MOLECULAR PHARMACOLOGY, 20:8–15

Irreversible Inactivation of the Opiate Receptors in the Neuroblastoma × Glioma Hybrid NG108-15 by Chlornaltrexamine

ROBERT FANTOZZI,[1] DEBRA MULLIKIN-KILPATRICK, AND ARTHUR J. BLUME

Department of Physiological Chemistry and Pharmacology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Received November 10, 1980; Accepted February 26, 1981

SUMMARY

FANTOZZI, R., D. MULLIKIN-KILPATRICK, AND A. J. BLUME. Irreversible inactivation of the opiate receptors in the neuroblastoma × glioma hybrid NG108-15 by chlornaltrexamine. *Mol. Pharmacol.* **20**:8–15 (1981).

Chlornaltrexamine irreversibly inactivates opiate receptors in intact NG108-15 cells in a concentration- and time-dependent fashion. This inactivation is seen as a loss in the number of receptors per cell (decrease in B_{max}), and is quantitatively the same whether based on the number of specific binding sites for the agonist opioid peptide [D-Ala^2-Met^5] enkephalinamide or those for the opiate antagonist naltrexone. Inactivation appears to require direct interaction of chlornaltrexamine with these receptors as naltrexone and [D-Ala^2-Met^5]enkephalinamide protect against the irreversible effects of chlornaltrexamine. Furthermore, treatment with chlornaltrexamine does not decrease muscarinic or prostaglandin receptors or diazepam binding sites in NG108-15. In intact NG108-15 cells, the opioid peptide [D-Ala^2-Met^5]enkephalinamide can inhibit 80 ± 10% of the accumulation of cyclic AMP that is stimulated by prostaglandin E_1. In control cells, the action of this peptide is cooperative and the half-maximum effect (K_{inh}) occurs with 3.3 nM peptide. Under identical conditions, the half-maximum receptor occupancy by the peptide (K_D) is 23 nM. After the opiate receptor number is significantly decreased by previous incubation with chlornaltrexamine, both the maximal effects of prostaglandin E_1, as stimulator of cyclic AMP accumulation, and the opioid peptide, as inhibitor of this accumulation, are maintained. In the case where the receptor number has been reduced by 95%, although there is a small change (≤3-fold) in the K_D and K_{inh} values for this opioid peptide, there is no significant change in the K_D/K_{inh} ratio which remains ≃7. These results indicate that the high density of opiate receptors which exist in intact NG108-15 cells is not necessary for the maximum inhibitory effect of this enkephalinamide on adenylate cyclase. In addition, the mode of action of this peptide also appears to be relatively independent of the density of the opiate receptors in these cells.

INTRODUCTION

Many extracellular chemical signals act as regulators of the synthesis of the intracellular second messenger cyclic AMP (1). Much effort has recently been directed at elucidating the relationships which exist among the number of specific binding sites for a given agonist on a cell, occupation of these sites by the agonist, and the maximal effects that agonist has on the activity of the enzyme adenylate cyclase. Early work by Jard *et al.* (2) with the antidiuretic hormone receptor showed that the effect of an agonist need not be linearly related to occupancy of its receptor and demonstrated ratios for half-maximal receptor occupation (K_D) to activation of adenylate cyclase (K_{act}) of <1 to >1. The *beta*-adrenergic receptor coupled to adenylate cyclase from different sources exhibits a similar wide range in K_D/K_{act} ratios, even when one considers only a single agonist (3). A great diversity also exists in the dependency of agonist action on receptor number. In C6 rat glioma cells, there is little relationship between the maximal catecholamine activation of the enzyme and the number of *beta*-adrenergic receptors (4), yet a direct relationship exists between these two parameters in VA_2 human lung cells (5) or S49 lymphoma cells (6).

Although there is an equally large number of hormones and neurotransmitters which inhibit as well as activate adenylate cyclase, most of the effort described above has dealt preferentially with the "activating" group of signals. In order to pursue similar questions regarding receptor-mediated inhibitions of adenylate cyclase, an experimental system was needed which allowed not only for the monitoring of agonist binding and regulation over enzyme activity, but also offered a way to modulate the

[1] Permanent address: Department of Pharmacology and Toxicology, University of Florence, Florence, Italy.

0026-895X/81/010008-08$02.00/0

number of agonist-binding sites. NG108-15 tissue culture cells appear to offer such a model system involving an opiate receptor coupled adenylate cyclase. The ability of opiates to inhibit cyclic AMP accumulation was first convincingly demonstrated in the intact mouse neuroblastoma × rat glioma hybrid cell NG108-15 (7). The basis for the action is an opiate-directed inhibition of adenylate cyclase activity, a process which has in fact been measured *in vitro* with membrane preparations (7). The interaction of the opiate with its receptor [which can be monitored with a variety of radioactive opiates (8–10)] is required for these effects and is competitively blocked when these sites are occupied by specific opiate antagonists (7). Furthermore, a number of opiate antagonists have been described now which appear to decrease irreversibly the number of opiate-binding sites in brain (11–14). One of these agents, CNA,[2] an analogue of the reversible opiate antagonist naltrexone (Fig. 1), has been postulated to act on opiate receptors as an irreversible alkylating agent (11, 12, 15).

This report demonstrates that chlornaltrexamine acts with some selectivity as an irreversible antagonist for the opiate receptors in NG108-15 and has been used to alter the number of opiate receptors per cell. The effect of this modulation of receptor density on opiate binding and inhibition of cyclic AMP accumulation is detailed.

MATERIALS AND METHODS

Materials. Radioactive ligands were obtained as follows: [^{3}H]QNB (16 Ci/mmol), Amersham Corp. (Arlington Heights, Ill.); [^{3}H]diazepam (76.8 Ci/mmol), [^{3}H]-cyclic AMP (32.3 Ci/mmol), [^{3}H]PGE$_1$ (89.5 Ci/mmol), [^{3}H]Dala^{2}met^{5}amide (30 Ci/mmol), and [^{3}H]naloxone (50 Ci/mmol), New England Nuclear (Boston, Mass.); and [^{3}H]naltrexone (25 Ci/mmol), National Institute of Drug Abuse (National Institutes of Health, Bethesda, Md.). The phosphodiesterase inhibitor Ro20-1724, diazepam, etorphine hydrochloride, and PGE$_1$ were all gifts from Hoffmann-La Roche Inc. (Nutley, N. J.). Naltrexone hydrochloride was a gift from Endo Laboratories, Inc. (Garden City, N. Y.). Atropine sulfate and Dala^2-met^{5}amide were purchased from Sigma Chemical Company (St. Louis, Mo.) and Boehringer Mannheim Biochemicals (Indianapolis, Ind.), respectively. Our initial supply of CNA was a generous gift from Professors P. S. Portoghese and A. E. Takemori (Departments of Medicinal Chemistry and Pharmacology, University of Minnesota, Minneapolis, Minn.). Quantities of CNA (dihydrochloride salt) necessary for these studies were synthesized for us by Dr. E. Mohacsi, Department of Chemical Research, Hoffman-La Roche Inc. Stock solutions of CNA were made in acidified anhydrous ethanol and stored at −20° (12).

Cells. Mouse neuroblastoma × rat glioma hybrid cells (clone NG108-15) (8) were grown in supplemented Dulbecco's modified Eagle's media as previously described (16). One day after reaching confluency, the cells were detached (by shaking), washed twice with sucrose buffer (0.32 M sucrose, 20 mM Tris·HCl pH 7.4, 5 mM glucose, 10 mM $MgCl_2$) and used immediately.

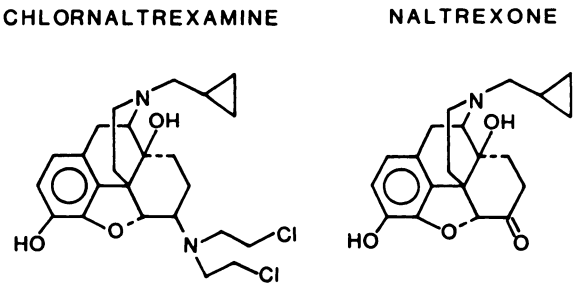

FIG. 1. *Molecular structures of chlornaltrexamine and naltrexone.*

CNA treatment. After washing, cells from 10–20 culture flasks were suspended in Na^+ buffer (135 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid·Tris buffer pH 7.4, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM KCl, and 5.5 mM glucose) (17) at 37° with various concentrations of CNA (see text) for 60 min unless otherwise noted in the text. Afterwards, free CNA was separated from the cells by washing the cells with 200 volumes of Na^+ buffer three times. Finally, the cells were suspended in Na^+ buffer at concentrations of 10–20 × 10^6 cells/ml. CNA was added to the cells such that the final concentration of ethanol was always 1%, and 1% acidified ethanol was used as control treatment. The specific binding of [^{3}H]Dala^{2}met^{5}amide and [^{3}H]naltrexone to untreated, unwashed cells incubated in Na^+ buffer gave B_{max} and K_D values of 530 ± 120 fmoles/10^6 cells and 22 ± 2 nM for the opioid peptide and 437 ± 70 fmoles/10^6 cells and 25 ± 5 nM for [^{3}H]naltrexone. These values are not significantly different from those obtained with treated, washed cells (Table 1). Cell viability after CNA treatment was ≥90%.

Radioreceptor assays. The specific binding of [^{3}H]-Dala^{2}met^{5}amide and [^{3}H]naltrexone to intact cells (defined as the difference ± 10 μM etorphine or naltrexone)

TABLE 1

Effect of CNA on opiate receptors in intact NG108-15 cells

Cells were treated with CNA for 60 min at 37° and then washed as described under Materials and Methods.

CNA treatment	Opiate receptors	Dala^{2}met^{5}amide		Naltrexone	
		B_{max}	K_D	B_{max}	K_D
	% control	fmoles/10^6 cells	nM	fmoles/10^6 cells	nM
None (control)	100	500 ± 160[a]	23 ± 1.4[a] 34 ± 1.7[b]	460 ± 85[a]	25 ± 5[a]
CNA, 0.5 μM	45 ± 2	230[a]	27[a]	200[a]	40[a]
CNA, 2.0 μM	20 ± 3	—	50[b]	—	—
CNA, 5.0 μM	10 ± 3	—	74[b]	—	—

[a] Affinity (K_D) and B_{max} determined by Scatchard analysis of the saturation-binding isotherm of the ^{3}H-ligand. Percentage of opiate receptors is obtained by comparing B_{max} of control and CNA-treated cells.

[b] Affinity (K_D) determined from the ED_{50} of the ligand as competitor of [^{3}H]naltrexone binding according to the formula $K_D = ED_{50}/1 + ([\text{naltrexone}]/K_D \text{ naltrexone})$ (18). Percentage of opiate receptors is obtained by comparing the binding of subsaturating concentrations of [^{3}H]naltrexone, assuming CNA changes only the receptor density.

[2] The abbreviations used are: CNA, chlornaltrexamine, 6β-[*N,N*-bis(2-chloroethyl)amino]-17-(cyclopropylmethyl)-4,5 α-epoxy-3,14-dihydroxymorphinan; QNB, quinuclidinyl benzilate; PGE$_1$, prostaglandin E$_1$; Dala^{2}met^{5}amide, [D-Ala^2-met^5]-enkephalinamide (Tyr-D-Ala-Gly-Phe-Met-NH_2); Ro20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; n_H, Hill coefficient.

was monitored after 20-min incubations in Na^+ or sucrose buffer (see text) at 37° in reaction volumes of 0.5 ml with ~1 × 10^6 cells, as described previously (16). Assays were terminated by separation of cells from buffer by rapid (≤1 min) centrifugation. Under these conditions, specific binding is proportional to cell number (0.5–2 × 10^6 cells per reaction (data not shown) and for both ^{3}H-ligands is ≥85% of the total binding. The specific binding of ^{3}H-ligands to NG108-15 cell membranes was measured as follows: membranes were isolated from homogenized cells as described[3] and suspended in 50 mM Tris·HCl, pH 7.4, at protein concentrations of 3–5 mg/ml. Membranes (150–250 μg of protein per reaction) were incubated in 50 mM Tris·HCl, pH 7.4, in final volumes of 0.1 ml with [^{3}H]-Dala^{2}met^{3}amide at 32° for 20 min,[3] with [^{3}H]diazepam for 15 min at 4° (19), or with [^{3}H] PGE_1 as above, but with 10 mM $MgCl_2$ in the buffer for 10 min at 37° (20). For [^{3}H]QNB binding, membranes were suspended at 37° for 60 min in 50 mM phosphate buffer, pH 7.4, in a final volume of 1 ml (21). All incubations were performed ±10 μM of the appropriate nonradioactive ligand and terminated by filtration over Whatman GF/B filters. Protein was measured by the method of Lowry *et al.* (22). All binding assays were performed in triplicate and repeated at least twice, and the results given are the mean. Replicate values within an experiment differed by ≤10%.

Cyclic AMP determinations. After washing, control and CNA-treated cells (0.5–1 × 10^6 cells) were incubated for 10 min at 37° with 5 μM PGE_1 in Na^+ buffer (final volume, 0.5 ml) in the presence of 0.7 mM Ro20-1724 and increasing concentrations of Dala^{2}met^{5}amide (17). Reactions were terminated by addition of trichloroacetic acid and cyclic AMP was determined by the protein-binding method of Gilman (23), as modified by Brostrom and Kon (24). Assays were performed in triplicate and repeated at least twice; results given are the mean of values that varied ≤5%. The "basal" concentration of cyclic AMP/10^6 cells in the absence of PGE_1 and Dala^{2}met^{5}amide is 376 ± 76 pmoles/10^6 cells.

RESULTS

CNA interaction with opiate receptors. The ability of CNA to compete with another opiate for binding to the opiate receptors on intact NG108-15 cells was examined (Fig. 2). When cells are mixed together in sucrose buffer at 37° with CNA and [^{3}H]Dala^{2}met^{5}amide (4 nM), CNA competes for binding with this stable opioid peptide in a noncooperative manner ($n_H \simeq 1.2$) and with an ED_{50} value of 30 nM (Fig. 2, inset A). Naltrexone, an opiate antagonist which binds reversibly, has a $n_H = 1.0$ and an $ED_{50} = 34$ nM when incubated with cells and this opioid peptide under similar conditions.

We next determined whether or not the interaction of CNA and the cells is reversible (Fig. 2, inset B). Here cells were incubated for 60 min with different concentrations of CNA, or naltrexone as control, washed free of any unbound ligand and the binding of [^{3}H]-Dala^{2}met^{5}amide and [^{3}H]naltrexone was then monitored

[3] N. E. Larsen, D. Mullikin-Kilpatrick, and A. J. Blume, manuscript submitted for publication.

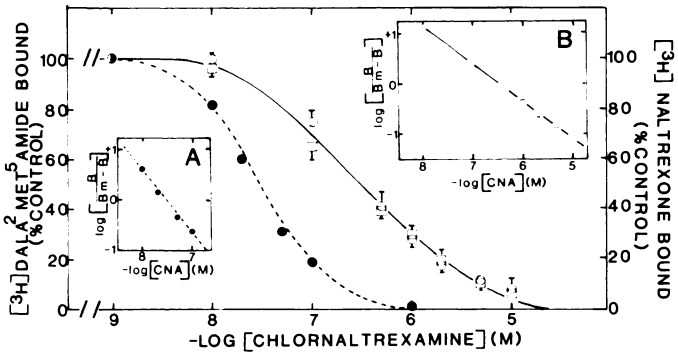

FIG. 2. *CNA inhibition of opiate binding in intact NG108-15 cells*
The competition of CNA with [^{3}H]Dala^{2}met^{5}amide (4 nM) binding was monitored by mixing cells, CNA, and ^{3}H-ligand together in sucrose buffer at 37° for 20 min (●). The specific binding of the peptide in the absence of CNA (70 fmoles/10^6 cells) is taken as 100%; *inset A* is a Hill plot of these data. The irreversible decreases in opiate binding produced by CNA were assessed in cells incubated for 60 min in Na^+ buffer at 37° with the concentrations of CNA noted along the abscissa. After the incubation, the cells were washed free of unbound drug and assayed in Na^+ buffer at 37° for the specific binding of 8 nM [^{3}H]Dala^{2}met^5 amide (□) and 4 nM [^{3}H]naltrexone (○). Here the specific binding of [^{3}H] Dala^{2}met^{5}amide and [^{3}H]naltrexone to cells not treated with CNA is 120 and 110 fmoles/10^6 cells, respectively, and is taken as 100%; *inset B* is a Hill plot of these data.

in Na^+ buffer using subsaturating concentrations of the radioligands. Such treatment of the cells with ≤25 μM naltrexone does not alter the binding of either ^{3}H-ligand after the cells have been washed free of unbound naltrexone. In contrast, treatment with CNA causes a lowering in the binding of both ^{3}H-ligands even after the removal of unbound CNA. The ED_{50} concentration of CNA which produces this irreversible effect is approximately the same regardless of whether one assays for the binding of the agonist [^{3}H]Dala^{2}met^{5}amide ($ED_{50} = 355$ nM; $n_H = 0.81$) or the antagonist [^{3}H]naltrexone ($ED_{50} = 375$ nM; $n_H = 0.84$). It is equally clear that the average ED_{50} value for irreversible action (i.e., 365 ± 14 nM) is ~10-fold larger than the ED_{50} value found for the competition by CNA of [^{3}H]Dala^{2}met^{5}amide binding to these receptors. Possible reasons for this discrepancy will be discussed later.

The irreversible inactivation of the opiate binding to NG108-15 produced by CNA is time- as well as concentration-dependent. When cells are mixed with 0.5μM CNA at 37° in Na^+ buffer for various times and then washed free of unbound CNA, the binding of a subsaturating concentration of [^{3}H]Dala^2-met^{5}amide decreases with a $t_{1/2}$ of ~2 min and the maximal effect, which is a reduction in binding of 65%, occurs within 20 min (Fig. 3).

The irreversible effects of CNA on both ligand affinity (K_D) and the number of receptor sites per cell (B_{max}) is evident by analyzing the saturation binding isotherm of the opioid peptide [^{3}H]Dala^{2}met^{5}amide. Scatchard plots of these data show that 0.5 μM CNA produces an irreversible decrease of ~60% in receptor B_{max} without significantly changing the peptide affinity at the remaining sites (K_D = 23 and 27 nM in control and CNA-treated cells, respectively) (Fig. 4). Furthermore, CNA-treatment also decreases in an irreversible manner the B_{max} for

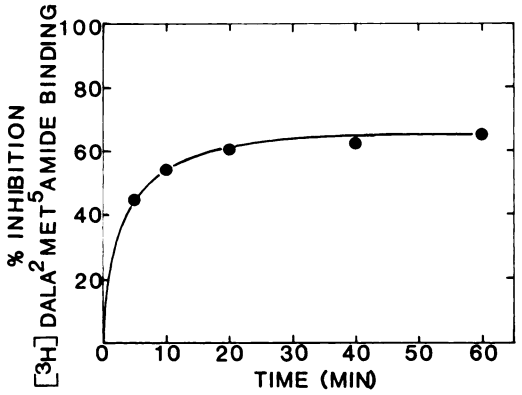

FIG. 3. *Time course of the irreversible inactivation of [^{3}H] Dala^{2}met^{5}amide binding by CNA*

Cells were incubated in Na^+ buffer at 37° with or without 0.5 μM CNA. At different times, the cells were washed free of unbound drug (see Materials and Methods) and assayed for ^{3}H-opioid peptide binding. These assays were performed in Na^+ buffer at 37° by using 8 nM [^{3}H]-Dala^{2}met^{5}amide. The results are expressed as percentage inhibition of the peptide binding that occurs to control cells incubated similarly.

[^{3}H]-naltrexone while changing the affinity of this antagonist only ~30% (Table 1). The fact that the percentage reduction in agonist- and antagonist-binding sites is the same for a given concentration of CNA agrees with the proposed identity of the opiate agonist- and antagonist-binding sites on NG108-15 (8–10). When cells are treated with high concentrations of CNA (2–25 μM), the binding of subsaturating concentrations of ^{3}H-opiates is reduced by 80–95%. In addition, the affinity of the remaining sites for Dala^{2}met^{5}amide (as judged from competition with [^{3}H]naltrexone) is not changed enough to account for these losses in [^{3}H]peptide binding. With a receptor loss of 90 ± 3%, we find only a 2 to 3-fold loss in peptide

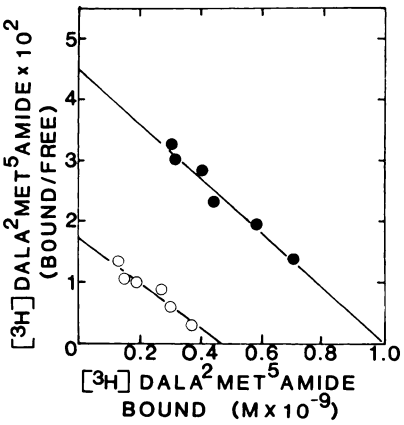

FIG. 4. *[^{3}H]Dala^{2}met^{5}amide binding to NG108-15 cells after treatment with CNA*

Cells were mixed with either no CNA (control) or 0.5 μM CNA at 37° for 60 min and then washed free of unbound drug (see Materials and Methods). The specific binding of Dala^{2}met^{5}amide in control (●) and CNA-treated cells (○) was monitored in Na^+ buffer at 37° with 9 nM ^{3}H-labeled peptide and increasing concentrations of nonradioactive peptide. The data were then analyzed according to Scatchard (25). The maximal number of binding sites per 10^6 cells (B_{max}) and the equilibrium dissociation constant (K_D) in control cells were B_{max} = 500 fmoles/10^6 cells, K_D = 23 nM and in CNA treated cells, B_{max} = 230 and K_D = 27 nM.

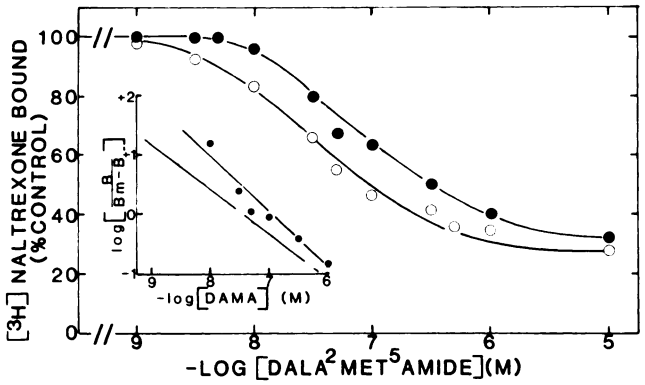

FIG. 5. *Competition by Dala^{2}met^{5}amide for [^{3}H]naltrexone binding to control and CNA-treated cells*

Cells were treated with (●) or without (○) 5 μM CNA at 37° for 60 min in Na^+ buffer and then washed free of unbound ligand. The cells were then incubated at 37° in Na^+ buffer for 20 min with [^{3}H]naltrexone (5 nM) and increasing concentrations of Dala^{2}met^{5}amide (*DAMA*). The results are expressed as the percentage of specific binding seen in the absence of any peptide. [^{3}H]Naltrexone binding to the CNA-treated cells is 14 fmoles/10^6 cells and to non-CNA-treated cells is 170 fmoles/10^6 cells. The *inset* is a Hill plot of the data. The ED_{50} and n_H values obtained for control and CNA-treated cells are 40 nM and 0.8, and 89 nM and 1.0, respectively.

affinity (Table 1 and Fig. 5). We conclude from the above that the major action of CNA is to cause irreversible losses in opiate-binding sites.

The selectivity of the action of CNA on intact cells has been evaluated by using concentrations of the drug which inactivate ~90% of the opiate receptors. After treatment of cells with 5 μM CNA, the cells were washed, homogenized, and the membrane fraction was isolated. These membrane preparations were then analyzed for the following sites shown to exist on various neuroblastoma cells: opiate receptors (8–10), muscarinic cholinergic receptors (21), PGE_1 receptors[4] (27), and diazepam-binding sites[4] (19) (Table 2). Membranes isolated from 5 μM CNA-treated cells show the expected ≥90% loss in opiate binding, yet only a 13% loss in the binding of the specific muscarinic antagonist QNB, and no loss in PGE_1 binding. The binding of diazepam is actually increased after the CNA-treatment. Data to be presented later also indicate that such CNA-treatment does not significantly disturb the functional coupling of PGE_1 receptors to the catalytic moiety of adenylate cyclase.

To confirm that the inactivation of opiate receptors in NG108-15 by CNA requires the binding of CNA to these receptors, intact cells were incubated with and without naltrexone, Dala^{2}met^{5}amide, or dextrorphan and then challenged with CNA. In these experiments, CNA was always used at 1 μM, and when incubated with cells alone caused a 75% loss in ^{3}H-ligand specific binding (Table 3), in good agreement with the results shown earlier in Fig. 2. As shown by the data in Table 3, the presence of naltrexone decreases, in a dose-dependent manner, the loss in receptor binding induced by CNA. Fifty per cent

[4] D. Lichtshtein and A. J. Blume, unpublished observations. In NG108-15, the displacement of specific [^{3}H]diazepam binding by various ligands suggests that these binding sites are similar to those in peripheral tissues as described by Braestrup and Squires (26).

TABLE 2

Selectivity of CNA inactivation of opiate receptors

Cells were treated with and without 5 μM CNA at 37° for 60 min and then washed three times, homogenized, and membranes were prepared as described under Materials and Methods. Specific binding of each radioligand was then determined (see Materials and Methods) by using the concentration of [^{3}H]ligand noted.

Receptor/binding sites	^{3}H-Ligand		Ligand binding	
	Ligand	Concentration	Pre-CNA	Post-CNA
		n M	*fmoles/mg protein*	*% pre-CNA*
Opiate	[^{3}H]Dala^{2}met^{5}amide	5.4	398	7
Cholinergic (muscarinic)	[^{3}H]QNB	0.14	60	87
PGE_1	[^{3}H]PGE_1	3.9	39	97
Benzodiazepine	[^{3}H]Diazepam	3.8	33	169

protection occurs with concentrations of naltrexone of 0.1 μM, and complete protection is observed when naltrexone is present at a 10-fold excess over CNA. The opioid peptide, at 1:1 ratio, also appears to give about 70% protection. With both the opioid peptide and naltrexone, the degree of protection is approximately the same, regardless of whether [^{3}H]agonist or [^{3}H]antagonist specific binding is being evaluated. It has not been possible to determine if higher concentrations of opioid peptide afford 100% protection, since it is not possible to wash out all of the peptide and retain 100% of control binding. The difficulties with the peptide may simply be related to its slower rate of dissociation from these receptors than is seen for naltrexone dissociation (9, 10). Nevertheless, it is clear that in the presence of this peptide, the action of CNA is significantly reduced. It has been reported that the affinity of dextrorphan for these opiate receptors is approximately 500 times poorer than that of naloxone (7), which has similar affinity as does naltrexone for these receptors. This would mean that 1 μM concentrations of dextrorphan should not provide any significant protection against the action of 1 μM CNA on NG108-15 opiate receptors. In accordance with the above, we find that 1 μM dextrophan does not afford any significant protection (Table 3). Therefore, it appears that CNA interaction with NG108-15 opiate receptors is a prerequisite for its irreversible inactivation of these receptors.

TABLE 3

Protection from CNA inactivation

Cells were incubated for 20 min at 37° in Na^+ buffer with various concentrations of ligands, after which time 1 μM CNA was added, and the incubation was continued for 60 min at 37°. After this time, the cells were washed as described. All washed cells were assayed subsequently for the specific binding of 7 nM [^{3}H]Dala^{2}met^{5}amide and 3 nM [^{3}H]naloxone (see Materials and Methods). Control cells were treated identically but were incubated without any ligands. Washing the cells up to seven times did not modify the specific binding of ^{3}H opiates to control cells. The specific binding of ^{3}H opiates to control cells was taken as 100%.

Treatment		^{3}H-Ligand binding	
		[^{3}H]Dala^{2}met^{5}amide	[^{3}H]Naloxone
		% control	
——[a]	——	100[b]	100[c]
Naltrexone, 10 μM[a]	——	100	100
Dextrorphan, 1 μM[a]	——	100	100
——[a, d]	CNA, 1 μM	25	25
Dextrorphan, 1 μM[a]	CNA, 1 μM	25	25
Naltrexone, 0.1 μM[a]	CNA, 1 μM	61	66
Naltrexone, 1 μM[a]	CNA, 1 μM	82	87
Naltrexone, 10 μM[a]	CNA, 1 μM	100	100
——[a]	——	100[e]	100[f]
Dala^{2}met^{5}amide, 1 μM[d]	——	58	60
——[d]	CNA, 1 μM	25	25
Dala^{2}met^{5}amide, 1 μM[d]	CNA, 1 μM	47	48

[a] Washed three times.
[b] 100 fmoles/10^6 cells.
[c] 93 fmoles/10^6 cells.
[d] Washed five to seven times.
[e] 107 fmoles/10^6 cells.
[f] 89 fmoles/10^6 cells.

Effects of CNA on Dala^{2}met^{5}amide-directed inhibition of cyclic AMP synthesis in intact NG108-15. Opiates decrease the cyclic AMP concentration of NG108-15 cells in the absence or presence of PGE_1 (7). This action is attributable to the inhibition by opiates of both basal and PGE_1-activated adenylate cyclase activity (7, 17). The studies reported here confine themselves to an analysis of the effect of CNA on intact cells whose cyclic AMP synthesis has been stimulated by PGE_1. These experiments are done in the presence of a potent inhibitor (Ro20-1724) of the enzyme 3′:5′-cyclic-AMP phosphodiesterase (EC 3.1.4.17), so as to block cyclic AMP degradation. Under these conditions, in control cells, the stable opioid peptide Dala^{2}met^{5}amide decreases in a dose-dependent manner the levels of cyclic AMP elevated by PGE_1. Half-maximal inhibition (K_{inh}) occurs at 3.3 nM peptide and the maximal inhibition found with saturating concentrations of peptide is 80 ± 10% (Fig. 6). Moreover, this inhibition appears cooperative (n_H = 2.2; Table 4) and is directed through the opiate receptor. The presence of increasing concentrations of naltrexone increases the K_{inh}, and at high concentrations can block all peptide action (Fig. 6). If we assume that agonist and antagonist compete in a reversible manner for binding and use the equation $K_B = \frac{[B]}{CR - 1}$ (18) (which is a rearrangement of the expression described by Schild [28]), naltrexone acts as a blocker of opiate action with an affinity (K_D) of 30 nM. This calculated affinity agrees with that obtained

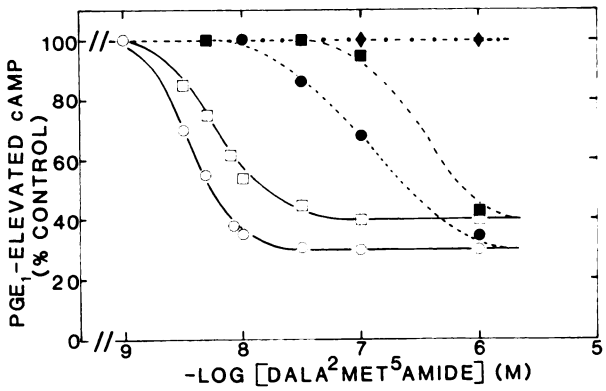

FIG. 6. *Dala²met⁵amide inhibition of PGE_1-stimulated cyclic AMP (cAMP) synthesis in control and CNA-treated cells*

Cells were incubated with or without 2 μM CNA in Na^+ buffer washed free of unbound drug and subsequently assayed in Na^+ buffer for PGE_1-stimulated cyclic AMP synthesis in the presence of increasing concentrations of Dala²met⁵amide (see Materials and Methods). The results are expressed as percentage of the value seen in the absence of peptide, which in control cells = 2621 ± 160 and in CNA-treated cells = 2995 ± 275 pmoles/10^6 cells. Naltrexone (≤10 μM) does not alter these control values. As judged from [³H]Dala²met⁵amide (9 nM) and [³H]naltrexone (5 nM) binding, this CNA-treatment reduced the number of opiate receptors per cell to 20% of control value. Control cells assayed without (○), or with 1 μM (●), or 10 μM (▼) naltrexone. CNA-treated cells assayed without (□), or with 1 μM (■) or with 10 μM (▲) naltrexone.

from direct measurement of [³H]naltrexone binding to intact NG108-15 cells (Table 1).

Treatment of the cells with ≤25 μM CNA does not significantly alter the PGE_1 elevation of cyclic AMP accumulation from its control value of 2621 ± 160 pmoles/10^6 cells. With cells which have had their opiate receptor density reduced by 80% by 2 μM CNA-treatment, the opioid peptide Dala²met⁵amide still reduces cyclic AMP accumulation in a cooperative manner (n_H = 1.8) with a K_{inh} of 5.8 nM (Fig. 6, Table 4). In addition, the maximal inhibitory effect of the peptide is ~90% of that seen in control cells. Furthermore, both the maximal inhibiting effect and the cooperative nature of the action of this opioid peptide are maintained in cells whose receptor population has been reduced 95 ± 1% by treatment with 25 μM CNA (Table 4). Such CNA-treated cells show only a 3-fold increase in the K_{inh} for Dala²met⁵amide. The actions of the opioid peptide on cells with ≤20% of the control opiate receptor density are still mediated through opiate receptors. In these cells, naltrexone increase the K_{inh} of the peptide, blocks all observable effects of the opioid peptide at high enough concentrations, and exhibits an affinity of 20 ± 5 nM as an opiate antagonist (Fig. 6).

TABLE 4

Effects of CNA on Dala²met⁵amide inhibition of PGE_1-elevated cyclic AMP concentrations in intact NG108-15 cells

Cells were incubated with or without CNA at 37° for 60 min in Na^+ buffer and then washed.

CNA treatment	Opiate receptors[a]	Action of Dala²met⁵amide[b]	
		K_{inh}	n_H
	% control	nM	
None (control)	100	3.3 ± 0.3	2.2
CNA, 2 μM	20 ± 3	5.8 ± 0.1	1.8
CNA, 10 μM	10 ± 3	7.1 ± 0.2	1.8
CNA, 25 μM	5 ± 1	10.0 ± 1.4	1.5

[a] After CNA treatment, the percentage of receptors remaining was determined based on a comparison with control cells of the binding of subsaturating concentrations of [³H]Dala²met⁵amide and [³H]naltrexone. The conclusions were independent of the ³H-ligand used.

[b] Control and CNA-treated cells were incubated with 5 μM PGE_1 and varying concentrations of Dala²met⁵amide at 37° for 10 min and cyclic AMP (picomoles per 10^6 cells) determined. K_{inh} = concentration of peptide needed to half-maximally decrease PGE_1-elevated cyclic AMP levels. n_H = Hill coefficient for peptide action calculated from the dose-response curve.

DISCUSSION

We find that CNA not only competes with other ligands for the opiate receptors in intact NG108-15 cells, but produces a blockade of both [³H]Dala²met⁵amide and [³H]naltrexone binding which is not reversed by removal of the free drug. Naltrexone, an opiate antagonist with which CNA has many structural similarities (Fig. 1), does not produce any such irreversible effects. The irreversible actions of CNA are both concentration- and time-dependent, and are independent of whether one assays for the binding of an opiate agonist or an antagonist.

Analysis of cells treated with CNA reveals that the irreversible loss in opiate binding is due to a loss in the number of opiate receptors per cell, and not due to a significant loss in ligand affinity. The action of CNA, even when used at 1–5 μM, shows selectivity for the opiate receptors of NG108-15 leaving intact PGE_1 and muscarinic cholinergic receptors and diazepam-binding sites. Naltrexone and Dala²met⁵amide protect NG108-15 opiate receptors against the effects of CNA. Naltrexone, at 0.1 μM, affords about 50% protection against 1 μM CNA. Dextrorphan, which has an affinity for these receptors that is at least 100 times poorer than that of naltrexone, does not protect against CNA action even when present at 1 μM. Based on our protection experiments, we believe that the site of action of CNA is the opiate-binding site *per se*, and that CNA must bind to this site to be able to inactivate these receptors. The actual nature of the modification of the opiate-binding sites in NG108-15 cells by CNA is unknown. However, work with CNA and rat brains by Portoghese *et al.* (12) indicates that an aziridinium ion of CNA is most likely to be the active agent responsible for an irreversible alkylation of opiate receptors. Such a reaction is also likely to be responsible for the modification of the NG108-15 opiate receptors, and in both systems the receptor modification appears to prevent ligand binding.

The discrepancy observed between the ability of CNA to compete for the binding of [³H]opioid peptide (ED_{50} ≃ 30 nM) and irreversibly inactivate the opiate-binding site (ED_{50} ≃ 355 nM) is not due to the presence of Na^+ in the latter assays or its absence in the former assays. Sodium does not change the ability of CNA to compete for binding to or irreversibly modify NG108-15 opiate receptors.[5] A similar lack of action of Na^+ on CNA

[5] A. J. Blume, unpublished observations.

interaction with rat brain opiate receptors has been reported (11, 12). This is not unexpected, since CNA appears to be a relatively pure opiate antagonist (11, 12, 15) and it is the binding of opiate agonists which is greatly reduced by Na^+ (29). Most likely, the discrepancy we observed is due to transformation of CNA in aqueous solutions of neutral pH into products other than the active aziridinium ion, some of which retain high affinity but bind reversibly. In addition, it is also possible that CNA itself can reversibly bind to these opiate receptors.

We have used CNA in this work to investigate the relationship among the opiate receptor density, opiate binding, and opiate action in intact NG108-15 cells. The action of opiates followed here was their ability to inhibit cyclic AMP accumulation stimulated by PGE_1 (30). This effect has been shown previously to require ligand interaction with a receptor site (7) and result from an inhibition of adenylate cyclase activity (17). To allow a direct comparison between the observed receptor occupancy and opiate regulation of cyclic AMP synthesis, both of these phenomena have been evaluated under identical experimental conditions. In NG108-15, the B_{max} of opiate receptors is 483 ± 127 fmoles/10^6 cells and the stable opioid Dala^{2}met^{5}amide produces an 80 ± 10% reduction in PGE_1-elevated steady-state levels of cyclic AMP under our conditions. The opioid-directed inhibition occurs in a cooperative fashion ($n_H \geq 2.0$) and with a K_{inh} of 3.3 mM; yet under identical assay conditions the K_D for peptide binding is 23 nM.

We have treated cells with various concentrations of CNA so as to reduce the number of opiate-binding sites down to ≃5% of the control value. Despite these drastic reductions in the number of opiate receptors per cell, cyclic AMP concentrations are raised to the same maximal level by PGE_1. The absence of any irreversible CNA effects on cyclic AMP synthesis indicates that in NG108-15 this drug acts as an opiate antagonist and its action is relatively specific and leaves intact all the components needed to couple PGE_1 receptors to the catalytic moiety of adenylate cyclase. Moreover, the reduction in opiate receptors of NG108-15 by CNA has only little effect on the maximum action of the opioid peptide. There is actually less than a 15% reduction in maximal opiate action which accompanies ≃90% loss in receptor number. This apparent independence of the maximum agonist effect from the existing number of receptors is similar to that observed with catecholamine activation of adenylate cyclase through the *beta*-adrenergic receptors in C6 rat glioma cells (4). However, there are other opiate receptor coupled systems in which the receptor number and agonist action are more directly related. For instance, in the neuroblastoma clone N18TG-2, the number of opiate receptors is very much lower than in the NG108-15 hybrid cell line and the maximum opiate inhibition of N18TG-2 adenylate cyclase is considerably less than that observed in the hybrid cells (7). Of course, in cell lines without any opiate receptors, like the C6BU-1 rat glioma, there is no demonstrable opiate regulation of adenylate cyclase (7). It is important to point out that, although we find that the high density of opiate receptors in NG108-15 is not required for the full efficacy of opiate regulation of adenylate cyclase in these cells, we do not have any evidence that all of these receptors are not normally used to regulate this enzyme.

A large reduction in the number of opiate receptors also has little effect on the cooperative nature of opiate inhibition of cyclic AMP accumulation. Pre-CNA, opioid peptide action has a n_H of 2.2; after 10 μM CNA treatment, it has a n_H of 1.8. Before or after CNA treatment, we find no such cooperation in peptide binding. Sharma *et al.* (7) had originally reported that although opiate-binding *in vitro* was noncooperative, inhibition of adenylate cyclase by opiates *in vitro* was cooperative.

Finally, CNA treatment appears to have little if any effect on the discrepancy that normally exists between the concentrations of peptide required for half-maximal equilibrium occupation of the opiate-receptor sites (K_D) and those which produce half-maximal inhibitory effects on cyclic AMP accumulation (K_{inh}) (Fig. 7). In control NG108-15 cells suspended in Na^+ buffer, the K_{inh} for Dala^{2}met^{5}amide is 3.3 nM, but its K_D is 23 nM. This indicates a K_D/K_{inh} ratio of ~7. Maximal opiate effects on adenylate cyclase do not apparently require equilibrium occupation of all the opiate receptors. With cells treated with 10 μM CNA and having only ≃10% of the maximal number of opiate receptors, the K_{inh} and K_D for the opioid peptide measured under identical conditions are now 7.1 and 50 nM, respectively. Therefore, despite the drastic reduction in B_{max}, the K_D/K_{inh} ratio remains unchanged at ~7. Although there is a small change in ligand affinity for the receptor which accompanies the action of CNA, the method by which opiates produce their effects on the catalytic moiety of adenylate cyclase appears to have been conserved after CNA treatment.

K_D/K_{inh} ratio of <1 to >1 have been previously reported for opiate action *in vitro* on the adenylate cyclase activity in NG108-15 cell free homogenates (7). However, the work presented in this paper is the first *in situ* demonstration of a similar discrepancy between agonist receptor occupation and agonist-mediated inhibition of adenylate cyclase activity. Although the presence of a

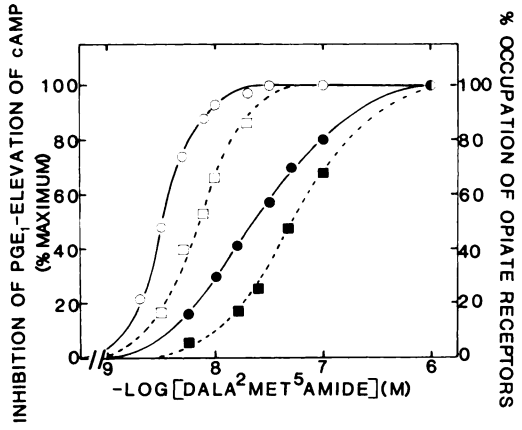

FIG. 7. *Comparison of the isotherms for opioid peptide binding and opioid peptide inhibition of cyclic AMP synthesis*

The isotherms for occupation of opiate receptor (●, ■) and inhibition of PGE_1-stimulated cyclic AMP synthesis (○; □) by Dala^{2}met^{5}amide were compared in control (●; ○) and 10 μM CNA-treated (■; □) NG108-15 cells. In this case, the CNA-treatment had decreased the receptor number per cell to 10% of control value.

K_D/K_{inh} difference for opiates would fit with the proposed indirect regulation of the catalytic moiety of adenylate cyclase by opiate receptors (17), much more information is needed before the actual mechanisms involved are clarified.

REFERENCES

1. Robison G. A., R. W. Butcher, and E. W. Sutherland. Cyclic AMP *Annu. Rev. Biochem.* **37**:149–174 (1968).
2. Jard, S., C. Roy, T. Barth, R. Rajerison, and J. Bockaert. Antidiuretic hormone-sensitive kidney adenylate cyclase. *Adv. Cyclic Nucleotide Res.* **5**: 31–52 (1975).
3. Maguire, M. E., E. M. Ross, and A. G. Gilman. β-Adrenergic receptor: ligand binding properties and the interaction with adenylate cyclase. *Adv. Cyclic Nucleotide Res.* **8**:1–83 (1977).
4. Terasaki, W. L., J. Linden, and G. Brooker. Quantitative relationship between β-adrenergic receptor number and physiologic responses as studied with a long-lasting β-adrenergic antagonist. *Proc. Natl. Acad. Sci. U. S. A.* **76**:6401–6405 (1979).
5. Venter, J. C. High efficiency coupling between beta-adrenergic receptors and cardiac contractility: direct evidence for "spare" beta-adrenergic receptors. *Mol. Pharmacol.* **16**:429–440 (1979).
6. Johnson, G. L., H. R. Bourne, M. K. Gleason, P. Coffino, P. A. Insel, and K. L. Melmon. Isolation and characterization of S49 lymphoma cells deficient in β-adrenergic receptors: relation of receptor number to activation of adenylate cyclase. *Mol. Pharmacol.* **15**:16–27 (1979).
7. Sharma, S. K., M. Nirenberg, and W. A. Klee. Morphine receptors as regulators of adenylate cyclase activity. *Proc. Natl. Acad. Sci. U. S. A.* **72**:590–594 (1975).
8. Klee, W. A., and M. Nirenberg. A neuroblastoma × glioma hybrid cell line with morphine receptors. *Proc. Natl. Acad. Sci. U. S. A.* **71**:3474–3477 (1974).
9. Blume, A. J. Opiate binding to membrane preparations of neuroblastoma × glioma hybrid cells NG108-15: effects of ions and nucleotides. *Life Sci.* **22**: 1843–1852 (1978).
10. Blume, A. J., G. Boone, and D. Lichtshtein. Regulation of neuroblastoma × glioma hybrid opiate receptors by Na^+ and guanine nucleotides, in *Modulators, Mediators, and Specifiers in Brain Function* (Y. H. Ehrlich, J. Volavka, L. G. Davis, and E. G. Brunngraber, eds.). Plenum Press, New York, 163–174 (1979)
11. Portoghese, P. S., D. L. Larson, J. B. Jiang, A. E. Takemori, and T. P. Caruso. 6β-[N,N-Bis(2-chloroethyl)amino]-17-(cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxymorphinan (chlornaltrexamine), a potent opioid receptor alkylating agent with ultralong narcotic antagonist activity. *J. Med. Chem.* **21**:598–599 (1978).
12. Portoghese, P. S., D. L. Larson, J. B. Jiang, T. P. Caruso, and A. E. Takemori. Synthesis and pharmacologic characterization of an alkylating analogue (chlornaltrexamine) of naltrexone with ultralong-lasting narcotic antagonist properties. *J. Med. Chem.* **22**:168–173 (1979).
13. Portoghese, P. S., D. L. Larson, L. M. Sayre, D. S. Fries, and A. E. Takemori. A novel opioid receptor site directed alkylating agent with irreversible narcotic antagonistic and reversible agonistic activities. *J. Med. Chem.* **23**:233–234 (1980).
14. Pasternak, G. W., S. R. Childers, and S. H. Snyder. Naloxazone, a long-acting opiate antagonist: effects on analgesia in intact animals and on opiate receptor binding *in vitro*. *J. Pharmacol. Exp. Ther.* **214**:455–462 (1980).
15. Caruso, T. P., A. E. Takemori, D. L. Larson, and P. S. Portoghese. Chloroxymorphamine, an opioid receptor site-directed alkylating agent having narcotic agonist activity. *Science (Wash. D. C.)* **204**:316–318 (1979).
16. Blume, A. J., J. Shorr, J. P. M. Finberg, and S. Spector. Binding of the endogenous non-peptide morphine-like compound to opiate receptors. *Proc. Natl. Acad. Sci. U. S. A.* **74**:4927–4931 (1977).
17. Blume, A. J., D. Lichtschtein, and G. Boone. Coupling of opiate receptors to adenylate cyclase: requirement for Na^+ and GTP. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5626–5630 (1979).
18. Williams, L. T., and R. J. Lefkowitz. *Receptor Binding Studies in Adrenergic Pharmacology*. Raven Press, New York (1978).
19. Baraldi, M., A. Guidotti, J. P. Schwartz, and E. Costa. GABA receptors in clonal cell lines: a model for study of benzodiazepine action at molecular level. *Science (Wash. D. C.)* **205**:821–823 (1979).
20. Lefkowitz, R. J., D. Mullikin, C. L. Wood, T. B. Gore, and C. Mukherjee. Regulation of prostaglandin receptors by prostaglandins and guanine nucleotides in frog erythrocytes. *J. Biol. Chem.* **252**:5295–5303 (1977).
21. Burgermeister, W., W. L. Klein, M. Nirenberg, and B. Witkop. Comparative binding studies with cholinergic ligands and histrionicotoxin at muscarinic receptors of neural cell lines. *Mol. Pharmacol.* **14**:751–767 (1978).
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
23. Gilman, A. G. A protein binding assay for adenosine 3′:5′-cyclic monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**:305–312 (1970).
24. Brostrom, C. O., and C. Kon. An improved protein binding assay for cyclic AMP. *Anal. Biochem.* **58**:459–468 (1974).
25. Scatchard, G. The attraction of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
26. Braestrup, C., and R. F. Squires. Specific benzodiazepine receptors in rat brain characterized by high-affinity [^{3}H]diazepam binding. *Proc. Natl. Acad. Sci. U. S. A.* **74**:3805–3809 (1977).
27. Brunton, L. L., R. A. Wiklund, P. M. Van Arsdale, and A. G. Gilman. Binding of [^{3}H]prostaglandin E_1 to putative receptors linked to adenylate cyclase of cultured cell clones. *J. Biol. Chem.* **251**:3037–3044 (1976).
28. Schild, H. O. pAx and competitive drug antagonism. *Br J. Pharmacol.* **4**:277–280 (1949).
29. Simon, E. J., J. M. Hiller, J. Groth, and I. Edelman. Further properties of stereospecific opiate binding sites in rat brain: on the nature of the sodium effect. *J. Pharmacol. Exp. Ther.* **192**:531–537 (1975).
30. Lichtschtein, D., G. Boone, and A. J. Blume. A physiological requirement of Na^+ for the regulation of cAMP levels in intact NG108-15 cells. *Life Sci* **25**: 985–992 (1979).

Send reprint requests to: Arthur J. Blume, Department of Physiological Chemistry and Pharmacology, Roche Institute of Molecular Biology, Nutley, N. J. 07110.