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Guanine Nucleotides Inhibit Binding of Agonists and Antagonists to Soluble Opiate Receptors*

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The guanine nucleotides GDP, GTP, and guanosine-5'-(β,γ-imido)triphosphate inhibit binding of opiates and opioid peptides to receptors solubilized from membranes of neuroblastoma × glioma NG108-15 hybrid cells. The inhibition reflects decreased affinity of receptors for opioid ligands. Whereas in membranes, only opioid agonist binding is sensitive to guanine nucleotide inhibition, both agonist and antagonist binding is reduced in the case of soluble receptors. Furthermore, soluble receptors are more sensitive to the effects of guanine nucleotides than are membrane-bound receptors. These observations are consistent with the suggestion that solubilized receptors may be complexes of an opiate binding protein and a guanine nucleotide-sensitive regulatory component.

Neuroblastoma × glioma hybrid NG108-15 cells contain membrane-bound opiate receptors (1) which mediate agonist specific inhibition of adenylate cyclase (2). The binding affinity of opioid agonists to these receptors is lowered by guanine nucleotides and sodium ions (3) and coupling to adenylate cyclase requires GTP (4). These effects, in turn, are presumably mediated by a membrane-bound, guanine nucleotide-sensitive regulatory protein which is an essential component of the hormone-responsive adenylate cyclase system (5).

We have recently shown (6) that opiate receptors from NG108-15 hybrid cells can be solubilized in an active form by the novel detergent, CHAPS,[1] a zwitterionic derivative of cholic acid (7). We now report that solubilized opiate receptors exhibit sensitivity to guanine nucleotides which decrease affinity of the receptors for opioid ligands. The solubilized receptor, which can be separated from fluoride-stimulated adenylate cyclase activity (6), may thus be part of a complex containing both an opiate binding protein and a guanine nucleotide-sensitive regulatory component.

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[1] The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DALAMID, D-Ala^2-enkephalin(5-L-methionineamide); GppNHp, guanosine-5'-(β,γ-imido)triphosphate.

EXPERIMENTAL PROCEDURES

Materials—Nucleotides and analogs were obtained from Sigma. CHAPS (7) was obtained from Dr. Leonard Hjelmeland (National Institute of Child Health and Human Development). [^{3}H]Etorphine (41.6 Ci/mmol), [^{3}H]diprenorphine (18 Ci/mmol), and DALAMID (16 Ci/mmol) were purchased from Amersham. Nonradioactive DALAMID was obtained from Calbiochem. Other opiates were from Dr. Robert Willete (National Institute of Drug Abuse). All other chemicals were analytical reagent grade unless otherwise specified. Neuroblastoma × glioma hybrid NG108-15 cells were grown in as described previously (1, 6).

Opioid Binding—Binding assays were performed with either solubilized receptors or intact membranes using the gel filtration method described previously (6). Assay mixtures contained either 2 nM [^{3}H]DALAMID, 1 nM [^{3}H]etorphine or 1 nM [^{3}H]diprenorphine, and 1 mM CHAPS, 10 mM Tris·HCl, pH 7.5. Specific binding is defined as the difference between the amount of radioactive ligand bound in the presence of 1 μM nonradioactive ligand after a 10-min incubation at 37°C.

Protein was measured by the method of Lowry *et al.* (8), with bovine serum albumin as a standard.

Solubilization—Receptors were solubilized from NG108-15 membranes according to the procedure described previously (6) with slight modification. Frozen, packed NG108-15 cells were suspended in 9 volumes of cold 0.32 M sucrose, 10 mM Tris·HCl, pH 7.5, and disrupted with 25 strokes of a ground-glass tissue grinder. The homogenate was centrifuged at 105,000 × *g* (40,000 rpm) for 30 min at 4°C. The pellet was resuspended in 2 volumes of cold 10 mM Tris·HCl, pH 7.5. After adding CHAPS to a final concentration of 10 mM, the suspension was rehomogenized and centrifuged at 105,000 × *g* for 60 min at 4°C. After centrifugation, the supernatant includes a floating layer of cloudy material on its surface. This material can be separated from the clear supernatant fraction as described previously (6); however, we have found that more efficient separation is achieved by gel filtration of the entire supernatant on columns of Sepharose 4B (or 6B) equilibrated in a buffer containing 1 mM CHAPS in 10 mM Tris·HCl, pH 7.5. The cloudy material, which is excluded from the gel, is eluted in the void volume and thereby separated from solubilized membrane proteins. Solubilized receptors are detected by assaying aliquots of each fraction for opiate binding and the appropriate fractions are pooled. Except where specifically stated, all of the binding experiments described below were done with solubilized, partially purified opiate receptors from NG108-15 hybrid cells.

RESULTS

The effects of GTP on binding of [^{3}H]DALAMID to solubilized receptors are illustrated in Fig. 1. In the absence of Na^+ (*open circles*), GTP has little effect on binding at concentrations less than 20 μM; binding is inhibited about 25% by 100 μM GTP. In the presence of 90 mM NaCl, binding is half-maximally inhibited by 10 μM GTP. Fig. 1 also shows that without added GTP, DALAMID binding is inhibited by the addition of salt to the assay mixture. This reduction in binding by NaCl is due partly to increased ionic strength and is not sodium-specific; similar amounts of Tris·HCl cause a similar reduction in binding (data not shown). The ability of sodium to enhance inhibition of binding by GTP is shared at least in part by other monovalent cations (Table I). In the presence of 50 μM GTP, all of the cations tested (as chlorides) enhanced inhibition of [^{3}H]DALAMID binding by GTP, but Na^+ was most effective. The salt dependence of GTP-induced inhibition was examined further by measuring binding in the presence and absence of 20 μM GTP at various concentrations of NaCl (Fig. 2). Some inhibition occurs in the absence of Na^+, with half-maximal inhibition occurring between 10–20 mM NaCl.

To study the ability of nucleotides other than GTP to

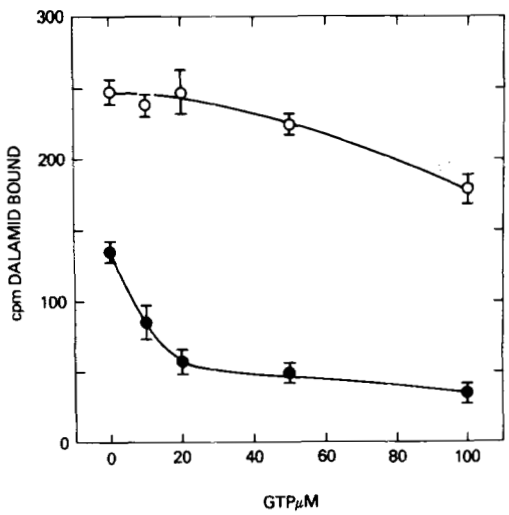

FIG. 1. **Inhibition by GTP of specific [^{3}H]DALAMID binding to solubilized opiate receptors in the absence (*open circles*) or presence (*closed circles*) of 90 mM NaCl.** Assays were performed as described under "Experimental Procedures." Each *point* represents the mean ± S.E. for quintuplicate determinations.

TABLE I

Effects of monovalent cations on opiate binding to solubilized receptors in the presence of GTP

Solubilized receptors (236 μg of protein) from NG108-15 membrane were assayed for specific [^{3}H]DALAMID binding as described under "Experimental Procedures," in the presence of 50 μM GTP and the ions listed (10 mM). Data are mean ± S.E. counts per min specifically bound for quadruplicate determinations.

Cation	[^{3}H]DALAMID specifically bound
	cpm
None	238 ± 8
Na^+	117 ± 20
Li^+	162 ± 8
K^+	153 ± 7
$Choline^+$	155 ± 13
NH_4^+	144 ± 5

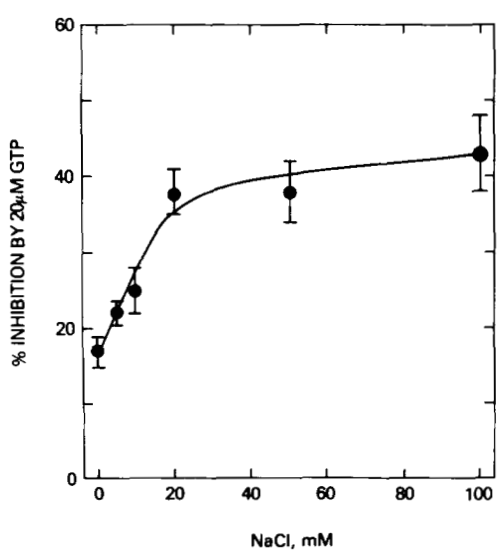

FIG. 2. **Effect of NaCl on GTP-induced inhibition of specific [^{3}H]DALAMID binding to solubilized opiate receptors.** Binding was measured in the presence and absence of 20 μM GTP at indicated salt concentrations. The *ordinate* is expressed as the per cent inhibition by GTP of specific [^{3}H]DALAMID binding. Each *point* represents mean ± S.E. for quadruplicate determination.

inhibit binding to solubilized opiate receptors, we measured specific binding of [^{3}H]etorphine in the presence of 10 mM NaCl and various nucleotides. As shown in Table II, GTP, GDP, and GppNHp all inhibited binding. The nonhydrolyzable analog GppNHp was slightly less effective than GTP or GDP. ATP and UTP inhibited binding slightly at 100 μM, but were ineffective at lower concentrations (data not shown). GMP and CTP had no effect. Similar results were obtained when [^{3}H]DALAMID binding was assayed in the presence of these nucleotides. Thus, the inhibition of binding by nucleotides is specific for guanosine di- and triphosphates.

Fig. 3 shows that inhibition of binding by GppNHp is due principally to reduced affinity of the receptors for opiate ligands. Specific binding of [^{3}H]DALAMID to soluble receptors was measured with (*closed circles*) or without (*open circles*) 50 μM GppNHp. The binding data were analyzed according to the method of Scatchard (9) as shown in the *inset* of Fig. 3. The apparent K_d for DALAMID increased from 7 nM to 17 nM in the presence of 50 μM GppNHp. The number of binding sites, however, was constant within the limits of experimental error (289 fmol/mg of protein without GppNHp and 252 fmol/mg of protein with GppNHp). The

TABLE II

Effect of nucleotides on [^{3}H]etorphine binding to solubilized opiate receptors

Solubilized receptors (146 μg of protein) from NG108-15 membranes were assayed for specific binding of [^{3}H]etorphine as described under "Experimental Procedures" in the presence of 10 mM NaCl and 100 μM nucleotide. Data are mean ± S.E. counts per min specifically bound for quadruplicate determinations.

Nucleotide	[^{3}H]Etorphine specifically bound
	cpm
None	525 ± 23
GTP	159 ± 30
GDP	178 ± 16
GMP	483 ± 20
GppNHp	283 ± 25
ATP	436 ± 26
UTP	435 ± 17
CTP	526 ± 27

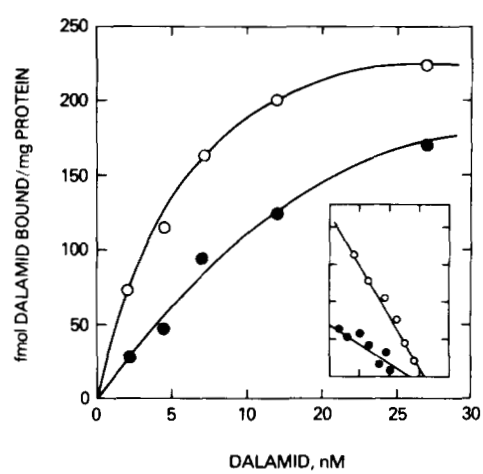

FIG. 3. **Binding of DALAMID to solubilized opiate receptors in the absence (*open circles*) and presence (*closed circles*) of 50 μM GppNHp.** All assays were performed in the presence of 10 mM NaCl and 2 nM [^{3}H]DALAMID. Nonradioactive DALAMID was added to achieve total ligand concentrations indicated. Data are means of quadruplicate determinations. An analysis according to the method of Scatchard (9) is shown in the *inset*. The *abscissa* is femtomoles of DALAMID bound, ranging from 0 to 80. The *ordinate* is the ratio of bound DALAMID to free DALAMID, ranging from 0 to 0.025. Lines of best fit were calculated by the least squares method.

relatively low affinities of the soluble receptor for DALAMID in this experiment compared to the value published previously (2 nM) (6) is due to the presence of 10 mM NaCl in these assays.

Previous reports showed that Na^+ and guanine nucleotides selectively inhibit binding of opiate agonists, but not antagonists, to NG108-15 membrane-bound receptors (3). We also found that binding of etorphine, a potent opioid agonist, to membrane-bound receptors was inhibited 46% in the presence of GTP whereas binding of diprenorphine, a closely related antagonist, was unaffected (Table III), even in the presence of 1 mM CHAPS (data not shown). In contrast, there was no agonist specificity of the GTP-induced inhibition of binding to solubilized receptors (Table III) since both etorphine and diprenorphine binding are inhibited equally. Furthermore, agonist binding to solubilized receptors was more sensitive to GTP than binding to membrane-bound receptors.

DISCUSSION

Opiate receptors in NG108-15 hybrid cells are coupled, as inhibitors, to adenylate cyclase (2, 10) and may be so coupled in brain as well (11). Hormonal control of adenylate cyclase involves a complex system believed to consist of at least three components: a receptor, a catalytic unit, and a regulatory coupling component which is sensitive to guanine nucleotides. It had previously been found, and we confirm here, that membrane-bound opiate receptors are sensitive to guanine nucleotides in the presence of Na^+ (3). Our results show that the binding affinity of opioids to receptors solubilized from NG108-15 hybrid cell membranes is significantly reduced by guanosine di- and triphosphates. Other nucleotides tested, including ATP, CTP, UTP, and GMP, are without appreciable effect. Guanine nucleotide inhibition requires the presence of Na^+ or other monovalent cation for maximal activity. Each of the opioid ligands tested gave similar results. Interestingly, we find that soluble opiate receptors are more sensitive to GTP inhibition than are the membranes of NG108-15 cells from which they are prepared.

The results described above suggest that the solubilized opiate receptors which we can detect are coupled to a guanine nucleotide regulatory protein. Receptors which are not so coupled may also have been solubilized, but either because of instability or reduced binding affinity may not have been observed in our assays. The 10–20% yield of soluble receptors which we have so far obtained (6) may therefore represent that fraction of receptor which is, on the average, coupled to the regulatory protein in the untreated membrane. In the β-adrenergic system, agonists (but not antagonists) induce the coupling of receptors to the nucleotide regulatory protein (12).

TABLE III

Differential effects of GppNHp on agonist and antagonist binding to NG108-15 membrane-bound and solubilized receptors

Specific binding of [^{3}H]etorphine and [^{3}H]diprenorphine to membranes (115 μg of protein) and solubilized receptors (119 μg of protein) from NG108-15 hybrid cells was measured as described under "Experimental Procedures," in the presence and absence of 100 μM GTP. Data are mean ± S.E. for quadruplicate determinations.

Ligand	Specific binding			
	Membrane-bound receptor		Solubilized receptor	
	Control	GTP	Control	GTP
	fmol/mg protein			
Etorphine (agonist)	609 ± 21	330 ± 12	102 ± 5	24 ± 2
Diprenorphine (antagonist)	704 ± 49	774 ± 20	91 ± 3	20 ± 5

In unpublished experiments, we found that treatment of membranes with opioid agonists prior to solubilization increases the yield of soluble receptors and Simon *et al.* (13) found that opiate receptors from rat brain can be solubilized with BRIJ-36T only as agonist-receptor complexes. These observations are consistent with the suggestion that free opiate receptors are either inherently unstable or of low affinity. It will be of interest to examine the GTP sensitivity of opiate receptors solubilized from amphibian (14) and rodent (6, 15) brains.

In contrast to our experience with soluble opiate receptors, Jeffery *et al.* have reported that β-adrenergic receptors, solubilized from turkey erythrocyte membranes with digitonin, are no longer sensitive to GTP inhibition (16). However, Welton *et al.* (17) found that the ^{125}I-glucagon which remains protein-bound after solubilization of liver plasma membranes is dissociated by GTP. Thus if, as is commonly supposed, GTP inhibition of hormone binding is mediated by a specific regulatory protein (5), soluble opiate and glucagon receptors are coupled to such a protein whereas soluble β-adrenergic receptors are not.

In our earlier studies (6), we found that the binding affinities of the receptor for a series of opioid ligands were unaffected by solubilization. Thus, soluble opiate receptors were indistinguishable from membrane-bound material by this criterion. The present finding that binding to soluble receptors of an almost pure antagonist (diprenorphine) (18) and of the agonists, etorphine and DALAMID, are inhibited by GTP was therefore unexpected since, in membranes, diprenorphine binding is unaffected by guanine nucleotides (Table III). This lack of discrimination between diprenorphine and etorphine by the opiate receptors after solubilization may reflect changed properties of either the opioid binding or guanine nucleotide regulatory proteins, or of both. Whatever the mechanism, the observation establishes a second criterion by which reconstitution of complete receptor function may be assayed. Thus, both restoration of agonist-antagonist discrimination and of coupling of receptor occupancy to adenylate cyclase can now be studied in reconstituted systems.

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