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Multiple Agonist-Affinity States of Opioid Receptors: Regulation of Binding by Guanyl Nucleotides in Guinea Pig Cortical, NG108-15, and 7315c Cell Membranes

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SUMMARY

Multiple affinity states of opioid receptors of the μ and δ types have been identified in membranes prepared from cells which bear only one type of opioid receptor (μ receptors in 7315c cells, δ receptors in NG 108-15 cells), and in guinea pig cortical membranes where both types of receptors were present in the membrane preparations. States of μ and δ receptors which have agonist affinities too low to be identified by radiolabeled agonist have been measured indirectly by agonist competition for sites labeled by radioactive antagonist. Using analogues of guanyl nucleotides, we have examined the competition of the μ and δ agonists DAGO and DSLET against [^{3}H]DIP or [^{3}H]NAL binding to opioid receptors and identified several agonist affinity states. In the absence of added nucleotide, competition of DSLET for [^{3}H]DIP binding to δ opioid receptors revealed the presence of two binding sites with differing apparent agonist affinities. Addition of GDPβS produced a steep monophasic curve which was best fit by a one-site model. In contrast, in the presence of added GTP or GTPγS, two affinity states were again apparent for DSLET competition at the δ receptor. The competition curve with GTP was shifted to the right relative to that produced in the absence of added guanyl nucleotide, indicating the presence of a lower apparent affinity state than any observed under other treatment conditions. DAGO competed against [^{3}H]DIP or [^{3}H]NAL binding to μ receptors over a wide concentration range in the absence of added guanyl nucleotide, consistent with the occupation by this ligand of more than one agonist affinity state of the μ receptor. However, when GDPβS was added to the incubation mixture, only a single binding site was identified. Two μ receptor affinity states were again observed in the presence of added GTP or GTPγS. One of these had significantly lower apparent affinity than those states detected in the absence of added nucleotide or with GDPβS. Pertussis toxin treatment resulted in a monophasic agonist competition curve which was best fitted by a single-site model in both 7315c and NG108-15 cell membranes. Addition of 100 μM GTP did not affect the agonist K_{app} or B_{max} after pertussis toxin treatment, suggesting that sites labeled under these conditions were not functionally associated with a G protein. In general, the effects of guanyl nucleotides were qualitatively similar at μ and δ receptors. The multiple apparent affinity states of each type of receptor probably reflect the preferential occurrence of different forms of agonist-receptor-G protein-guanyl nucleotide complex depending on the agonist or antagonist properties of the ligand and the guanyl nucleotides present.

Several reports of guanyl nucleotide regulation of opioid agonist binding in brain membrane preparations have suggested that these nucleotides act to decrease the binding of agonists to their receptors (1-4). The extent of inhibition for the agonists tested varied according to receptor type (3) and depended upon cationic content of the incubation buffer (2). In general, the effect of GTP addition to a membrane preparation appears to be a reduction in agonist affinity as a result of increased agonist dissociation rate (1, 5). The effects of guanyl nucleotides in regulating opioid agonist binding show several similarities to their effects on agonist binding at other receptors coupled to guanyl nucleotide-binding proteins (6, 7). Recent characterization of the proteins which bind these guanyl nucleotides (G

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ABBREVIATIONS: G_i, inhibitory guanine nucleotide-regulatory protein; G_o, guanine nucleotide-regulatory protein of unknown function; B_{max}, density of binding sites; BSA, bovine serum albumin; DADLE, [D-Ala^2-D-Leu^5]enkephalin; DAGO, Tyr-D-Ala-Gly-(Me)Phe-Gly-ol; DIP, diprenorphine; DMEM, Dulbecco's modified Eagle's essential medium; DSLET, [D-Ser^2,Leu^5]enkephalin-Thr; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; GDPβS, guanosine-5'-*O*-(2-thiodiphosphate); GTPγS, guanosine-5'-*O*-(3-thiotriphosphate); HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ICI 174,864,allyl$_2$-Tyr-(α-aminoisobutyric acid)$_2$-Phe-Leu-OH; K_{app}, apparent dissociation constant (macroscopic dissociation constant); K_D, equilibrium dissociation constant of labeled ligand; K_i, equilibrium dissociation constant of competing ligand; NAL, naloxone; U50,488H, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate hydrate; DPDPE, [D-Pen^2-D-Pen^5]enkephalin.

proteins) and the model proposed to describe the cycle of interaction of these proteins, nucleotides, and associated regulatory receptors (8) suggest that the assumption of a single affinity state of a particular receptor in the absence of added guanyl nucleotide and another in its presence may be too simplistic. Gilman (8) presented a cyclic scheme of G protein activation in which, depending on the nucleotides present, agonist may be bound to free receptor, receptor coupled to GDP-occupied G protein, receptor coupled to G protein from which GDP has dissociated, and receptor coupled to G protein occupied by GTP. The latter form is presumed to be unstable, resulting in the liberation of the α subunit of the G protein in association with GTP. This is the component of the system essential for activation of the effector system. It is also possible that the agonist-receptor complex liberated by dissociation from the G-GTP may exhibit an affinity different from that existing at its original formation, and certainly different from that observed when it is associated with the GDP- or GTP-G protein. Assuming that this cycle is continuously operative during the course of a binding incubation, at equilibrium, one is likely to label several forms of the receptor complex, each of which may have a distinct affinity. To further complicate the model, more than one G protein may be capable of interacting with opioid receptors. In NG108-15 cells, G_i appears to couple opioid receptors to adenylate cyclase (9). However, another G protein, G_o, which may also be involved in mediation of some opioid effects (10), has also been described in NG108-15 cells. This protein is presumed to be regulated in a manner similar to that of G_i. It is not clear if receptor bound to G_o has an affinity for agonist different from that of receptor bound to G_i.

Since addition of guanyl nucleotides lowers agonist affinity beyond that reliably detectable by a radiolabeled agonist binding paradigm (11), we have sought to exploit the relative insensitivity to guanyl nucleotides of opioid antagonist binding to label all available opioid receptors in guinea pig cortical membrane preparations. The effects of various guanyl nucleotides were evaluated by measurement of competition against labeled antagonist binding by selective opioid agonists over a broad concentration range in the presence and absence of added nucleotides. We have used three guanyl nucleotides in these studies, two of which are the nonhydrolyzable analogues of GTP and GDP. Incubations have been conducted in the presence of physiological concentrations of cations (12) in view of the synergistic effects of sodium and guanyl nucleotides on opioid agonist binding (2), and the requirement for GTP, sodium, and magnesium in activating adenylyl cyclase (13). We have used the nonselective antagonist [^{3}H]DIP to label each type of opioid receptor in guinea pig cortical membranes, and included blocking concentrations of site-selective opioids to preclude binding of the antagonist to sites not currently being studied. We have also used membranes prepared from cells which bear only μ (7315c) or δ (NG108-15) opioid receptors. The 7315c cells contain a homogeneous population of μ receptors (14) coupled to adenylyl cyclase (15) via a guanyl nucleotide-binding protein (16). Similarly, δ receptors in NG108-15 cells are coupled to adenylyl cyclase (17) via a guanyl nucleotide-binding protein (9). Experiments examining the effects of guanyl nucleotides on membranes prepared from these cells yield findings similar to those found for brain membranes, under conditions where there can be no complications arising from existence of other opioid receptor types. Pertussis toxin has been used to treat NG108-15 and 7315c cells in order to determine the agonist affinity for opioid receptors which are presumably uncoupled from their G-proteins (18).

Materials and Methods

Chemicals. Chemicals and reagents were obtained from the following sources: [^{3}H]DAGO (60 Ci/mmol), [^{3}H]DADLE (35 Ci/mmol), and [^{3}H]DIP (46.5 Ci/mmol) from Amersham Corp. (Arlington Heights, IL); [^{3}H]NAL (16–39 Ci/mmol) from New England Nuclear (Boston, MA); GTP, hypoxanthine, aminopterin, thymidine, BSA (fraction V), HEPES, GTP, and DSLET from Sigma Chemical Co. (St. Louis, MO); unlabeled DIP from Research Technology Branch, National Institute on Drug Abuse (Rockville, MD); unlabeled NAL from Endo Labs (Garden City, NJ); DPDPE, DADLE, DAGO, and ICI174,864 from Cambridge Research Biochemicals LtD. (Atlantic Beach, NY); GTPγS, and GDPβS from Boehringer Mannheim Biochemicals (Indianapolis, IN); DMEM and glutamine from Biofluids (Rockville, MD); fetal calf serum from KC Biochemicals (Lenexa, KS); and U50,488H from The Upjohn Co. (Kalamazoo, MI). Pertussis toxin was generously donated by Dr. T. E. Cote (Uniformed Services University of the Health Sciences).

Preparation of NG108-15 membranes. NG108-15 cells were grown as described previously (14). Briefly, the cells were grown in 75-cm^2 plastic tissue culture flasks containing DMEM, 0.1 μM hypoxanthine, 10 μM aminopterin, 17 μM thymidine, 2 nM glutamine, 0.1 M glucose, and 10% fetal calf serum at 37° in a humidified atmosphere of 10% CO_2 and 90% air. For studies involving pertussis toxin-treated NG108-15 membranes, cells were incubated in the presence of 30 ng/ml pertussis toxin for 36 hr. Prior to membrane preparation, cells were removed from flasks by rinsing with modified Krebs-HEPES buffer (25 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH adjusted to 7.4). This cell suspension was placed in plastic tubes and centrifuged twice at 450 rpm (40 × g) for 2 min. The pellet was disrupted by homogenization using a Teflon-glass homogenizer (four passes at speed setting 70 on a T-line laboratory stirrer; Thomas Scientific, Philadelphia, PA). The suspension was then washed twice with buffer and centrifuged at 10,000 × g for 15 min.

Preparation of 7315c membranes. The 7315c tumor was maintained by serial implantation in the peritoneal cavity of female Buffalo rats (40–60 g; National Cancer Institute, Frederick, MD). Membranes were prepared as previously described (15). Briefly, tumors were removed and minced finely with a razor blade in 10 ml of DMEM supplemented with 25% BSA. The cell suspension was filtered through gauze to remove large tissue fragments and red blood cells, and was centrifuged twice at 200 × g for 5 min. For studies involving pertussis toxin-treated membranes, 7315c cells were incubated at a concentration of 2 × 10^6 cells/ml DMEM/BSA in the presence of 30 ng/ml pertussis toxin. After this treatment, the cells were washed twice in DMEM/BSA and used to prepare membranes. Membranes were prepared from untreated or pertussis toxin-treated cells by a resuspension in 20 volumes of buffer containing 6 mM Tris-HCl (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, and 250 mM sucrose. The suspension was disrupted with two to three bursts of a Polytron generator (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged for 10 min at 1000 rpm (120 × g) and the resultant supernatant was then centrifuged at 15,000 rpm (25,000 × g) for 30 min. The pellet was resuspended in 6 mM Tris-HCL (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, and 10% glycerol. Membranes were stored under liquid nitrogen for later use. Prior to use the membranes were thawed and rinsed three times with modified Krebs-HEPES buffer.

Preparation of brain membranes. Male Hartley guinea pigs (200–500 g; Controlled Animal Management and Marketing, Wayne, NJ) were sacrificed by decapitation and their brains were removed to ice. Cortices were dissected and homogenized in 10 volumes of cold modified Krebs-HEPES buffer with a Teflon glass homogenizer driven by a T-Line laboratory stirrer (Thomas Scientific) at a setting of 70. The

homogenate was centrifuged at 27,000 × *g* for 15 min at 4°. The pellet was resuspended in 20 volumes of buffer and held on ice for 60 min to facilitate dissociation of any endogenous ligand. The suspension was then washed three times by centrifugation at 27,000 × *g* for 15 min at 4° and resuspended in ice-cold buffer. Membranes were stored frozen in modified Krebs-HEPES buffer at −70° at a concentration of 2% (w/v).

Binding assay. Cell or guinea pig cortical membranes were thawed at room temperature and homogenized by five strokes in a Teflon glass homogenizer. Binding assays were conducted in modified Krebs-HEPES buffer at 37°. The total volume in each tube was 0.5 or 1.0 ml. The final cortical membrane concentration after all additions were made was 1% (w/v) corresponding to 300–500 μg of protein/sample. Triplicate samples of membrane suspension were preincubated for 5 min with or without nonradioactive competing drug and guanyl nucleotide. Radiolabeled ligand was then added and the incubation continued for 20 min, by which time all radioligands used had achieved equilibrium. In experiments using the hydrolyzable guanyl nucleotides, a second addition was made at 10 min incubation time. The incubation was terminated by the addition of 4 ml of ice-cold buffer and rapid filtration through Whatman GF/B glass fiber filter paper using a Brandel (Gaithersburg, MD) Cell Harvester. The filters were washed with an additional 8 ml of buffer and transferred to scintillation vials. Absolute ethanol (0.5 ml) and Beckman EP Ready-Solv (2.75 ml) were added to vials which were then counted at an efficiency of about 30%. For saturation binding experiments, binding was measured at 15–18 concentrations of radioactive labeled ligand between 0.05 and 50 nM. For competition experiments, 21 concentrations of nonradioactive DIP or 28 concentrations of unlabeled DAGO or DSLET were used against a single concentration of radioligand chosen to be below the K_D of that ligand for the site being studied. When [^{3}H]DIP was employed as the primary ligand, its concentration was always 1.0 nM in order for sufficient dpm to be obtained for analysis. When a nonselective labeled ligand was employed in brain membrane experiments, site-selective blockers for the sites not being studied were used as follows: for μ, 5 μM DAGO; for δ, 5 μM ICI 174,864; for κ, 1 μM U50,488H. These concentrations of blocking agents were chosen from analysis of their potencies as competitors of each labeled ligand in the presence and absence of GTP under our incubation conditions. In saturation experiments for [^{3}H]DADLE, the blocking concentration of DAGO was 10 nM. Total [^{3}H]DIP binding was equivalent to that bound to μ, δ, and κ sites plus that defined as nonspecific. For graphical presentation, the nonspecific binding, as determined by the LIGAND computer program (19), was subtracted from total binding before plotting. The value for nonspecific binding for each fit shown is provided in the figure legends. Nonspecific binding as determined by 1.0 μM concentrations of antagonist was always approximately equal to that remaining in the presence of the highest concentration of competing agonist, and corresponded well to that computed by the LIGAND program. The estimated reliability of a reported value is indicated as a standard error of the parameter estimate calculated by the LIGAND program of the pooled data from two or more independent experiments, each of which contained triplicate samples at each of 21 or 28 different concentrations of unlabeled competing ligand.

Analysis of binding data. Saturation and displacement data were analyzed by the use of the computer program LIGAND (19). This program utilizes a nonlinear least squares curve-fitting algorithm and assumes the simultaneous contribution of one or more binding sites. All curves of a single treatment were modeled together to produce a set of parameter estimates and the associated standard errors of these estimates. The K_D values reported are the equilibrium dissociation constants derived by the program for the labeled ligands and the K_i values are the dissociation constants derived for the unlabeled ligands. In experiments where agonists have been used to compete against antagonist, our results are consistent with previous studies suggesting the existence of several bound forms of ligand. The K_i values calculated by LIGAND in this situation are therefore effective binding constants in which two or more sequential equilibrium reactions may participate. Since the estimated K_i values cannot be assigned to a specific reaction, we have chosen to describe such values as apparent dissociation constants (K_{app}). Nonspecific binding was always analyzed as a fitted parameter. In order to determine whether data from a guanyl nucleotide treatment produced a fit significantly different from that for control (or another guanyl nucleotide-treated) set of curves, curves were first fitted allowing all parameters to be computed iteratively. Then, the curves were fitted to fixed parameters obtained from control (or other treatment) curves. Curves were considered to be significantly different if the comparison of these fits yielded an F value associated with a $p < 0.05$.

Results

Determination of effective concentrations of guanyl nucleotides. We tested the effects of several guanyl nucleotides on the ability of μ and δ agonists to compete against [^{3}H]NAL binding in 7315c cell membranes, or against [^{3}H]DIP binding in NG108-15 and guinea pig cortical membranes. In order to ensure that the concentrations of guanyl nucleotides chosen were maximally effective, the effects of a range of concentrations of nucleotide on the binding of 1 nM [^{3}H]NAL or [^{3}H]DIP were tested in the absence or presence of either a low or high concentration of agonist. For μ binding, 20 and 500 nM concentrations of DAGO were used. For δ binding, 10 and 500 nM DSLET were used. The nonhydrolyzable analogues of GTP and GDP, GTPγS and GDPβS, were tested over a range from 0.01 μM to 100 μM, and GTP was tested over a range from 0.1 μM to 300 μM. Maximal reversal of agonist inhibition of ^{3}H-antagonist binding to both μ and δ sites in guinea pig cortical membranes and in 7315c and NG108-15 cell membranes was achieved by 10 μM for the nonhydrolyzable analogues and by 100 μM for GTP. These concentrations were chosen to further explore the pattern of agonist competition against antagonist binding.

Effects of guanyl nucleotides on radiolabeled agonist binding in guinea pig cortical membranes. Initial experiments in which we examined saturation binding of the μ-selective agonist [^{3}H]DAGO to guinea pig cortical membranes in the presence and absence of 100 μM added GTP showed that this manipulation lowered the K_D for [^{3}H]DAGO from 6.7 ± 1.1 nM to about 20 nM (n = 2). The standard error of the estimate of affinity in the presence of nucleotide was extremely large due to the reduction in bound dpm to values barely above nonspecific binding levels, making reliable quantitation impossible. The density of binding in the absence of added GTP was 30 ± 3.0 fmol/mg of protein and was not apparently changed in the presence of GTP. To confirm this affinity shift, we also performed competition of DAGO against [^{3}H]DAGO in the presence and absence of GTP and obtained similar results. In these experiments, the K_i for DAGO was shifted from 6.6 ± 1 nM to 21 ± 5 nM (n = 2). We also performed saturation analysis of δ binding in guinea pig cortical membranes using [^{3}H]DADLE in the presence of a blocking concentration of DAGO to prevent labeling of μ receptors. In the absence of added GTP, the K_D for [^{3}H]DADLE was 3.6 ± 0.6 nM, whereas in its presence this value was 20 ± 9.0 nM (n = 4). The B_{max} value was 20 ± 2.0 fmol/mg of protein in the absence of added GTP and 20 ± 7.0 fmol/mg of protein in its presence. Although the errors determined for the δ receptor binding parameters in the presence of GTP were much lower than those obtained for the μ site, the actual number of specific dpm was again very low.

Also, for both μ and δ receptors, the affinities calculated for the agonists in the presence of GTP were approximately equivalent to the highest concentrations giving measurable specific binding in the saturation binding experiments, making an alternate method of measuring agonist affinity under these circumstances necessary. We therefore decided to use labeled antagonist to bind to receptors, measuring agonist affinity in the presence and absence of guanyl nucleotides by competition experiments.

Effects of guanyl nucleotides on radiolabeled antagonist binding. In order to confirm that guanyl nucleotides had no effect on antagonist affinity for μ or δ receptors, we constructed saturation and competition curves for antagonists in the presence and absence of GTP. There was no significant change in the affinity of naloxone or DIP for μ-binding sites or of DIP for δ-binding sites (Table 1). We also tested GTPγS in saturation experiments on guinea pig cortical membrane preparations and found no differences in affinity compared to GTP or no added nucleotide (data not shown).

Guanyl nucleotide regulation of agonist apparent affinity at μ receptors. Having ascertained that the affinities of labeled NAL or DIP were not affected by the addition of guanyl nucleotide, we used these antagonists to radiolabel opioid-binding sites and examined the pattern of agonist competition produced in the presence and absence of various guanyl nucleotides. In the absence of any added guanyl nucleotide, DAGO competed against ^{3}H-antagonist binding to μ sites over 3.5 orders of magnitude (Fig. 1). In individual experiments, the control curves could be fit to one- or two-site models by the computer program LIGAND. However, when large numbers of control curves were modeled together simultaneously, the preferred model was for two sites, with a relatively smaller proportion existing in the lower affinity state (Tables 2 and 3). The shallow nature of the displacement curve supported the existence of multiple binding sites for DAGO in the absence of added guanyl nucleotide. The K_{app} values for these two sites were about 7 and 450 nM in 7315c cells and about 60 and 850 nM in guinea pig cortical membranes. The addition of GTP or GTPγS caused a slight rightward shift in DAGO competition curves, with a greater proportion of sites now appearing to be in the lower affinity state. This can be visualized as a slight plateau in the GTPγS curve as shown in the computer-generated plot in Fig. 1. In both cell and brain membranes, the lower affinity state of the μ receptor in the presence of added guanyl nucleotide had a K_{app} in the μM range (Tables 2 and 3). Although there is a relatively high error associated with the characterization of the state with very low apparent affinity, the results suggest that there is an increase in the number of low affinity receptors over those labeled in the absence of added nucleotide. The K_{app} of the higher affinity site in each tissue was only slightly increased by guanyl nucleotides. We also examined the competition of [^{3}H]NAL by DAGO in the presence and absence of GTP (n = 2) or GTPγS (n = 1) in cortical membranes and found a pattern similar to that observed when [^{3}H]DIP was used. In control membranes, DAGO competed against [^{3}H]NAL over 3 orders of magnitude and could be fit equally well to a one-site (K_{app} = 55 ± 10 nM) or a two-site (K_{app} values = 34 ± 12 and 1500 ± 1360 nM) model (n = 3). In GTP- or GTPγS- treated membranes, a two-site model was clearly preferred (K_{app} values = 23 ± 8 nM and 1700 ± 1300 nM). GTP and GTPγS curves were found to be significantly different from control but were not significantly different from one another.

In contrast to the complex competition curve observed with GTP and GTPγS, DAGO competition against ^{3}H-antagonist or in the presence of GDPβS yielded a competition curve which was best fit by a single-site model, with a K_{app} for DAGO of about 90 nM in cortical membranes and about 350 nM in 7315c cell membranes (Tables 2 and 3). The curves generated by the addition of GDPβS were slightly to the right of the control curve and somewhat steeper in shape, consistent with the labeling of a single type of receptor. In order to confirm that

TABLE 1

Effects of GTP on antagonist binding to μ and δ receptors in guinea pig cortical, 7315c, and NG108-15 membranes

Membranes were incubated for 20 min at 37° in modified Krebs buffer in the absence or presence of guanyl nucleotide. The number of independent replicate experiments is indicated in the table (N). Results were analyzed by a nonlinear curve-fitting algorithm (19). The tabulated values are the estimates of K_D or K_i ± the standard error of the estimate from a combined analysis of N independent experiments. All data obtained in the presence or absence of added nucleotide were analyzed together, and in each case a single-site model fitted the data better than more complex models. In saturation experiments, binding was measured in the presence or absence of 100 μM GTP. In competition experiments, the binding of 1 nM labeled antagonist was competed for by increasing concentrations of homologous unlabeled ligand in the presence or absence of 10 μM GTPγS.

Tissue	Experiment type	Labeled ligand	Receptor type	Guanyl nucleotide	K_i or K_D	N
					nM	
Guinea pig cortex	Saturation	DIP	μ	absent	0.39 ± 0.04	3
				present	0.35 ± 0.02	3
	Competition	DIP	μ	absent	0.20 ± 0.03	2
				present	0.19 ± 0.02	2
	Saturation	DIP	δ	absent	1.2 ± 0.23	3
				present	1.3 ± 0.17	3
	Competition	DIP	δ	absent	0.88 ± 0.09	2
				present	0.84 ± 0.08	2
7315c	Saturation	NAL	μ	absent	0.93 ± 0.3	2
				present	1.00 ± 0.45	2
	Competition	NAL	μ	absent	2.0 ± 0.5	2
				present	1.5 ± 1.0	2
NG108-15	Saturation	DIP	δ	absent	1.2 ± 1.0	2
				present	1.2 ± 0.5	2
	Competition	DIP	δ	absent	2.3 ± 1.0	3
				present	3.6 ± 1.0	3

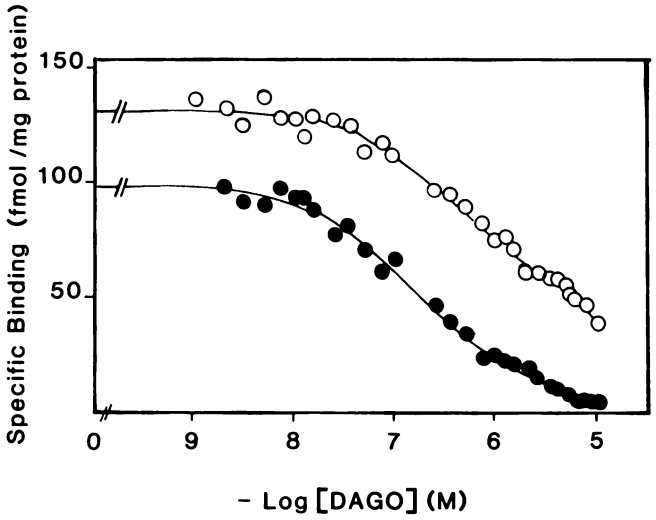

Fig. 1. Competition for [^{3}H]DIP binding to μ opioid sites in guinea pig cortical membranes by unlabeled DAGO in the presence (○) or absence (●) of 10 μM GTPγS. To prevent binding to δ or κ sites, 5 μM ICI 174,864 and 1 μM U50,488H were included in the incubation. The lines were traced from the LIGAND program best fits. The optimal models for these data were for two binding sites in either the presence or absence of added nucleotide. Nonspecific binding was a modeled parameter equal to 19% of total binding in the presence and 23% in the absence of added nucleotide. For direct comparison between treatments, this parameter has been subtracted in these plots. Data shown are from a single experiment which was representative of pooled data from three experiments for GTPγS addition and eight experiments for no added nucleotide.

the effect of GDPβS differed from that of GTP and GTPγS, we attempted to fit the two-site models which best fit these curves to the data obtained in the presence of GDPβS in both types of membranes. The GDPβS data were also compared with the optimum model for the control data. In each case, the curves were found to be significantly different from one another based upon the p value ($p < 0.05$) associated with the F test for each comparison.

Guanyl nucleotide regulation of agonist binding at δ receptors. When used to compete against [^{3}H]DIP, the δ receptor-preferring peptide DSLET clearly discriminated two classes of δ sites in the absence of added guanyl nucleotide in both guinea pig cortical and NG108-15 cell membranes under our binding conditions. The K_{app} values of DSLET at these sites were about 5 nM and 400 nM for both types of membranes (Tables 1 and 2). When GTP or GTPγS was added, two binding sites were still identifiable, but each with a slightly lowered affinity for DSLET (Tables 2 and 3, Fig. 2). At the δ receptor, the higher affinity site in the presence of GTP or GTPγS had a K_{app} for DSLET of about 2- to 5-fold higher than in its absence. The lower affinity site had a K_{app} which was shifted about 10-fold higher in the presence of added GTP or GTPγS. In contrast, when binding was measured in the presence of GDPβS, a single-site computer model produced the best fit, with a K_{app} of 20–40 nM (Fig. 3). Again, when computer fits for sets of like curves were compared successively to fits in which the parameters were constrained to values obtained from fits of control curves or those generated in the presence of other nucleotides, only the GTP and GTPγS curves were found not to differ significantly from one another. All other treatments produced optimal fits which differed significantly at $p < 0.05$.

Effects of guanyl nucleotides on agonist binding in pertussis toxin-treated NG108-15 and 7315c cell membranes. In order to determine the agonist K_{app} for the receptor uncoupled from its guanyl nucleotide-binding protein, competition studies were performed on pertussis toxin-treated membranes. [^{3}H]NAL displaced by DAGO in pertussis toxin-treated 7315c membranes yielded a monophasic competition curve which was fitted better by a single-site model than a two-site model. The single apparent agonist affinity state was approximately 10-fold lower than the high affinity state observed in control 7315c membranes (compare Tables 2 and 4). Similar results were obtained in pertussis toxin-treated NG108-15 membranes. [^{3}H]DIP binding competed for by DSLET resulted in a monophasic curve with a substantially lower agonist affinity than the high affinity state observed in control NG108-15 membranes (compare Tables 2 and 4). The pertussis toxin treatment of both the 7315c and NG108-15 cells appeared to decrease the total number of binding sites labeled by the ^{3}H-

TABLE 2

Opioid binding by NG108-15 and 7315c cell membranes in the presence and absence of guanine nucleotides

Cell membranes were incubated for 20 min at 37° in modified Krebs buffer in the absence or presence of 100 μM GTP or 10 μM guanine nucleotide analogues. The number of independent replicate experiments under each condition is indicated in the table (N). Results were analyzed by a nonlinear curve-fitting algorithm (19). The tabulated values are the estimates of K_i or B_{max} (± standard error of the parameter estimate) from a combined analysis of the results from N independent experiments. The concentration of [^{3}H]NAL was 1 nM and of [^{3}H]DIP was 1 nM.

Cell membrane	Radioligand	Unlabeled agonist	Guanine nucleotide	N	Agonist K_{app}	B_{max}
					nM	fmol/mg protein
7315c	NAL	DAGO	none	8	7 ± 2	28 ± 3
					465 ± 220	16 ± 20
			GDPβS	3	348 ± 110	32 ± 2
			GTP	3	18 ± 7	12 ± 7
					5200 ± 4000	51 ± 6
			GTPγS	3	36 ± 15	41 ± 4
					3600 ± 1200	60 ± 4
NG108-15	DIP	DSLET	none	8	3 ± 2	94 ± 9
					428 ± 52	28 ± 9
			GDPβS	3	20 ± 3	100 ± 3
			GTP	3	7 ± 5	100 ± 20
					4600 ± 2900	52 ± 14
			GTPγS	3	18 ± 4	52 ± 3
					5100 ± 4000	57 ± 10

TABLE 3

Opioid binding in guinea pig cortical membranes in the presence and absence of guanyl nucleotides

Parameters were derived by the computer program LIGAND (19), which models N independent experiments simultaneously and provides estimates of K_i and B_{max} ± standard error of these estimates. Other details of analysis and incubation procedures are provided under Materials and Methods.

Receptor type	Agonist	Guanyl nucleotide	N	Agonist K_{app}	B_{max}
				nM	fmol/mg protein
μ	DAGO	none	8	60 ± 5.4	65 ± 1.3
				850 ± 160	37 ± 4.1
		GDPβS	3	95 ± 35	53 ± 5.3
		GTP	3	40 ± 30	35 ± 7.1
				2900 ± 1500	45 ± 9.5
		GTPγS	3	99 ± 22	60 ± 3.6
				5200 ± 1800	51 ± 7.1
δ	DSLET	none	5	4.6 ± 3.1	53 ± 11
				370 ± 546	25 ± 9.9
		GDPβS	3	39 ± 8.2	61 ± 3.1
		GTP	3	26 ± 8.5	44 ± 4.0
				3200 ± 1100	39 ± 3.9
		GTPγS	3	15 ± 4.7	45 ± 4.0
				2700 ± 2400	37 ± 12

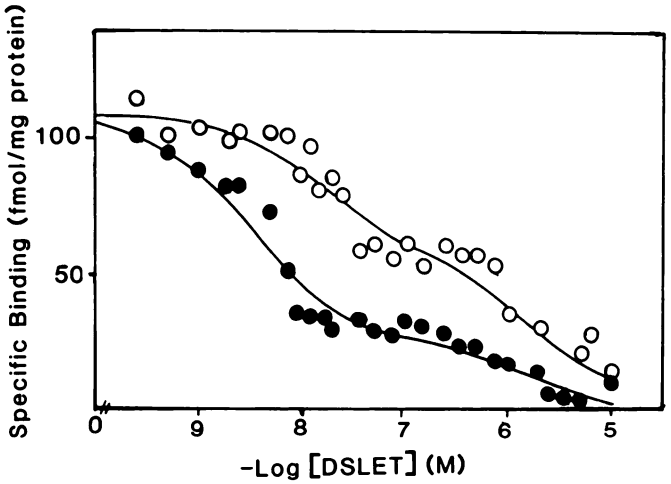

Fig. 2. Competition for [^{3}H]DIP binding to δ opioid sites in NG108-15 cell membranes by unlabeled DSLET in the presence (○) or absence (●) of 10 μM GTPγS. The lines were traced from the best fits generated by the LIGAND program. The optimal fits produced from these data were for two binding sites under each condition. Nonspecific binding was a modeled parameter equal to 36% of total binding in the presence and 37% in the absence of added nucleotide. For more direct comparison between treatments, this parameter has been subtracted in these plots. Other details of the binding are provided under Materials and Methods. Data are from a single experiment of three in the presence of GTPγS and eight in its absence which yielded similar results.

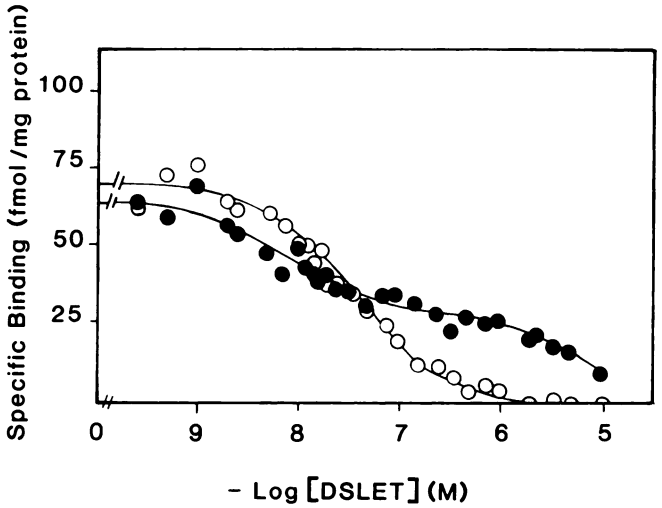

Fig. 3. Competition for [^{3}H]DIP binding to δ opioid sites in guinea pig cortical membranes by unlabeled DSLET in the presence (○) or absence (●) of 10 μM GDPβS. To prevent binding to μ or κ sites, 5 μM DAGO and 1 μM U50,488H were included in the incubation. The plot was traced from the LIGAND program-generated best fits. The optimal models for these data were for two binding sites in the absence and one binding site in the presence of nucleotide. Nonspecific binding was a modeled parameter equal to 37% of total binding in the presence and 40% in the absence of added nucleotide. For direct comparison between curves, this parameter has been subtracted in these plots. Data are from a single experiment which was representative of three experiments performed in the presence of added nucleotide and five in its absence.

antagonist. [^{3}H]NAL binding competed for by DAGO in control 7315c cell membranes resulted in a calculated total site density of 44 fmol/mg of protein. However, in pertussis toxin-treated 7315c cell membranes, the receptor density was decreased to 35 fmol/mg of protein. In control NG108-15 cell membranes, DSLET competition for [^{3}H]DIP binding yielded a total site density of 122 fmol/mg of protein. In pertussis toxin-treated NG108-15 cell membranes the receptor density was about 50 fmol/mg of protein. Addition of 100 μM GTP did not induce any additional change in affinity of the agonist or the receptor density in the pertussis toxin-treated membranes from either 7315c or NG108-15 cell membranes (Table 4).

Discussion

The findings reported herein demonstrate that multiple affinity forms of both the μ and δ opioid receptors can be identified in cell and guinea pig cortical membrane preparations by radiolabeling receptors of a single type with antagonist and examining the pattern of competition over a wide range of agonist concentrations. When [^{3}H]DAGO and [^{3}H]DADLE were used to characterize binding to μ and δ receptors in saturation and competition experiments in guinea pig cortical membranes, the limited range of labeled agonist concentrations that could be used for the estimation of specific binding pro-

TABLE 4

Opioid binding by pertussis toxin-treated NG108-15 and 7315c cell membranes in the presence and absence of GTP

Cells were treated with 30 ng/ml pertussis toxin for 36 hr. Cell membranes were incubated for 20 min at 37° in the absence or presence of 100 μM GTP. The number of independent replicate experiments under each condition is indicated in the table (*N*). Results were analyzed by a nonlinear curve-fitting algorithm (19). The tabulated values are the estimates of K_i or B_{max} (± standard error of the parameter estimate) from a combined analysis of the results from *N* independent experiments. The concentration of [^{3}H]NAL was 1 nM and of [^{3}H]DIP was 1 nM.

Cell membrane	Radioligand	Unlabeled agonist	[GTP]	*N*	Agonist K_{app}	B_{max}
			μM		nM	fmol/mg protein
7315c	NAL	DAGO	none	3	88 ± 24	35 ± 3
			100	3	73 ± 17	35 ± 4
NG108-15	DIP	DSLET	none	3	100 ± 37	47 ± 4
			100	3	82 ± 28	56 ± 4

hibited detection or characterization of sites which had low affinities for these peptides in the presence of added guanyl nucleotide. The guanyl nucleotide-dependent generation of very low agonist affinity states of opioid receptors has therefore been demonstrated by agonist competition against labeled antagonist binding (14, 20). The nonselective antagonist [^{3}H]DIP was used in most experiments. Estimates of its binding to specific types of opioid receptors in guinea pig cortex membranes were made in the presence of selective blocking agents. Labeled ligand binding to δ receptors was prevented by coincubation with the antagonist, ICI 174,864 (21), since receptor occupation by the antagonist should be unaffected by added nucleotide. Inspection of competition curves for ICI 174,864 against [^{3}H]DIP revealed a plateau from 2 μM to 10 μM in the presence or absence of added GTP. We therefore chose a concentration of 5 μM to block δ receptor binding in subsequent experiments. Selective antagonists for μ and κ receptors were not available when these experiments were initiated. We therefore used the agonist U50,488H (22) to block [^{3}H]DIP binding to κ sites. We have previously shown that κ agonist binding is relatively insensitive to guanyl nucleotide regulation (11). When U50,488H competed for [^{3}H]DIP binding to κ receptors, a plateau was observed at concentrations above 500 nM in either the presence or absence of added GTP. We therefore chose 1 μM to block binding to κ receptors in subsequent experiments. DAGO was used at a concentration of 5 μM to block [^{3}H]DIP binding to μ receptors. This concentration was selected after inspection of DAGO competition curves in the presence and absence of added guanyl nucleotides, and is a compromise between the concentration needed to occupy all μ sites, including the low affinity sites apparent in the presence of GTP or GTPγS, and the concentration at which significant occupation of δ receptors might occur in the absence of added guanyl nucleotides. It is unlikely that the relatively low concentration of [^{3}H]DIP used in our studies labeled to any significant extent additional classes of binding sites such as ε or σ which have been proposed by other investigators, since approximately equal K_{app} values for DAGO were obtained in guinea pig cortical membranes in competition against either 1 nM [^{3}H]DIP or [^{3}H]NAL binding to μ receptors. NAL saturation binding experiments conducted after blockade of δ and κ receptors produced linear Scatchard plots with a K_D of about 1 nM in the presence or absence of added nucleotide, suggesting binding only to μ receptors at low NAL concentrations. NAL has low affinity for sites which have been classified as ε (K_e about 15–30 nM) (23, 24) and does not bind to σ receptors (25). Our estimates of DSLET affinity for δ receptors (high affinity K_{app} about 5 nM) made in the presence of 5 μM DAGO are comparable to those reported elsewhere (12, 20, 26) in studies in which DAGO was absent or present at a much lower concentration. The properties of δ receptors in NG108-15 and guinea pig cortical membranes appeared very similar. We therefore consider that a meaningful estimate of δ receptor properties in guinea pig cortical membranes can be obtained in the presence of this concentration of DAGO under our experimental conditions. In most cases, the agonist affinities measured in guinea pig cortical membranes correspond reasonably well to those made in membranes prepared from cells which contain only a single population of opioid receptors, suggesting that the binding conditions used for brain membranes were appropriate for the selective labeling of either μ or δ receptors. However, there were differences between 7315c cell membranes and guinea pig cortical membranes in the computed K_{app} values for DAGO in the absence of added nucleotide. The reasons for this are unclear. It is possible they reflect real differences in the properties of the μ-type receptors in each tissue, but other factors may have contributed to this apparent difference. The DAGO competition curves against [^{3}H]DIP binding in guinea pig cortex were very shallow, but no clear plateau discriminating two (or more) affinity states was apparent. The curves were best fit by a two-site model, but proportioning of the curve between the two affinity states and, thus, the assignment of K_{app} values to each is problematic under these circumstances. Alternatively, it is possible that residual GDP and/or GTP in guinea pig cortical membranes contributed more significantly to modifications of μ receptor apparent affinities than at δ receptors. We also cannot exclude the possibility that similar receptors in each tissue were interacting with different G proteins which had the ability to induce different agonist affinity states of the receptors under some circumstances.

Our studies confirm that a small fraction of μ and δ receptors is present in a state with relatively low affinity for agonist even in the absence of added guanyl nucleotide. This fraction was increased by the addition of nucleotides, and in the presence of GTP or GTPγS, an extremely low apparent affinity form was observed at both μ and δ receptors. These very low affinity forms are most likely GTP-induced forms of μ and δ receptors (rather than low affinity agonist binding to non-opioid, GTP-sensitive binding sites) since: (i) these sites bound [^{3}H]NAL or [^{3}H]DIP with high affinity (K_D values of 2 nM or less), (ii) full competition by the μ agonist DAGO but not by the δ-selective ligand ICI 174,864 was observed at low affinity μ sites, whereas full competition by the δ agonist, DSLET, but not by DAGO was observed at low affinity δ sites, and (iii) similar opioid antagonist-binding sites with low agonist affinity were present in parallel with the high affinity μ or δ receptors in brain tissue and in two cell types (one of non-neuronal origin), suggesting an association between the low affinity and high affinity states.

Our studies therefore suggest that transitions between the various agonist affinity states of μ and δ receptors are regulated by guanyl nucleotides, supporting the conclusions reached by several other groups (1, 4, 5, 13, 27). After treatment with pertussis toxin, which is known to induce functional inactivation of a subset of G proteins (18, 28), only a single agonist affinity form could be discerned. The most reasonable interpretation of these results is that the effects of guanyl nucleotides on opioid agonist binding are mediated through receptor-G protein interactions, essentially as described by Gilman (8). The analysis presented here assumes that equilibria exist between free ligand-receptor complexes and each of the G protein-guanyl nucleotide-associated forms of the receptor, the relative proportions of each form being determined by the agonist/antagonist properties of the ligand and the properties of the guanyl nucleotides present. We have employed the program LIGAND (19) to analyze our data. The model on which this program is based has been demonstrated to approximate the ternary complex formation involved in the interaction of hormone with β-adrenergic receptor and component X, which was tentatively identified as the "G site" (29). We extend this model to include a series of reactions, assuming that all states concomitantly labeled exist in a macroequilibrium:

$$\begin{array}{lll} H + R & \rightleftharpoons H \cdot R & (1) \\ \quad \downarrow\uparrow + G \cdot \mathrm{GDP} & \quad \downarrow\uparrow + G \cdot \mathrm{GDP} & \\ H + R \cdot G \cdot \mathrm{GDP} & \rightleftharpoons H \cdot R \cdot G \cdot \mathrm{GDP} & (2) \\ \quad \downarrow\uparrow - \mathrm{GDP} & \quad \downarrow\uparrow - \mathrm{GDP} & \\ H + R \cdot G & \rightleftharpoons H \cdot R \cdot G & (3) \\ \quad \downarrow\uparrow + \mathrm{GTP} & \quad \downarrow\uparrow + \mathrm{GTP} & \\ H + R \cdot G \cdot \mathrm{GTP} & \rightleftharpoons H \cdot R \cdot G \cdot \mathrm{GTP} & (4) \\ \quad \downarrow\uparrow - \mathrm{G} \cdot \mathrm{GTP} & \quad \downarrow\uparrow - \mathrm{G} \cdot \mathrm{GTP} & \\ H + R' & \rightleftharpoons H \cdot R' & (5) \\ \quad \downarrow\uparrow & \quad \downarrow\uparrow & \\ H + R & \rightleftharpoons H \cdot R & (6) \end{array}$$

where H indicates agonist, R receptor, and G guanyl nucleotide-binding protein. R' represents a hypothetical conformation of the receptor formed by the dissociation of G·GTP. The affinity of R' for H may differ from that of R, and R' is presumed to revert to R. This expanded form of the full ternary complex model (29) implies that some interaction of receptor with G protein can occur in the absence of agonist, although it is assumed that the balance of the equilibria is such that this interaction is greatly facilitated by agonist.

The model suggests that at least five forms of agonist-receptor complex should exist under various experimental conditions, although these forms do not necessarily differ with respect to agonist affinity. The K_{app} values that are generated in the analysis must be considered as macroscopic equilibrium constants describing the summed equilibria pertaining under any particular experimental condition. The presence or absence of added guanyl nucleotide will tend to shift the equilibria in favor of different reaction products. Interpretation of the observed K_{app} values is complicated by the possibility that receptor interactions with different G proteins might lead to the formation of analogous agonist-receptor-G protein-nucleotide forms which differ in their apparent affinity for agonist, although this possibility is not supported by direct evidence. The errors inherent in this type of analysis also make it unlikely that affinity states whose K_{app} values do not differ by a factor greater than 10-fold could be discriminated. Nevertheless, some generalizations can be made.

Antagonist binding affinity was unaffected by guanyl nucleotides or pertussis toxin treatment. The single observed affinity state probably indicates that antagonist-bound receptor does not interact with G proteins. Agonist binding was clearly more complex. At both μ and δ receptors, pretreatment with pertussis toxin resulted in the appearance of a single affinity form for agonists which was unaffected by GTP. It seems likely that this reflects the predominant occurrence of reaction (1) under these conditions, yielding $H \cdot R$. However, we cannot exclude the possibility that the predominant bound form after pertussis toxin treatment is $H \cdot R \cdot G$, in which the G protein has been modified so that it can no longer interact with guanyl nucleotides or activate effector systems. The effects of GDPβS differed significantly from those of GTP or GTPγS at both μ and δ receptors. At both types of receptor, a single agonist form was apparent, with a K_{app} value in each case intermediate between the high apparent affinity form in untreated membranes and the lowest affinity form induced by GTP treatment. It seems reasonable to conclude that this reflects the predominance of reaction (2), yielding $H \cdot R \cdot G \cdot$GDPβS under these conditions.

Two apparent agonist affinity states were apparent at μ and δ receptors after GTP or GTPγS treatments, and the effects of these nucleotides appeared identical. The lowest affinity state after GTP treatment, with K_{app} values in the range 1–10 μM for DAGO at μ receptors and DSLET at δ receptors, was only observed in the presence of GTP or its analogue. Presumably, either the very low affinity forms or the higher affinity forms of bound agonist observed in the presence of GTP, or both, reflect the formation of $H \cdot R \cdot G \cdot$GTP or $H \cdot R'$ complexes. Spain and Cosica (27) have suggested, on the basis of guanyl nucleotide effects on agonist dissociation rate, that GTP may act at more than one regulatory site to influence agonist-receptor-G protein interactions. Thus, more than one affinity state may be related to the formation of the $H \cdot R \cdot G \cdot$GTP complex. In membranes assayed in the absence of added nucleotide, two apparent agonist affinity states were also noted at both μ and δ receptors. The highest affinity form showed a lower K_{app} value than that observed after pertussis toxin treatment. It is thus unlikely to be the $H \cdot R$ form; we tentatively suggest that the $H \cdot R \cdot G$ form without associated nucleotide is the most probable primary product. The nature of the lower apparent affinity state or states observed under these conditions cannot be identified at present. Residual bound endogenous nucleotide may account in part for the presence of this or these forms.

Electrophysiological studies have demonstrated that the conductance properties of K^+ channels opened by agonist activation of μ or δ receptors in rat locus coeruleus and guinea pig submucous plexus are essentially identical (30). The coupling between opioid receptors and the K^+ channels in these tissues was shown to involve GTP and to be inhibited by pertussis toxin treatment. Our results demonstrating the similarity of guanyl nucleotide regulation of agonist affinity at μ and δ receptors in guinea pig cortical membranes and in 7315c and in NG108-15 cells in consistent with the view that these receptor types operate through very similar effector systems. Furthermore, the guanyl nucleotide regulation of μ and δ receptors in guinea pig cortex (where a major consequence of receptor

activation is presumed to be opening of K^+ channels) and the nucleotide regulation of δ receptors in NG108-15 cells or of μ receptors in 7315c cells (where in each case the major known consequence of receptor activation is inhibition of adenylyl cyclase) appear to be very similar. In each case, the essential primary action of opioids seems to be activation of one or more G proteins. The mechanisms by which G protein activation is achieved in ion channel-coupled receptors in cortex or in adenylyl cyclase-coupled receptors in the cell cultures cannot be distinguished on the basis of effects of nucleotides on agonist binding affinity.

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