Site of Pertussis Toxin-Induced ADP-Ribosylation on Gi Is Critical for Receptor Modulation of GDP Interaction with Gi

Marilyn E. Gosse, Elizabeth A. Frey, and Thomas E. Cote

Department of Pharmacology
Uniformed Services University of the Health Sciences
Bethesda, Maryland 20814-4799

In this study, the influence of the inhibitory μ-opioid receptor on the potencies of 5′-guanosine α–thiotriphoshate (GTPγS) and GDP at the inhibitory GTP–binding protein (Gi) were investigated in an adenylyl cyclase system. It was hoped that a receptor-mediated change in the potency of either GTPγS or GDP in affecting adenylyl cyclase activity may elucidate how a receptor alters cyclase activity via its G-protein. In an adenylyl cyclase system employing 5′-adenylyl imidodiphosphate as substrate, GTPγS, a nonhydrolyzable analog of GTP, inhibited forskolin-stimulated adenylyl cyclase activity in the absence of morphine; morphine failed to significantly affect the apparent potency of GTPγS. GDP blocked the GTPγS-induced inhibition of adenylyl cyclase; morphine profoundly diminished the ability of GDP to block the inhibitory effect of GTPγS. The IC_{50} values of GTPγS were 0.02 ± 0.01, 0.18 ± 0.04, and 2.2 ± 0.5 μM in the absence of other drugs, in the presence of a combination of 100 μM GDP and morphine, and in the presence of 100 μM GDP, respectively. GDP blocked the inhibitory effect of GTPγS (0.3 μM) in a concentration-dependent manner; the EC_{50} for GDP was 16 ± 2.6 μM in the absence of morphine and 170 ± 32 μM in the presence of morphine. Exposure of 7315c cells to pertussis toxin for 3 h resulted in a small decrease in the potency of GTPγS in inhibiting cyclase. However, the relative potency of GDP in blocking the GTPγS-mediated inhibition of cyclase was increased: the EC_{50} values of GDP were 11 ± 4 and 0.81 ± 0.2 μM in untreated and pertussis toxin-treated membranes, respectively. In untreated membranes, there was a brief lag in the GTPγS-induced inhibition of adenylyl cyclase; morphine diminished this lag. In membranes treated with pertussis toxin, there was an exaggerated lag in the onset of GTPγS inhibition of adenylyl cyclase activity; morphine could no longer affect this lag. Thus, uncoupling the μ-opioid receptor from Gi appeared to increase the affinity of Gi for GDP. These data suggest that the effect of an inhibitory receptor is to decrease the affinity of Gi for GDP by virtue of its interaction with the carboxy-terminal region of Giα. Since intracellular concentrations of GTP and GDP are both approximately 100 μM, occupancy of the μ-opioid receptor by an agonist would greatly increase the chances that GTP, rather than GDP, would interact with and activate Gi. (Molecular Endocrinology 3: 315–324, 1989)

0888-8809/89/0315-0324$02.00/0
Molecular Endocrinology

INTRODUCTION

The signal transduction mechanism of hormones that regulate adenylyl cyclase has been studied extensively since the discovery that GTP was required for maximal glucagon stimulation of adenylyl cyclase activity (1). The subsequent discovery that GTP was also required for receptor-mediated inhibition of adenylyl cyclase activity, light activation of cGMP phosphodiesterase, receptor-mediated stimulation of phosphatidylinositol metabolism, and, most recently, receptor-mediated alterations in ion channel activity has led to the realization that multiple GTP-binding proteins are involved in a variety of signal transduction systems (2–14). The purification of a number of these GTP-binding proteins has allowed investigators to study the ability of various purified receptors to stimulate GTPase activity associated with these G-proteins after the reconstitution of these purified components in phospholipid vesicles (see Refs. 13 and 14 for comprehensive reviews).

From studies performed on purified receptors and G-proteins reconstituted in phospholipid vesicles, it has been proposed that a fundamental function of a receptor is to enhance the exchange of GDP for GTP by diminishing the binding of GDP to its G-protein (13–15). To date, this proposal has never been tested in an adenylyl cyclase system. Therefore, in the current study, we have investigated the ability of the well characterized μ-opioid receptor (16–19) to alter the influence of GDP on adenylyl cyclase in membranes prepared from 7315c tumor cells. In this model system, 5′-guanosine α-thiotriphosphate (GTPγS) was used to inhibit forskolin-stimulated adenylyl cyclase activity. 5′-

Adenylyl imidodiphopsphate [App(NH)p] was used as a substrate for adenylyl cyclase, since its use obviates the phosphorylation of GDP to GTP (20, 21). In this system we have demonstrated that activation of the μ-opioid receptor with morphine diminished by approximately 10-fold the potency of GDP in blocking the inhibitory effect of GTPγS.

We have further demonstrated that treatment of 7315c cells with pertussis toxin, a treatment that covalently modifies Gi at a site critical for interaction with inhibitory receptors (22–30), resulted in a 10-fold greater potency in the ability of GDP to block GTPγS-induced inhibition of adenylyl cyclase and an exaggerated lag in the onset of GTPγS-induced inhibition of adenylyl cyclase. In untreated membranes, activation of the μ-opioid receptor with morphine diminished the lag in the GTPγS-induced inhibition of adenylyl cyclase activity. Taken together these data suggest that Gi binds GDP with high affinity. The interaction of the unoccupied μ-opioid receptor with Gi decreases the affinity of Gi for GDP, while activation of the μ-opioid receptor by an agonist decreases further the affinity of Gi for GDP, insuring that GTP, rather than GDP, will bind to and activate Gi.

RESULTS

It has been proposed that a primary effect of an agonist is to enhance the exchange of GDP for GTP at the G-protein (13–15, 31). Therefore, in the current study, it was of interest to determine if stimulation of the μ-opioid receptor could influence the interaction of either GTP or GDP with Gi, as assessed by changes in adenylyl cyclase activity. In a previous study of the 7315c cell, GTP was shown to cause a concentration-dependent inhibition of adenylyl cyclase activity in the presence of morphine when ATP was used as the substrate for cAMP; the potency (IC_{50}) of GTP was 1 μM (16). In the current study, GTP was shown to cause a similar concentration-dependent inhibition of adenylyl cyclase activity in the presence of morphine when App(NH)p was used as substrate; the IC_{50} of GTP was 0.69 ± 0.14 μM (Fig. 1A). In contrast, GDP failed to support morphine-induced inhibition of adenylyl cyclase (Fig. 1B). However, GDP was able to shift the concentration-response curve of GTP to the right; a 7.1 ± 1.2-fold higher concentration of GTP ($P < 0.05$ *vs.* no GDP) was required to inhibit adenylyl cyclase activity when 100 μM GDP was included in the assay (Fig. 1C). In the presence of morphine and 10 μM GTP, GDP caused a concentration-dependent blockade of the morphine-induced GTP-dependent inhibition of adenylyl cyclase (Fig. 1D). Half-maximal blockade occurred at a GDP concentration of 81 ± 10 μM. These data are consistent with the hypothesis that the hydrolysis of GTP to GDP at Gi terminates the activation of Gi by GTP (13–15).

It has been shown previously that Gi can be activated by nonhydrolyzable GTP analogs in the absence of an inhibitory agonist (8 27–30, 32). As is shown in the *inset* of Fig. 2, GTPγS inhibited forskolin-stimulated adenylyl cyclase activity with IC_{50} values of 45 ± 2.9 and 42 ± 6.5 nM in the absence and presence of 10 μM morphine, respectively. Thus, stimulation of the opioid receptor had no significant effect on the activation of Gi by GTPγS.

Although morphine failed to significantly affect the ability of GTPγS to inhibit forskolin-stimulated adenylyl cyclase activity, morphine profoundly diminished the potency of GDP in blocking the inhibitory effect of GTPγS (Fig. 2; GDP *vs.* GDP plus morphine, $P < 0.05$). GTPγS inhibited adenylyl cyclase activity with IC_{50} values of 0.02 ± 0.01, 0.18 ± 0.04, and 2.2 ± 0.5 μM in the absence of other drugs, in the presence of a combination of 100 μM GDP and morphine, and in the presence of 100 μM GDP, respectively. Morphine had a similar effect on the ability of GDP to block the inhibitory effect of guanylyl imidodiphosphate [Gpp(NH)p], another nonhydrolyzable GTP analog, on forskolin-stimulated adenylyl cyclase activity (not shown); Gpp(NH)p inhibited adenylyl cyclase activity with IC_{50} values of 0.13 ± 0.04, 2.0 ± 1.5, and 19 ± 5.9 μM in the absence of other drugs, in the presence of a combination of 100 μM GDP and morphine, and in the presence of 100 μM GDP, respectively.

The time course of GTPγS-induced inhibition of adenylyl cyclase activity revealed an initial lag during the first 3 min of the assay (Figs. 3A, *inset*, 5 and 6). From 3–12 min, the GTPγS-induced inhibition of cyclase was linear; the inclusion of GDP (10 μM) in the assay partially blocked the GTPγS-induced inhibition of cyclase. The ability of GDP to cause a concentration-dependent blockade of the GTPγS-induced inhibition of forskolin-stimulated adenylyl cyclase activity was also investigated in the absence and presence of morphine; the EC_{50} values of GDP were 16 ± 2.6 and 170 ± 32 μM in the absence and presence of morphine, respectively (Fig. 3A; $P < 0.05$). 5′-Guanosine β-thiodiphospate (GDPβS), an analog of GDP that is a poor substrate for phosphorylation, was also tested for its ability to block the inhibitory effect of GTPγS (Fig. 3B); the EC_{50} values of GDPβS were 50.7 ± 5.5 and 290 ± 85 μM in the absence and presence of morphine, respectively ($P < 0.05$). Thus, stimulation of the μ-opioid receptor diminished by approximately 10-fold the potency of GDP in blocking the interaction of GTPγS with Gi.

Since agonist activation of the μ-opioid receptor caused a 10-fold decrease in the ability of GDP to interact with Gi, we speculated that the opioid receptor itself may have a negative influence on the interaction of GDP and Gi. Previous studies have suggested that pertussis toxin, by virtue of its ADP ribosylation of the α-subunit of Gi (Giα), uncouples Gi from its interaction with the receptor but does not prevent guanine nucleotides from interacting with Gi or Gi from inhibiting adenylyl cyclase activity (22–30). Therefore, in the present study pertussis toxin was tested for its ability to affect the interaction of GDP with Gi. As has been

shown previously (8), treatment of 7315c cells for 3 h with pertussis toxin (30 ng/ml) abolished the ability of morphine to inhibit adenylyl cyclase activity (data not shown). Pertussis toxin treatment also increased by approximately 2-fold ($P < 0.05$) the IC_{50} of GTPγS in inhibiting adenylyl cyclase activity (Fig. 4A). In membranes from cells incubated for 3 h in the absence of pertussis toxin, GTPγS inhibited adenylyl cyclase activity with an IC_{50} of 19 ± 1.5 nM; in membranes prepared from pertussis toxin-treated cells, GTPγS inhibited adenylyl cyclase activity with an IC_{50} of 44 ± 1.6 nM. Pertussis toxin also appeared to diminish the efficacy of GTPγS in inhibiting adenylyl cyclase. In membranes from cells incubated for 3 h in the absence of pertussis toxin, GTPγS inhibited forskolin-stimulated adenylyl cyclase activity by 81 ± 1.1%; in membranes from cells incubated for 3 h in the presence of pertussis toxin, GTPγS inhibited forskolin-stimulated adenylyl cyclase by 54 ± 4.4%. In the same membranes, GDP was tested for its ability to block the inhibitory effect of GTPγS. The EC_{50} values of GDP in blocking the inhibitory effect of 0.3 μM GTPγS were 11 ± 4 and 0.81 ± 0.20 μM in control and pertussis toxin-treated membranes, respectively ($P < 0.05$; Fig. 4B).

In stimulatory systems, nonhydrolyzable GTP analogs have been reported to stimulate adenylyl cyclase activity after an initial lag; the inclusion of a stimulatory agonist in the assay system has been shown to abolish the lag, presumably by enhancing the release of GDP from Gs (13). In the current study, the time course of the inhibitory effect of GTPγS on forskolin-stimulated adenylyl cyclase activity was investigated (Fig. 5A). In the absence of GTPγS, forskolin caused a linear stimulation of adenylyl cyclase activity; cAMP was formed at the rate of 64 ± 6.1 pmol cAMP/(mg protein·min). The inclusion of GTPγS diminished the rate of formation of cAMP. During the first 3 min of the assay, cAMP was formed at a rate of approximately 41 ± 10 pmol cAMP/(mg protein·min); between 3 and 10 min, cAMP was formed at the rate of approximately 22 ± 7.5 pmol cAMP/(mg protein·min).

Morphine was tested for its ability to affect the lag in the GTPγS-induced inhibition of adenylyl cyclase (Fig. 6). In these experiments ATP was used as substrate to enhance the level of cAMP formed at the early time points [lower amounts of cAMP are made when App(NH)p is used as substrate]. In this study morphine had no effect on the formation of cAMP in the presence of forskolin, but significantly enhanced the inhibition of adenylyl cyclase by GTPγS at 1 min.

The time course of the inhibitory effect of GTPγS on forskolin-stimulated adenylyl cyclase activity was also investigated in pertussis toxin-treated membranes (Fig. 5B). In the absence of GTPγS, forskolin caused a linear stimulation of adenylyl cyclase activity; cAMP was formed at a rate of 55 ± 2.7 pmol cAMP/(mg protein·min). There was a prolonged lag before GTPγS caused a substantial inhibition of adenylyl cyclase activity. During the first 7 min of the assay, cAMP was formed at the rate of 44 ± 2.6 pmol/(mg protein·min) in the presence of GTPγS; from 7–10 min, cAMP was formed at the rate of 17 ± 1.8 pmol/(mg protein·min). Thus, in pertussis toxin-treated membranes, there is a prolonged lag in the GTPγS-induced inhibition of adenylyl cyclase activity. This lag accounts for the diminished efficacy of GTPγS in inhibiting adenylyl cyclase activity in pertussis toxin-treated membranes and is consistent with the proposal that GDP is bound with greater avidity to Gi in pertussis toxin-treated membranes than in control membranes.

DISCUSSION

A fundamental assumption of the current hypothesis of signal transduction by G-proteins is that activation of a membrane receptor by its agonist in some way causes GDP to be replaced by GTP at the G-protein (13, 15). The question then becomes what property of Gi becomes altered that results in the preferential binding of GTP over GDP at the G-protein. In 1978 Cassel and Selinger (15) discovered that stimulation of the β-adrenergic receptor on turkey erythrocyte membranes enhanced the release of [^{3}H]GDP from the membranes. Subsequently, inhibitory receptors were also shown to enhance the release of [^{3}H]GDP from mammalian cell membranes (33). More recently, carbachol complexed to the muscarinic cholinergic receptor has been shown to reduce the affinity of Gi for GDP when both purified components were reconstituted in phospholipid vesicles (34). On the basis of these studies, it has been proposed that the primary function of an agonist-activated receptor is to enhance the release of GDP from its G-protein. The results of the present study are consistent with this proposal and demonstrate, for the first time, the relevance of guanine nucleotide exchange at Gi to the inhibition of adenylyl cyclase activity. In the present study, agonist activation of the μ-opioid receptor caused a small, but insignificant, increase in the potency of GTPγS in inhibiting adenylyl cyclase activity. We assume that GTPγS accurately reflects how GTP acts at Gi, except that GTPγS remains active at Gi for an extended period of time by virtue of its resistance to hydrolysis. On the other hand, agonist activation of the μ-opioid receptor had a striking effect on the ability of GDP to block the GTPγS-induced inhibition of adenylyl cyclase activity. Since the intracellular concentrations of both GTP and GDP are approximately 100 μM in the few tissues in which they have been measured (20, 21, 35), agonist activation of the receptor would insure that GTP, rather than GDP, would bind to and activate Gi. Conversely, in the absence of an agonist, Gi would have an enhanced affinity for GDP and thus maintain the system in an inactive state.

Since an opioid agonist must act through its receptor to affect the interaction of guanine nucleotides with Gi, it was of interest to determine if the μ-opioid receptor itself (unoccupied by an agonist) could exert a negative influence on the interaction of GDP with Gi. Pertussis toxin has been shown to prevent the interaction of

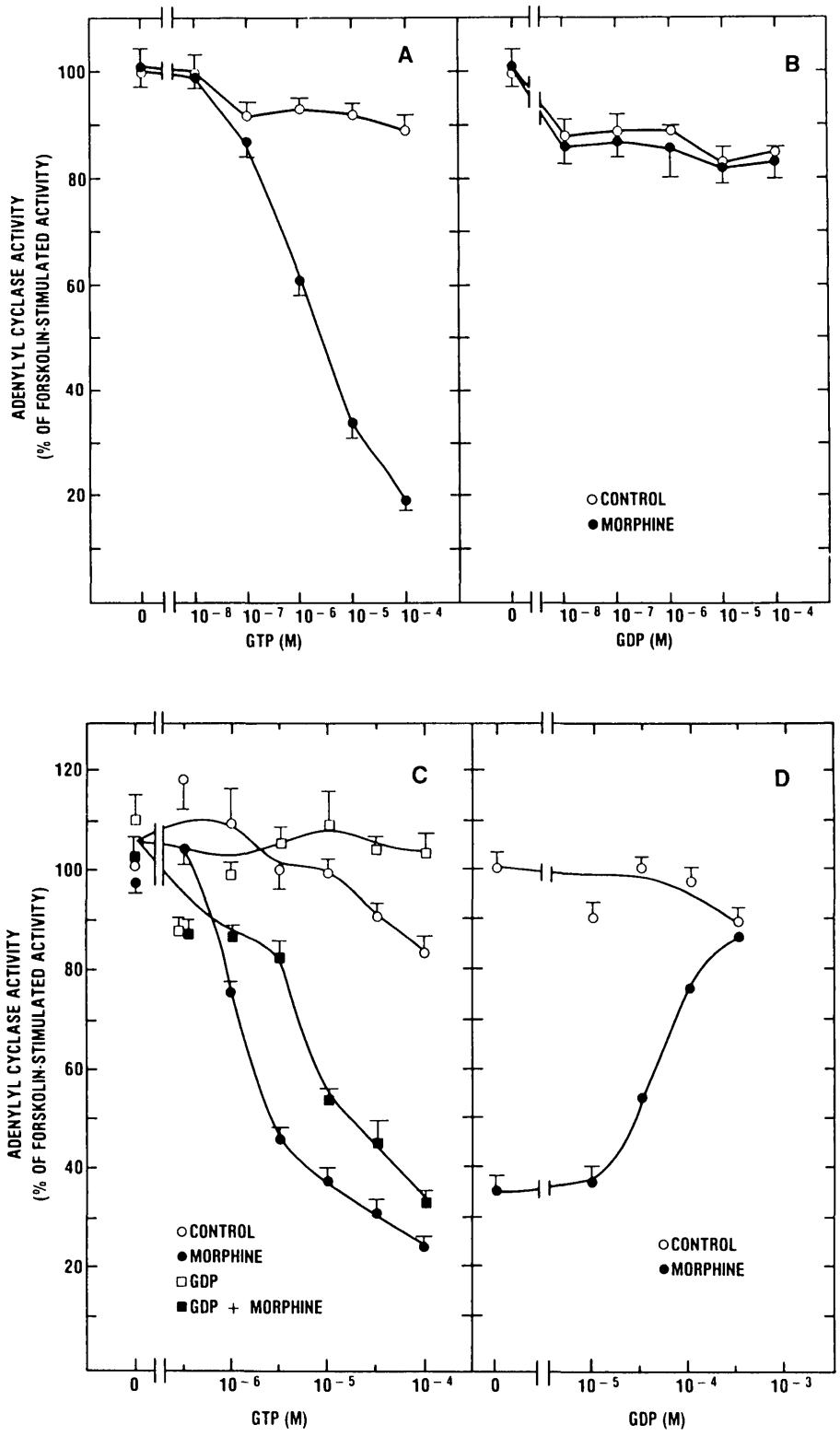

Fig. 1. GTP Is Required for Opioid Inhibition of Adenylyl Cyclase Activity; GDP Blocks the Inhibitory Effect of GTP in the Presence of Morphine

Adenylyl cyclase activity was determined in the presence of App(NH)p and 10 μM forskolin as described in *Materials and Methods*. A, GTP was tested in the absence (○) or presence of 10 μM morphine (●). B, GDP was tested in the absence (○) or presence of 10 μM morphine (●). Data represent the mean ± SEM (n = 3) obtained in a representative experiment expressed as a percentage of the activity determined in the presence of 10 μM forskolin alone. Data presented in A and B were from the same experiment. In four independent experiments, adenylyl cyclase activity was 2.5 ± 0.3 and 43 ± 4.1 pmol cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively. C, GTP was tested in the absence of other drugs (○), in the presence of 10 μM morphine (●), in the presence of 100 μM GDP (□), or in the presence of a combination of 10 μM morphine and 100 μM GDP (■). Data represent the mean ± SEM (n = 3) obtained in a single experiment expressed as a percentage of the activity determined in

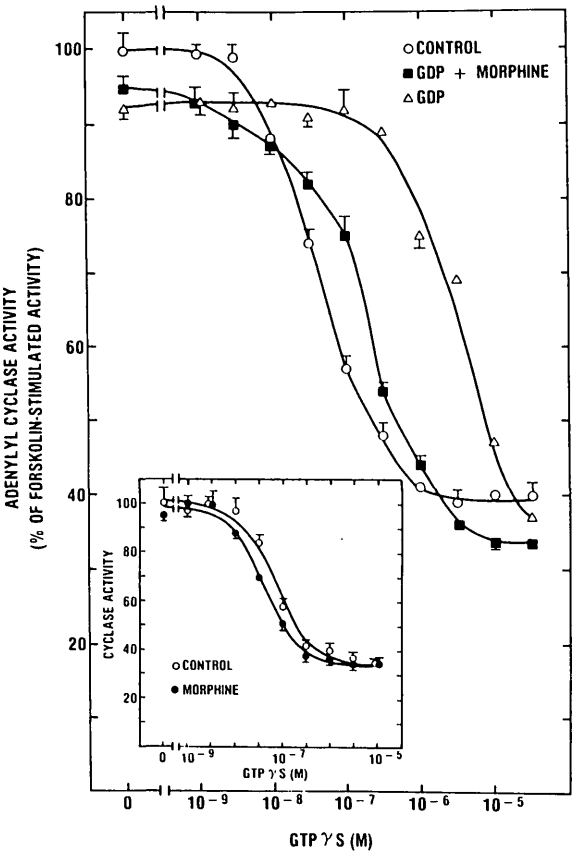

Fig. 2. Morphine Diminishes the Ability of GDP to Block GTPγS-Induced Inhibition of Forskolin-Stimulated Adenylyl Cyclase Activity

Adenylyl cyclase activity was determined in the presence of App(NH)p and 10 μM forskolin as described in *Materials and Methods*. GTPγS was tested alone (○), in the presence of 100 μM GDP (△), or in the presence of a combination of 100 μM GDP and 10 μM morphine (■). Data represent the mean ± SEM (n = 3) obtained in a representative experiment, expressed as a percentage of the activity determined in the presence of 10 μM forskolin. In three independent experiments, adenylyl cyclase activities were 2.0 ± 0.1 and 41 ± 4.3 pmol cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively. *Inset*, GTPγS was tested in the absence (○) and presence of 10 μM morphine(●). Data represent the mean ± SEM (n = 3) obtained in a single experiment, expressed as a percentage of the activity determined in the presence of 10 μM forskolin. In three independent experiments, adenylyl cyclase activity was 1.7 ± 0.7 and 31 ± 2.2 pmol cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively.

receptors and Gi by virtue of the ADP ribosylation of the cysteine residue near the carboxy-terminus of Giα (22–30). Apparently, the carboxy-termini of Gs_α, Gi_α, Go_α the α-subunit of the GTP-binding protein with unknown funtion (Go_α), and the α-subunit of transducin (Gt_α) are all critical for each G_α to interact with its appropriate receptor (36). In the current study, 7315c cells were incubated with pertussis toxin (30 ng/ml) for 3 h, since these conditions were previously found to abolish the ability of morphine to inhibit adenylyl cyclase activity and to reduce by more than 70% the radiolabeling of Gi_α upon subsequent exposure to [^{32}P]NAD and pertussis toxin (8). Pertussis toxin had only a small effect on the IC_{50} of GTPγS in inhibiting adenylyl cyclase. However, the efficacy of GTPγS was substantially diminished. A comparison of the time courses of GTPγS-induced inhibition of adenylyl cyclase in control and pertussis toxin-treated membranes revealed that the difference in the efficacy of GTPγS in the two experimental groups was due to an exaggerated lag in the onset of inhibition of cyclase in the pertussis toxin-treated membranes. The most striking effect of pertussis toxin treatment was the enhanced apparent potency of GDP in blocking the inhibitory effect of GTPγS; the EC_{50} of GDP was reduced by approximately 10-fold after pertussis toxin treatment. We suspect that the pertussis toxin-induced tighter binding of GDP to Gi explains the exaggerated lag in the GTPγS-induced inhibition of cyclase; consistent with this proposal was the finding that activation of the receptor in control membranes diminished the lag in the activation of Gi by GTPγS. The enhanced apparent potency of GDP and the exaggerated lag in the onset of cyclase inhibition in the pertussis toxin-treated membranes are consistent with the notion that the unliganded μ-opioid receptor itself exerts a negative influence on the interaction of GDP with Gi by interacting with the carboxy-terminal of Gi_α (36); the binding of an agonist to the receptor amplifies the negative influence of the receptor.

Support for the notion that an unliganded receptor can influence the interaction of guanine nucleotides with its G-protein is provided from studies of the Unc cell line. Unc cells contain a mutant form of Gs that is uncoupled from its receptor due to a point mutation near the carboxy-terminus of the α_s subunit; although uncoupled from its receptor, this mutant Gs remains coupled to adenylyl cyclase (36). Studies performed on these cells over a decade ago revealed that a nonhydrolyzable GTP analog was less effective in stimulating cyclase in Unc membranes than in wild type membranes (37).

The current report of the negative influence of the μ-opioid receptor on the interaction of GDP with Gi may be related to a recent study performed on the purified subunits of Go, Go_α, and βγ (38). In this study, it was discovered that βγ enhanced the binding of GDP to Go_α by more than 100-fold. Interestingly, the βγ-subunit

the presence of 10 μM forskolin. In three independent experiments, adenylyl cyclase activity was 2.7 ± 3 and 34 ± 5 pmol cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively. D, GDP was tested in the presence of 10 μM GTP (○) or in the presence of a combination of 10 μM GTP and 10 μM morphine (●). Data represent the mean ± SEM (n = 3) obtained in a single experiment, expressed as a percentage of the activity determined in the presence of 10 μM forskolin. In three independent experiments, adenylyl cyclase activity was 2.0 ± 0.1 and 17 ± 0.9 pmol cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively.

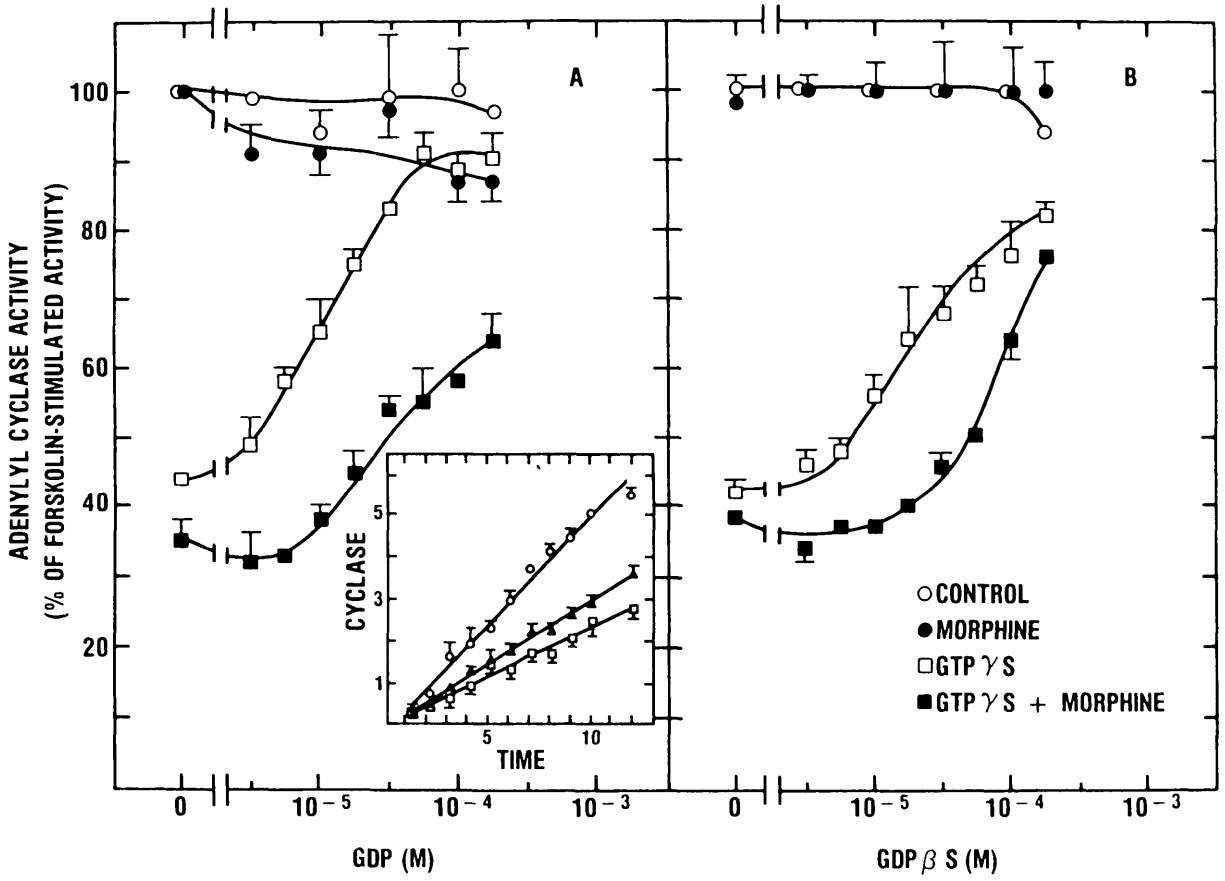

Fig. 3. Morphine Diminishes the Potency of GDP and GDPβS in Blocking the GTPγS-Induced Inhibition of Adenylyl Cyclase Activity

Adenylyl cyclase activity was determined in the presence of App(NH)p and 10 μM forskolin as described in *Materials and Methods*. A, GDP was tested alone (○), in the presence of morphine (●), in the presence of 0.3 μM GTPγS (□), or in the presence of a combination of 10 μM morphine and 0.3 μM GTPγS (■). Data represent the mean ± SEM (n = 3) obtained in a representative experiment expressed as a percentage of the activity determined in the presence of 10 μM forskolin. In three independent experiments, adenylyl cyclase activities were 2.3 ± 0.3 and 40 ± 4 pmoles cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively. A, *Inset*, The time course of the formation of cAMP was determined in the presence of 10 μM forskolin (○), 10 μM forskolin and 0.3 μM GTPγS (□), or a combination of 10 μM forskolin, 0.3 μM GTPγS, and 10 μM GDP (▲). B, GDPβS was tested alone (○), in the presence of morphine (●), in the presence of 0.3 μM GTPγS (□), or in the presence of a combination of 10 μM morphine and 0.3 μM GTPγS (■). Data represent the mean ± SEM (n = 3) obtained in a single experiment expressed as a percentage of the activity determined in the presence of 10 μM forskolin. In three independent experiments, adenylyl cyclase activity was 2.1 ± 0.2 and 34 ± 2.6 pmol cAMP/mg protein·min, respectively.

was found to increase the affinity of GDP for Go_{α}, in part by increasing the rate of association of GDP with Go_{α}. In contrast, $\beta\gamma$ slightly diminished the binding of GTPγS to Go_{α}. If the findings of the current study bear any relationship to the findings obtained with purified Go_{α} and $\beta\gamma$, it is conceivable that the main influence of a receptor on a G-protein is to somehow disrupt the interaction of G_{α} with $\beta\gamma$, thus enhancing the release of GDP and the binding of GTP. The binding of GTP would result in a conformational change in the α-subunit, causing a dissociation of the $\beta\gamma$-subunit and a modulation of the activity of the effector by α·GTP (39).

MATERIALS AND METHODS

Materials and Chemicals

Drugs and chemicals were obtained from the following sources: GTP, GDP, and 5′-adenylyl imidodiphosphate, Sigma (St. Louis, MO); GTPγS and GDPβS, Boehringer Mannheim (Indianapolis, IN); Gpp(NH)p, ICN Chemical Co. (Irvine, CA); [^{3}H]cAMP, DuPont-New England Nuclear (Boston, MA); and pertussis toxin, List Biologicals (Campbell,CA).

Preparation and Treatment of Tissue

Membranes were prepared from 7315c tumor cells as previously described (16, 40). Occasionally, 7315c cells were incubated for 3 h at 37 C in an atmosphere of 95% air and 5% CO_2 with or without pertussis toxin (30 ng/ml) in Eagles' Minimum Essential Medium supplemented with penicillin (10,000 U/ml) and streptomycin (100 mg/ml). All membrane preparations were stored in liquid nitrogen.

Assay of Adenylyl Cyclase Activity

Adenylyl cyclase activity was assayed essentially as previously described (8, 16, 40), except that App(NH)p was substituted for ATP to prevent the conversion of GDP to GTP (20, 21). Briefly, the assay system for the determination of adenylyl cyclase activity contained 80 mM Tris-HCl (pH 7.4), 10 mM

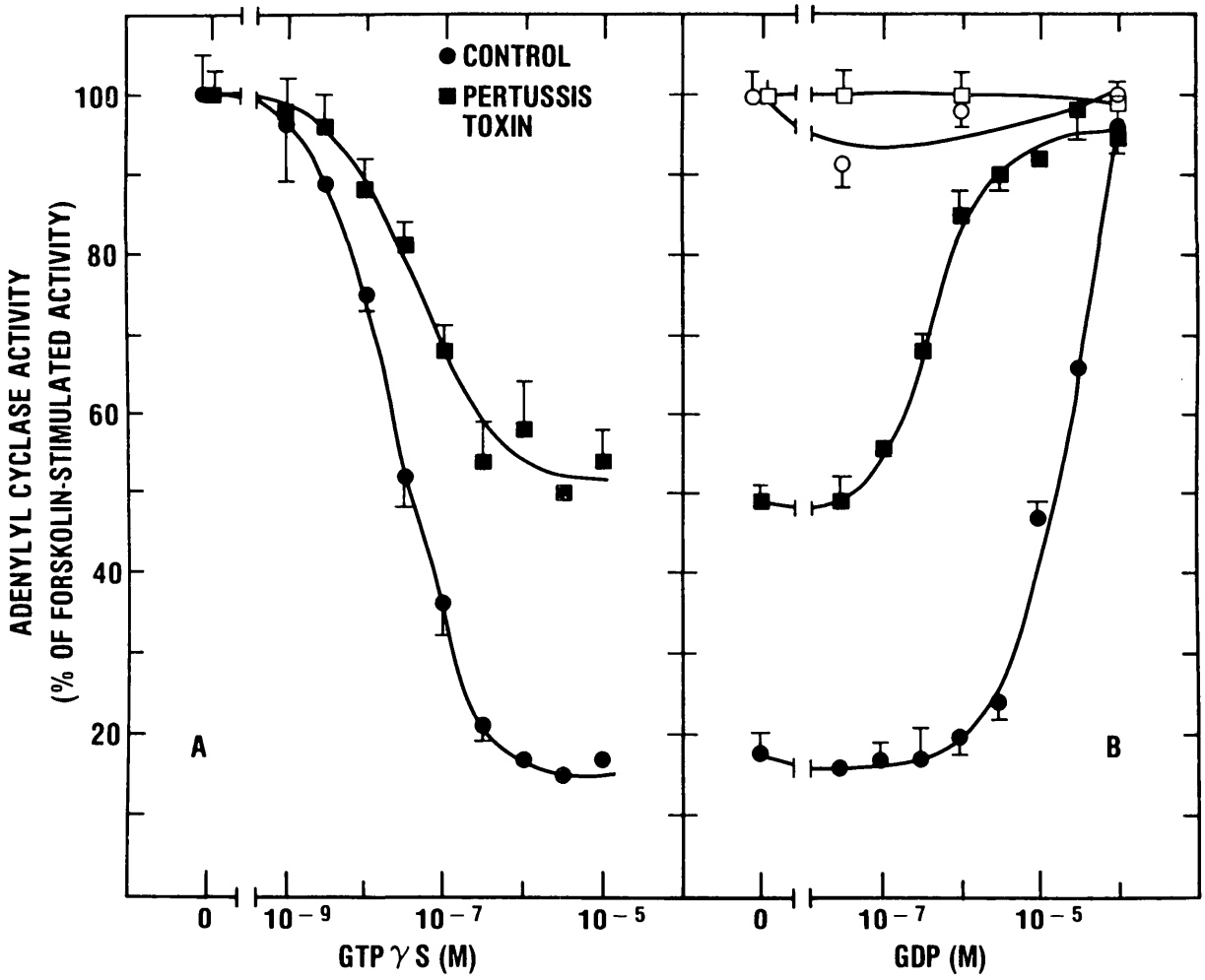

Fig. 4. Pertussis Toxin Diminishes the Potency of GDP in Blocking GTPγS-Induced Inhibition of Adenylyl Cyclase Activity

7315c cells were incubated for 3 h in the absence or presence of pertussis toxin (30 ng/ml); membranes were prepared from these cells, and adenylyl cyclase activity was determined in the presence of App(NH)p and 10 μM forskolin as described in *Materials and Methods*. A, GTPγS was tested in control (●) and pertussis toxin-treated (■) membranes. In three independent experiments performed on control membranes, adenylyl cyclase activities were 4.2 ± 0.9 and 61 ± 9 pmol cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively. In three independent experiments performed on membranes treated with pertussis toxin, adenylyl cyclase activities were 2.5 ± 0.8 and 58 ± 4.6 pmol cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively. Depicted are the results obtained in a single experiment, expressed as a percentage of the activity determined in the presence of 10 μM forskolin. B, GDP was tested in control (● and ○) and in pertussis toxin-treated membranes (■ and □) in the absence (○ and □) and presence of 0.3 μM GTPγS (● and ■). In three independent experiments performed on control membranes, adenylyl cyclase activities were 3.0 ± 1.4 and 72 ± 11 pmol cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively. In three independent experiments performed on membranes treated with pertussis toxin, adenylyl cyclase activities were 1.7 ± 0.6 and 55 ± 2.6 pmol cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively.

theophylline, 1 mM $MgSO_4$, 0.8 mM EGTA, 30 mM NaCl, 0.25 mM App(NH)p, the indicated drugs, and between 15 and 30 μg of membrane protein. Routinely, forskolin (10 μM) was included to stimulate adenylyl cyclase activity and amplify the inhibitory effects of drugs acting through Gi. The components were added to each assay tube (final volume, 0.06 ml) on ice; the reaction was initiated by placing the tubes in a water bath at 30 C. After 10 min, the assay was terminated by placing the tubes in a boiling water bath for 1 min. The amount of cAMP formed was determined by the protein binding assay of Brown *et al.* (41). In 45 experiments, adenylyl cyclase activity was 2.4 ± 0.2 and 40 ± 3 pmol cAMP/mg protein·min in the absence and presence of 10 μM forskolin, respectively. Initial experiments were performed to insure that GDP was not phosphorylated to GTP when App(NH)p was substituted for ATP as the substrate for the formation of cAMP and to validate that App(NH)p could be used in our assay system. Under these assay conditions, less than 5% of the GDP was phosphorylated to GTP, as determined by the method of White (42). Under these assay conditions, the formation of cAMP was linear for at least 12 min. Each experiment was performed at least three times; each figure depicts a representative experiment. The results presented in the text are the mean ± SEM derived from at least three independent experiments. Student's *t* test was used to determine the statistical significance of differences between the means of experimental groups.

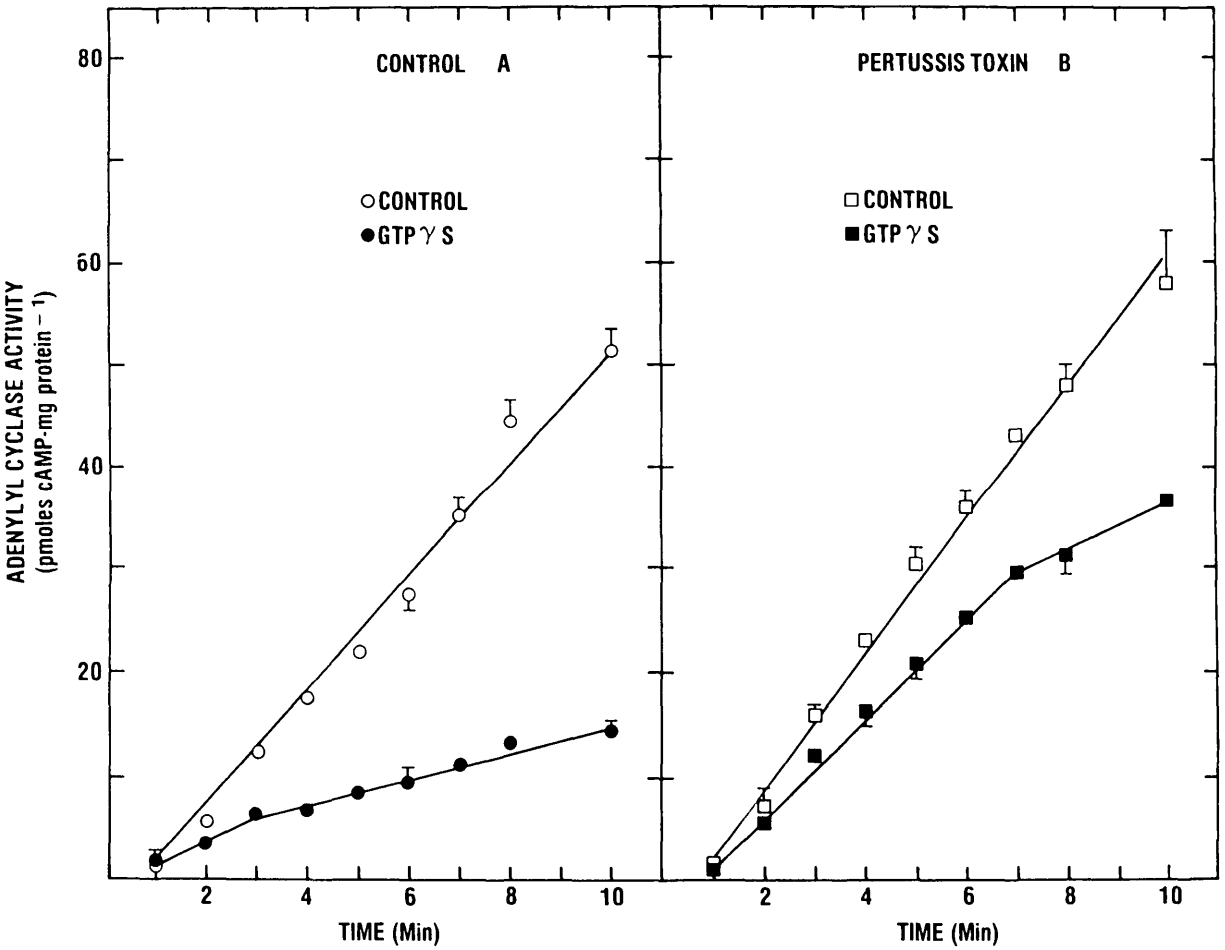

Fig. 5. Pertussis Toxin Prolongs the Lag in the GTPγS-induced Inhibition of Adenylyl Cyclase Activity

7315c cells were incubated for 3 h in the absence or presence of pertussis toxin; membranes were prepared from these cells, and adenylyl cyclase activity was determined in the presence of App(NH)p and 10 μM forskolin as described in *Materials and Methods*. A, In control membranes, the time course of the formation of cAMP was determined in the absence (○) and 0.3 μM GTPγS (●). Data represent the mean ± SEM (n = 3) obtained in a representative experiment, expressed as a percentage of the activity determined in the presence of 10 μM forskolin. B, In pertussis toxin-treated membranes, the time course of the formation of cAMP was determined in the absence (□) and presence of 0.3 μM GTPγS (■). Data represent the mean ± SEM (n = 3) obtained in a representative experiment, expressed as a percentage of the activity determined in the presence of 10 μM forskolin.

Assay of the Conversion of GDP to GTP

Various concentrations of GDP, spiked with approximately 50,000 cpm [^{3}H]GDP, were incubated in the presence and absence of 7315c membranes under conditions indentical to those used in the adenylyl cyclase assay. Aliquots of the reaction mixture were spotted on Polygram Gel 300 polyethyleneimine chromatography plates (Brinkmann Instruments, Westbury, NY.) and analyzed for [^{3}H]GDP and [^{3}H]GTP as described by White (42). Under these assay conditions, 6.4 ± 3.6%, 4.8 ± 3.0%, and 3.0 ± 1.8% [^{3}H]GDP were converted to [^{3}H]GTP at [^{3}H]GDP concentrations of 1, 10, and 100 μM, respectively. The inclusion of morphine with 100 μM [^{3}H]GDP did not affect the rate of conversion of [^{3}H]GDP to [^{3}H]GTP.

Acknowledgments

Received September 12, 1988. Accepted November 14, 1988.

Address requests for reprints to: Dr. Thomas E. Cote, Department of Pharmacology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814-4799.

This work was supported by Grant GM-35199 from the NIH. The costs of publication of this article were defrayed in part by the payment of page charges. This article must, therefore, be hereby marked "advertisement" in accordance with 18 U.S.A. Section 1734 solely to indicate this fact.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the DoD or USUHS. The experiments reported herein were conducted according to the principles set forth in the "Guide for Care and Use of Laboratory Animals," Institute of Animal Resources, National Research Council, DHEW Publication no. (NIH) 74-23.

REFERENCES

1. Rodbell M, Birnbaumer L, Pohl SL, Krans HMJ 1971 The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanyl nucleotides in glucagon action. J Biol Chem 246:1877–1882
2. Northup JK, Sternweis PC, Smigel MD, Schleifer LS, Ross EM, Gilman AG 1980 Purification of the regulatory com-

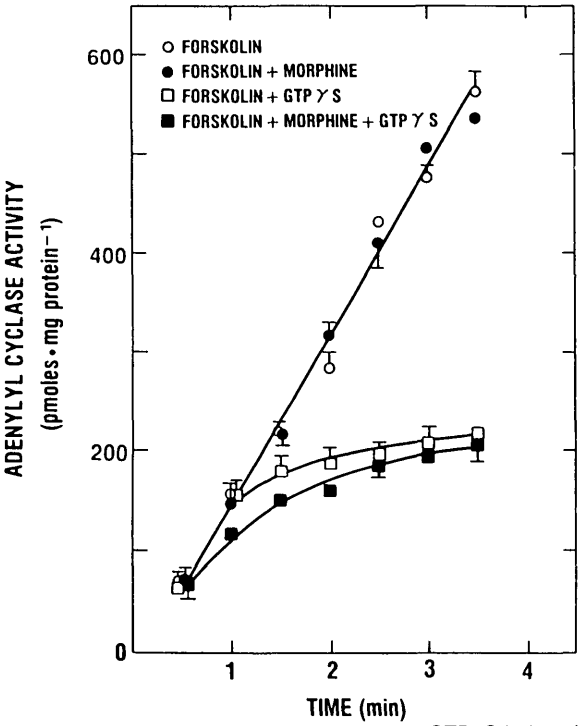

Fig. 6. Morphine Diminishes the Lag in the GTPγS-Induced Inhibition of Adenylyl Cyclase Activity

7315c membranes were preincubated for 2 min in the absence (○ and □) or presence (● and ■) of 10 μM morphine. The adenylyl cyclase assay was initiated by the addition of either 0.25 mM ATP and 10 μM forskolin (circles) or a combination of 0.25 mM ATP, 10 μM forskolin, and 10 μM GTPγS (squares). Triplicate samples of each assay condition were placed in a 100 C water bath at the indicated time points. Data represent the mean ± SEM (n = 3) of a single experiment representative of three.

ponent of adenylate cyclase. Proc Natl Acad Sci USA 77:6516–6520
3. Bokoch GM, Katada T, Northup JK, Hewlett EL, Gilman AG 1983 Identification of the predominant substrate for ADP-ribosylation by islet-activating protein. J Biol Chem 258:2072–2075
4. Codina J, Hildebrandt J, Iyengar R, Birnbaumer L, Sekura RD, Manclark CR 1983 Pertussis toxin substrate, the putative Ni of adenylyl cyclases, is an α/β heterodimer regulated by guanine nucleotide and magnesium. Proc Natl Acad Sci USA 80:4276–4280
5. Bitensky MW, Wheeler GL, Aloni B, Ventury S, Matuo Y 1978 Light- and GTP-activated photoreceptor phosphodiesterase: regulation by a light-activated GTPase and identification of rhodopsin as the phosphodiesterase binding site. Adv Cyclic Nucleotide Res 9:553–572
6. Cockcroft S, Gomperts BD 1985 Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature 314:534–536
7. Litosch I, Wallis C, Fain JN. 1985 5-Hydroxytryptamine stimulates inositol phosphate production in a cell-free system from blowfly salivary glands. J Biol Chem 260:5464–5471
8. Aub DL, Frey EA, Sekura RD, Cote TE. 1986 Coupling of the thyrotropin-releasing hormone receptor to phospholipase C by a GTP-binding protein distinct from the inhibitory or stimulatory GTP-binding protein. J Biol Chem 261:9333–9340
9. Aub DL, Gosse ME, Cote TE 1987 Regulation of thyrotropin-releasing hormone receptor binding and phospholipase C activation by a single GTP-binding protein. J Biol Chem 262:9521–9528
10. Pjaffinger PJ, Martin JM, Hunter DD, Nathanson NM, Hille B 1985 GTP-binding proteins couple cardiac muscarinic receptors to a K channel. Nature 317:536–538
11. Breitwieser GE, Szabo G 1985 Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. Nature 317:538–540
12. Holz GG, Rane SG, Dunlap K 1986 GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. Nature 319:670–672
13. Gilman AG 1987 G proteins: transduction of receptor-generated signals. Annu Rev Biochem 56:615–649
14. Birnbaumer L, Codina J, Mattera R, Yatani A, Scherer N, Toro M-J, Brown AM 1987 Signal transduction by G proteins. Kidney Int 32:S-14–S-37
15. Cassel D, Selinger Z 1978 Mechanism of adenylate cyclase activation through the β-adrenergic receptor: catecholamine-induced displacement of bound GDP by GTP. Proc Natl Acad Sci USA 75:4155–4159
16. Frey EA, Kebabian JW 1984 A μ-opiate receptor in 7315c tumor tissue mediates inhibition of immunoreactive prolactin release and adenylate cyclase activity. Endocrinology 115:1797–1804
17. Puttfarcken P, Werling LL, Brown SR, Cote TE, Cox BM 1986 Sodium regulation of agonist binding at opioid receptors. I. Effects of sodium replacement on binding at μ- and δ-type receptors in 7315c and NG108-15 cells and cell membranes. J Pharmacol Exp Ther 30:81–89
18. Tocque B, Jacobson AE, Rice KC, Frey EA 1987 The μ-opioid receptor in the 7315c tumor cell. Eur J Pharmacol 143:127–130
19. Werling LL, Puttfarcken PS, Cox BM 1988 Multiple agonist affinity states of opioid receptors: regulation of binding by guanyl nucleotides in guinea pig cortical, NG108-15, and 7315c cell membranes. Mol Pharmacol 33:423–431
20. Chen TC, Cote TE, Kebabian JW 1980 Endogenous component of the striatum confers dopamine-sensitivity upon adenylate cyclase activity: the role of endogenous guanyl nucleotides. Brain Res 181:139–149
21. Cote TE, Chen TC, Kebabian JW 1980 Guanosine triphosphate: an endogenous compound in the rabbit cerebellar cortex which couples the beta-adrenergic receptor to adenylate cyclase. Brain Res 181:127–138
22. Katada T, Ui M 1979 Effect of *in vivo* pretreatment of rats with a new protein purified from *Bordetella* pertussis on *in vitro* secretion of insulin: role of calcium. Endocrinology 104:1822–1827
23. Katada T, Ui M 1979 Islet-activating protein: enhanced insulin secretion and cyclic AMP accumulation in pancreas islets due to activation of native calcium ionophores. J Biol Chem 254:469–479
24. Katada T, Ui M 1981 Islet activating protein: a modifier of receptor-mediated regulation of rat islet adenylate cyclase activity. J Biol Chem 256:8310–8317
25. Murayama T, Ui M 1983 Loss of the inhibitory function of the guanine nucleotide regulatory component of adenylate cyclase. J Biol Chem 258:3319–3326
26. Kurose H, Katada T, Amano T, Ui M 1983 Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via α-adrenergic, cholinergic, and opiate receptors in neuroblastoma × glioma hybrid cells. J Biol Chem 258:4870–4875
27. Cote TE, Frey EA, Sekura RD 1984 Altered activity of the inhibitory guanyl nucleotide-binding component (Ni) induced by pertussis toxin. Uncoupling of Ni from receptor with continued coupling of Ni to the catalytic unit. J Biol Chem 259:8693–8698
28. Hildebrandt JD, Sekura RD, Codina J, Iyengar R, Manclark CR, Birnbaumer L 1983 Stimulation and inhibition of adenylyl cyclases mediated by distinct regulatory proteins. Proc Natl Acad Sci USA 302:706–709
29. Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham

DE 1987 The $\beta\gamma$ subunits of GTP-binding proteins activate the muscarinic K^+ channels in heart. Nature 325:321–326
30. Yatani A, Codina J, Sekura RD, Birnbaumer L, Brown AM 1987 Reconstitution of somatostatin and muscarinic receptor mediated stimulation of K^+ channels by isolated G_K protein in clonal rat anterior cell membranes. Mol Endocrinol 1:283–289
31. Michel T, Lefkowitz RJ 1982 Hormonal inhibition of adenylate cyclase. α_2-adrenergic receptors promote release of [^{3}H] guanylyl imidodiphosphate from platelet membranes. J Biol Chem 257:13557–13563
32. Cote TE, Grewe CW, Tsuruta K, Stoof JC, Eskay RL, Kebabian JW 1982 D-2 dopamine receptor-mediated inhibition of adenylate cyclase activity in the intermediate lobe of the rat pituitary gland requires GTP. Endocrinology 110:812–819
33. Murayama T, Ui M 1984 [^{3}H]GDP release from rat and hamster adipocyte membranes independently linked to receptors involved in activation or inhibition of adenylate cyclase. J Biol Chem 259:761–769
34. Tota MR, Kahler KR, Schimerlik MJ 1987 Reconstitution of the purified porcine atrial muscarinic acetylcholine receptor with purified porcine atrial inhibitory guanine nucleotide binding protein. Biochemistry 26:8175–8182
35. Heald PJ 1957 Guanosine di- and tri-phosphates in the phosphate metabolism of cerebral tissues promoted by electrical pulses. Biochem J 67:529–536
36. Sullivan KA, Miller RT, Masters SB, Beiderman B, Heideman W, Bourne HR 1987 Identification of receptor contact site involved in receptor-G protein coupling. Nature 330:758–760
37. Haga T, Ross EM, Anderson HJ, Gilman AG 1977 Adenylate cyclase permanently uncoupled from hormone receptors in a novel variant of S49 mouse lymphoma cells. Proc Natl Acad Sci USA 74:2016–2020
38. Higashijima T, Ferguson KM, Sternweis PC, Smigel MD, Gilman AG 1987 Effects of Mg^{2+} and the $\beta\gamma$-subunit complex on the interactions of guanine nucleotides with G proteins. J Biol Chem 262:762–766
39. Miller RT, Masters SB, Sullivan KA, Beiderman B, Bourne HR 1988 A mutation that prevents GTP-dependent activation of the α chain of Gs. Nature 334:712–715
40. Cote TE, Grewe CW, Kebabian JW 1981 Stimulation of a D-2 dopamine receptor in the intermediate lobe of the rat pituitary gland decreases the responsiveness of the β-adrenoceptor: biochemical mechanism. Endocrinology 108:420–426
41. Brown BL, Albano JDM, Ekins RP, Sgherzi AM, Tampion WA 1971 Simple and sensitive saturation assay method for the measurement of adenosine 3′,5′-monophosphate. Biochem J 121:561–562
42. White AA 1975 Separation and quantification of ^{32}P-labeled guanine nucleotides by thin layer chromatography. J Chromatogr 104:184–188

Annual Conference of the Society for the Study of Fertility

The Annual Conference of the Society for the study of Fertility will be held at the University of Kent at Canterbury on July 5–7, 1989. The main symposium of the conference will be on the subject "Control of gene expression in reproductive tissues," and will include speakers such as N. Hecht (USA), N. Josso (Paris), M. Parker (London), S. Harris (Edinburgh) and J. Pollard (USA). Participants are invited to submit abstracts for either oral or poster communication on this subject, or any topic related to reproduction or fertility. Further information can obtain from Dr. N. Jenkins, Conference Secretary, University of Kent at Canterbury Kent, United Kingdom.