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Guanine nucleotide exchange-independent activation of Gs protein by β_2-adrenoceptor[†]

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

Ankara University Faculty of Medicine, 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), Sıhhiye 06100 Ankara, Turkey. Department of Pharmacology (P.M., C.A) Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy.

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Running title: Guanine nucleotide exchange-independent activation of Gs

Address correspondence to: Dr. H. Ongun Onaran, Ankara Üniversitesi Tıp Fakültesi, Farmakoloji ve Klinik Farmakoloji Ab.D. Sıhhiye 06100, Ankara, TURKEY. Tel +90 312 311 8829, Fax: +90 312 310 6268, e-mail: onaran@dialup.ankara.edu.tr.

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Abbreviations:

β-AR: β-adrenoceptor; CTX: cholera toxin; HEK: Human embryonic kidney cell.

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Abstract

β_2-Adrenoceptor-mediated activation of Gs 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 β_2-adrenoceptor-Gs system, heterologously expressed in cyc^- or HEK-293 cells, can be activated in the presence of GDP or its phosphorylation-resistant analogue GDPβS. The potency and maximal ability of GDP to activate Gs and adenylyl cyclase were identical to those of GTP. GDP-mediated activation of adenylyl cyclase, like that mediated by GTP: 1) was concentration-dependent, 2) required high magnesium concentrations, 3) was inhibited by inverse agonists, and 4) was correlated with the efficacy of receptor ligands used to stimulate the receptor. UDP did not block the GDP-mediated activation, although it completely blocked the formation of a small amount of GTP (~5% of GDP) from GDP. Moreover, the activation of Gs 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. We therefore concluded that GDP itself could mediate β-adrenoceptor-induced activation of Gs-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-Gs-adenylyl cyclase system, where nucleotide-exchange is not necessary, and GDP and GTP play identical roles in receptor-induced Gs protein activation.

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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 receptor-mediated exchange of GDP with GTP on the nucleotide-binding site is necessary and sufficient to initiate the activation process. Deactivation, in turn, 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_α-GTP), thus the strength of the signal transmitted to the inside of the cell (see Gilman, 1987; Hamm, 1998 for review). 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. Accordingly, 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 receptor's ligand to displace GDP from G_α. More rigorously, the sensitivity of receptor-mediated G protein activation to the presence of GDP should be inversely correlated with the ligand's ability to reduce the affinity of GDP for G_α. 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_2-AR-Gs system by evaluating the sensitivity of ligand-induced adenylyl cyclase activity (which is well known to be mediated by Gs protein) to the presence of GDP. To our 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 (unpublished observation).

There is a number of experiments in the literature showing that Gs (or Gi) 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; Lutz et al., 2002; Seifert et al., 1998, Hatley et al., 2003). These reports include observations dating from late 1970s to the present day. Still, though,

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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 Schimada, 1983; Kikkawa et al., 1990). Thus, the true nature and significance of GDP-mediated activation of the G protein has 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 to 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.

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Materials and Methods

*Plasmid constructs***.** The cDNA encoding the long form of rat $G_{\alpha s}$ was in the pCDNA 3.1 (+) vector (Invitrogen, Carlsbad CA). Construction of cDNA's encoding the fusion proteins of human β-AR or δ-opioid receptor and the long form of rat $G_{\alpha s}$ was described elsewhere (Molinari et al., 2003).

*Cell culture and transfection***.** We used following cell systems throughout the present experiments: 1) native S49 cells, 2) cyc^- cells transfected with $G_{\alpha sL}$, 3) cyc^- cells transfected with β-AR-$G_{\alpha s}$ fusion protein, 4) HEK 293 cells transfected with β-AR, 5) HEK 293 cells transfected with β-AR-$G_{\alpha s}$ fusion protein, and finally 6) HEK 293 cells transfected with δ-opiod receptor-$G_{\alpha s}$ fusion protein. All the cell lines were cultured in Dulbecco's MEM with 10% fetal calf serum in standard conditions. cyc^- cells were transfected by electroporation essentially as described (Gonzales et al., 1992). HEK-293 cells were transfected with FuGENE. Stable clones were selected in the culture medium containing 600-700 μg/ml G418 as described (Ugur et al., 2003).

Cholera toxin treatment. cyc^- cells stably transfected with cDNA encoding $G_{\alpha s}$ were incubated with holotoxin (at a final concentration of 1 μg/ml) in standard cell culture conditions for 2 hours, which led a considerable increase in basal intracellular cAMP accumulation. We did not check the completeness of the ADP-ribosylation, for 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 before (Ugur and Onaran, 1997). Briefly, membranes were mixed in 96 well plates (0.25-2μg protein/well) with indicated additives in 75 μl, then the assay was initiated by adding 25 μl adenylyl cyclase assay buffer (final concentrations unless indicated otherwise; 50 mM Tris HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 250 μM ATP and 1 mM isobutylmethylxanthine) and terminated by adding 100 μl 0.2 N HCl. Before adding the ATP-containing adenylyl cyclase buffer, cell membranes and relevant additives were incubated together for 1-2 minutes. Preincubation time with GTPγS was 15 minutes, since our kinetic experiments showed that adenylyl cyclase activation in the presence of GTPγS reached to a peak in 15 minutes. All incubations were at 37 °C and total assay time was 5 minutes. Time-dependent cAMP accumulation was determined by sampling the reaction mixture at indicated time points. In this

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case, the reaction was started by adding membranes to the rest of the mixture. Amount of accumulated cAMP was measured by radioimmunoassay in all experiments as described before (Ugur 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 RIA determination.

We used an indirect approach to measure GDP-displacing ability of receptor ligands based on the fact that Al/F can activate the G protein only in its GDP-bound form. We incubated cell membranes with relevant ligands for 5 minutes in the absence of guanine nucleotides to let GDP to dissociate from Gs, and than determined cyclase activity after adding $NaF+AlCl_3$ as described above. We interpreted ligand-dependent "inhibition" of Al/F-induced cyclase activity as ligand's ability to displace GDP from Gs. 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 10-15 times through a 26-gauge syringe tip in a hypotonic buffer) were centrifuged at 400 xg for 5 min, and the supernatant was repelleted at 100.000 xg. 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 $MgCl_2$, protease mixture and 25% (w/v) sucrose and stored at –70 °C. Protein concentrations were determined by the Bradford assay. Radioligand binding assays and immunoblottings 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, 10 mM $MgCl_2$ in a final volume of 250 µL containing 1-2 µg 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). For immunobloting, 5 µg of membrane protein was separated by sodiumdodecylsulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose or PVDF paper (Biorad or Millipore). The proteins were detected with the polyclonal, affinity-purified RM antibody (1 µg/ml), which recognizes the carboxy-terminal decapeptide of $G\alpha_s$ and Enhanced Chemiluminesence (Amersham, Arlington Heights, IL) as described by the manufacturer. Horseradish peroxidase-conjugated goat anti-rabbit antibodies (developed in the M.B.T.D.R unit of Ankara University) were used to visualize the relevant bands.

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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) and a weakly basic anion exchanger column (Waters, Protein-Pak DEAE-5PW). Fractions were collected in a volume of 500 µl and radioactivity in the fractions was determined in a scintilation counter (Wallac Microbeta 1450 Trilux).

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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 $G_{\alpha s}$ in cyc$^-$ (which does not express Gs intrinsically) or in HEK-293 cells. Expression of the fusion protein in the selected clones was roughly 4 and 40 pmol/mg membrane protein in cyc$^-$ and HEK-293 cells, respectively, as assed by [^{125}I]-iodocyanopindolol saturation-binding. Integrity of the fusion protein in the cell membranes was assessed by immunoblotting (not shown).

We measured stimulated-adenylyl cyclase activity as a readout for Gs 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 did not exhibit any 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, GDP, added in excess amount, did not have any 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 (figure 1A, 1B) as assessed by means of a F statistics based on the evaluation of extra-residual 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-$G_{\alpha s}$ fusion protein in the presence of 100 μM isoproterenol (with a maximal response not different from that mediated by GTP under identical conditions) (figure 1C). Indeed, the latter observation is consistent 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).

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A full comparison of GDP- or GTP-mediated cyclase activation in HEK-293 cell membranes expressing the fusion protein is given in figure 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 (non-fused) human β_2-AR (figure 2B), 2) cyc^- cell membranes that express $G_{\alpha s}$ heterologously but β-AR endogenously (figure 2C), or 3) wild type S49 cell membranes that express both Gs and β-AR (400 fmol/mg) endogenously (figure 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 $G_{\alpha s}$ protein. Note that the absolute value of 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 the general pattern remained almost the same (compare figure 2A, 2B, 2C and 2D). Unlike GDP, GMP did not show an 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 μM. Such a bell-shaped tendency was also evident in GTPγS-induced activation of adenylyl cyclase in HEK-293 membranes that express β-AR-Gs fusion (figure 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 Gs. 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, Boehringer-Manheim or ICN), 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 Gs mediated adenylyl cyclase activation.

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Adenylyl cyclase activation observed in the presence of GDP or GTP (with or without agonist) was Mg-dependent, and its sensitivity to [Mg^{2+}] was the same for GDP and GTP (figure 3A), whereas the activation was invariant with respect to the salt (NaCl or KCl) used in the assay buffer (figure 3B). The only difference between the presence of potassium and sodium was that the inhibitory effect of inverse agonist (±)-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, 1996). Such a conversion must be nearly complete and almost instantaneous to be an explanation for the present results, since 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 almost equal level of adenylyl cyclase activation throughout the concentration-response curves (see figures 2 and 6). Under the assumption that only GTP is the activating nucleotide, 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 $G_{\alpha s}$. 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 μM), 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 minutes, respectively. UDP, which is known to inhibit nucleotide diphosphate kinase activities (Kimura and Schimada,

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1983) or local transphosphorylation of GDP on the nucleotide binding site of G_α (Kikkawa et al., 1990; Kowluru, 1996), totally blocked the conversion in 5 minutes (which was the maximum incubation time used in the present assays) (figure 4A), but did not affect GDP- (or GTP-) mediated activation of adenylyl cyclase significantly (figure 4B, 4C). 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 cells (data not shown), whereas the pattern of GDP- or GTP-mediated activation was still the same. 3) GDPβS, a phosphorylation-resistant analogue of GDP (Kowluru, 1996; Eckstein et al., 1979), was able to mediate adenylyl cyclase activation as much as GTP or GDP (figure 4D). 4) In the absence of ATP but in the presence of 500 μM App(NH)p, a substrate for adenylyl cyclase but not a phosphate donor, GDP was still able to support receptor-induced cyclase activation (figure 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 as 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 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-Gs fusion protein. Results are shown in figure 5A: The process of cAMP accumulation was identical for GTP and GDP, and did not depart from linearity in either case during 5 minutes of observation; steady-state adenylyl cyclase activity was achieved almost instantaneously with the addition of either nucleotide. This implies that either the local conversion

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from GDP to GTP does not occur and binding of GDP directly activates Gs, or 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 Gs, which is also an interesting possibility. In order 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 Gs. In cyc$^-$ cells expressing the long form of $G_{\alpha s}$, cyclase activity was about 4 times higher in CTX-treated membranes than in the control membranes in the presence, but not in the absence of added GTP. Therefore, comparison of the reactivities of CTX-treated and untreated membranes could provide information about 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 did not see any such "report from G_s" (i.e. signal amplification by CTX) in a wide range of GDP concentrations (figure 5C), whereas the amplification was obvious in the presence of added GTP (figure 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-Gs 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 cannot result from its conversion to GTP on the G_α binding site or in 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 (figure 5B).

Receptor- dependence of cyclase activation in the presence of GDP or GTP.

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 which inhibits basal activity of β-AR (Samama et al, 1994; Chidiac et al., 1994), suppressed the cyclase activity observed in the presence of GDP (or GTP) (figure 6A). 2) No nucleotide-mediated cyclase activity was observed when $G_{\alpha s}$ was fused to δ-opioid receptor instead of β-AR (figure 6B). Note that δ-opioid receptor is not actually

coupled to Gs. And, 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) (figure 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 (figure 7) (see discussion).

Two apparently different components of adenylyl cyclase activity can be distinguished by close inspection of figures 1, 2, 6: The one that depends on the presence of guanine nucleotides and the activity of the cognate receptor (β_2-AR), and the other 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 figure 7 below) and the ability of inverse agonists to inhibit nucleotide-dependent adenylyl cyclase activity (figure 6). This invariant background was about 200 pmol/mg/min in β-AR-Gs system. Its magnitude was apparently related with the total only by whether the Gs is fused to a receptor or not, regardless of the identity of the fused receptor (compare figure 2A, 2B, 2C and 6B). We did not further investigate this activity and concentrated only on the nucleotide- and receptor-dependent one.

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Discussion

In the present study we showed that β-AR, Gs 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. Following is a brief discussion of 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 GDP-bound form of the G protein is inactive, receptor stimulation "catalyses" the exchange of GDP with GTP on the G protein yielding the active GTP-bound state, and that GTPase activity of G_α is the turn-off mechanism for the activation (see Gilman, 1987; Hamm, 1998 for review).

Experimental foundations of the current paradigm for G protein activation can be roughly summarized as follows: 1) G_α subunits bind GDP or GTP (or their analogues) and hydrolyze the bound GTP to GDP in the presence of Mg (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 nonhydrolysable analogues of GTP (GTPγS or Gpp(NH)p) are able to activate the G protein (Graziano et al., 1989). Likewise, nonhydrolysable GTP analogues 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 (Cerione et al., 1984; Brandt et al., 1983), 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_α (Johnson et al., 1978), or by mutations in the G_α 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 RGS proteins that increase the GTPase activity

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inhibits 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). And finally, 6) crystal structures of G proteins have revealed that there are common conformational changes in the active G_α subunit (bound with GTPγS or GDP-AlF_4) compared to its GDP-bound inactive form (Noel et al., 1993; Lambright et al., 1994; Coleman et al., 1994).

The present observations, on the other hand, can be summarized as follows: 1) adenylyl cyclase could be activated by β-AR 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, like the GTP-mediated one, required the receptor activity: Minimizing receptor signal by using inverse agonists or by fusing "wrong" receptor (δ-opioid) to Gs prevented nucleotide-mediated activation. 4) This activation could still be observed when GDPβS 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 attributed to the conversion of GDP to GTP by any mechanism whatsoever (see results). Among all 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 Gs and β-AR endogenously (figure 2D), 2) cyc^- cells that express β-AR endogenously but $G_{\alpha s}$ heterologously (figure 2C), 3) HEK cells that express β-AR heterologously but $G_{\alpha s}$ endogenously (figure 2B), or 4) HEK or cyc^- cells that express β-AR-$G_{\alpha s}$ fusion construct heterologously (figure 1, figure 2A), in which the pattern of activation by GDP or GTP with or without agonist were all the same. This also showed that the observed

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phenomenon did not depend on whether the Gs protein or β-AR was overexpressed by heterologous transfection, or whether they were fused together. Therefore, we confidently used 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 β_2-AR, Gs 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 which is active independently of the identity of the bound nucleotide (i.e. GDP or GTP); the active receptor, when bound to Gs, can transmit a conformational signal to $G_{\alpha s}$, which results in a Gs state that can fruitfully interact with adenylyl cyclase even when it is bound with GDP (see figure 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 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) (figures 2, 3, 4, 5, 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 GTPγS (or GppNHp), may not be an activating nucleotide by itself, but like GDP, requires the intervention of the receptor in order for its activating potency to become apparent. However, binding of a nucleotide (either GDP or GTP) seems to be necessary for Gs to adopt this hypothetical state, since no receptor-induced cyclase activation was observed in the absence of the nucleotides (see figures 2 and 6).

An independent experimental support for the latter hypothesis that GTP-Gs may not be active in the absence of receptor intervention can be found in the functional behavior of an interesting mutant of

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$G_{\alpha s}$, where Gln^{170} 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 (see reference Warner and Weinstein, 1999 for details). 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 WT, which should lead to a higher cyclase activity, if the GTP-bound form of Gs were active by its own. However, it was not so experimentally: cyclase activating ability of the mutant and WT were exactly the same in the presence of GTP alone. These results are consistent with the idea that GTP binding to Gs is not a sufficient condition for activating Gs, but an additional activating signal from the receptor is required. Indeed, by using environmentally-sensitive fluorophore-labeled guanine nucleotides, Remmers et al. (Remmers et al., 1994; Remmers and Neubig, 1996) have shown that, compared to GTPγS, the conformational effect of GTP on purified Go 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 Go (i.e. by converting inactive GTP-Go to active GTP-Go as the authors have stated), which suggests that the above hypothesis may also apply to Go 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 β2-AR-Gs-cyclase system.

The mechanism we proposed here, based on the hypothesis that guanine-nucleotide exchange is neither a requirement nor sufficient for Gs activation, seems to be the most plausible explanation for the present observations. Other explanations such as GDP-induced disinhibition of Gi protein (Piacentini et al., 1996; Lutz et al., 2002) or conversion of GDP to GTP does not explain the present results as a whole. However, there are still two compelling arguments that suggest the guanine nucleotide exchange to be 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 was also shown here under the same experimental conditions, in which GDP activated adenylyl cyclase as efficiently as GTP (figure 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) may 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 observations 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 in 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 classical paradigm, toxin-induced reduction of intrinsic GTPase activity enhances the lifetime of the GTP-bound form of G_α, and hence, produces constitutive activation. It is just as likely, however, that the primary effect of ADP-ribosylation (or modification of GTPase activity in general) may be to trigger an active state of GTP-bound $G_{\alpha s}$ in the absence of receptor stimulus. Thus, we may speculate that the GTPase-incompetence of the Gs 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 inhibition of GTPase and enhanced ability of GTP to activate Gs 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. GTPγS or Gpp(NH)p).

In conclusion, the present results show that β-AR activates Gs (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 $G_{\alpha s}$ subunit. The hypothesis that the Gs protein can assume an active conformation under the influence of β-AR regardless of the identity of the bound nucleotide (GTP or

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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_2-AR, Gs (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 test, which may eventually help understand the molecular mechanism(s) of receptor-mediated activation of Gs or G proteins in general.

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Footnotes

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Send reprint requests to: Dr. H. Ongun Onaran, Ankara Üniversitesi Tıp Fakültesi, Farmakoloji ve Klinik Farmakoloji Ab.D. Sıhhiye 06100, Ankara, TURKEY. Tel +90 312 311 8829, Fax: +90 312 310 6268, e-mail: onaran@dialup.ankara.edu.tr.

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Figure Legends

Figure 1

Isoproterenol-induced adenylyl cyclase activation in cell membranes. Adenylyl cyclase activity was measured in cyc$^-$ (1 μg/well) **(A)** and HEK-293 (0.5 μg/well) **(B)** cell membranes expressing β_2-AR-$G_{\alpha s}$ fusion protein in the presence of indicated concentrations of isoproterenol and 1 μM GTP. Solid lines indicate the non-linear 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. Panel **(C)** shows adenylyl cyclase activity measured in the membranes of cyc$^-$ cells expressing β_2-AR-$G_{\alpha s}$ fusion protein in the presence of indicated concentrations of GDP and 100 μM isoproterenol. For comparison, the levels of adenylyl cyclase activity measured in the presence of GTP (with or without isoproterenol) are also shown as empty circles and extended dotted lines. Data are mean values of 4-6 experiments ± S.E.M.

Figure 2

Sensitivity of adenylyl cyclase activation to GDP, GTP or GTPγS. Adenylyl cyclase activity was measured in HEK-293 cell membranes overexpressing β_2-AR-$G_{\alpha s}$ fusion protein **(A)**, β_2-AR **(B),** in $G_{\alpha s}$-transfected cyc$^-$ cell membranes **(C),** or in S49 cell membranes that express both β-AR and $G_{\alpha s}$ endogenously (**D**) in the presence of indicated concentrations of GDP, GTP with or without 100 μM isoproterenol as indicated. The activity in the absence of agonist was not shown in (D) as there was no measurable nucleotide-dependent activity in the absence of agonist in S49 cell membranes. Sensitivity of adenylyl cyclase activity to GTPγS in HEK-293 cell membranes expressing β_2-AR-$G_{\alpha s}$ fusion protein is shown in panel **(E).** Membranes were incubated 15 minutes with GTPγS before starting adenylyl cyclase assay. Two, 1 or 0.5 μg membrane protein/well was used in case of S49, cyc$^-$ or HEK cells, respectively to optimize the measurement sensitivity. See methods for more detail. Solid lines in

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all panels indicate non-linear regression of an arbitrarily modified gamma density function. Data are mean values of 4-11 determinations ± S.E.M.

Figure 3

Mg- or monvalent ion-dependence of adenylyl cyclase activation in HEK-293 cell membranes expressing β-AR-Gs. **(A)** Adenylyl cyclase activity was measured in HEK-293 cell membranes expressing β_2-AR-$G_{\alpha s}$ fusion protein in the presence of GTP (10 μM) or GDP (10 μM) with or without isoproterenol (ISO) at the indicated concentrations of Mg as log[molar]. Solid lines indicate nonlinear regression of the four-parameter logistic equation. Data are mean values of a quadruplicate experiment ± 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: GDP or GTP 10 μM, ICI118551 (ICI) 1 μM and isoproterenol (ISO) 100 μM. The absence of receptor ligands was indicated as 'cntr'. Data are mean values of 3 experiments ± S.E.M.

Figure 4

Control experiments for conversion of GDP to GTP in HEK 293 membranes expressing β_2-AR-$G_{\alpha s}$ fusion protein. **(A)** HPLC chromatograms showing the conversion of ^{32}P-labeled GDP (100 μM) to GTP at indicated times and conditions in cyclase buffer at 37 ºC. UDP was used at a concentration of 1 mM. **(B)** Adenylyl cyclase activity in the presence of 100 μM isoproterenol and 10 μM GDP or GTP at indicated concentrations of UDP, or **(C)** in the presence of 1 mM UDP and 100 μM isoproterenol at indicated concentrations of GDP. **(D)** Adenylyl cyclase activity in the presence of 10 μM GTP (empty bars) or 10 μM GDPβS (shaded bars) with or without 100 μM isoproterenol. **(E)** Adenylyl cyclase activity was measured exactly as above except that ATP was replaced with 500 μM App (NH)p, and

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isoproterenol-induced activity was shown as fold over basal activity measured in the presence of 10 μM of the indicated nucleotides nucleotide. Data are mean values of 3-4 experiments ± S.E.M.

Figure 5

Control experiments for conversion of GDP to GTP. **(A)** Time dependent-cAMP accumulation in HEK-293 cell membranes expressing β-AR-Gs measured right after addition of 10 μM GDP or GTP to the membranes that were preincubated (1 min) with or without isoproterenol as indicated. **(B)** isoproterenol-induced adenylyl cyclase activity in CTX-treated (CTX+) membranes in the presence of GDP. **(C, D)** Effect of CTX treatment on adenylyl cyclase activity in the presence of GDP **(C)** or GTP **(D)** in cyc^- cell membranes expressing $G_{\alpha s}$. Adenylyl cyclase activity was determined in the cell membranes prepared from CTX-treated (CTX+) or untreated (CTX-) cells. Dotted curve in panel B is reproduced from panel C to show the basal activity of CTX+ membranes in the presence of GDP. Data are mean values of 3 experiments ± S.E.M.

Figure 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 **(A)** in the presence of the inverse agonist ICI118551 (1 μM) in β-AR-Gs fusion system or **(B)** in a system where Gs was fused to a "wrong" receptor (δ-opioid, indicated as δ-OR-Gs). Nucleotide-mediated activation disappeared almost completely in either case. Data are mean values of 3-6 experiments ± 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-Gs. Adenylyl cyclase activity was determined in

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the presence of different ligands (each point) and 10 μM GDP (abscissa) or GTP (ordinate). Cyclase activity was presented as fold-over-basal. Following ligands were used at the indicated saturating concentrations (in the order of intrinsic activity): Epinephrine (100 μM), isoproterenol (100 μM), cimaterol (100 μM), clenbuterol (100 μM), dobutamine (100 μM), alprenolol (1 μM), cyanopindolol (1μM), propranolol (1 μM), sotalol (1 μM), ICI-118,551 (1 μM), timolol (1 μM). Solid line is the y = x line. Data are mean values of 3 experiments ± S.E.M.

Figure 7

GDP dissociating effect of β-AR Ligands. **A)** AlF ($AlCl_3$ + NaF)-induced adenylyl cyclase activity was determined after 5-minute incubation with indicated ligands in the absence or presence of 10 μM GDP in the HEK-293 cell membranes expressing β-AR-Gs. 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 methods for details). Data are mean values of a quadruplicate experiment ± S.E.M. **B)** Ability of ligands to displace GDP from Gs (as determined in panel A) was plotted against the ability of ligands to activate adenylyl cyclase in the presence of 10 μM GTP in the same membrane system. GDP-displacing effect of the ligands was shown (abscissa) as the relative inhibition of AlF-induced response (calculated from panel A). Ligand-induced activation was given as (ordinate) the adenylyl cyclase activity relative to the basal level (measured in the presence of 10 μM GTP alone). Each point represents a ligand (used at the same concentrations as in figure 6). The abbreviations are: Epi: epinephrine, ISO: isoproterenol, Cim: cimaterol, Clen: clenbuterol, Dob: dobutamine, Alp: alprenolol, Cyp: cyanopindolol, Prop: propranolol, ICI: ICI118551, Tim: timolol, Sot: sotalol and none: no addition of ligand.

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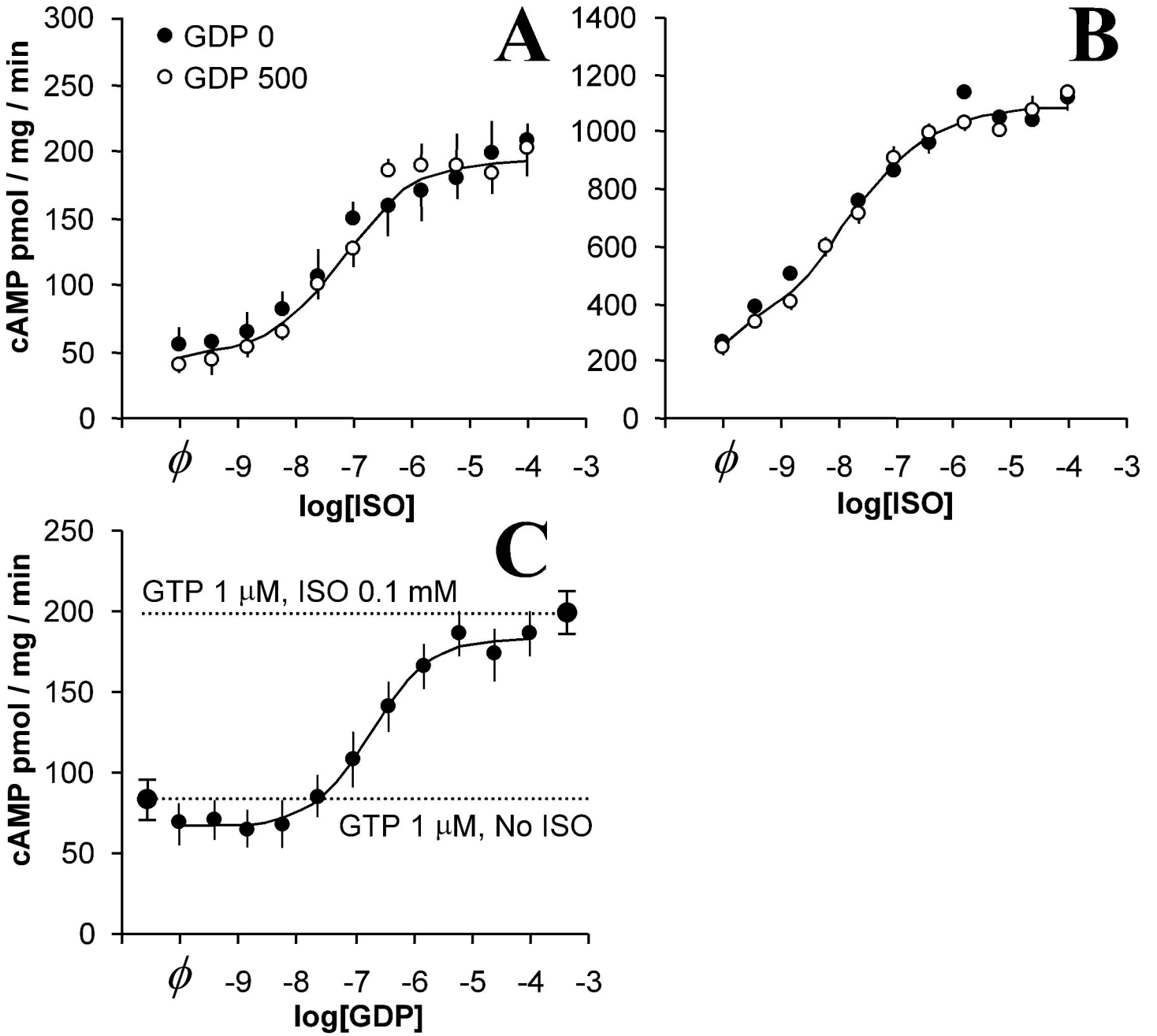
Figure 8

A rough schematic summary of the present hypothesis for receptor-induced Gs 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 text for the implications.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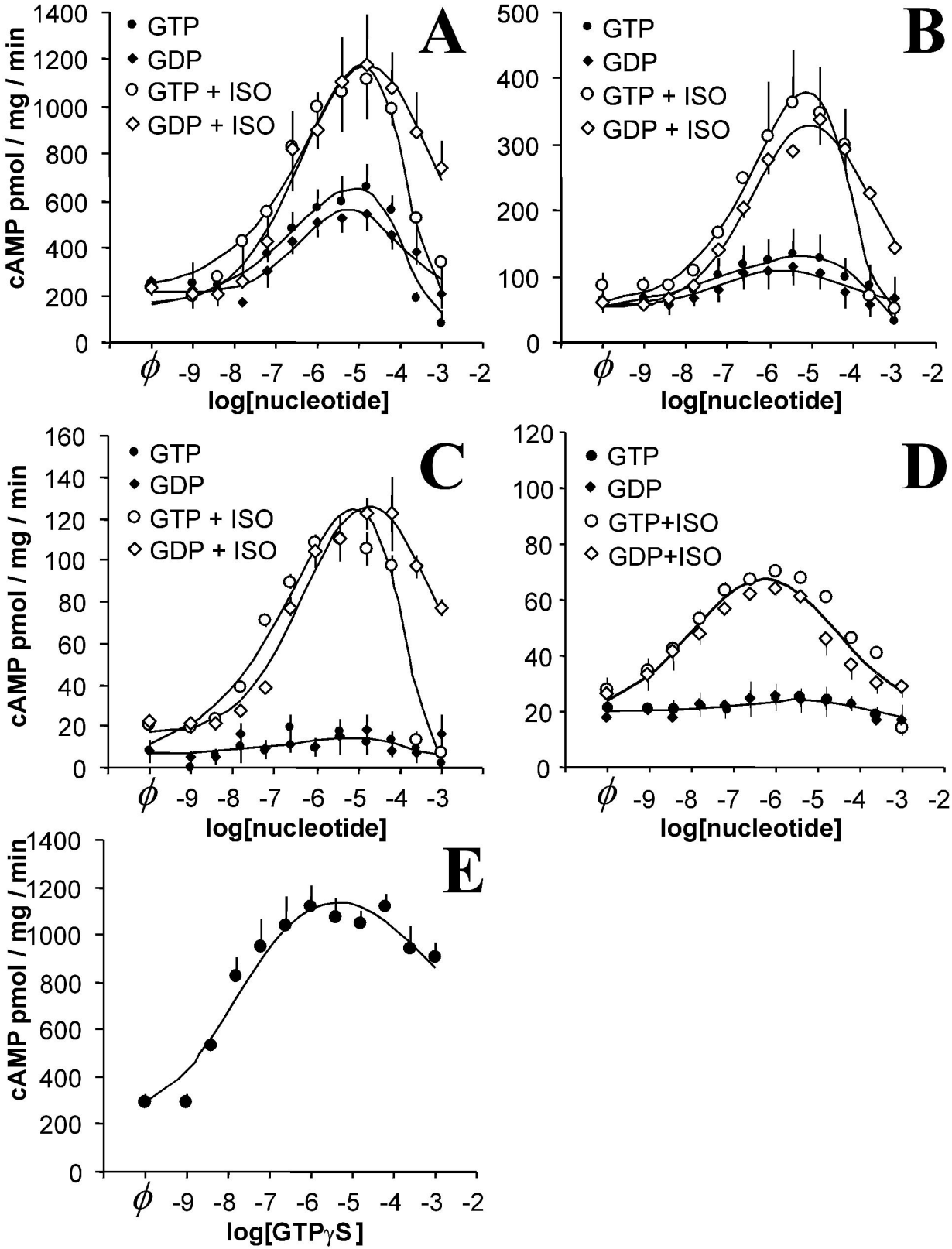
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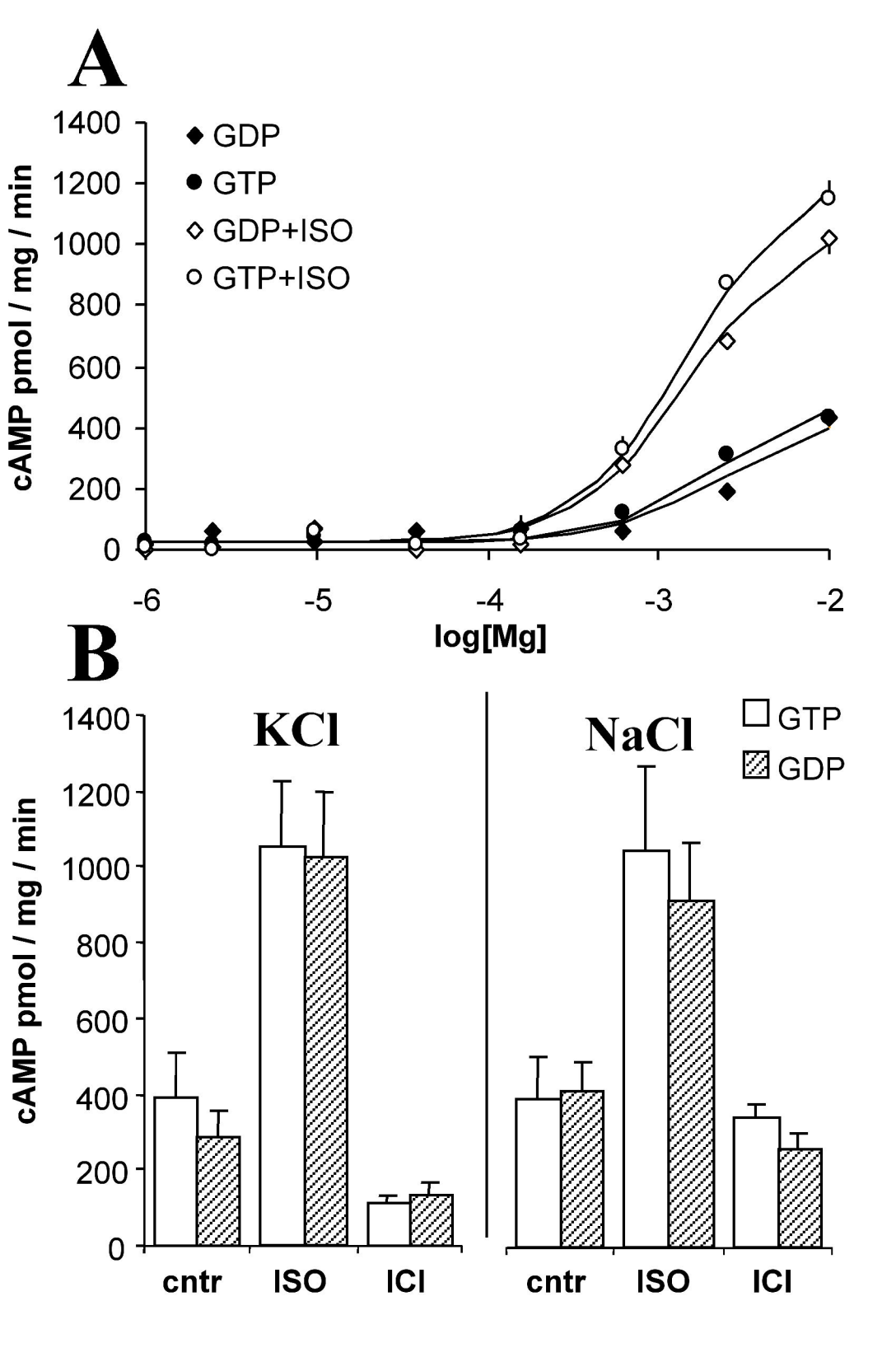
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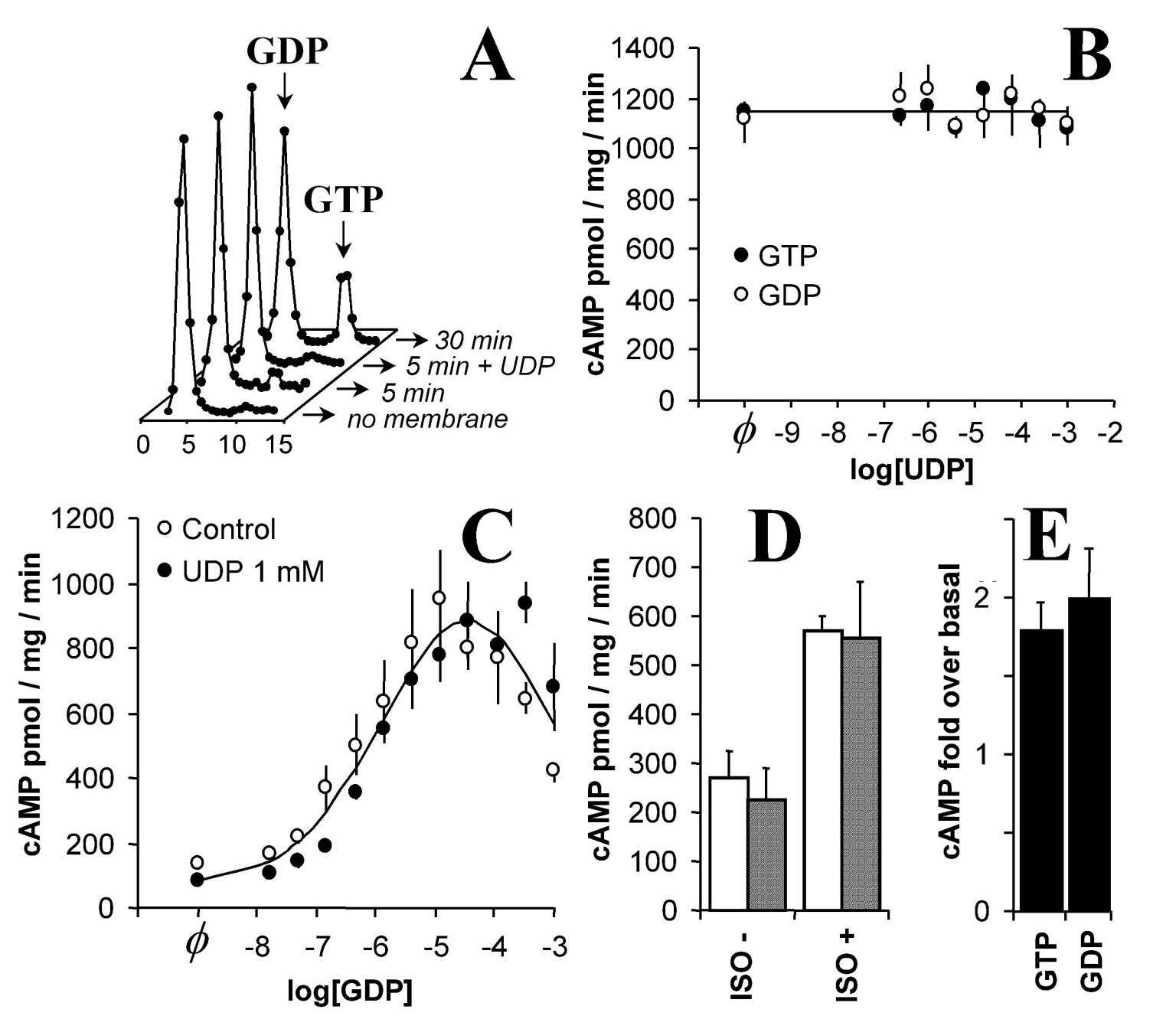
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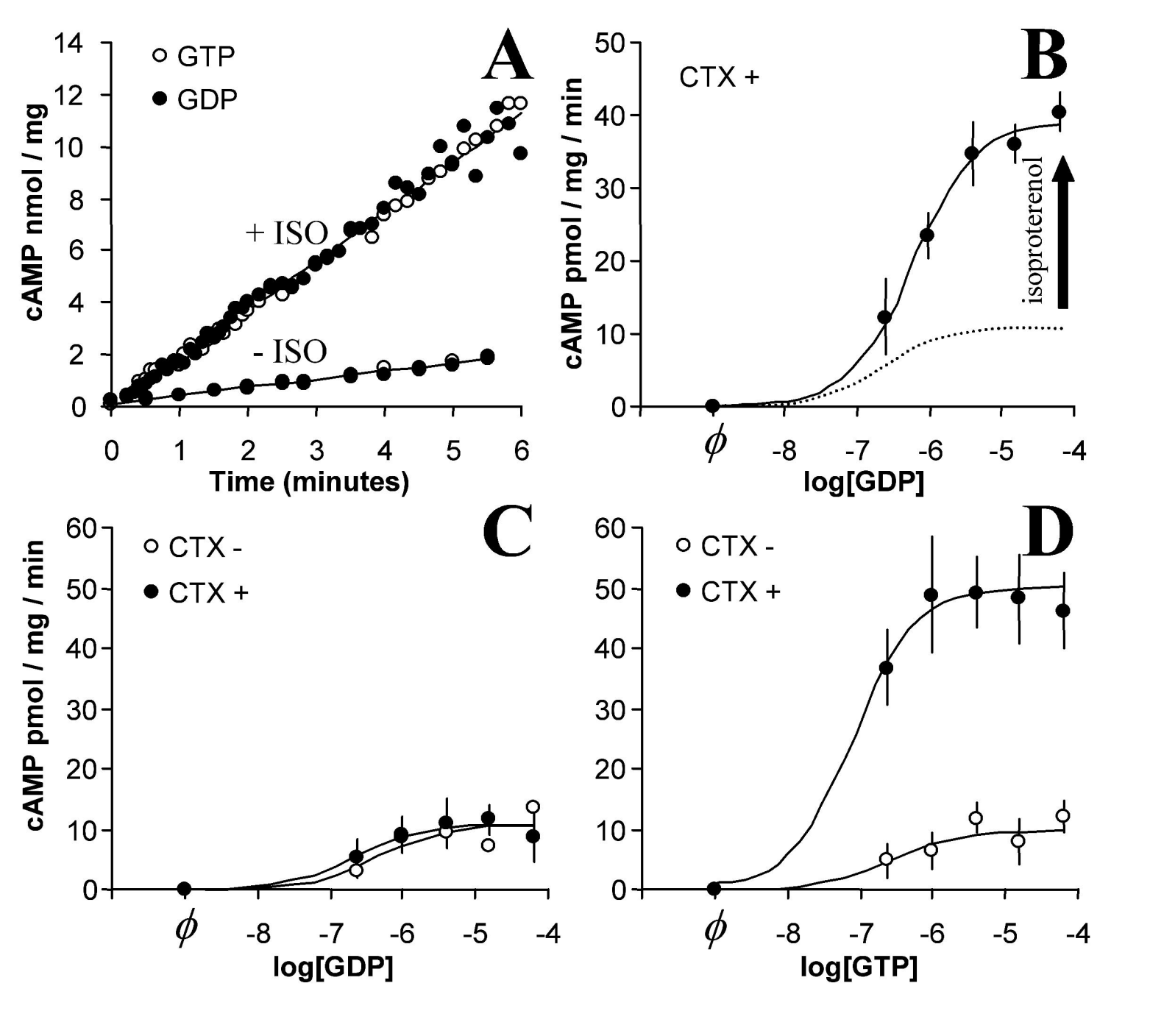
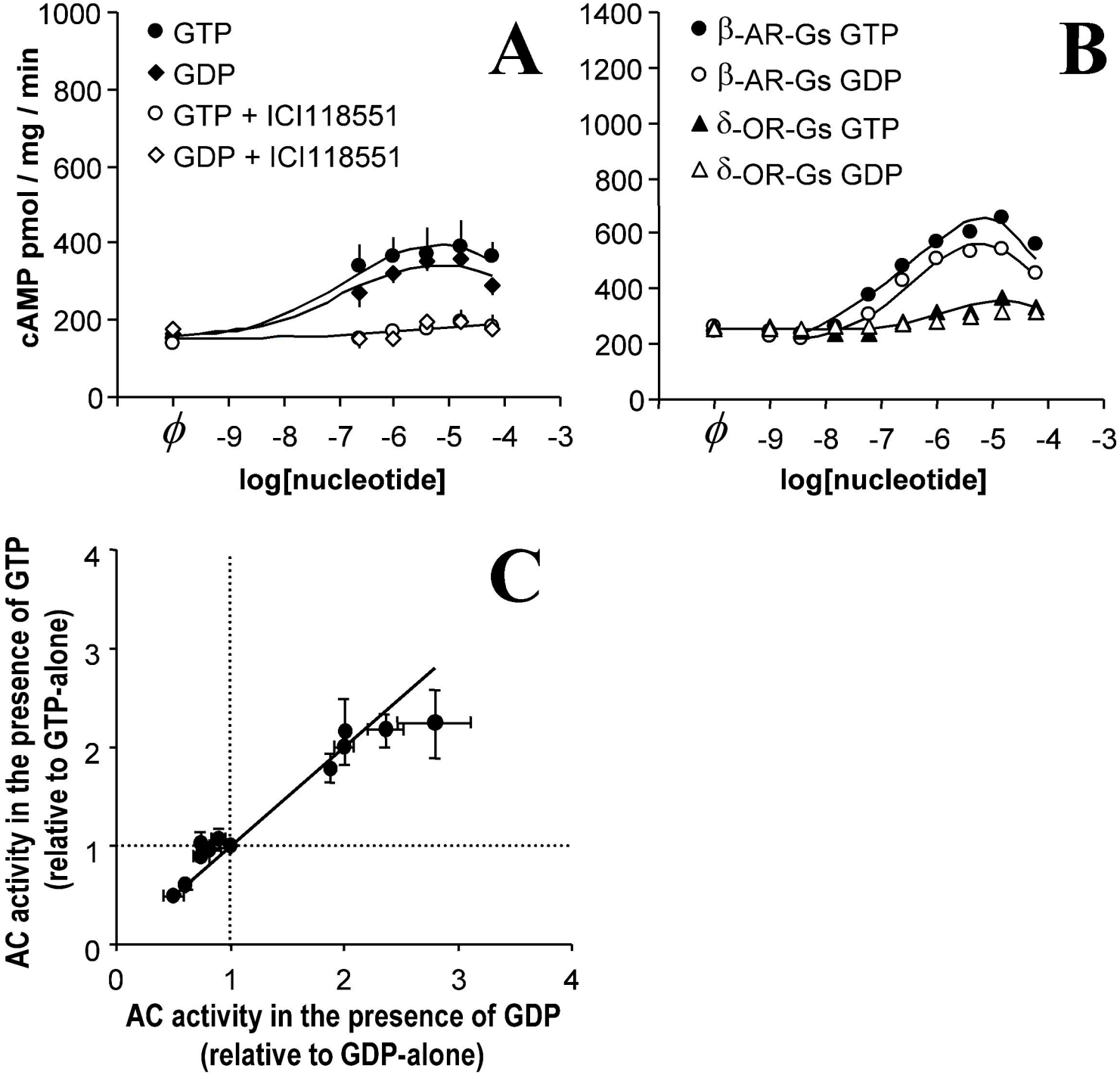
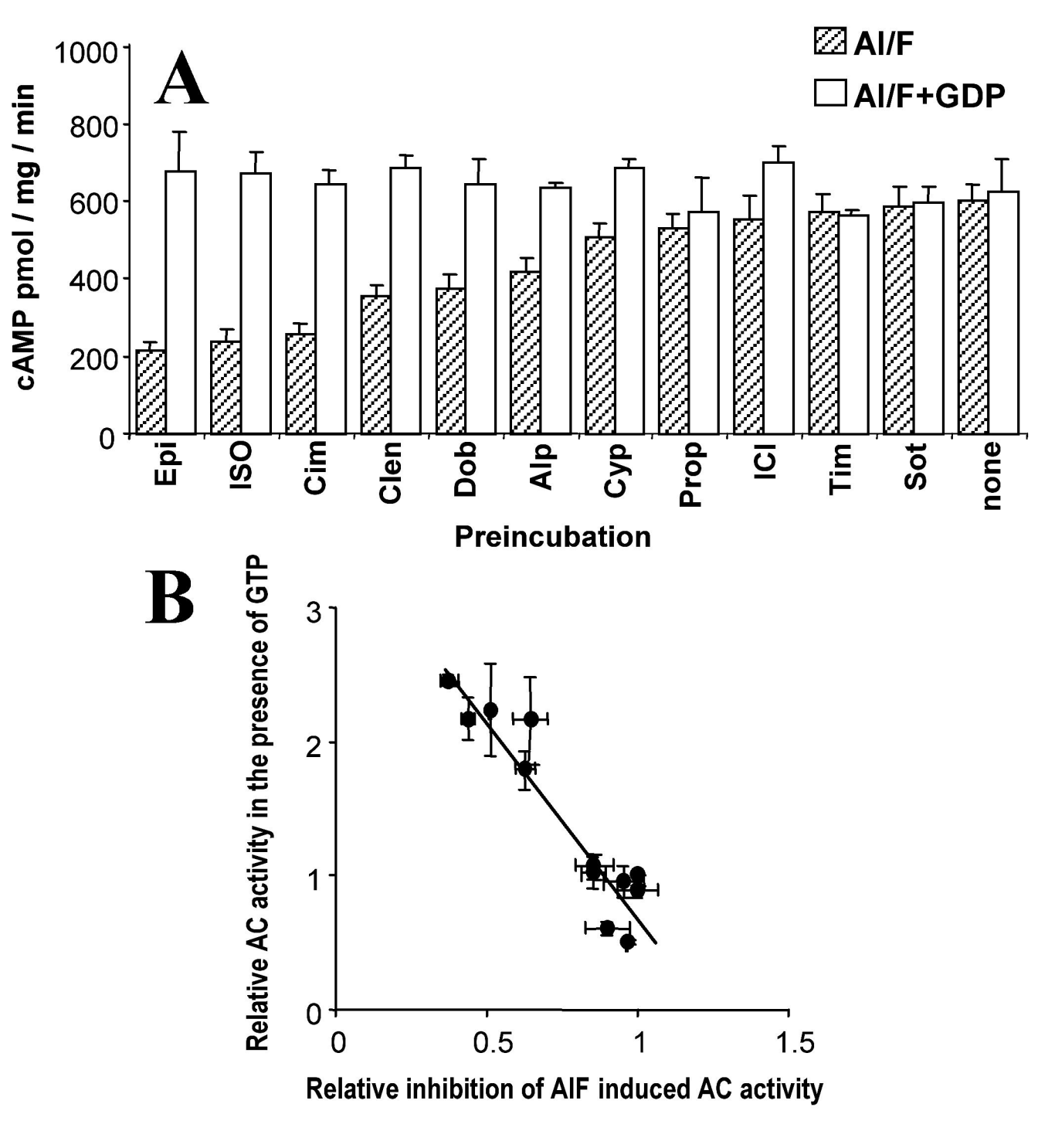
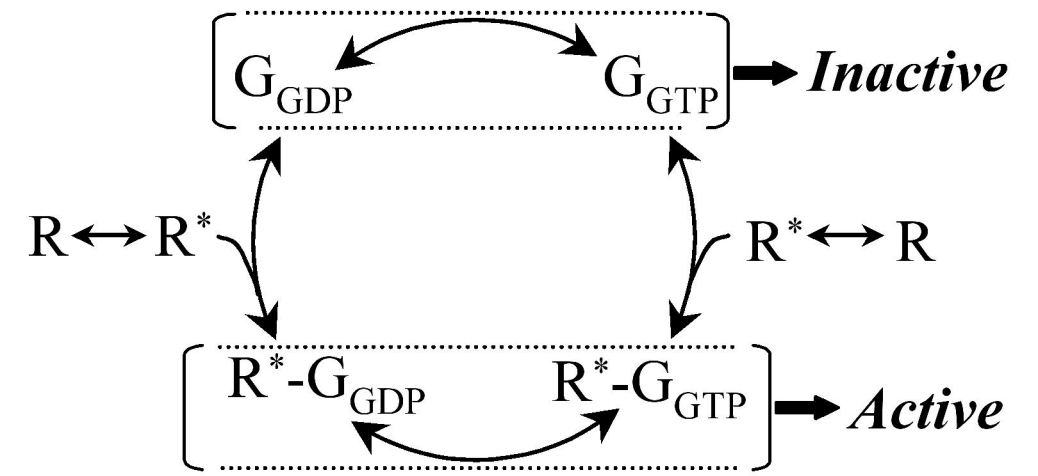
GTP
GDP


A
GTP
GDP
+ ISO
- ISO
cAMP nmol / mg
Time (minutes)
B
CTX +
isoproterenol
cAMP pmol / mg / min
log[GDP]
C
CTX -
CTX +
cAMP pmol / mg / min
log[GDP]
D
CTX -
CTX +
log[GTP]




A
AlF
AlF+GDP
cAMP pmol / mg / min
1000
800
600
400
200
0
Epi
ISO
Cim
Clen
Dob
Alp
Cyp
Prop
ICI
Tim
Sot
none
Preincubation
B
Relative AC activity in the presence of GTP
3
2
1
0
0
0.5
1
1.5
Relative inhibition of AlF induced AC activity


G_{GDP}
G_{GTP}
Inactive
$R \leftrightarrow R^*$
$R^* \leftrightarrow R$
$R^*\text{-}G_{GDP}$
$R^*\text{-}G_{GTP}$
Active