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GUANINE NUCLEOTIDE-MEDIATED INHIBITION OF OPIOID AGONIST BINDING

MODULATORY EFFECTS OF IONS AND OF RECEPTOR OCCUPANCY

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Abstract—We have analysed the potency of GTP, GDP and their analogues in reducing [^{3}H]DADLE binding to opioid receptors in NG 108-15 cell membranes. Under conditions where non-specific hydrolysis and transphosphorylation is inhibited, the following rank order of potency was found: GDP ≥ GTPγS > GTP > GDPβS ≥ GDPNH$_2$ > GppNHp ≫ GMP. Remarkably, the slopes for the inhibition curves of GTP, GDP and their thiosubstituted analogues, but not of GDPNH$_2$ and GppNHp, were extremely shallow, indicating either negative cooperativity or the existence of two states for the guanine nucleotide binding proteins, that both can mediate the effect of nucleotides on agonist receptor binding. The potencies of the different guanine nucleotide analogues, except that of GppNHp, were increased by the presence of sodium or chloride ions in the assay medium. Magnesium also affected GTP-mediated inhibition of opioid agonist binding since it decreased the IC_{50} of the nucleotide and steepened the slope of the inhibition curve. The IC_{50}s of nucleotides and the slopes of their inhibition curves were also dependent on the extent of receptor occupancy by the agonist. From these data we conclude that (1) either diphospho- or triphosphonucleotides can regulate agonist binding. (2) Magnesium, sodium and chloride, by acting at different components of the receptor/G protein complex produce similar effects on nucleotide mediated regulation of agonist binding. (3) A mutual influence exists between receptor occupancy by agonists and G protein-mediated guanine nucleotide effect on the receptor.

A variety of hormone or neurotransmitter receptors interact on the plasma membrane with GTP-binding regulatory proteins (G proteins).† Binding of guanine nucleotides to the α subunit of G proteins produces a decrease in the affinity of the receptor for its agonist (see reviews [1–4]). It is believed that agonists stabilize the ternary complex (HRG) between hormone, receptor and G protein, whereas guanine nucleotides destabilize this complex, yielding a low affinity agonist–receptor complex (HR) [5–7]. Different guanine nucleotides display different abilities to activate G proteins. GTP and its analogues, GppNHp and GTPγS are activators, whereas GDP and its analogues GDPNH$_2$ and GDPβS are unable to activate and act as competitive inhibitors of guanine triphosphates. The relationship between ability to induce an active state of the G protein and ability to promote the negative allosteric effect on agonist binding for various nucleotide analogues is not yet clear. The majority of studies have shown little or no differences between activating or inactivating guanine nucleotides in their ability to regulate receptor affinity [8–10]. Furthermore, in general the rank order of potency of guanine nucleotides for reducing agonist binding does not correlate with their potency for G protein activation, either examined in membrane preparation or in purified preparations of G proteins in solution [11]. Therefore it is not clear whether transmission of the allosteric effect of nucleotides onto the receptor involves or not the formation of the active state of the G protein. For the glucagon receptor in rat liver membranes (a receptor linked to G_s activation) it has been recently shown that, under condition in which the non-specific hydrolysis of GTP is inhibited, GDP is more potent than both GTP and GppNHp in reducing agonist affinity [12]. This difference in potency was further amplified by cholera toxin treatment of the membranes, a procedure which suppresses the GTPase activity of G_s [13]. The authors suggest that for receptor coupled to G_s the destabilization of the high-affinity ternary complex is preferentially mediated by GDP, and thus, by an inactive state of the G protein, and that the domain of the G protein involved in the interaction with the receptor is distinct and independent from that involved in the interaction with the effector system [12].

We have measured the potency of several guanine nucleotides in reducing opioid binding either in the presence and absence of a number of ions which

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† Abbreviations used: G proteins:guanine nucleotide binding, regulatory proteins; G_s: stimulatory- and G_i inhibitory to adenylate cyclase activity, G_o of other function; AppNHp: 5′-adenylyl imidodiphosphate; GppNHp: 5′-guanylylimidodiphosphate; GDPNH$_2$: guanylyl 5′-phosphoramidate; GTPγS: guanosine 5′-O-(3-thio-triphosphate); GDPβS: guanosine 5′-(2-thiodiphosphate); EGTA: ethylene-glycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid); DADLE: [D-Ala^2,D-Leu^5]enkephalin; IC_{50}: concentration that cause 50% of maximal inhibition.

are important for ligand-G protein interactions. We report here that (1) for the regulation of opioid agonist binding there is no preferential role for either diphospho- or triphosphonucleotides; (2) sodium, chloride and magnesium by apparently acting through different mechanisms, increase the potency of guanine nucleotides; (3) there is mutual influence between receptor occupancy by agonists and guanine nucleotide effect at G proteins.

MATERIALS AND METHODS

Materials

[^{3}H]DADLE (40 Ci/mmol) was purchased from Amersham Buchler GmbH (Braunschweig, F.R.G.); the unlabeled peptides DADLE and bestatin were obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland). GTP, GDP (Tris-salts), GMP, ATP (Na^+-salts), AppNHp (Li^+-salt), bacitracin, phosphocreatine (Tris-salt) and creatine phosphokinase (2500 U/mg) were obtained from Sigma Chemie (Deisenhofen, F.R.G.); GppNHp, GDPβS and GTPγS (Li^+-salt) from Boehringer (Mannheim, F.R.G.). Pertussis toxin was obtained from List Biological Labs (Campbell, CA, U.S.A.). All other chemicals were from either Merck (Darmstadt, F.R.G.), Sigma Chemie or Roth (Karlsruhe, F.R.G.) of the highest grade of purity available.

Experimental procedures

Cells and membrane preparation. NG 108-15 cells were a gift from Dr Nirenberg (National Institutes of Health, Bethesda, MA, U.S.A.) and grown in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (Gibco, Karlsruhe, F.R.G.) as described elsewhere [14]. For pertussis toxin treatment, confluent monolayers (10–15 × 10^6 cells) were incubated with the toxin (10 ng/ml) in growth medium 24 hr prior to cell harvesting. Cells were harvested in Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate buffered saline (Gibco), washed once in this buffer by low speed centrifugation and frozen as pellets at −70°. For membrane preparation, the frozen cell pellet was thawed (1 ml per 10^6 cells) in Tris–HCl (5 mM, pH 7.5, 1 mM DTT, 1 mM EGTA, herein referred to as buffer HB) containing 0.32 mM of sucrose. The cells were homogenized in this buffer by 20 strokes in a Dounce homogenizer (pestel A), and then centrifuged at 1000 *g* for 10 min. The pellet was resuspended in HB/0.32 mM sucrose and centrifuged a second time at 1000 *g* for 10 min. The combined supernatants were centrifuged in buffer HB without sucrose and recentrifuged at 40,000 *g* for 30 min. The final pellet was resuspended in buffer HB and stored as aliquots corresponding 2×10^7 cells (equivalent to 2 mg protein)/ml at −70° until use. Protein concentration was determined by the method of Peterson [15] using bovine serum albumin as standard.

Purification of nucleotides. The purity of the nucleotides was examined by HPLC as described previously [16]. GDP and GTP were both 99% pure and used without further purification. GppNHp and GTPγS exhibited a detectable degree of contamination (10% and 15%, respectively) with the corresponding diphosphate analogue, and were purified by column chromatography as described previously [16]. Briefly, concentrated solutions of the nucleotide were applied onto Diethylaminoethyl-Sephadex A 25 columns (A25, Pharmacia, Freiburg, F.R.G.), and eluted with an ammonium carbonate gradient. The elution profile was monitored by UV absorption at 254 nm. Using this procedure we were able to obtain a highly purified preparation of GppNHp, but not of GTPγS, since re-examination of the purified peak of this nucleotide revealed the same degree of GDP contamination as prior to purification. We suspect that the spontaneous hydrolysis of GTPγS to GDP is regulated by an equilibrium between the two compounds, since the 15% contamination with GDP of GTPγS preparations was stable even after prolonged storage. We did not attempt to investigate this problem further, and it has to be kept in mind that as much as 15% of the GTPγS preparation used in this study was actually GDP. GDPβS exhibited a low contamination with GMP (5–8%) which was not necessary to remove since the monophosphonucleotide has very low affinity to G proteins.

Stability of nucleotides. The stability of guanine nucleotides during the incubation with membranes was examined by thin layer chromatography as described previously [17, 18]. In the presence of ATP and AppNHp and absence of Mg^{2+}, guanine nucleotides were found to be stable (less than 10% degradation) for 60 min of incubation time at 30°. A stable concentration of GTP can be achieved also in the presence of Mg^{2+} when phosphocreatine (5 mM) and creatine phosphokinase (50 U/ml) were added.

Opioid binding studies. In a reaction volume of 300 μl [^{3}H]DADLE (1.5 nM, unless otherwise stated) was incubated at room temperature with membranes (30 μg protein) in potassium phosphate buffer (70 mM, pH 7.5, herein referred to as KPi buffer) containing AppNHp (1 mM), ATP (0.5 mM), bestatin (10 μM) and bacitracin (0.5 mg/ml). The amount of specific [^{3}H]DADLE binding and the effect of GTP on binding in KPi buffer was not different compared to that measured in Tris–Hepes (pH 7.5, 25 mM). To study the effect of Na^+ and Cl^- ions, we replaced 50 mM of KPi with either 50 mM of sodium phosphate buffer (pH 7.5) or 50 mM of KCl, and thus maintained in all experiments a constant ionic strength. Mg^{2+} was used as $MgSO_4$ or $MgCl_2$, and the free concentration of Mg^{2+} in the presence of 1 mM EDTA was calculated using LIGAND [19]. Guanine nucleotides were added at different concentrations, ranging from 1 nM to 1 mM. The reaction lasted for 1 hr and was stopped by addition of 2 ml of ice-cold KPi buffer and immediate filtration over GF/B Whatman glass fiber filters using a "cell harvester" (Brandel, Gaitherburgh, MA, U.S.A.). The filters were washed three times with 2 ml of ice-cold KPi buffer, and the amount of radioactivity retained by the filter was determined in a Packard 1500 scintillation counter, using 3 ml of Scintigel (Roth) per filter. Reactions were carried out as duplicate or triplicate determinations and the non-specific binding of [^{3}H]DADLE was measured in the presence of 10 μM unlabeled DADLE.

Data analysis. The data were analysed by the curve fitting program ALLFIT [20]. This program was used to determine the IC_{50} and the slope factor of the concentration response curves for guanine nucleotide mediated inhibition of opioid binding. These curves were further analysed with two mass action law-derived models describing the interaction of the nucleotide with, respectively, one or two binding sites:

model 1: $E/E_{max} = \alpha_1/(1 + K_{d_1}/[G])$

model 2:

$$E/E_{max} = \alpha_1/(1 + K_{d_1}/[G]) + \alpha_2/(1 + K_{d_2}/[G])$$

where: E/E_{max} is the fractional effect of the guanine nucleotide on agonist binding (assumed to be proportional to the fraction of bound nucleotide); α_1 and α_2 are the proportion of effect due to the high and low affinity site, respectively; K_{d_1} and K_{d_2} are the corresponding dissociation constants; [G] is the free concentration of guanine nucleotide. Curve fitting was accomplished using the algorithm of the computer program LIGAND [19] and the choice between the two models was based on the "extra sum of squares" principle [19].

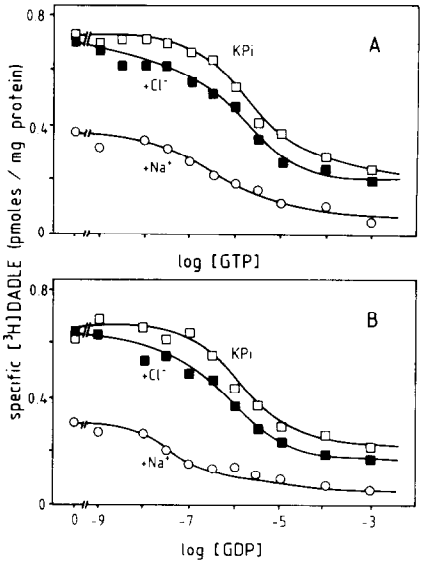

Fig. 1. Inhibition of specific [^{3}H]DADLE binding to opioid receptors in membranes from NG108-15 cells by increasing concentrations of GTP (upper panel) or GDP (lower panel). The reaction was carried out as described in "methods" in the absence (□) or presence of 50 mM Na^+ (○) or 50 mM Cl^- (■). Data points are means of duplicate determinations from one representative experiment, which has been repeated at least three times.

RESULTS

As documented previously, membranes from NG108-15 cells can rapidly alter the concentration of either natural or stable guanine nucleotide analogues by a combination of non-specific NTPase and transphosphorylation activities [18]. To obtain a stable concentration of a defined guanine nucleotide species during the opioid binding reaction, we included millimolar concentrations of adenosine nucleotides. The presence of ATP and AppNHp did not affect [^{3}H]DADLE binding (data not shown). Addition of guanine nucleotides to the opioid binding reaction resulted in a decrease of specific [^{3}H]DADLE binding which was explained by an apparent interconversion from high into low affinity state of opioid receptors. Complete conversion of the high affinity sites cannot be achieved even at the highest concentration of the nucleotides (not shown here [14, 18, 21]). To study the concentration–response relation of this nucleotide effect and to compare the potencies of several analogues, we examined the inhibitory effect of various concentrations of nucleotides on the specific binding obtained at a single, low concentration of agonist (1.5 nM [^{3}H]DADLE).

Effect of Na^+ and Cl^- on the potency of nucleotides to inhibit [^{3}H]DADLE binding

In the absence of chloride and sodium ions, the addition of GTP and GDP to the binding reaction produced a concentration-dependent reduction of tracer binding that reached an apparent plateau at millimolar concentrations of the nucleotides, and a maximal inhibition corresponding to 60–70% of the binding observed in the absence of nucleotides (Fig. 1A and B). The addition of either Cl^- (50 mM) or Na^+ (50 mM) to the reaction shifted to the left the apparent IC_{50} of the nucleotides. The shift produced by Na^+ was more pronounced than that produced by Cl^-. Na^+, but not Cl^-, produced in addition a direct inhibition of opioid receptor binding. Although both ions potentiated the inhibitory effect of nucleotides there was no apparent additivity upon addition of the two ions (as NaCl) simultaneously (data not shown). The effects of the ions were not limited to the natural guanine nucleotides but were also apparent for other guanine nucleotide analogues with the exception of GppNHp. The magnitude of the decrease of IC_{50} produced by the ions was dependent on the nucleotide investigated, being the larger, the greater was the actual potency of the nucleotide; however, there was no systematic difference between nucleotide di- and triphosphates in their sensitivity to Na^+ or Cl^- (see data summarized in Table 1).

Difference in the potencies between di- and triphosphonucleotides

To evaluate whether there was a systematic difference in potency between diphosphate or triphosphate guanine nucleotide analogues in inhibiting agonist binding to its receptor, we analysed the concentration–response curves of several guanine nucleotide pairs in the presence of either Na^+ or Cl^-. A clear difference between di- and triphosphonucleotides was apparent only for stable guanine nucleotide analogues (Fig. 2A and B). However, while GTPγS was more potent than GDPβS, $GDPNH_2$ was more active than GppNHp. The potencies of the various nucleotide analogues in the

Table 1. IC_{50}s and slope factor of the concentration–response curves of different guanine nucleotides in inhibiting [^{3}H]DADLE binding

	KP_i-buffer			+ 50 mM Cl			+ 50 mM Na^+		
	IC_{50} (μM)	Slope factor	N	IC_{50} (μM)	Slope factor	N	IC_{50} (μM)	Slope factor	N
GTP	3.8 ± 1.6	0.47 ± 0.08	3	1.42 ± 0.024	0.6 ± 0.04	10	0.52 ± 0.12	0.64 ± 0.07	3
GDP	2.1 ± 0.5	0.67 ± 0.07	3	0.52 ± 0.06	0.55 ± 0.03	12	0.1 ± 0.05	0.63 ± 0.03	3
GTPγS	2.8 ± 0.6	0.59 ± 0.002	2	0.58 ± 0.06	0.55 ± 0.02	7	0.22 ± 0.072	0.57 ± 0.14	3
GDPβS	11.7 ± 6	0.47 ± 0.09	1	3.8 ± 1.4	0.65 ± 0.14	4	2.43 ± 1.4	0.52 ± 0.03	2
GppNHp	22.1 ± 9.5	1.4 ± 0.6	2	14.0 ± 5	0.98 ± 0.4	4	26.4 ± 7.3	1.0 ± 0.3	3
$GDPNH_2$	7.1 ± 1.3	1.4 ± 0.3	1	4.97 ± 1	1.2 ± 0.3	1	1.44 ± 0.62	1.7 ± 0.2	2

Concentration response curves of several guanine nucleotides for inhibition of [^{3}H]DADLE (1.5 nM) binding to membrane from NG 108-15 cells were obtained in the absence and presence of Na^+ (50 mM) or Cl^- (50 mM). Means ± SE of computer fitted parameters (ALLFIT, see "methods") from N independent experiments are listed. In the case where only one experiment was performed (N = 1) the indicated error corresponds to that of the computed fit.

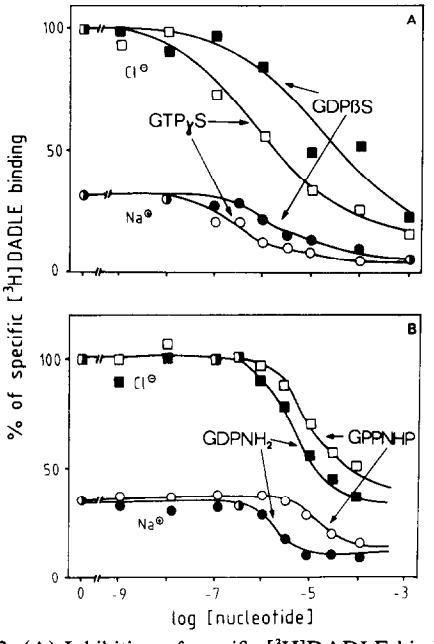

Fig. 2. (A) Inhibition of specific [^{3}H]DADLE binding by increasing concentrations of GTPγS (open symbols) and GDPβS (solid symbols). The reaction was carried out in the presence of 50 mM Cl^- (□, ■) or 50 mM Na^+ (○, ●). (B) Inhibition of [^{3}H]DADLE binding by increasing concentrations of GppNHp (open symbols) and $GDPNH_2$ (solid symbols) in the presence of 50 mM Cl^- (□, ■) or 50 mM Na^+ (○, ●). Data points are means of duplicate determinations from one representative experiment, that was repeated at least twice. 100% corresponds to 671 fmol/mg protein (A), and 541.67 fmol/mg protein (B).

absence and presence of Na^+ and Cl^- are summarized in Table 1. In the absence of Na^+ and Cl^-, the differences in potencies for the different guanine nucleotides were only small, whereas in the presence of Cl^-, and more clearly, of Na^+, substantial differences in potencies between guanine nucleotides were observed. The rank order of potencies of nucleotides in the presence of Na^+ was: GDP ≥ GTPγS > GTP > GDPβS > $GDPNH_2$ > GppNHp. GMP (not shown) was over an order of magnitude less potent than GppNHp, and its potency was also unaffected by ions. It is clear from this rank order of potency that there are no systematic differences in potency between guanine nucleotide di- and triphosphates.

Effect of Mg^{2+} on the potency of GTP

We have shown previously that the addition of Mg^{2+} to the binding reaction greatly accelerates the non-specific hydrolysis of nucleotides even in the presence of ATP and AppNHp [18]. This hydrolysis could be overcome by the addition of creatine phosphokinase and phosphocreatine as GTP regenerating system. However, under these conditions, the effect of Mg^{2+} ions can only be tested on GTP. Figure 3A shows that Mg^{2+} increased both the potency and the maximal effect produced by GTP. The inhibitory effect of GTP was also observed (although reduced) in the presence of 1 mM EDTA, suggesting either that the effect of GTP is transmitted to the receptor even the absence of free Mg^{2+}, or that nanomolar concentrations of Mg^{2+}, that could be present as contaminant even under this condition, are sufficient to maintain an effect of GTP. The effect of Mg^{2+} on the ability of GTP to inhibit agonist binding was apparently additive with that of Cl^- (Fig. 3B).

Effect of pertussis toxin treatment of NG 108-15 cells

Pertussis toxin treatment of NG 108-15 cells greatly reduced the effect of guanine nucleotides on agonist binding. As shown in Fig. 4, the binding of DADLE in membranes prepared from pertussis toxin treated cells was even lower than that observed in control cells at maximal concentration of guanine nucleotides. This binding activity was minimally affected by GTP, GDP and GTPγS. Thus, following pertussis toxin-mediated modification of G proteins the effect of nucleotides was abolished, regardless of their ability to serve as a substrate for the GTPase activity of G proteins.

Mass action law analysis of guanine nucleotide inhibition curves

A striking characteristic of the concentration–

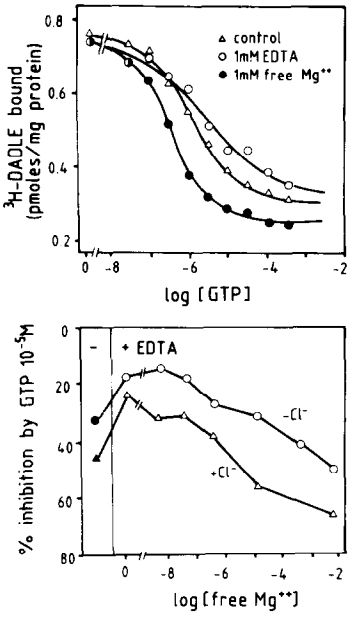

Fig. 3. (A) Inhibition of [^{3}H]DADLE binding by increasing concentrations of GTP in the absence (△) and presence of 1 mM EDTA (○) or 1 mM EDTA + 10 mM $MgSO_4$ (●). (B) Effect of 10 μM GTP on [^{3}H]DADLE binding, (expressed as per cent of inhibition), in the absence (solid symbols) or presence of 1 mM EDTA, and increasing concentrations of $MgSO_4$ (open symbols). The reaction was performed in the absence (○, ●) and presence (△, ▲) of 50 mM Cl^-. [^{3}H]DADLE binding in the absence of GTP was 614.4 fmol/mg in the absence and 631.1 fmol/mg in the presence of Cl^-, respectively.

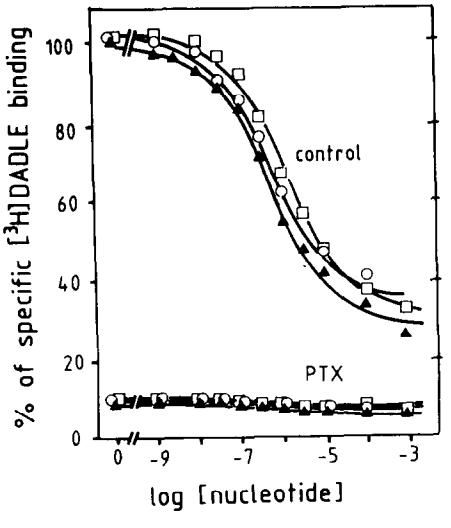

Fig. 4. Inhibition of [^{3}H]DADLE binding by increasing concentrations of GTP (■), GDP (▲) and GTPγS (○) in membranes from cells that were (PTX) or not (control) treated with pertussis toxin as described in "methods". Data are expressed as per cent of specific [^{3}H]DADLE binding in control cells in the absence of nucleotides (100% = 813 fmol/mg protein). The data are means of three independent experiments.

Table 2. Mass action law analysis of guanine nucleotide concentration–response curves in inhibiting [^{3}H]DADLE binding

	K_{d_1} (μM)	K_{d_2} (μM)	α_1 (%)	N
GTP	0.66 ± 0.092	37.8 ± 9.45	80.8 ± 9.3	3
GDP	0.28 ± 0.058	44 ± 9.7	83.0 ± 2.8	4
GTPγS	0.214 ± 0.026	72.1 ± 20.5	74.4 ± 1.4	4
GTPβS	0.724 ± 0.036	234 ± 104	73.9 ± 0.75	2
GppNHp	8.16 ± 2.4	—	100	3
$GDPNH_2$	5.98 ± 0.6	—	100	1

Inhibition of [^{3}H]DADLE binding by increasing concentrations of guanine nucleotide in the presence of Cl^- (50 mM) were analysed by computer according to models, based on the mass action law (see data analysis). A model based on two different affinity components (K_{d_1} and K_{d_2}) described the data significantly better for GTP ($P \leq 0.001$), GDP ($P \leq 0.008$), GTPγS ($P < 0.001$) and GDPβS ($P < 0.002$). For GppNHp and $GDPNH_2$ however a two-site-fit was either not possible or did not provide a significant improvement of the fit ($P = 0.6$). α_1 represents the % of total effect mediated through the high affinity component (displaying an affinity of K_{d_1}). The mean values (± SE) of the computer-fitted parameters from N experiments are listed in the table.

response curves of guanine nucleotide-mediated inhibition of [^{3}H]DADLE binding was their shallow slope factors. As summarized in Table 1, all guanine nucleotides, with the exception of GppNHp and $GDPNH_2$, inhibited [^{3}H]DADLE binding with a slope factor significantly lower than 1. This suggests that the interaction of nucleotide with the binding site that mediates the allosteric effect to the opioid receptor is complex and may involve two affinity components. Computer analysis of the concentration response curves for GTP, GDP, GTPγS, and GDPβS indicated that a model based on two affinity sites provided a significantly better description of the data (Table 2). In contrast the concentration response curves for GppNHp and $GDPNH_2$ were not better fitted with a model based on two sites as expected from their slope factors. The differences between high and low affinity sites for natural or thio-substituted nucleotides was of two to three orders of magnitudes and only 20–30% of the total nucleotide effect was mediated by the low affinity site.

Effect of receptor occupancy on guanine nucleotide concentration–response curves

To test whether the guanine nucleotide inhibition curves are changed by different degrees of initial occupancy of the receptor by the agonist, we examined the concentration–response curves of GTP, GDP, and GTPγS obtained at four different concentrations of [^{3}H]DADLE. As shown in Fig. 5, both slope factor and apparent potencies of guanine nucleotide were dependent on the concentration of opioid agonist used. This suggests that the proportion of high affinity component of the nucleotide effect and/or the apparent affinities of guanine nucleotides were altered by increasing the concentration of the tracer. Indeed, mass action law analysis of these curves indicated that for GTP and GDP, the proportion of effect that is mediated through the high

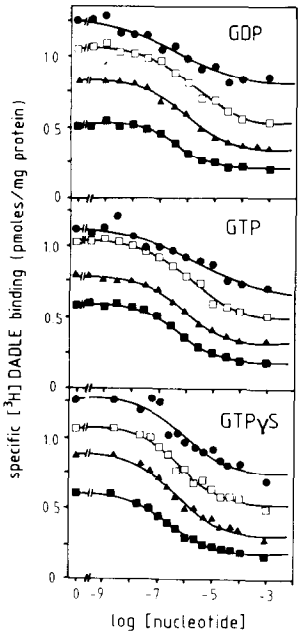

Fig. 5. Concentration–response curves of guanine nucleotide-mediated inhibition of [^{3}H]DADLE binding measured in the presence of 50 mM Cl^- with four different [^{3}H]DADLE concentrations: (■) 0.6 nM; (▲) 2 nM; (□) 5 nM and (●) 12 nM. Data points are mean values of duplicate determinations and the curves were fitted with ALLFIT as described in "methods".

affinity component was progressively reduced as the concentration of the agonist (and thus receptor occupancy) was increased. At high [^{3}H]DADLE concentration (12 nM) where nearly complete occupancy is attained, only 40% of the effect of GDP or GTP arises from the nucleotides' action at their high affinity site. At the lowest tracer concentration used (0.6 nM; corresponding to a fractional opioid receptor occupancy of 0.45) the total GDP effect and about 80% of the GTP effect occurred at the high affinity site for nucleotides. For GTPγS this effect was less pronounced: the high affinity site accounted for 80% of the GTPγS action at high opioid receptor occupancy and for 60% when the fractional opioid receptor occupancy was reduced to 0.45.

DISCUSSION

In this study we have investigated in detail the potencies of a number of GTP and GDP analogues in inhibiting opioid agonist binding. We also investigated the influence of ions (Cl^-, Na^+ and Mg^{2+}) and of different concentrations of [^{3}H]DADLE on the guanine nucleotide effect on receptor binding.

Rank order of potency of the nucleotides

Under conditions where non-specific hydrolysis and transphosphorylation of guanine nucleotides during incubation with membranes was minimized [18], we found the following rank order of potency for guanine nucleotide-mediated inhibition of opioid agonist binding: GDP ≥ GTPγS > GTP > GDPβS ≥ $GDPNH_2$ > GppNHp ≫ GMP. In several studies on guanine nucleotide-mediated regulation of G protein-coupled receptors the two stable GTP analogues GTPγS and GppNHp were found to be more effective than GTP or GDP [22–24]. However, since in most of these studies the non-specific degradation of the nucleotides was not inhibited, the rank orders reported therein may reflect the stability of nucleotides against non-specific NTPases. In a study where this degradation was controlled, the most potent nucleotide was found to be GDP [12]. Since, in addition, the hydrolysis resistant analogue GppNHp was very weak, the authors suggested that GDP and not GTP plays a key role in the G protein mediated allosteric regulation of receptor binding. Unfortunately GTPγS was not investigated in that study. From the data presented here, there is no indication for a preferential role of diphospho- compared with trisphosphonucleotides. In fact, while GDP was slightly more potent than GTP, and $GDPNH_2$ was more potent than GppNHp, GDPβS was clearly weaker than GTPγS. GTP was active in inhibiting opioid binding even in the presence of 1 mM EDTA, a condition under which hydrolysis at the G protein is greatly reduced. We thus conclude that the effect of GTP on agonist binding does not require its conversion to GDP. A further indication for this contention is that the nonhydrolysable analogue GTPγS was one of the most potent nucleotides in this study.

The 100-fold difference in potency between GppNHp and GTPγS and the intermediate potency of GTP observed in the present study, is in agreement with the apparent affinities of the nucleotide for purified Gα [11] and with the 100-fold difference in potency of the two stable nucleotides for the inhibition of DADLE-stimulated GTPase activity in NG108-15 cells [16]. The actual values of the affinities for Gα and the apparent K_i for enzyme inhibition are, however, significantly smaller than the IC_{50}s for the regulation of receptor binding described here. A similar discrepancy between the affinity of guanine nucleotide to G proteins and the potency in inhibiting receptor binding has been recently reported for the muscarinic receptor system [25], where the IC_{50} of GTP and GDP were approximately 20-fold higher than the apparent dissociation constants of these nucleotides. Thus, the effect of nucleotides on receptor agonist binding seems to require full occupancy at the nucleotide binding site.

Ion regulation of nucleotide effect

It is well known that NaCl is required for agonist-mediated inhibition of adenylate cyclase [2] and that this salt inhibits agonist binding to several receptors that are coupled to G_i/G_o [26]. We have shown before that the effect of NaCl on opioid receptor binding is synergistic with that of GTP [14] and that it is specific for the cation since it could not be mimicked by KCl. Recent data indicate that Cl^- and other monovalent anions, but not Na^+ modulate the affinity of guanine nucleotides for purified G proteins [11]. Here we show that not only Na^+ but also Cl^- increase the potency of guanine nucleotides to inhibit opioid receptor binding in membranes from NG108-15 cells, although only Na^+, and not Cl^-, directly affects opioid agonist binding in the absence of

nucleotides. The effects of Na^+ and Cl^- on the potency of guanine nucleotide-mediated regulation of opioid binding were not additive, nevertheless the two ions may not act at the same site. In fact, whereas Cl^- can be expected to act directly at the level of G protein [11], the Na^+ effect is unlikely to occur at a G protein for two reasons: (a) as mentioned above no effect of this cation was detected on purified G proteins [11] and (b) investigations on the Na^+ effect by target size analysis [27] suggested that it is mediated by a membrane component different from G proteins. In the study of Higashijima *et al.* mentioned above, Cl^- increased the affinity of GTP, but decreased that of GDP. The authors therefore assumed that Cl^- acts through a facilitation of the dissociation of $\beta\gamma$ from the $\alpha\beta\gamma$ complex of G proteins [11]. In our study no difference in the effect of Cl^- on the potency of diphospho- or triphosphonucleotides was observed. It is, however, interesting to note that the potency of the weakest nucleotide GppNHp was only marginally increased by Cl^- and seemed insensitive to Na^+. Zajac and Roques [28], by investigating the effect of GppNHp in crude rat brain membranes found that δ-opioid binding was only inhibited by this nucleotide in the presence of NaCl. This apparant discrepancy to our result may arise from the different membrane used (different content of the various G proteins). On the other hand it has to be noted that the degradation of GppNHp to $GDPNH_2$ [18] was not counteracted in their study, so that the effect may be due to $GDPNH_2$, a nucleotide that is also in our study sensitive to the action of NaCl.

There are multiple effects of Mg^{2+} on G protein–receptor interactions that occur in the nanomolar, micromolar and millimolar range of the cation [3]. Mg^{2+} at millimolar concentrations has been reported to antagonize the guanine nucleotide effect on agonists binding for several receptors [10, 29–31]. This antagonism might be explained by its ability to stimulate nonspecific nucleotide hydrolysis [18, 32]. Other studies in which the stability of GTP was ensured, report either no effect [12] or a reduction in the IC_{50} for GTP [33] in the presence of millimolar concentrations of Mg^{2+}. Here we show that Mg^{2+} is indeed facilitatory to the effect of guanine nucleotide inhibition of opioid binding and this effect occurred at micromolar concentrations of free cation. Thus, it is distinct from the requirement of nanomolar concentrations of Mg^{2+} for GTPase activity of G proteins [34] and rather correlates with the effect of Mg^{2+} in promoting hormone receptor-mediated increase of nucleotide binding to G proteins [34]. Furthermore, we show here that the effect of Cl^- is not competitive but actually additive to that of Mg^{2+}. This is consistent with the fact that the effect of Mg^{2+} and that of Cl^- are qualitatively different. Indeed, although both ions produced a decrease in the IC_{50} of GTP, Mg^{2+} and not Cl^- steepened the slope of the nucleotide inhibition curve of opioid binding.

Shallowness of guanine nucleotide inhibition curves

An important finding of this study is that the concentration–response curves for guanine nucleotide-mediated reduction of agonist binding were extremely shallow. Notable exceptions were $GDPNH_2$ and GppNHp, for which steep slopes were observed. Complex kinetics of guanine nucleotide binding even to purified $G\alpha$ subunits have been described and explained by a tight binding of endogenous GDP [35]. This mechanism could also be the reason for the shallow curve of the nucleotide effect on opioid binding described here. However, preincubation of the membranes with high concentrations of ammonium sulfate, a procedure that has been demonstrated to release GDP from G proteins [35], did not alter the slope of the inhibition curves (data not shown). In addition, if the presence of slowly exchangeable GDP were the reason for the flatness of the curves, this phenomenon should also be observed for GppNHp and $GDPNH_2$, which is not the case. Mass action law analysis of the inhibition curves of [^{3}H]DADLE binding by guanine nucleotides were consistent with a model based on two affinity sites (or states) for the interaction of the nucleotides except for GppNHp and $GDPNH_2$. The difference in affinity between the two nucleotide binding sites was in the range of two to three orders of magnitude. Therefore, it is unlikely that this heterogeneity can be explained by differential affinities of guanine nucleotides for G_i and G_o (both present in membranes from NG108-15 cells), since with purified material it has been shown that the difference in affinity of nucleotides for these two G proteins is usually lower than one order of magnitude [7]. The existence of a different type of G protein, not yet characterized, that has very low affinity for guanine nucleotides and interacts with opioid receptors seems also unlikely, since, as shown here, PTX treatment of the cells abolished the nucleotide effect in a non-competitive way and almost completely. Furthermore, we have previously shown that the magnitude of the GTP effect on opioid binding is indeed dependent on the amount of PTX substrate in the membrane, since the reduced amount of G proteins in the microsomal membranes from NG108-15 cells is correlated with a lower effect of GTP on the receptors present in that membrane preparation [21]. The possibility that G proteins bear a second low affinity binding site seems also unlikely as indicated by cross-linking experiments with affinity labeling guanine nucleotides on purified subunits [36]. Thus, it is conceivable that the shallowness of the guanine nucleotide inhibition curve does not arise from two independent nucleotide binding sites, but indicates the existence of two different states of G proteins both mediating the allosteric effect of nucleotides on agonist occupied receptors. In favour of this is the fact that the high affinity component and the proportion of effect mediated through that high affinity component were changed as the occupancy of the opioid receptor was increased. These two distinct states of G proteins may result from dissociation or association of the $\beta\gamma$ subunit to $G\alpha$ in the membranes, for which dependency from agonist occupancy of the receptor has been shown [1, 3, 4]. It has been recently reported for example that the addition of $\beta\gamma$ potentiates the guanine nucleotide effect on receptors reconstituted with purified $G\alpha_i$ or $G\alpha_o$ in liposomes [37]. We may therefore hypothesize that the holocomplex $\alpha\beta\gamma$ represents the high affinity component, and that the free α-subunit is responsible for the low affinity effect of nucleotides on agonist

binding. According to the ternary complex model in its simple form [5] binding of guanine nucleotides to G proteins results in a dissociation of the receptor/G protein complex, thereby producing a low affinity receptor state. This model does not predict a shallow slope for the inhibition of agonist binding by increasing concentrations of the nucleotides, and, in fact, no such effect was observed by Rojas and Birnbaumer [12] for the guanine nucleotide effect on glucagon receptor (a receptor linked to G_s). We therefore suggest that an extension of the ternary-complex model (that would include perhaps the role of $\beta\gamma$ subunit) is necessary for its application to nucleotide-mediated regulation of agonist binding at the family of receptors coupled to G_i/G_o.

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