation of high-affinity agonist-receptor complexes.

INTRODUCTION

Opiate agonists and antagonists interact with a single population of stereospecific receptors in neuroblastoma × glioma hybrid NG108-15 cells (1, 2). Whereas opiate agonists inhibit the activity of the receptor coupled adenylate cyclase, opiate antagonists have no observable effect on adenylate cyclase activity but do block the action of opiate agonists (3). The coupling of these receptors with the catalytic moiety of adenylate cyclase is regulated by guanine nucleotides and divalent and monovalent cations (4–6). These three types of regulators modify the binding of ligands to the opiate receptor and can distinguish between the binding of an opiate agonist and that of an opiate antagonist (2, 6, 7).

To gain information concerning the site of action of guanine nucleotides and monovalent and divalent cations, as well as to obtain an insight into the mechanism by which opiates regulate adenylate cyclase activity, we have examined the effects of a sulfhydryl-modifying reagent on ligand binding to these receptors. The involvement of sulfhydryl groups in maintenance of structure and function has been observed for many membrane-bound systems, including acetylcholine (8), insulin (9), and *beta*-adrenergic (10, 11) receptors. The interaction of opiate ligands with their receptors in rat brain membranes has also been proposed to be regulated by sulfhydryl groups (12, 13). As postulated, there is a sulfhydryl site closely associated with the opiate receptor binding site (12). It is possible that another one exists which is involved specifically in the formation of a high-affinity

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opiate agonist-receptor complex (13, 14). In the *beta*-adrenergic receptor-coupled adenylate cyclase system, the independent guanine nucleotide-sensitive membrane component (i.e., coupler) responsible for transferring information from the receptor to the catalytic moiety of cyclase is required for formation of a high affinity agonist: receptor complex and is modified by sulfhydryl reagents such as NEM[1] (15–17).

We report here that NEM differentially influences the binding of agonist and antagonist ligands to the opiate receptors of NG108-15. The data are discussed in terms of various components needed for high-affinity agonist binding and those thought to be required for opiate regulation of adenylate cyclase.

MATERIALS AND METHODS

Cells. Mouse neuroblastoma × rat glioma hybrid clone NG108-15 (1) were used and grown as described previously (7) with the following modifications: the cells were passaged by 1:12 splits, allowed to grow to confluence (~6 days), and harvested 1 day after becoming confluent. Cells for use in experiments were harvested without trypsin (by shaking), and the cell pellets were washed once with serum-free media and twice with choline buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/HCl buffer, pH 7.4, 5.5 mM glucose, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 130 mM choline chloride). Washed cell pellets from 10 flasks were combined and stored frozen in liquid nitrogen until use.

Membrane preparation. Frozen pellets were thawed at room temperature and then suspended in 15 ml of sucrose buffer (0.32 M sucrose, 1 mM $MgCl_2$, 5 mM Tris/HCl buffer, pH 7.4). The cells were then homogenized (Teflon-glass) with cell breakage being monitored by phase-contrast microscopy. Upon release of ≥90% of intact cell nuclei, the homogenate was centrifuged (800 × *g* for 5 min at 4°) to eliminate nuclei and intact cells, and the resulting supernatant was centrifuged (39,000 × *g* for 30 min at 4°). The final pellet was suspended in 50 mM Tris/HCl buffer so as to give a protein concentration of 3–5 mg/ml. Protein was measured by the method of Lowry *et al.* (18).

Binding assays. Membranes (100–400 mg of protein per assay) were incubated in a final volume of 0.1 ml in 50 mM Tris/HCl buffer (pH 7.4) 1 mM EDTA, and 0.2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid for 20 min at 32° with the following additions: one of two radiolabeled opiate antagonists (i.e., [^{3}H]naltrexone or [^{3}H]naloxone) or the ^{3}H-labeled, stable opioid peptide agonist Dala^{2}met^{5}amide; up to eight different concentrations of a nonradioactive opiate agonist or antagonist; and monovalent cations and GTP. Incubations were terminated by the addition of 4 ml of cold 50 mM Tris/HCl buffer and filtering the sample over Whatman GF/B glass fibers (under vacuum) and washing with an additional 8 ml of cold buffer. All ^{3}H-labeled ligand binding described refers to specific ligand binding (the amount that is prevented by 10^{-5} M etorphine HCl). The specific binding for all three ^{3}H-labeled ligands is 85–95% of the total binding. For certain competition binding experiments, as noted, the data were analyzed by the nonlinear least-squares curve-fitting program described by Hancock *et al.* (19). Control membranes contained 10 mM DTT, which was added prior to any NEM additions. No differences in ^{3}H-labeled agonist or ^{3}H-labeled antagonist binding was observed due to the presence of 10 mM DTT.

Materials. [^{3}H]Naltrexone (25 Ci/mmole) was a gift from the National Institute on Drug Abuse, Bethesda, Md; [^{3}H]naloxone (50 Ci/mmole) and [^{3}H]Dala^{2}met^5-amide (30 Ci/mmole) were obtained from New England Nuclear Corporation, Boston, Mass., and [^{3}H]etorphine (42 Ci/mmole) from Amersham Corporation, Arlington Heights, Ill. Naltrexone HCl and naloxone HCl were gifts from Endo Pharmaceuticals, Garden City, N. Y.; cyclazocine and pentazocine were gifts from Sterling-Winthrop Research Institute, Rensselaer, N. Y. Dala^{2}met^{5}amide and Dala^{2}Dleu^{5}enkephalin were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and NEM, DTT, and GTP were purchased from Sigma Chemical Company, St. Louis, Mo. Etorphine HCl, dihydromorphine, and normorphine were provided by Hoffmann-La Roche Inc., Nutley, N. J., and the human β-endorphin was kindly provided by J. Meienhofer, Peptide Synthesis Division, Hoffmann-La Roche, Inc. All other chemicals were of highest available purity.

RESULTS

Treatment of NG108-15 membranes with NEM reduced the specific binding of nonsaturating concentrations of the opioid peptide agonist [^{3}H]Dala^2-met^{5}amide and the opiate antagonist [^{3}H]naltrexone (Fig. 1). The decreases in binding of both of these ligands that were induced by NEM were dependent upon the time of treatment with NEM (Fig. 1A) as well as on the concentration of NEM (Fig. 1B). Of major importance was the finding that the binding of the peptide agonist appeared more sensitive than the binding of the antagonist naltrexone to NEM treatment. With a 2 mM concentration of NEM, at 32° [^{3}H]naltrexone binding decreased with time monotonically and with a pseudo-first order rate constant of 0.021 min^{-1} (i.e., a $t_{1/2}$ = 34 min). In contrast, the binding of the ^{3}H-labeled peptide agonist had decreased to ~20% of its initial value after only 20 min of such NEM treatment. It then declined further more slowly over the next 60 min. The more rapid disappearance in peptide binding had a pseudo-first order rate constant of ≥0.1 min^{-1} (i.e., a $t_{1/2} \leq 8$ min). Its slower rate of disappearance was not significantly different from that noted above for the disappearance of ^{3}H-labeled antagonist binding. In addition, when the incubation time with NEM was kept constant, the concentration of NEM which reduced binding of the ^{3}H-labeled agonist peptide by 50% (i.e., 0.4 mM) was 2.5 times lower than that needed to reduce the binding of the ^{3}H-labeled antagonist by 50% (i.e., 1 mM). We also monitored the loss in binding of the opiate agonist [^{3}H]etorphine (at 2.6 nM) and the opiate antagonist [^{3}H]naloxone (at 6.4 nM) during incubation of NG108-15 membranes with 2 mM NEM at 32°.

[1] The abbreviations used are: NEM, *N*-ethylmaleimide; Dala^{2}met^{5}amide, [D-Ala^2-Met^5]-enkephalinamide (H_2N-Tyr-D-Ala-Gly-Phe-Met-NH_2); DTT, dithiothreitol; Dala^{2}Dleu^{5}enkephalin, H_2N-Tyr-D-Ala-Gly-Phe-D-Leu.

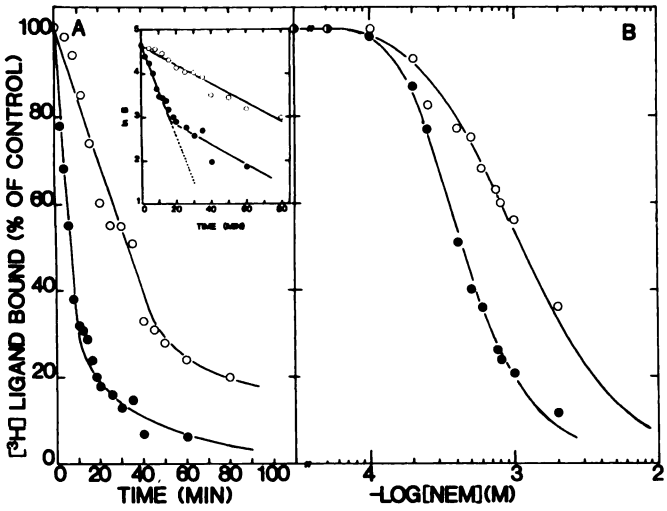

FIG. 1. *Inactivation of [^{3}H]naltrexone and [^{3}H]Dala^{2}met^{5}amide binding by NEM*

A. Membranes were allowed to react with 2 mM NEM at 32° for the indicated times, and the reaction was stopped by the addition of DTT (see Materials and Methods). Specific binding of 5.6 nM [^{3}H]naltrexone (○) or 8.6 nM [^{3}H]Dala^{2}met^{5}amide (●) was assayed as described under Materials and Methods. Control binding is the amount of ligand bound to membranes treated first with DTT and then NEM (i.e., control membranes). *Inset*, Semilogarithmic representation of the same data; B = per cent of control binding. The pseudo-first order rate constant for the loss of binding (k_{ob}) is defined as $\ln(B/B_0) = -k_{ob} \cdot t$, with B_0 for $t_0 = 0$.

B. Membranes were incubated at 32° for 15 min with the NEM concentrations indicated, and the reactions were stopped by the addition of DTT. Specific binding of 2.6 nM [^{3}H]naltrexone (○) and 2.7 nM [^{3}H]Dala^{2}met^{5}amide (●) was assayed as described above.

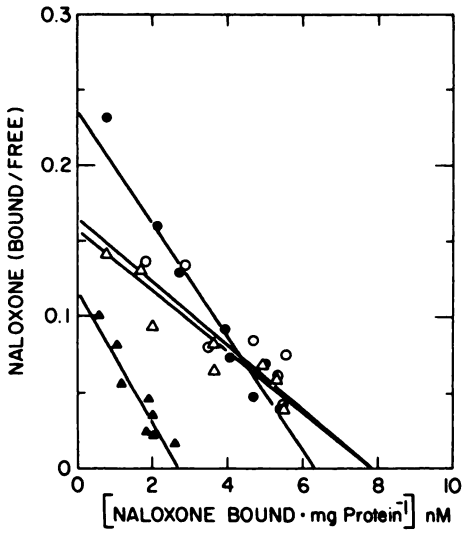

FIG. 2. *Effects of NEM on the binding of the opiate antagonist [^{3}H]naloxone*

Membranes were treated with 2 mM NEM or with 10 mM DTT and 2 mM NEM (control membranes) for 15 or 80 min at 32° and then DTT was added to those without DTT so as to stop the reaction (see Materials and Methods). NEM-treated and control membranes were then assayed for [^{3}H]naloxone specific binding (5–115 nM), and the data were normalized per milligram of protein and then plotted according to Scatchard. Control membranes: 15 min (○), 80 min (△); NEM-treated membranes: 15 min (●), 80 min (▲) contained 235, 201, 261, and 246 μg of protein, respectively. The correlation coefficients (r) for the best-fit straight lines were 0.9 (○), 0.93 (△), 0.98 (●), and 0.94 (▲). For the 15- and 80-min control membranes, the apparent K_D and B_{max} values were 43.5 nM and 0.8 pmole/mg of protein and 55.5 nM and 0.8 pmole/mg of protein, respectively. With the 15- and 80-min NEM-treated membranes these values were 26.9 nM and 0.64 pmole/mg of protein and 23.3 nM and 0.27 pmole/mg of protein, respectively. This experiment was duplicated, and average values are given in Table 1 and Fig. 3.

Although the binding of both of these ligands decayed in a pseudo-first order manner, [^{3}H]etorphine binding decayed with a $t_{1/2}$ of 24 min whereas the $t_{1/2}$ for [^{3}H]-naloxone binding was 50 min (data not shown).

One possible interpretation of these data is that there are two NEM-sensitive processes required for opiate agonist binding but that only one of these plays a role in the binding of opiate antagonists. As a first step in testing such a hypothesis, we analyzed the binding isotherm of the pure opiate antagonist [^{3}H]naloxone in membranes before and after treatment with 2 mM NEM for up to 80 min (Fig. 2). Scatchard analysis of the data indicated that after 15 min of NEM treatment, when the binding of the ^{3}H-labeled opioid peptide agonist has been reduced to 20% of its control value (see Fig. 1A), there is only a 22% loss in the number of naloxone binding sites. Moreover, the apparent affinity of naloxone is approximately 50% better after this treatment [i.e., change in K_D from 34 ± 11 nM (n = 3) to 19.4 ± 6 nM (n = 3)]. However, after continued treatment with NEM there are larger losses in the number of binding sites for naloxone, with only 30% of the control B_{max} still in existence after 80 min. Yet, even after such prolonged treatment with NEM, the apparent K_D of naloxone is 23.3 ± 11 nM (n = 2). Therefore, only a small increase in antagonist affinity and a decrease in the number of antagonist binding sites is seen after NEM treatment (Table 1). We also determined the effect these treatments with NEM have on the B_{max} of another pure opiate antagonist, naltrexone. We found that NEM also reduces the B_{max} for naltrexone without significantly altering its affinity (Table 1). When the effects of NEM on the number of receptor sites for both of these opiate antagonists were plotted versus time on the same graph, the losses in B_{max} for both ligands could be described by a single pseudo-first order rate constant of 0.016 min^{-1} ($t_{1/2}$ = 38 min) (Fig. 3). We therefore propose that there is an NEM-sensitive site associated with the ligand-binding domain of the NG108-15 opiate receptor and that, when modified by NEM, these receptors can no longer bind antagonists.

Assuming that both opiate antagonists and agonists bind to the same receptor site in these membranes (as has been reported previously [1, 2]), we should expect to find that the B_{max} for agonist sites is also decreased by NEM treatment. The observed rate of reactivity of NEM with the site controlling the number of antagonist binding sites could account for all the slow losses in [^{3}H]naltrexone binding as well as the slow rate of disappearance in the binding of the ^{3}H-labeled opioid peptide agonist shown in Fig. 1A. Analyses of saturation binding isotherms for the peptide agonist [^{3}H]Dala^{2}met^{5}amide shows that (a) decreases in the B_{max} of the peptide are readily evident after NEM treatment; (b) the longer the treatment with NEM, the larger the decrease in the B_{max}

TABLE 1

Summary of the effects of NEM on the affinity of ^{3}H-labeled ligands for the opiate receptors in NG108-15 membranes

Membranes were assayed for saturation binding of either [^{3}H]Dala^{2}met^{5}amide (0.3–115 nM), [^{3}H]naloxone (6–130 nM), or [^{3}H]naltrexone (11.6 nM with increasing concentrations of naltrexone). Apparent affinity constants (nanomolar K_D) were obtained by Scatchard analysis of the data. In most cases, the experiments were repeated; averages were calculated ± standard error of the mean, and the number of replicate experiments is given in parentheses. Appropriate control membranes (see Materials and Methods) were routinely incubated for 15 min at 32°.

^{3}H Labeled ligand	Apparent K_D at time of treatment with NEM before DTT addition		
	0 min	15 min	80 min
Agonist:			
Dala^{3}met^{5}amide	5.5 ± 1.8 (7)	37.2 ± 7.6 (7)	ND[a]
Antagonist			
Naloxone	34 ± 11 (3) 44 ± 11 (3)[b]	19.4 ± 6 (3)	23.3 ± 11 (2)
Naltrexone	45 (1)	45 (1)	65 (1)

[a] ND, Not determined.

[b] Control membranes incubated for 80 min.

of the peptide; (c) although the B_{max} can vary as much as 2-fold among individual membrane preparations, when assayed in the same preparation, the B_{max} values for the opioid peptide agonist and the antagonist naloxone are the same before as well as after different NEM treatments; and (d) the time course of the loss in B_{max} for the agonist peptide in the presence of 2 mM NEM approximates that seen for the loss in B_{max} for the two opiate antagonists similarly treated (Fig. 3). The above data indicate that a single modification of the opiate receptor by NEM is capable of preventing the binding of either opiate agonist or antagonist ligands.

Finding an NEM inactivation rate for agonist binding sites which has a $t_{1/2}$ of 38 min does not account for the rapid loss in ^{3}H-labeled peptide binding ($t_{1/2} \leq 8$ min) that is observed upon treatment of membranes with 2 mM NEM (see Fig. 1A). However, an explanation for the rapid loss is evident upon comparison of the actual binding isotherms of the ^{3}H-labeled opioid peptide Dala^{2}met^{5}amide to control and NEM-treated (2 mM, 15 min) membranes (Fig. 4, Table 1). Before NEM treatment, the apparent K_D for this peptide is 5.5 ± 1.8 nM (n = 7); after a 15-min 2 mM NEM treatment the apparent K_D is 37.2 ± 7.6 nM (n = 7). Therefore, NEM treatment causes not only a loss in the number of binding sites for the agonist peptide but also a significant loss (approximately 7-fold) in peptide affinity. With regard to NEM

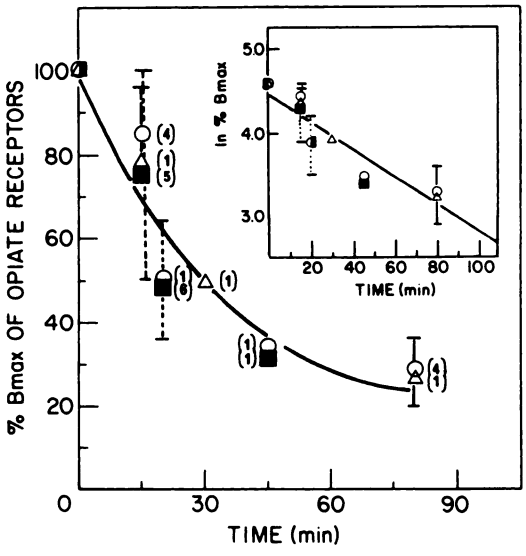

FIG. 3. *Loss of opiate receptors produced by NEM*

Membranes, including paired controls, were allowed to react with 2 mM NEM for up to 80 min. Afterward, the number of receptor sites for [^{3}H]naltrexone (△), [^{3}H]naloxone (○), and [^{3}H]Dala^{2}met^{5}amide (■) were determined from Scatchard plots of the saturation-binding isotherms. The numbers obtained from NEM-treated and paired control membranes were compared to obtain the percentage of receptor sites remaining after NEM treatment (i.e., % B_{max}). The data from a number of experiments were averaged and are given ± standard error of the mean; the number of experiments is listed in parentheses. The *inset* represents a semilogarithmic plot of the data, and the best-fit straight line (r = 0.91) indicates that ~90% of the opiate receptors are inactivated by NEM in a "one-hit" process with a $t_{1/2}$ of 38 min and with a pseudo-first order rate constant of 0.016 min^{-1} (see legend to Fig. 1).

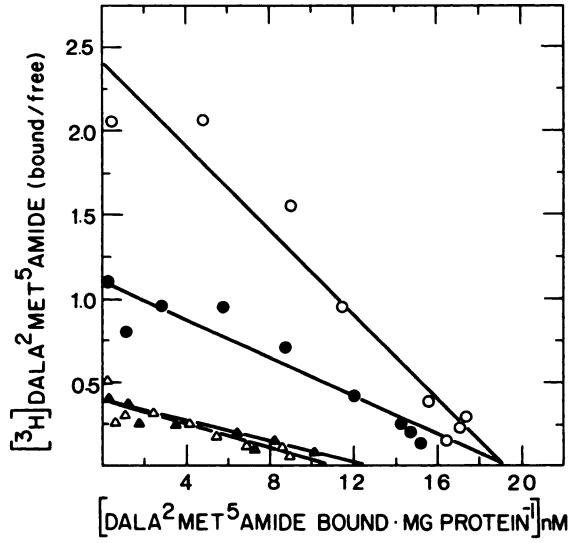

FIG. 4. *Effect of NEM on the binding of the opioid peptide agonist [^{3}H]Dala^{2}met^{5}amide*

Membranes were treated with 2 mM NEM for 15 min at 32° and then DTT was added to stop the reaction. These NEM-treated membranes, along with appropriate control membranes (see Materials and Methods), were then assayed for [^{3}H]Dala^{2}met^{5}amide specific binding (0.3–115 nM) with or without 100 μM GTP. After the data were normalized per milligram of protein they were plotted according to Scatchard: control membranes, 200 μg of protein per reaction assayed without GTP (○) or with GTP (●); NEM-treated membranes, 190 μg of protein per reaction assayed without GTP (△) or with GTP (▲). The results of one representative experiment are shown in which the B_{max} (picomoles per milligram of protein) and apparent K_D (nanomolar) for control membranes were 1.9 and 7.9 nM minus GTP, and 1.9 and 17.6 nM plus GTP, respectively. In the NEM-treated membranes without GTP, B_{max} = 1.1 and K_D = 28.6 nM; with GTP, B_{max} = 1.3 and the K_D = 34.5 nM. This study was repeated at least three times and the average calculated B_{max} and K_D for all of these independent experiments were as follows: in control membranes without GTP (n = 7) B_{max} = 1.4 ± 0.5 and K_D = 5.5 ± 1.9 nM; with GTP (n = 3) the values are B_{max} = 1.1 ± 0.4 and K_D = 14 ± 3.9 nM. In contrast, in the NEM-treated membranes without GTP (n = 7), B_{max} = 1.1 ± 0.4 and K_D = 37.2 ± 7.6 nM; with GTP (n = 3) B_{max} = 1.2 ± 0.2 and K_D = 33 ± 17 nM.

sensitivity, the loss in agonist peptide affinity, rather than the inactivation of the binding site, is the more sensitive process. The rate of change in peptide affinity would appear to be fast enough to account for the rapid losses in ^{3}H-labeled agonist opioid peptide binding described earlier (see Fig. 1A). As these saturation studies indicate a B_{max} for opiate receptors of ~1.0 ± 0.3 pmole/mg of protein, the opiate receptor concentrations in these assays is routinely 2–3 nM. If the receptor concentration in the assay is lowered, the apparent K_D becomes significantly better (Fig. 5). This means that we probably underestimated the actual loss in agonist affinity caused by NEM, since our apparent agonist K_D in control membranes is higher than the true K_D (estimated at zero receptor concentration).

Another binding method which has proven useful in illustrating basic differences between agonist and antagonist ligands is the study of the competition by nonradiolabeled ligands for ^{3}H-labeled antagonist binding. We followed such a protocol to determine whether selective losses in opiate agonist affinity are indeed brought about by NEM treatment. In these studies, the radiolabeled ligand was [^{3}H]naloxone and the membranes were treated for 15 min with 2 mM NEM so as to cause only small losses (20–30%) in receptor numbers. The results of a typical experiment using control and NEM-treated membranes to study the ability of Dala^{2}met^{5}amide or naltrexone to compete for [^{3}H]naloxone binding are shown in Fig. 6. Clearly, NEM treatment causes the IC_{50} concentration for the agonist peptide to increase dramatically whereas that for the antagonist naltrexone is unaltered. Taking into account the changes in K_D for [^{3}H]naloxone that accompany such NEM treatments (see Table 1 and Fig. 2), we calculate that this NEM treatment causes a 7-fold decrease in the affinity of this peptide agonist [i.e., change in K_D from 10.9 ± 4.8 nM (n = 6) to 75.3 ± 42 nM (n = 6)] without significantly changing the affinity of the antagonist naltrexone (Table 2). As there has recently been much discussion about different endogenous opiates and possible differences in their mode of action, we decided to test a number of these ligands by competition binding before and after NEM treatment (Table 2). We found that NEM decreases the NG108-15 receptor's affinity for the following opiate agonists: (a) enkephalin peptides; (b) normorphine and dihydromorphine, which are purported to act preferentially at μ opiate receptors; (c) Dala^{2}Dleu^5, which acts preferentially at δ opiate receptors; and (d) etorphine, which acts equally well at all opiate receptors.

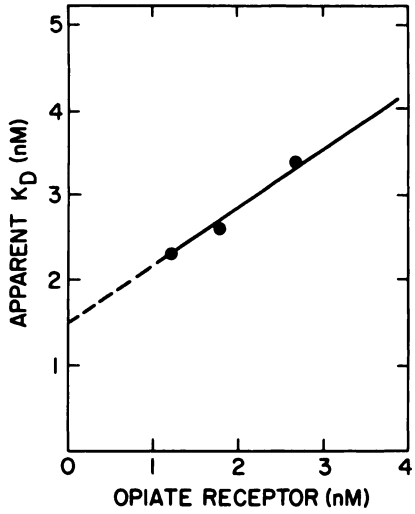

FIG. 5. *Dependence of the apparent K_D of [^{3}H]Dala^{2}met^{5}amide upon receptor concentration*

The specific binding of [^{3}H]Dala^{2}met^{5}amide (0.4–16.5 nM) was assayed in 50 mM Tris-HCl buffer using 170, 220, and 340 μg of protein of non-NEM, non-DTT-treated membranes. All of the data were analyzed by the method of Scatchard to obtain apparent K_D values and the maximal number of binding sites per milligram of protein (i.e., B_{max}) at each protein concentration. For this particular membrane preparation, B_{max} was determined to be 0.74 ± 0.04 pmole/mg of protein and to be independent of the protein concentration of the assay. The concentration of the membrane-bound opiate receptors in these assays in terms of nanomoles per liter is given along the abscissa and was calculated as follows: receptor concentrations (nanomolar) = B_{max}(nanomoles per milligram of protein) × milligrams of protein per assay × 1/reaction volume in liters.

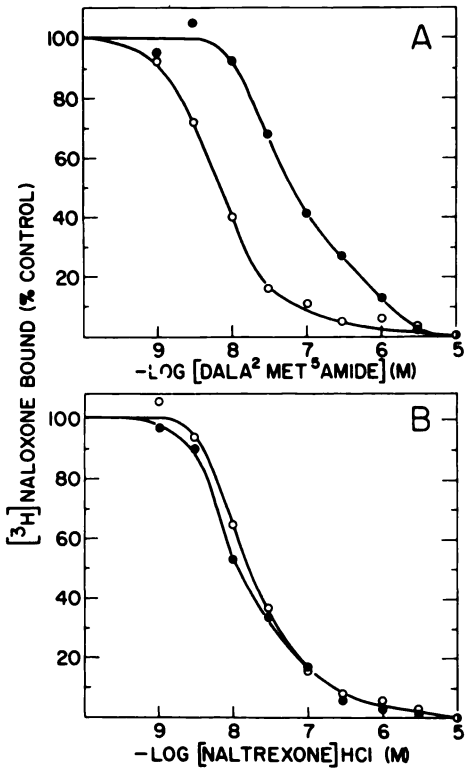

FIG. 6. *Demonstration of the selective loss in agonist affinity produced by NEM treatment based on ligand competition of the [^{3}H] naloxone binding*

Membranes were treated with 2 mM NEM for 15 min at 32° and the reaction was stopped by DTT. The appropriate control membranes (see Materials and Methods) and NEM-treated membranes were then assayed for the binding of the opiate antagonist [^{3}H]naloxone (5–8 nM) in the presence of increasing concentrations of the opioid peptide agonist Dala^{2}met^{5}amide (*panel A*) or the opiate antagonist naltrexone (*panel B*). ○, control membranes; ●, NEM-treated membranes. Analysis of these representative curves by a nonlinear least-squares curve-fitting program (19) showed the following: the apparent K_D of Dala^{2}met^{5}amide changes from 4.3 ± 0.5 nM (± confidence limits of the computed fit) to 40.7 ± 16 nM due to NEM treatment. In contrast, the apparent K_D of the antagonist naltrexone goes from 10.9 ± 1.7 nM to 7.4 ± 0.6 nM after NEM treatment. This experiment was replicated at least three times and the average K_D values are listed in Table 2.

TABLE 2

Summary of the selective loss in opiate agonist affinity induced by NEM treatment

The affinity of various ligands for the opiate receptor was judged by their ability to compete for the specific binding of [^{3}H]naloxone (6–8 nM) using 150–250 μg of membrane protein per reaction. Each ligand was tested at at least eight different concentrations. NEM-treated membranes were incubated with 2 mM NEM for 15 min at 32° and the reaction was stopped by addition of 10 mM DTT. Paired control membranes were similarly incubated in buffers containing both NEM and DTT (see Materials and Methods). The apparent K_D values ± standard error of the mean were determined from the equation $K_D = IC_{50} + (1 + \text{concentration } [^3\text{H}]\text{naloxone}/K_D\ [^3\text{H}]\text{naloxone})$. The K_D values for [^{3}H]naloxone in control and NEM-treated membranes were taken as 40 and 20 nM, respectively.

Opiate	Apparent K_D		
	Control	Post-NEM treatment	K_D Ratios: post-NEM/control
Antagonist: naltrexone	14.4 ± 4 (3)	12.9 ± 3.6 (3)	0.9
Mixed antagonist-agonist: cyclazocine	7.7 (1)	6.1 (1)	0.8
Mixed agonist-antagonist: pentazocine	176 ± 75 (3)	339 ± 16 (3)	1.9
Agonist			
β-Endorphin	26.5 ± 9.5 (2)	39.7 ± 2.1 (2)	1.5
Dala^{2}Dleu^{5}enkephalin	5.8 ± 2.2 (2)	26.4 ± 9.4 (2)	4.5
Dala^{2}met^{5}amide	10.9 ± 4.8 (6)	75.3 ± 42 (6)	6.9
Etorphine	3.4 ± 1 (3)	7.5 ± 4 (3)	2.2
Dihydromorphine	131 ± 57 (3)	353 ± 98 (3)	2.7
Normorphine	631 ± 38 (2)	2983 ± 37 (3)	4.7

A loss in affinity due to NEM treatment also is seen with β-endorphin. However, as the observed change is less than 2-fold, we do not yet know whether the difference is significant. In addition, we find that even with the mixed opiate agonist-antagonist pentazocine there is an observable NEM-directed loss in affinity. In contrast, NEM is not observed to cause losses in the affinity of the opiate antagonist-agonist cyclazocine or the pure antagonists naltrexone and naloxone.

NG108-15 opiate binding sites are known to be modulated by both monovalent cations as well as by guanine nucleotides (2, 6, 7). We were interested to see whether one or both of these sensitivities would be changed in membranes which had been treated with NEM. For these experiments, the membranes were again treated with 2 mM NEM for only 15 or 20 min so as to produce the lowered agonist affinity state without any large changes in receptor number. Initially, the continued presence of the sensitivity to Na^+ was judged by competition binding experiments (Fig. 7). These studies show clearly that Na^+ can decrease up to ~100-fold the ability of Dala^2-met^{5}amide to compete for [^{3}H]naltrexone binding in NEM-treated membranes. The magnitude of the loss in agonist affinity induced by Na^+ in these studies is dose-dependent, being less at 10 than at 400 mM Na^+. The order of specificity for cations to produce such effects on NEM-treated membranes, as judged from direct inhibition of ^{3}H-labeled opioid peptide agonist binding, is $Na^+ > Li^+ > K^+ \simeq$ choline. The respective cation ED_{50} values are 7, 80, 150, and 200 mM (Fig. 8A). It is important to note that this is the same rank order found in control membranes for the selective cation inhibition of ^{3}H-labeled opioid agonist binding and effective coupling of these opiate receptors to adenylate cyclase (21). However, after NEM treatment, the EC_{50} value for Na^+ is 7 mM, whereas before NEM treatment it is 100 mM (Fig. 8A). After NEM treatment sodium does not inhibit antagonist binding and in fact enhances the binding of [^{3}H]naltrexone by ~2-fold.[2] Last, as judged by competition experiments, the losses in agonist affinity induced by 400 mM Na^+ or Li^+ (Fig. 8B) are quantitatively the same whereas the same amount of choline induces a smaller loss in agonist affinity.

With regard to nucleotide sensitivity, we find that GTP (100 μM) inhibits [^{3}H]Dala^{2}met^{5}amide binding in control NG108-15 membranes through a reduction in the apparent affinity of the peptide agonist for the opiate receptor. Scatchard analysis reveals an average 2-fold loss in agonist K_D and no significant change in the number of binding sites in the presence of GTP (Fig. 4). When these saturation binding studies are repeated using membranes incubated with 2 mM NEM for 15 min we find that the binding of this agonist peptide is no longer significantly influenced by the presence of 100 μM GTP (Fig. 4).

The above studies indicate that the lowered agonist affinity state of the receptor induced by NEM treatment has lost its nucleotide regulation yet is more sensitive to monovalent cation regulation.

DISCUSSION

Modification of the opiate receptors in NG108-15 membranes by NEM occurs by at least two processes which are clearly distinguishable. One of these processes results in a loss of binding sites and can be followed by monitoring the reduction in the number of opiate receptors. This particular action of NEM is dependent upon the time of treatment with NEM as well as on the concentration of NEM used. The essential nature of this site, in its unmodified form relative to the binding function of the receptor, is illustrated by the fact that NEM reduces with a single rate constant, and in an apparent "one-hit" fashion, the number of binding sites for both the opiate antagonists [^{3}H]naloxone and [^{3}H]naltrexone and the opioid peptide agonist [^{3}H]Dala^{2}met^{5}amide. Upon incubation of membranes with 2 mM NEM at 32° the $t_{1/2}$ for the loss in receptor number is ~38 min. The occurrence of an —SH group which is essential to the ligand-binding site of opiate receptors was originally proposed by Simon and Groth (12) for the opiate receptors in rat brain. The NG108-15 opiate receptors can be viewed in the same fashion, although the actual nature of the modified group must be verified. In addition, the localization of such a group within the physical binding domain of the receptor for opiates can be only tentative at this time, as two physically distinct domains interacting allosterically could also account for these observations.

The other modification of the opiate receptor produced by NEM is a selective loss in affinity for opiate agonists. The rate at which NEM causes this event appears to be faster than its rate of inactivation of receptor binding

[2] N. E. Larsen, D. Mullikin-Kilpatrick, and A. J. Blume, unpublished results.

sites. Upon incubation of membranes with 2 mM NEM, the loss in affinity for agonist ligands appears complete within 20–30 min; at this time less than 25% of the receptor sites have been inactivated. What is of major importance here is that these losses in agonist affinity occur without any concomitant loss in affinity for opiate antagonists. In fact, there is a slight increase in antagonist affinity as observed for the interaction of the receptor with [^{3}H]naloxone. With the opioid peptide agonist Dala^{2}met^{5}amide the loss in affinity produced by NEM has been documented both by direct saturation binding experiments using the ^{3}H-labeled peptide and in competition binding studies which monitor the ability of the peptide to compete for the binding of the ^{3}H-labeled antagonist naloxone. Both methods indicate that this type of NEM modification causes about a 7-fold loss in affinity for this peptide agonist. When the competition studies were extended to a larger number of opiate receptor specific ligands the following became evident about this NEM event: (a) there is a loss in affinity with all of the opiate agonist ligands tested; (b) the degree of loss in agonist affinity is not a constant but changes with the agonist; (c) there is no significant loss observable with the two pure opiate antagonists, naloxone and naltrexone; and (d) for ligands with both agonist and antagonist properties, certain ones such as pentazocine (which are mostly "agonistic") show an affinity loss whereas those such as cyclazocine (which are mostly "antagonistic") do not show any affinity loss. On the basis of the above findings, we propose that the loss in affinity is a general property of opiate agonists, including those in the enkephalin, endorphin, and alkaloid classes.

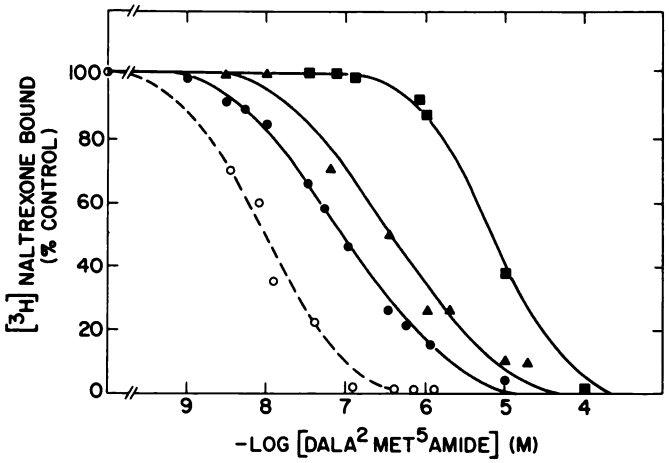

FIG. 7. *Effect of NEM on the ability of Dala^{2}met^{5}amide to compete for the binding of the antagonist [^{3}H]naltrexone in the absence or presence of NaCl*

Membranes were treated with 2 mM NEM for 15 min at 32°. These membranes, along with appropriate control membranes (see Materials and Methods), were assayed for specific [^{3}H]naltrexone binding (8.8 nM) in buffer with the concentrations of NaCl noted below. NEM-treated membranes (——) were assayed in buffer alone (●) or with 10 mM (▲) or 100 mM (■) NaCl; control membranes (- - -) were assayed in buffer alone (○).

Unfortunately, there are not many data concerning the location of either of these two different NEM-sensitive sites at this time. We do find that the NEM site related to agonist high-affinity binding can be modified without eliminating the sensitivity of opiate binding to monovalent cations. Before, as well as after NEM treatment, Na^+ selectively decreases the affinity of agonist ligands for these receptors. In addition, both the magnitude of the change in affinity induced by Na^+ and the selectivity (i.e., $Na^+ \geq Li^+ \gg K^+$ = choline) of this regulation by cations have not been changed by NEM treatment. The only change that we do observe in Na^+ action accompanying NEM treatment is that the binding of agonists actually becomes more sensitive to cation reduction (i.e., there is a decrease in the sodium ED_{50} value). Similar observations have been made with rat brain receptors (13). Very recently, we have found that the presence of Na^+ and/or opiate antagonists during NEM treatment

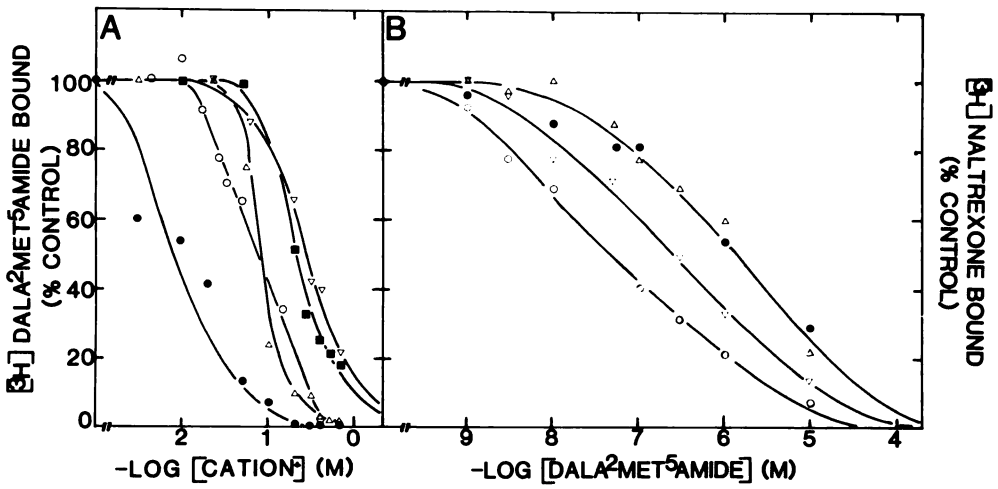

FIG. 8. *Specificity of the Na^+ effect on Dala^{2}met^{5}amide binding to NEM-treated membranes*

A. Control and NEM-treated membranes (2 mM NEM, 15 min, 32°) were assayed for specific binding of [^{3}H]Dala^{2}met^{5}amide (17.7 nM) with increasing concentrations of various monovalent cations. Control membranes with increasing NaCl (○); NEM-treated membranes with increasing NaCl (●), LiCl (△), KCl (■), or choline chloride (▽).

B. NEM-treated membranes were assayed for specific binding of [^{3}H]naltrexone (9.2 nM) with increasing concentrations of Dala^{2}met^{5}amide in buffer alone (○) or in buffer plus 0.4 M NaCl (●), 0.4 M LiCl (△), or 0.4 M choline chloride (▽).

significantly protects the binding site but not the formation of high-affinity agonist receptor complexes from NEM inactivation.[3] Taken together, the above data suggest that the NEM-sensitive site regulating the number of opiate receptors may be closely related to, if not actually part of, the ligand-binding domain of the receptor. From our previous studies showing that Na^+ induces an increase in the dissociation rate of ^3H-labeled opiate agonists from the NG108-15 receptors (7), we can infer that Na^+ is an allosteric effector of the ligand-binding site of these opiate receptors. The data shown here indicate further that Na^+ still can affect a modified form of the ligand binding site (i.e., one that does not have the normal high opiate agonist affinities). The above, plus the protection by Na^+ of the ligand-binding site from NEM inactivation, would be consistent with the site of action of Na^+ being on the opiate receptor, as originally proposed for rat brain opiate receptors by Simon and Groth (12). However, further studies are clearly needed to eliminate the possibility that Na^+ action is mediated allosterically through some other receptor-associated membrane component. In contrast, the NEM-sensitive site mediating opiate agonist high affinity would have to be distal to the ligand-binding site of the receptor. In this regard, it is interesting to note that, along with the loss in agonist affinity, there is a loss in the sensitivity of agonist binding to guanine nucleotides. As proposed earlier for these NG108-15 opiate receptors (2, 5–7), the guanine nucleotide sensitivity of opiate binding and opiate regulation of adenylate cyclase is a reflection of the interaction of the receptor with some type of nucleotide regulatory component in the membranes. Furthermore, this component was hypothesized to play an essential role in transferring information from the agonist-receptor complex to the catalytic moiety of adenylate cyclase. Other receptor-coupled adenylate cyclase systems have been shown to contain a guanine nucleotide regulatory component that is sensitive to NEM, confers guanine nucleotide sensitivity specifically on the binding of agonist ligands, and is required for the formation of the high-affinity agonist-binding state (11, 15–17, 22). Our data suggest an involvement of such a membrane component in the opiate receptor-adenylate cyclase system.

In summary, all of our results are consistent with the following model. (a) An NEM-sensitive site (probably an —SH group) associated with the receptor's ligand-binding domain exists and is essential for the binding of both opiate agonists and antagonists; modification of it by NEM results in the inability to bind these ligands. (b) A second NEM-sensitive site (also probably an —SH group) is involved in the formation of a high-agonist affinity, nucleotide-sensitive state of the receptor. This site is involved only in agonist ligand binding. (c) The location of this second NEM-sensitive group is distal to the receptor's binding site. *A priori*, this site could be physically located on the receptor or on some membrane component which binds nucleotides and interacts with the opiate receptor.

[3] N. E. Larsen, D. Mullikin-Kilpatrick, and A. J. Blume, manuscript in preparation.

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