

Guanine Nucleotide Exchange-Independent Activation of G_s Protein by β_2 -Adrenoceptor

Özlem Uğur, Şükrü Sadık Öner, Paola Molinari, Caterina Ambrosio, Kemal Sayar, and H. Ongun Onaran

Department of Pharmacology and Clinical Pharmacology (Ö.U., Ş.S.Ö., K.S., H.O.O.) and Molecular Biology and Technology Research and Development Unit (H.O.O.), Ankara University Faculty of Medicine, Ankara, Turkey; and Department of Pharmacology, Istituto Superiore di Sanità, Rome, Italy (P.M., C.A.)

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ABSTRACT

β_2 -Adrenoceptor-mediated activation of G_s and adenylyl cyclase or other receptor-mediated G protein activations is believed to occur by receptor-catalyzed replacement of GDP with GTP on the α -subunit of the G protein. Here we showed that a β_2 -adrenoceptor- G_s system, heterologously expressed in cyc[−] or human embryonic kidney (HEK)-293 cells, can be activated in the presence of GDP or its phosphorylation-resistant analog, guanosine 5'-O-(2-thiodiphosphate) (GDP β S). The potency and maximal ability of GDP to activate G_s and adenylyl cyclase were identical to those of GTP. GDP-mediated activation of adenylyl cyclase, similar to that mediated by GTP, was concentration-dependent, required high magnesium concentrations, was inhibited by inverse agonists, and was correlated with the efficacy of receptor ligands used to stimulate the receptor. UDP did not block the GDP-mediated activation, although it com-

pletely blocked the formation of a small amount of GTP (~5% GDP) from GDP. Moreover, the activation of G_s in the presence of GDP was insensitive to cholera toxin treatment of the cells, whereas that observed in the presence of GTP was amplified by the treatment, which showed that the activation observed in the presence of GDP was not mediated by GTP. Therefore, we concluded that GDP itself could mediate β -adrenoceptor-induced activation of G_s -adenylyl cyclase system as much as GTP. We discuss the results in the context of the current paradigm of receptor-mediated G protein activation and propose an additional mode of activation for β_2 -adrenoceptor- G_s -adenylyl cyclase system where nucleotide exchange is not necessary and GDP and GTP play identical roles in receptor-induced G_s protein activation.

Heterotrimeric G proteins and their cognate heptahelical membrane receptors constitute the largest family of transmembrane signaling proteins. According to the accepted model of G protein activation, the GTP-bound form of G protein is active, whereas the GDP-bound form is inactive and the receptor-mediated exchange of GDP with GTP on the nucleotide-binding site is necessary and sufficient to initiate the activation process. In turn, deactivation is achieved by the hydrolysis of bound GTP to GDP. Fine-tuning of the rates of the elementary steps involved in this activation-deactivation cycle determines the average activity of the G protein (i.e., the amount of accumulated $G\alpha$ -GTP), thus the strength of the signal transmitted to the inside of the cell (for review

see Gilman, 1987; Hamm, 1998). This scenario implies that GTP is the natural activator of the G proteins, whereas GDP is their universal inhibitor, and that GTPase reaction is the off-switch for the G protein-mediated signal transduction. Therefore, the presence of excess GDP is expected to inhibit receptor-induced G protein activation, at least partly, by competing with GTP and the potency of this inhibitory effect should be correlated with the ability of the receptor's ligand to displace GDP from $G\alpha$. Viewed from a more rigorous standpoint, the sensitivity of receptor-mediated G protein activation to the presence of GDP should be inversely correlated with the ability of the ligand to reduce the affinity of GDP for $G\alpha$. Based on this obvious inference, we designed experiments to compare GDP-displacing abilities of different β_2 -adrenoceptor (β -AR) ligands in native or artificially fused β -AR- G_s system by evaluating the sensitivity of ligand-induced adenylyl cyclase activity (which is well known to be mediated by G_s protein) to the presence of GDP. To our

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ABBREVIATIONS: β -AR, β_2 -adrenoceptor; HPLC, high-pressure liquid chromatography; GTP γ S, guanosine 5'-3-O-(thio)triphosphate; CTX, cholera toxin; HEK, human embryonic kidney; GDP β S, guanosine 5'-O-(2-thiodiphosphate); Gpp(NH)p, guanosine 5'-(β , γ -imido)triphosphate; App(NH)p, 5'-adenylyl- β , γ -imidodiphosphate.

surprise, however, neither in native nor in fused system expressed in S49 cyc⁻ lymphoma cells (will be referred to as cyc⁻ throughout the text), GDP inhibited ligand-induced adenylyl cyclase activity, despite the fact that even full agonists were not able to reduce the binding affinity of GDP to zero, as assessed by competition-binding assays (Ö. Uğur and H. O. Onaran, unpublished observation).

A number of experiments in the literature show that G_s (or G_i) protein can indeed be activated in the presence of GDP (Iyengar and Birnbaumer, 1979; Schneyer et al., 1984; Harding and Harris, 1986; Quist et al., 1992; Piacentini et al., 1996; Seifert et al., 1998; Hatley et al., 2003; Lutz et al., 2002). These reports include observations dating from late 1970s to the present day. However, the activation in the presence of GDP is considered as either inconsequential or artifactual (i.e., explained by the conversion of GDP to GTP by nucleotide diphosphate kinase activity during the experiments) (Kimura and Shimada, 1983; Kikkawa et al., 1990). Thus, the true nature and significance of GDP-mediated activation of the G protein have never been fully understood. The possibility that G proteins can be activated in the presence of GDP may call for a need for a revision of the established paradigm of the activation of G proteins summarized above and may shed light on the understanding of the molecular mechanism of receptor-mediated activation of G proteins. Therefore, we further investigated and characterized the β -AR-mediated adenylyl cyclase activation observed in the presence of GDP.

Materials and Methods

Plasmid Constructs. The cDNA encoding the long form of rat G_{α_s} was in the pCDNA3.1(+) vector (Invitrogen, Carlsbad, CA). Construction of cDNA encoding the fusion proteins of human β -AR or δ -opioid receptor and the long form of rat G_{α_s} is described elsewhere (Molinari et al., 2003).

Cell Culture and Transfection. We used the following cell systems throughout the present experiments: 1) native S49 cells; 2) cyc⁻ cells transfected with G_{α_{sL}}; 3) cyc⁻ cells transfected with β -AR-G_{α_s} fusion protein; 4) HEK-293 cells transfected with β -AR; 5) HEK-293 cells transfected with β -AR-G_{α_s} fusion protein; and 6) HEK-293 cells transfected with δ -opioid receptor-G_{α_s} fusion protein. All of the cell lines were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum in standard conditions. cyc⁻ cells were transfected by electroporation essentially as described previously (Gonzales et al., 1992). HEK-293 cells were transfected with FuGENE 6 (Roche, Mannheim, Germany). Stable clones were selected in the culture medium containing 600 to 700 μ g/ml G418 as described previously (Uğur et al., 2003).

Cholera Toxin Treatment. cyc⁻ cells stably transfected with cDNA encoding G_{α_s} were incubated with holotoxin (at a final concentration of 1 μ g/ml) under standard cell culture conditions for 2 h, which led to a considerable increase in basal intracellular cAMP accumulation. We did not check the completeness of the ADP-ribosylation, because the amplification of the GTP-mediated response in the cell membranes was the only requirement for the purpose of the present experiments (see *Results*).

Adenylyl Cyclase Assays. Adenylyl cyclase activity was measured essentially as described previously (Uğur and Onaran, 1997). In brief, membranes were mixed in 96-well plates (0.25–2 μ g of protein/well) with 75 μ l of the indicated additives, and then the assay was initiated by adding 25 μ l of adenylyl cyclase assay buffer (unless indicated otherwise, final concentrations were 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM MgCl₂, 250 μ M ATP, and 1 mM isobutylmethylxanthine) and terminated by adding 100 μ l of 0.2 N HCl.

Before adding the ATP-containing adenylyl cyclase buffer, cell membranes and relevant additives were incubated together for 1 to 2 min. Preincubation time with GTP γ S was 15 min, because our kinetic experiments showed that adenylyl cyclase activation in the presence of GTP γ S reached a peak in 15 min. All of the incubations were at 37°C, and total assay time was 5 min. Time-dependent cAMP accumulation was determined by sampling the reaction mixture at the indicated time points. In this case, the reaction was started by adding membranes to the rest of the mixture. The amount of accumulated cAMP was measured by radioimmunoassay in all of the experiments as described previously (Uğur and Onaran, 1997). Control experiments showed that different nucleotides (GMP, GDP, GTP, ATP, etc.) when used at the same concentrations as in the experiments did not interfere with the radioimmunoassay determination.

We used an indirect approach to measure GDP-displacing ability of receptor ligands based on the fact that AIF can activate the G protein only in its GDP-bound form. We incubated cell membranes with relevant ligands for 5 min in the absence of guanine nucleotides to let GDP dissociate from G_s and then determined cyclase activity after adding NaF + AlCl₃ as described above. We interpreted ligand-dependent "inhibition" of AIF-induced cyclase activity as the ligand's ability to displace GDP from G_s. As a normalizing control, we made the same experiment in the presence of GDP during incubation with ligands.

Membrane Preparation and Other Procedures. Homogenized cells (obtained by passing the cell suspension 10 to 15 times through a 26-gauge syringe tip in a hypotonic buffer) were centrifuged at 400g for 5 min, and the supernatant was repelleted at 100,000g. The pellet was washed once again, and the final pellet was resuspended in a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, protease mixture, and 25% (w/v) sucrose and stored at -70°C. Protein concentrations were determined by the Bradford assay (Bradford, 1976). Radioligand-binding assays and immunoblotting were performed by using standard methods. Radioligand-binding assays were performed in the binding buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM KCl, and 10 mM MgCl₂ in a final volume of 250 μ l containing 1 to 2 μ g of membrane protein at 27°C for 3 h. Reaction was stopped by filtration through Whatman GF/B filters by using a cell harvester (Skatron Instruments, Lier, Norway). For immunoblotting, 5 μ g of membrane protein was separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose or polyvinylidene difluoride paper [Bio-Rad (Hercules, CA) or Millipore (Billerica, MA)]. The proteins were detected with the polyclonal affinity-purified RM antibody (1 μ g/ml), which recognizes the carboxyl-terminal decapeptide of G_{α_s}, and enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) as described by the manufacturer. Horseradish peroxidase-conjugated goat anti-rabbit antibodies (developed in the Molecular Biology and Technology Research and Development Unit, Ankara University, Ankara, Turkey) were used to visualize the relevant bands.

Chromatographic separation of nucleotides was achieved by a continuous NaCl and pH gradient in 5 mM phosphate buffer using an HPLC system equipped with a UV-absorption detector (Jasco, Tokyo, Japan) and a weakly basic anion-exchanger column (Protein-Pak DEAE-5PW; Waters, Milford, MA). Fractions were collected in a volume of 500 μ l, and radioactivity in the fractions was determined in a scintillation counter (Wallac MicroBeta 1450 Trilux; Millipore Corp.).

Results

Effect of GDP on Agonist-Induced Activation of Adenylyl Cyclase. We stably expressed fusion protein of human β -AR and the long form of rat G_{α_s} in cyc⁻ (which does not express G_s intrinsically) or in HEK-293 cells. Expression of the fusion protein in the selected clones was roughly 4 and

40 pmol/mg of membrane protein in cyc^- and HEK-293 cells, respectively, as assessed by ^{125}I -iodocyanopindolol saturation binding. Integrity of the fusion protein in the cell membranes was assessed by immunoblotting (data not shown).

We measured stimulated adenylyl cyclase activity as a readout for G_s activation in cell membranes. Isoproterenol, a β -AR agonist, activated adenylyl cyclase in a concentration-dependent manner in the presence but not in the absence of 1 μM GTP in membranes prepared from cyc^- or HEK-293 cells expressing the fusion protein. Untransfected control cell membranes exhibited no agonist-mediated stimulation of cyclase activity. Considering that GTP is the activating nucleotide for G proteins and that G protein activation is achieved by receptor-induced exchange of GDP with GTP, the presence of excess GDP would be expected to inhibit β -AR-induced cyclase activation by competing with GTP. However, excess amounts of GDP had no effect on the adenylyl cyclase activation induced by isoproterenol in the presence of GTP; potency and maximal level of isoproterenol-induced adenylyl cyclase activities obtained in the absence or presence of 500 μM GDP were indistinguishable (Fig. 1, A and B) as assessed by the means of F statistics based on the evaluation of extraresidual variance due to parameter sharing in a family of nonlinear regressions (DeLean et al., 1978). Moreover, GDP (in the absence of added GTP) activated adenylyl cyclase in cyc^- membranes expressing β -AR- $\text{G}\alpha_s$ fusion protein in the presence of 100 μM isoproterenol (with a maximal response not different from that mediated by GTP under identical conditions) (Fig. 1C). Indeed, the latter observation is consis-

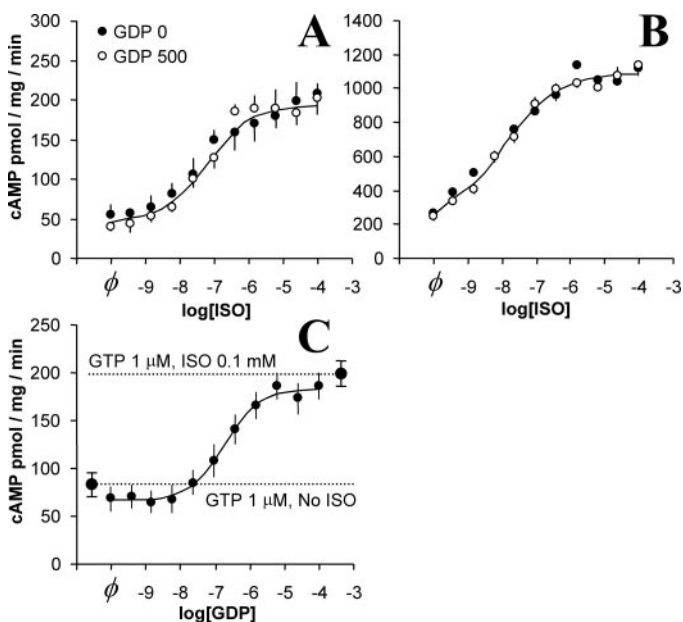


Fig. 1. Isoproterenol-induced adenylyl cyclase activation in cell membranes. Adenylyl cyclase activity was measured in cyc^- (1 $\mu\text{g}/\text{well}$) (A) and HEK-293 (0.5 $\mu\text{g}/\text{well}$) (B) cell membranes expressing β -AR- $\text{G}\alpha_s$ fusion protein in the presence of indicated concentrations of isoproterenol and 1 μM GTP. Solid lines indicate the nonlinear regression of the four-parameter logistic equation. There was no statistical difference between fitting two independent equations or single equation to the data obtained in the presence or absence of GDP. C shows adenylyl cyclase activity measured in the membranes of cyc^- cells expressing β -AR- $\text{G}\alpha_s$ fusion protein in the presence of indicated concentrations of GDP and 100 μM isoproterenol (ISO). For comparison, the levels of adenylyl cyclase activity measured in the presence of GTP (with or without isoproterenol) are also shown as open circles and extended dotted lines. The data are mean values of 4 to 6 experiments \pm S.E.M.

tent with the first one in the sense that GDP must mediate G protein activation as efficiently as GTP; otherwise, it would inhibit the adenylyl cyclase activation in the presence of GTP (see also below).

A full comparison of GDP- or GTP-mediated cyclase activation in HEK-293 cell membranes expressing the fusion protein is given in Fig. 2A, where similar potency for GDP and GTP in supporting cyclase activity is evident in the presence or absence of agonist. The same pattern of activation was observed in 1) HEK-293 cell membranes that heterologously express comparable amount of (nonfused) human β -AR (Fig. 2B); 2) cyc^- cell membranes that express $\text{G}\alpha_s$ heterologously but β -AR endogenously (Fig. 2C); or 3) wild-type S49 cell membranes that express both G_s and β -AR (400 fmol/mg) endogenously (Fig. 2D). This showed that the similarity of GDP and GTP in supporting G_s activation was not a peculiarity of the fusion protein or artificial overexpression of the receptor or $\text{G}\alpha_s$ protein. Note that the absolute value of

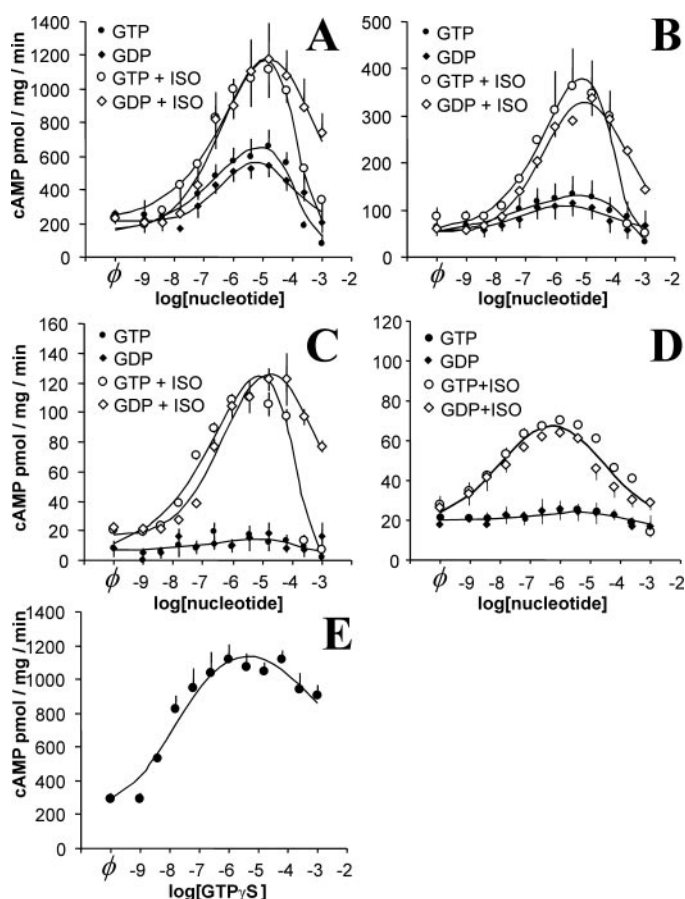


Fig. 2. Sensitivity of adenylyl cyclase activation to GDP, GTP, or $\text{GTP}\gamma\text{S}$. Adenylyl cyclase activity was measured in HEK-293 cell membranes overexpressing β -AR- $\text{G}\alpha_s$ fusion protein (A) and β -AR (B), in $\text{G}\alpha_s$ -transfected cyc^- cell membranes (C), or in S49 cell membranes that express both β -AR and $\text{G}\alpha_s$ endogenously (D) in the presence of indicated concentrations of GDP or GTP with or without 100 μM isoproterenol (ISO) as indicated. The sensitivity of adenylyl cyclase activity to $\text{GTP}\gamma\text{S}$ in HEK-293 cell membranes expressing β -AR- $\text{G}\alpha_s$ fusion protein is shown in E. Membranes were incubated for 15 min with $\text{GTP}\gamma\text{S}$ before starting adenylyl cyclase assay. Membrane protein (2, 1, or 0.5 μg) per well was used in the case of S49, cyc^- , or HEK-293 cells, respectively, to optimize the measurement sensitivity. See *Materials and Methods* for more detail. Solid lines indicate nonlinear regression of an arbitrarily modified Γ density function. Data are the mean values of 4 to 11 determinations \pm S.E.M.

overall signal and the relative magnitude of basal cyclase activity (in the absence of agonist but in the presence of GTP or GDP) diminished as the anticipated contribution of the receptor decreased (i.e., fusion > overexpression > normal expression) but that the general pattern remained almost the same (Fig. 2, compare A–D). Unlike GDP, GMP showed no activating effect in these experimental systems (data not shown).

In all instances, the concentration-response curves for the guanine nucleotides were bell-shaped with a descending arm starting from 10 μ M. Such a bell-shaped tendency was also evident in GTP γ S-induced activation of adenylyl cyclase in HEK-293 membranes that express β -AR- G_s fusion protein (Fig. 2E). High concentrations of the nucleotides inhibited both the nucleotide-independent background (see below) and forskolin-induced cyclase activity to the undetectable level (data not shown), suggesting that the effect might be on the cyclase molecule rather than on G_s . However, we did not investigate further the possible causes of this inhibitory effect seen at high nucleotide concentrations.

We reproduced the same results with GDP preparations obtained from three different sources [Sigma-Aldrich (St. Louis, MO), Roche Applied Science (Indianapolis, IN), or Valeant Pharmaceuticals (Costa Mesa, CA)], HPLC and NMR (P, C, and proton) analyses of which showed no significant contamination with GTP or with any other organic chemicals (results not shown) that may explain the observed similarity of GDP and GTP in supporting G_s -mediated adenylyl cyclase activation.

Adenylyl cyclase activation observed in the presence of GDP or GTP (with or without agonist) was magnesium-dependent, and its sensitivity to $[Mg^{2+}]$ was the same for GDP and GTP (Fig. 3A), whereas the activation was invariant with respect to the salt (NaCl or KCl) used in the assay buffer (Fig. 3B). The only difference between the presence of potassium and sodium was that the inhibitory effect of inverse agonist (\pm)-1-[2,3-(dihydro-7-methyl-1*H*-indene-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol (ICI-118,551) on the basal cyclase activity was more pronounced in the presence of K^+ . A more detailed analysis of the relationship between guanine nucleotide-mediated cyclase activation and ligand efficacy is given below.

GDP-Mediated Adenylyl Cyclase Activation Does Not Result from Conversion to GTP. Early observations that G_s or G_i could be activated in the presence of GDP (Iyengar and Birnbaumer, 1979; Schneyer et al., 1984; Harding and Harris, 1986; Quist et al., 1992; Piacentini et al., 1996; Lutz et al., 2002) have mostly been attributed to the conversion of GDP to GTP during the experiments (Kimura and Schimada, 1983; Kikkawa et al., 1990; Kowluru et al., 1996). Such a conversion must be nearly complete and almost instantaneous to be an explanation for the present results, because GTP and GDP supported the cyclase activation with almost identical potencies in the presence or absence of agonist. This implies that equal concentrations of GTP and GDP lead to an almost equal level of adenylyl cyclase activation throughout the concentration-response curves (see Figs. 2 and 6). Under the assumption that only GTP is the activating nucleotide, the observation of a given level of adenylyl cyclase activity with equal concentrations of GDP and GTP is possible only when the conversion of GDP to GTP is complete in the bulk solution or near the nucleotide-binding site (or even

at the nucleotide-binding site) of $G\alpha_s$. The following control experiments showed that the conversion in the bulk solution cannot explain the results. 1) HPLC analysis of nucleotides before and after incubation with HEK-293 cell membranes under identical conditions used in cyclase assays showed no obvious loss of GDP (100 μ M), which ruled out a bulk conversion of GDP to GTP in solution (data not shown). 2) Using 32 P-labeled GDP, we detected 5 or 21% conversion of GDP to GTP in 5 or 30 min, respectively. UDP, which is known to inhibit nucleotide diphosphate kinase activities (Kimura and Schimada, 1983) or local transphosphorylation of GDP on the nucleotide-binding site of $G\alpha$ (Kikkawa et al., 1990; Kowluru et al., 1996), totally blocked the conversion in 5 min (which was the maximum incubation time used in the present assays) (Fig. 4A) but did not affect GDP- (or GTP-) mediated activation of adenylyl cyclase significantly (Fig. 4, B and C). Moreover, in S49 (or in cyc^-) membranes, the conversion of GDP to GTP in the same conditions was even less (1.5% of initial GDP) than that observed in HEK-293 cells (data not shown), whereas the pattern of GDP- or GTP-mediated activation was still the same. 3) GDP β S, a phosphorylation-resistant analog of GDP (Eckstein et al., 1979; Kowluru et al., 1996), was able to mediate adenylyl cyclase activation as much as GTP or GDP (Fig. 4D). 4) In the absence of ATP but in the presence of 500 μ M App(NH)p, a substrate for adenylyl

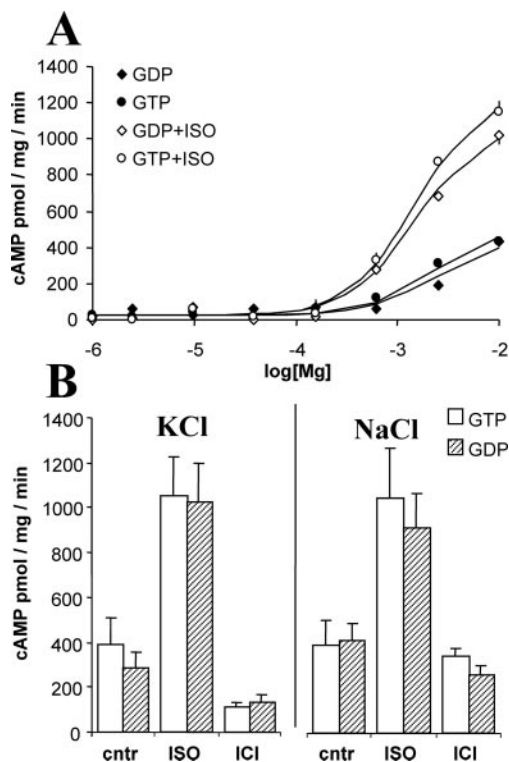


Fig. 3. Magnesium or monovalent ion dependence of adenylyl cyclase activation in HEK-293 cell membranes expressing β -AR- G_s . **A**, adenylyl cyclase activity was measured in HEK-293 cell membranes expressing β -AR- G_s fusion protein in the presence of GTP (10 μ M) or GDP (10 μ M) with or without isoproterenol (ISO) at the indicated concentrations of magnesium as log[molar]. Solid lines indicate nonlinear regression of the four-parameter logistic equation. Data are the mean values of a quadruplicate experiment \pm S.E.M. **B**, adenylyl cyclase activity was measured in the presence of 100 mM NaCl or KCl. Concentrations of the additives were as follows: 10 μ M GDP or GTP; 1 μ M ICI-118,551 (ICI), and 100 μ M ISO. The absence of receptor ligands was indicated as cntr. Data are the mean values of three experiments \pm S.E.M.

cyclase but not a phosphate donor, GDP was still able to support receptor-induced cyclase activation (Fig. 4E). Finally, further washing the membranes to remove loosely attached membrane proteins or contaminating nucleotides did not change the activation pattern observed in the presence of GDP (data not shown).

These findings clearly show that a bulk conversion of GDP to GTP cannot explain the present results. However, a locally complete in situ conversion still remains a possibility that can explain the results. This has always been a problem for obvious technical difficulties in measuring in situ concentration of GTP formed from GDP. Therefore, we tried to get around this problem by designing the following two experiments. The logic of the first experiment is as follows. If G_s (and adenylyl cyclase) activation were mediated only by GTP, then any finite-rate process that converts GDP to GTP would be expected to cause a lag that would make the kinetics of adenylyl cyclase activation different depending on whether the experiment was started in the presence of GDP or GTP. Therefore, we evaluated time-dependent cAMP accumulation as an integral measure of time-dependent adenylyl cyclase activity in the presence or absence of isoproterenol and in the presence of GDP or GTP in HEK-293 membranes expressing β -AR- G_s fusion protein. Results are shown in Fig. 5A; the process of cAMP accumulation was identical for GTP and GDP and did not depart from linearity in either case during 5 min of observation, and steady-state adenylyl cyclase activity was achieved almost instantaneously with the addition

of either nucleotide. This implies either that the local conversion from GDP to GTP does not occur and binding of GDP directly activates G_s or that the rate of the conversion is so fast that the formation of GTP is quasi-instantaneous and no GDP can stay in the vicinity of G_s , which is also an interesting possibility. To distinguish between these possibilities, we made the second experiment. We used cholera toxin (CTX)-treated membranes as a reporter of the type of nucleotide locally available to G_s . In cyc^- cells expressing the long form of G_{α_s} , cyclase activity was ~ 4 times higher in CTX-treated membranes than in the control membranes in the presence but not in the absence of added GTP. Therefore, the comparison of the reactivities of CTX-treated and untreated membranes could provide information on the presence of GTP available to G_s . In other words, adenylyl cyclase activity of CTX-treated membranes could be used as a bioassay in which G_s itself could report the presence of available local GTP in its microenvironment. In this bioassay, we saw no such "report from G_s " (i.e., signal amplification by CTX) in a wide range of GDP concentrations (Fig. 5C), whereas the amplification was obvious in the presence of added GTP (Fig. 5D). If the activity observed in the presence of GDP were actually mediated by local conversion into GTP, it should also have been amplified by CTX treatment to the same extent (unless we postulate that ADP-ribosyl- G_s can tell the difference between externally added or locally formed GTP). This inevitably means that added GDP did not provide appreciable amounts of GTP to G_s in the present experimental system. Therefore, we concluded that the effect of GDP could not result from its conversion to GTP on the $G\alpha$ -binding site or in

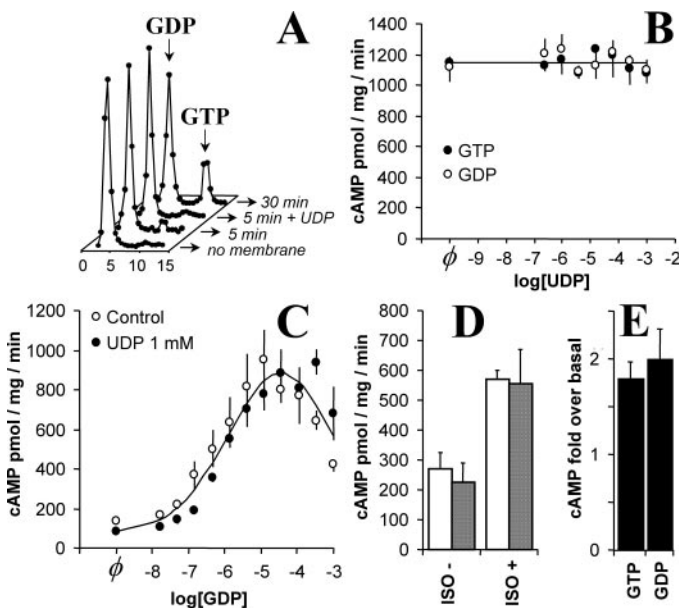


Fig. 4. Control experiments for conversion of GDP to GTP in HEK-293 membranes expressing β -AR- G_{α_s} fusion protein. A, HPLC chromatograms showing the conversion of 100 μ M 32 P-labeled GDP to GTP at the indicated times and conditions in cyclase buffer at 37°C. UDP was used at a concentration of 1 mM. B and C, adenylyl cyclase activity in the presence of 100 μ M isoproterenol (ISO) and 10 μ M GDP or GTP at the indicated concentrations of UDP (B) or in the presence of 1 mM UDP and 100 μ M isoproterenol at indicated concentrations of GDP (C). D, adenylyl cyclase activity in the presence of 10 μ M GTP (open bars) or 10 μ M GDP (shaded bars) with or without 100 μ M isoproterenol. E, adenylyl cyclase activity was measured exactly as above, except that ATP was replaced with 500 μ M App(NH)p and isoproterenol-induced activity was shown as -fold over basal activity measured in the presence of 10 μ M concentrations of the indicated nucleotides. Data are the mean values of three to four experiments \pm S.E.M.

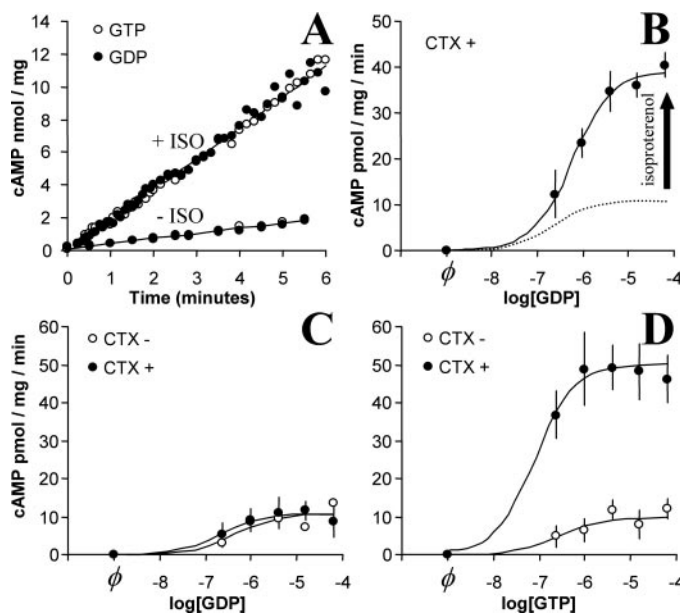


Fig. 5. Control experiments for conversion of GDP to GTP. A, time-dependent cAMP accumulation in HEK-293 cell membranes expressing β -AR- G_s measured right after the addition of 10 μ M GDP or GTP to the membranes that were preincubated (1 min) with or without isoproterenol as indicated. B, isoproterenol (ISO)-induced adenylyl cyclase activity in CTX-treated (CTX⁺) membranes in the presence of GDP. C and D, effect of CTX treatment on adenylyl cyclase activity in the presence of GDP (C) or GTP (D) in cyc^- cell membranes expressing G_{α_s} . Adenylyl cyclase activity was determined in the cell membranes prepared from CTX-treated (CTX⁺) or untreated (CTX⁻) cells. Dotted curve in B is reproduced from C to show the basal activity of CTX⁺ membranes in the presence of GDP. Data are the mean values of three experiments \pm S.E.M.

its vicinity by any mechanism whatsoever. Note that isoproterenol-induced adenylyl cyclase activation in the presence of GDP was still evident in CTX-treated membranes (Fig. 5B).

Receptor Dependence of Cyclase Activation in the Presence of GDP or GTP. The following results showed that the cyclase activity observed in the presence of GDP (or GTP) was strictly receptor-dependent. 1) ICI-118,551, an inverse agonist that inhibits basal activity of β -AR (Chidiac et al., 1994; Samama et al., 1994) suppressed the cyclase activity observed in the presence of GDP (or GTP) (Fig. 6A). 2) No nucleotide-mediated cyclase activity was observed when G_s was fused to δ -opioid receptor instead of β -AR (Fig. 6B). Note that δ -opioid receptor is not actually coupled to G_s. 3) The ability of different β -AR ligands to induce "GDP-mediated activation" was correlated with their ability to induce "GTP-mediated activation" (i.e., with their efficacies that are well known to be receptor-dependent) (Fig. 6C).

The present results, when considered as a whole, suggest that receptor-mediated activation of G_s (and thus adenylyl cyclase) is not necessarily achieved by receptor-mediated exchange of GDP with GTP. However, the ability of β -receptor ligands to induce cyclase activation in the presence of GDP (or GTP) was still found to be correlated with their "GDP-

displacing" ability in the present experimental system (Fig. 7) (see *Discussion*).

Two apparently different components of adenylyl cyclase activity can be distinguished by close inspection of Figs. 1, 2, and 6; one that depends on the presence of guanine nucleotides and the activity of the cognate receptor (β -AR) and another that does not. The latter activity can be observed in the absence of guanine nucleotides and is insensitive to the presence of agonist or inverse agonist, despite the ability of agonists to displace GDP from the G protein (see Fig. 7) and the ability of inverse agonists to inhibit nucleotide-dependent adenylyl cyclase activity (Fig. 6). This invariant background was ~ 200 pmol/mg/min in the β -AR-G_s system. Its magnitude was apparently related with whether the G_s was fused to a receptor or not, regardless of the identity of the fused receptor (compare Fig. 2, A–C, with Fig. 6B). We did not further investigate this activity and concentrated only on the nucleotide- and receptor-dependent one.

Discussion

In the present study, we showed that β -AR, G_s, and adenylyl cyclase system can be activated in the presence of GDP and that this activation could not be attributed to the conversion of GDP to GTP. The following is a brief discussion of

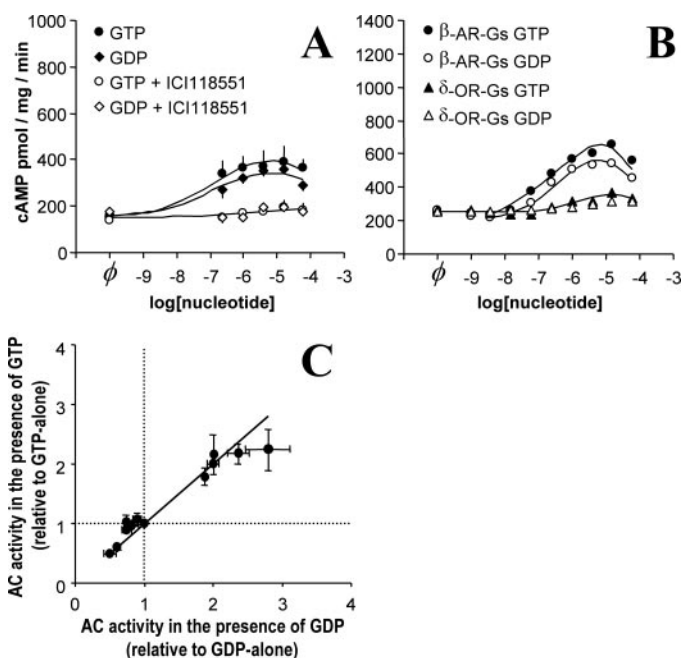


Fig. 6. Effect of receptor signal on adenylyl cyclase activity in the presence of GDP or GTP in HEK-293 cell membranes. Guanine nucleotide-mediated adenylyl cyclase activity was determined in the presence of the inverse agonist ICI-118,551 (1 μ M) in β -AR-G_s fusion system (A) or in a system where G_s was fused to a wrong receptor (δ -opioid, indicated as δ -OR-G_s) (B). Nucleotide-mediated activation disappeared almost completely in either case. Data are the mean values of 3 to 6 experiments \pm S.E.M. C, correlation between ligand-induced adenylyl cyclase activities measured in the presence of GDP or GTP in HEK-293 cell membranes expressing β -AR-G_s. Adenylyl cyclase activity was determined in the presence of different ligands (each point) and 10 μ M GDP (abscissa) or GTP (ordinate). Cyclase activity was presented as -fold-over-basal. The following ligands were used at the indicated saturating concentrations (in the order of intrinsic activity): 100 μ M epinephrine; 100 μ M isoproterenol; 100 μ M cimaterol; 100 μ M clenbuterol; 100 μ M dobutamine; 1 μ M alprenolol; 1 μ M cyanopindolol; 1 μ M propranolol; 1 μ M sotalol; 1 μ M ICI-118,551; and 1 μ M timolol. Solid line is the $y = x$ line. Data are the mean values of three experiments \pm S.E.M. AC, adenylyl cyclase.

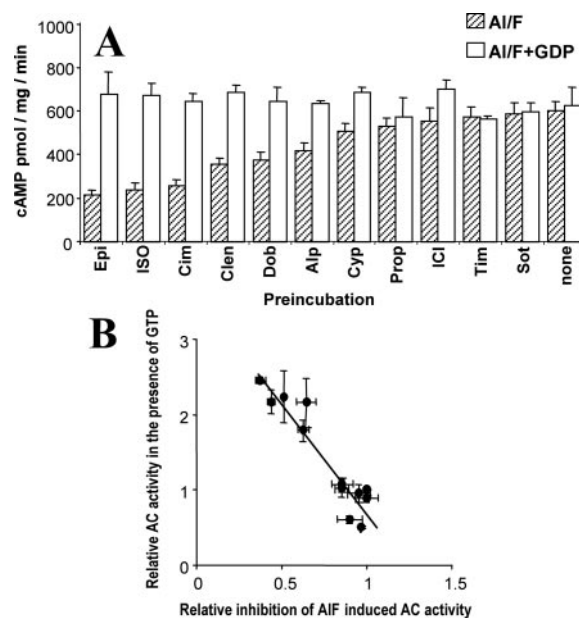


Fig. 7. GDP-dissociating effect of β -AR ligands. A, AIF ($\text{AlCl}_3 + \text{NaF}$)-induced adenylyl cyclase activity was determined after a 5-min incubation with the indicated ligands in the absence or presence of 10 μ M GDP in the HEK-293 cell membranes expressing β -AR-G_s. The difference between the cyclase activities observed in the presence and absence of GDP was attributed to the GDP-dissociating effects of the ligands (see *Materials and Methods* for details). Data are mean values of a quadruplicate experiment \pm S.E.M. B, ability of ligands to displace GDP from G_s (as determined in A) was plotted against the ability of ligands to activate adenylyl cyclase in the presence of 10 μ M GTP in the same membrane system. GDP-displacing effect of the ligands was shown (abscissa) as the relative inhibition of AIF-induced response (calculated from A). Ligand-induced activation was given as (ordinate) the adenylyl cyclase activity relative to the basal level (measured in the presence of 10 μ M GTP alone). Each point represents a ligand (used at the same concentrations as in Fig. 6). The abbreviations used are: Epi, epinephrine; ISO, isoproterenol; Cim, cimaterol; Clen, clenbuterol; Dob, dobutamine; Alp, alprenolol; Cyp, cyanopindolol; Prop, propranolol; ICI, ICI-118,551; Tim, timolol; Sot, sotalol; and none, no addition of ligand. AC, adenylyl cyclase.

the observed phenomenon and its implications that are apparently not concordant with the current paradigm of G protein-mediated signal transduction. By current paradigm, we mean the basic idea that the GDP-bound form of the G protein is inactive, that receptor stimulation “catalyzes” the exchange of GDP with GTP on the G protein, yielding the active GTP-bound state, and that GTPase activity of $G\alpha$ is the turn-off mechanism for the activation (for review see Gilman, 1987; Hamm, 1998).

Experimental foundations of the current paradigm for G protein activation can be roughly summarized as follows. 1) $G\alpha$ subunits bind GDP or GTP (or their analogs) and hydrolyze the bound GTP to GDP in the presence of magnesium (Cassel and Selinger, 1978; Higashijima et al., 1987). Receptor-induced G protein activation requires the presence of GTP (Rodbell et al., 1974; Hanoune et al., 1975). 2) Purified G proteins, when reconstituted with their effectors in the GDP-bound form, lack the ability to modify effector activity, whereas nonhydrolyzable analogs of GTP [$GTP\gamma S$ or $Gpp(NH)p$] are able to activate the G protein (Graziano et al., 1989). Likewise, nonhydrolyzable GTP analogs can permanently activate G proteins in the cell membrane, even in the absence of receptor stimulation (Schramm and Rodbell, 1975). 3) Steady-state rate of GTPase is limited by the dissociation rate of GDP, which holds the G protein in the GDP-bound form. Receptor activation increases the rate of GDP dissociation without affecting the intrinsic rate of GTPase and thus leads to the exchange of GTP for GDP in the presence of GTP (Higashijima et al., 1987). This is observed as an increased rate of steady-state GTPase (Brandt et al., 1983; Cerione et al., 1984), which is correlated with the efficacy of the ligand used to stimulate the receptor (Costa and Hertz, 1989). 4) Inhibition of the GTPase activity by CTX-mediated ADP ribosylation of $G\alpha$ (Johnson et al., 1978) or by mutations in the $G\alpha$ subunit (Graziano and Gilman, 1989) increases the constitutive activity of the G protein in the presence of GTP, whereas mutations or regulating factors, such as regulators of G protein signaling, that increase the GTPase activity inhibit G protein activation (Watson et al., 1996). 5) Fluoroaluminate ion (AlF_4^-), which imitates the γ -phosphate of GTP in the nucleotide-binding pocket of G protein, can activate the GDP-bound G protein in purified or native systems (Bigay et al., 1985; Higashijima et al., 1991). Finally, 6) the crystal structures of G proteins have revealed that there are common conformational changes in the active $G\alpha$ subunit (bound with $GTP\gamma S$ or $GDP-AlF_4^-$) compared with its GDP-bound inactive form (Noel et al., 1993; Coleman et al., 1994; Lambright et al., 1994).

On the other hand, the present observations can be summarized as follows. 1) Adenylyl cyclase could be activated in the presence of GDP in a concentration-dependent manner. 2) GDP had almost similar potency with GTP in this activation. 3) GDP-mediated activation of adenylyl cyclase, similar to GTP-mediated activation, required the receptor activity; minimizing receptor signal by using inverse agonists or by fusing “wrong” receptor (δ -opioid) to G_s prevented nucleotide-mediated activation. 4) This activation could still be observed when $GDP\beta S$ was used instead of GDP or in the presence of $App(NH)p$ (instead of ATP) or UDP (which fully blocked the minimal conversion of GDP to GTP in the present experimental system). When considered collectively, the present results strongly suggest that the observed activation cannot be at-

tributed to the conversion of GDP to GTP by any mechanism whatsoever (see *Results*). Among all of the control experiments, the one in which we used CTX-treated membranes as a bioassay tool (see *Results* for the logic) constitutes the most critical evidence for this conclusion.

We used two different cell systems (HEK-293 and S49 or its cyc^- variant) to rule out the possibility that the present phenomenon was a peculiarity of a particular cell line that hosts receptor, G protein, and adenylyl cyclase. Moreover, the present phenomenon was observable in the following expression combinations: 1) S49 cells, a native system that expresses G_s and β -AR endogenously (Fig. 2D); 2) cyc^- cells that express β -AR endogenously but $G\alpha_s$ heterologously (Fig. 2C); 3) HEK-293 cells that express β -AR heterologously but $G\alpha_s$ endogenously (Fig. 2B); or 4) HEK-293 or cyc^- cells that express β -AR- $G\alpha_s$ fusion construct heterologously (Figs. 1 and 2A) in which the patterns of activation by GDP or GTP with or without agonist were all the same. This also showed that the observed phenomenon did not depend on whether the G_s protein or β -AR was overexpressed by heterologous transfection or whether they were both fused together. Therefore, we confidently used the β -AR- $G\alpha_s$ fusion construct to maximize receptor signal in the relevant experiments.

Although the current paradigm that attributes G protein activation to the GTP bound form is an inevitable and firm inference made from the above-mentioned facts, it obviously does not explain the present observations made in the β -AR- G_s adenylyl cyclase system. Therefore, we propose an additional (but not necessarily alternative) mechanism as a plausible explanation for the present data. Activated β -AR can convert $G\alpha_s$ to a state that is active independently of the identity of the bound nucleotide (i.e., GDP or GTP); in other words, the active receptor, when bound to G_s , can transmit a conformational signal to $G\alpha_s$, which results in a G_s state that can fruitfully interact with adenylyl cyclase, even when it is bound with GDP (see Fig. 8 for a schematic representation of the idea). Inactivation, in turn, is simply achieved upon termination of the “activating” signal from the receptor (e.g., upon dissociation of agonist from receptor). The existence of such a state that is equally active in its GDP-bound form under the influence of the receptor implicitly means that the GDP-GTP exchange on $G\alpha_s$ is not necessary for β_2 -receptor-induced activation. In fact, we were unable to witness any advantage of GTP over GDP in supporting receptor-mediated adenylyl cyclase activity; the two nucleotides behaved almost identically in all respects (save the CTX effect, but see below) (Figs. 2–6). When the basal activity of the receptor was suppressed by inverse agonists or by modifying the receptor expression qualitatively or quantitatively, GTP-mediated activation was affected as much as the GDP-mediated one. This suggests that, in $G\alpha_s$, GTP, unlike $GTP\gamma S$ (or $Gpp(NH)p$),

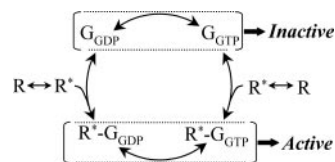


Fig. 8. A rough schematic summary of the present hypothesis for receptor-induced G_s activation with natural guanine nucleotides. R and R^* signify inactive and active receptor, respectively; the equilibrium between which is modified by receptor ligands. Other symbols are self-evident in the scheme. See *Discussion* for the implications.

may not be an activating nucleotide by itself but, similar to GDP, requires the intervention of the receptor for its activating potency to become apparent. However, the binding of a nucleotide (either GDP or GTP) seems to be necessary for G_s to adopt this hypothetical state because no receptor-induced cyclase activation was observed in the absence of the nucleotides (see Figs. 2 and 6).

An independent experimental support for the latter hypothesis that GTP- G_s may not be active in the absence of receptor intervention can be found in the functional behavior of an interesting mutant of G_{α_s} where Gln¹⁷⁰ in the helical domain was changed to Ala. This mutation resulted in a 4-fold increase in GDP dissociation rate without affecting the intrinsic GTPase or its coupling to receptor and effector (for details, see Warner and Weinstein, 1999). Hence, it is reasonable to expect that, in the presence of GTP, the GTP-bound form of the G protein accumulates more in Q170A-mutant than in the wild type, which should lead to a higher cyclase activity, if the GTP-bound form of G_s were active on its own. However, it was not so experimentally. The cyclase-activating ability of the mutant and wild type was exactly the same in the presence of GTP alone. These results are consistent with the idea that GTP binding to G_s is not a sufficient condition for activating G_s , but an additional activating signal from the receptor is required. Indeed, by using environmentally sensitive fluorophore-labeled guanine nucleotides, previous studies (Remmers et al., 1994; Remmers and Neubig, 1996) have shown that, compared with GTP γ S, the conformational effect of GTP on purified G_o protein is partial (independently of the GTPase) and that mastoparan, a receptor mimicking wasp venom peptide, can induce activation not only by facilitating the dissociation of GDP but also by inducing a conformational change on GTP-bound G_o (i.e., by converting inactive GTP- G_o to active GTP- G_o as the authors have stated), which suggests that the above hypothesis may also apply to G_o protein. However, different receptor-G protein-effector combinations should be studied in direct functional assays to generalize the hypothesis. Hence, at present, it should be limited to β -AR- G_s -cyclase system.

The mechanism we proposed here, based on the hypothesis that guanine-nucleotide exchange is neither required nor sufficient for G_s activation, seems to be the most plausible explanation for the present observations. Other explanations such as GDP-induced disinhibition of G_i protein (Piacentini et al., 1996; Lutz et al., 2002) or conversion of GDP to GTP do not explain the present results as a whole. However, there are still two compelling arguments that suggest that the guanine nucleotide exchange is a requirement for G_{α} activation and that GTP is the activating nucleotide. One is the strong correlation between the ability of ligand to induce GDP dissociation and ligand-induced activation of adenylyl cyclase. Such a correlation is also shown here under the same experimental conditions in which GDP activated adenylyl cyclase as efficiently as GTP (Fig. 6D). Thus, this correlation should not necessarily prove a causal link between receptor-induced GDP dissociation and activation but implies that these two processes (i.e., receptor-induced activation and increased rate of GDP dissociation) might be two different facets of the active conformation of the G protein. In other words, they may occur simultaneously as the protein enters into the active state, rather than being mechanistically dependent from one another. The second comes from the obser-

vations that mutations and modifications causing reduction or enhancement of GTPase activity also result, respectively, in enhancement (Graziano and Gilman, 1989) or inhibition (Watson et al., 1996; Warner and Weinstein, 1999) of GTP-mediated G protein activation. This suggests that the GTP-bound form of G_{α} is the active state and supports the idea that hydrolysis of bound GTP to GDP is the turn-off mechanism of activation. However, we show here (as others did under different experimental systems) that GDP does not turn off but actually sustains receptor-mediated activation of cyclase via G_s . We also show that ADP-ribosylation by CTX, a modification that diminishes GTPase activity, does not change GDP-mediated activation but dramatically amplifies that induced by GTP. According to the classic paradigm, toxin-induced reduction of intrinsic GTPase activity enhances the lifetime of the GTP-bound form of G_{α} and hence produces constitutive activation. However, it is just as likely that the primary effect of ADP-ribosylation (or modification of GTPase activity in general) may be to trigger an active state of GTP-bound G_{α_s} in the absence of receptor stimulus. Thus, we may speculate that the GTPase incompetence of the G_s protein may be conformationally (rather than causally) linked to its ability to assume a GTP-bound active state in the absence of receptor stimulus. In other words, there may not be a causal relationship between the inhibition of GTPase and enhanced ability of GTP to activate G_s protein. These two processes may simply be two different aspects of the same conformational perturbation imparted by the factors that eventually interfere with the GTPase activity. Such a perturbation can be achieved either by modifying the protein (i.e., ADP-ribosylation or mutagenesis) or the nucleotide chemistry [e.g., GTP γ S or Gpp(NH)p].

In conclusion, the present results show that β -AR activates G_s (and thus adenylyl cyclase) in the presence of excess GDP, which implies that the activation can occur without the involvement of a nucleotide exchange on the G_{α_s} subunit. The hypothesis that the G_s protein can assume an active conformation under the influence of β -AR, regardless of the identity of the bound nucleotide (GTP or GDP), seems to be a plausible explanation for the present results. However, it should be kept in mind that the observed phenomena may be a peculiarity of β -AR, G_s (long form), and adenylyl cyclase system and its generalization to other receptor and G protein systems obviously requires further studies. We believe that it is worth subjecting this hypothesis to further experimental testing, which may eventually help understand the molecular mechanism(s) of receptor-mediated activation of G_s or G proteins in general.

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Address correspondence to: Dr. H. Ongun Onaran, Ankara Üniversitesi Tıp Fakültesi, Farmakoloji ve Klinik Farmakoloji Ab.D. Sıhhiye 06100, Ankara, Turkey. E-mail: onaran@dialup.ankara.edu.tr
