Guanine nucleotide-independent inhibition of adenylate cyclase by a stimulatory hormone

Claudia GREINER and Karl H. JAKOBS

Pharmakologisches Institut der Universität Heidelberg, Im Neuenheimer Feld 366, D-6900 Heidelberg, Federal Republic of Germany

Stimulation of basal adenylate cyclase activity in membranes of neuroblastoma × glioma hybrid cells by prostaglandin E₁ (PGE₁) is half-maximal and maximal (about 8-fold) at 0.1 and 10 μM respectively. This hormonal effect requires GTP, being maximally effective at 10 μM. However, at the same concentrations that stimulate adenylate cyclase in the presence of GTP, PGE₁ inhibited basal adenylate cyclase activity when studied in the absence of GTP, by maximally 60%. A similar dual action of PGE₁ was observed with the forskolin-stimulated adenylate cyclase, although the potency of PGE₁ in both stimulating and inhibiting adenylate cyclase was increased and the extent of stimulation and inhibition of the enzyme by PGE₁ was decreased by the presence of forskolin. The inhibition of forskolin-stimulated adenylate cyclase by PGE₁ occurred without apparent lag phase and was reversed by GTP and its analogue guanosine 5'-[γ-thio]triphosphate at low concentrations. Treatment of neuroblastoma × glioma hybrid cells or membranes with agents known to eliminate the function of the inhibitory GTP-binding protein were without effect on PGE₁-induced inhibition of adenylate cyclase. The data suggest that stimulatory hormone agonist, apparently by activating one receptor type, can cause both stimulation and inhibition of adenylate cyclase, and that the final result depends only on the activity state of the stimulatory GTP-binding protein, G_s. Possible mechanisms responsible for the observed adenylate cyclase inhibition by the stimulatory hormone PGE₁ are discussed.

INTRODUCTION

The hormone-sensitive adenylate cyclase system is a multi-component system consisting of several interacting subunits embedded in the lipid bilayer of the plasma membrane. The components known so far are stimulatory and inhibitory hormone receptors, the adenylate cyclase catalyst and the two guanine nucleotide-binding regulatory proteins (G-proteins) G_s and G_i, acting as coupling components between stimulatory and inhibitory hormone receptors respectively, and the adenylate cyclase catalyst. Both of these proteins are heterotrimeric, consisting of a guanine nucleotide-binding α-subunit, which is different in G_s and G_i, and β- and γ-subunits, which are apparently very similar or identical in the two proteins. The G-proteins appear to oscillate between two activity states, an inactive GDP-bound form and an active GTP-bound form. Hormone–receptor complexes apparently activate the G-proteins by promoting the replacement of bound GDP by GTP in the guanine nucleotide-binding α-subunit (Birnbaumer *et al.*, 1985; Levitzki, 1986; Stryer & Bourne, 1986; Jakobs *et al.*, 1986; Gilman, 1987). Whereas the mechanism underlying inhibition of adenylate cyclase by an activated G_i-protein is still controversial, there is general agreement that stimulation of adenylate cyclase is caused by the GTP-bound α_s-subunit. The βγ-dimer, possibly released from the holo-G_s-protein during its activation, is assumed to act as an inhibitory constraint for the α_s-subunit.

When studied in a system reconstituted from purified

components (β-adrenoceptor, G_s-protein and adenylate cyclase), it has been shown that, for stimulation of adenylate cyclase by the agonist-occupied receptor, not only G_s but also a guanosine triphosphate is required (May *et al.*, 1985; Feder *et al.*, 1986). Membrane preparations used for study of adenylate cyclase regulation often contain small amounts of endogenous GTP or GDP. Therefore, addition of a stimulatory hormone usually causes at least a small activation of adenylate cyclase, even without GTP added. This stimulation can then be further increased by the addition of GTP or one of its poorly hydrolysable analogues. We report here that not only is the stimulation of adenylate cyclase in membranes of neuroblastoma × glioma hybrid cells by the hormonal factor prostaglandin E₁ (PGE₁) absolutely dependent on the presence of GTP, but that without GTP added the stimulatory hormone PGE₁ even causes inhibition of adenylate cyclase.

MATERIALS AND METHODS

Materials

PGE₁, 3-isobutyl-1-methylxanthine and phospho-creatine (Tris salt) were obtained from Sigma, Deisenhofen, Germany. ATP, GTP, guanosine 5'-[γ-thio]triphosphate (GTP[S]) and creatine kinase were purchased from Boehringer, Mannheim, Germany. Pertussis toxin was from List, Campbell, CA, U.S.A. Forskolin was kindly given by Dr. H. Metzger, Hoechst A.G., Frankfurt, Germany. [α-³²P]ATP was prepared enzymically (Walseth & Johnson, 1979).

Abbreviations used: PGE₁, prostaglandin E₁; G_s and G_i, the guanine nucleotide-binding regulatory protein that couples stimulatory and inhibitory hormone receptors, respectively, to adenylate cyclase; GTP[S], guanosine 5'-[γ-thio]triphosphate.

Cell culture and membrane preparation

The culture of neuroblastoma × glioma hybrid cells (NG 108-15 cells), kindly given by Dr. B. Hamprecht, Tübingen, Germany, was carried out essentially as described by Hamprecht *et al.* (1985). After growth to confluency in plastic culture flasks, the cells were mechanically detached from their substrate. Thereafter, the cells were washed with iso-osmotic NaCl solution containing 10 mM-triethanolamine/HCl, pH 7.4. The cells were then resuspended in an ice-cold medium containing 100 mM-NaCl, 0.5 mM-EDTA and 50 mM-KH₂PO₄, pH 7.0, at a concentration of 10⁸ cells/ml. The cells were disrupted by nitrogen cavitation. After disruption, the homogenates were supplemented with 2-mercaptoethanol (15 mM) and EDTA (2.5 mM) and centrifuged for 1 min at 1000 *g* to remove nuclei. The supernatant was re-centrifuged at 30000 *g* for 10 min at 4 °C. The pellet thus obtained was resuspended in 10 mM-triethanolamine/HCl, pH 7.4, containing 5 mM-EDTA. This centrifugation procedure was repeated twice more. The final pellet, representing the membrane preparation used, was resuspended in 10 mM-triethanolamine/HCl, pH 7.4, and frozen in small batches at -70 °C. Pertussis-toxin treatment of intact NG 108-15 cells was for 16 h at 37 °C at a concentration of 50 ng of toxin/ml. Washing of the toxin-treated cells and preparation of membranes were performed as described above for control cells.

Adenylate cyclase assay

Adenylate cyclase activity was determined with a reaction mixture containing, if not otherwise indicated, 50 μ M- $[\alpha$ -³²P]ATP (0.3 μ Ci/tube), 1 mM-3-isobutyl-1-methylxanthine, 2 mM-MgCl₂, 0.1 mM-cyclic AMP, 5 mM-phosphocreatine, 0.4 mg of creatine kinase/ml, 2 mg of bovine serum albumin/ml and the additions indicated in 50 mM-triethanolamine/HCl, pH 7.4, in a total volume of 100 μ l. Reactions were started by the addition of NG 108-15 cell membranes (20–40 μ g of protein/tube) to the pre-equilibrated reaction mixture and conducted for 10 min at 30 °C. Reactions were stopped and cyclic [³²P]AMP was isolated as described previously (Jakobs *et al.*, 1976). The assays were performed in triplicate, with an intra-assay variation of about 5% of the means, and were repeated several times with results similar to those shown herein. PGE₁ and forskolin were diluted with water from 10 mM stock solutions in ethanol. Control samples always received the same concentration of ethanol as the test tubes.

RESULTS

NG 108-15 cells contain receptors for PGE₁ causing stimulation of adenylate cyclase, as shown by studies in intact cells and cell-free preparations (Hamprecht *et al.*, 1985). In the NG 108-15 cell membrane preparation used, PGE₁ increased basal adenylate cyclase activity by maximally about 8-fold at a concentration of 10 μ M. Half-maximal stimulation was observed at 0.1 μ M-PGE₁ (Fig. 1). This activation of the adenylate cyclase by PGE₁ required the presence of GTP, being half-maximally and maximally effective at 0.1 and 10 μ M respectively. When GTP was not added to the reaction mixture for measurement of adenylate cyclase activity, PGE₁ was not just without stimulatory effect on adenylate cyclase, but the stimulatory hormone now decreased

Fig. 1. Influence of PGE₁ on basal adenylate cyclase activity in membranes of NG 108-15 cells

Adenylate cyclase activity was studied in membranes of NG 108-15 cells as described in the Materials and methods section in the absence (●) and the presence (■) of 10 μ M-GTP at the indicated concentrations of PGE₁.

basal enzyme activity. Half-maximal and maximal (about 60%) inhibition occurred at 0.03–0.1 μ M- and 1–10 μ M-PGE₁ respectively. This most surprising result prompted us to study whether such a bimodal regulation of adenylate cyclase activity by PGE₁ is also observed when the enzyme is stimulated by the diterpene forskolin. As illustrated in Fig. 2, PGE₁ also caused a bidirectional response of the adenylate cyclase in the presence of a maximally effective concentration of forskolin (100 μ M). In the presence of GTP (10 μ M), PGE₁ induced a further increase in activity by about 80%, with half-maximal and maximal effects observed at 0.01 μ M- and 1 μ M-PGE₁ respectively. In contrast, in the absence of GTP, PGE₁ decreased the forskolin-stimulated adenylate cyclase activity by maximally about 30%. As observed for stimulation of adenylate cyclase in the presence of GTP, the inhibition observed in the absence of GTP was half-maximal and maximal at 0.01 μ M- and 1 μ M-PGE₁ respectively.

Using adenylate cyclase stimulated by forskolin (10 μ M), we studied whether the inhibitory effect of PGE₁ observed in the absence of GTP is a time-dependent process. As shown in Fig. 3, the stimulation of adenylate cyclase by forskolin (10 μ M) was preceded by a lag phase of 2–3 min. However, the inhibition caused by PGE₁ (0.1 μ M) occurred without apparent lag phase. Even during the lag phase of forskolin action, PGE₁ inhibited cyclic AMP accumulation, and the decrease in adenylate cyclase activity caused by PGE₁ remained constant (about 30%) over the incubation period studied.

The inhibition of forskolin-stimulated adenylate cyclase by PGE₁ was reversed by adding GTP at rather low concentrations (Fig. 4). Whereas, in the absence of

Fig. 2. Influence of PGE_1 on forskolin-stimulated adenylate cyclase activity

Adenylate cyclase activity was determined in membranes of NG 108-15 cells in the absence (●) and the presence (■) of $10 \mu M$ -GTP at the indicated concentrations of PGE_1 . Forskolin ($100 \mu M$) was present under each condition.

Fig. 3. Time course of adenylate cyclase inhibition by PGE_1

Cyclic AMP accumulation was studied in membranes of NG 108-15 cells in the absence (○) and the presence (●) of $0.1 \mu M$ - PGE_1 for the indicated periods of time. Forskolin ($10 \mu M$) was present under each condition.

GTP, PGE_1 inhibited adenylate cyclase stimulated by forskolin ($20 \mu M$) by maximally 35 %, the inhibition was decreased to maximally 10 % at $0.1 \mu M$ -GTP. At $1 \mu M$ - and $10 \mu M$ -GTP, PGE_1 only increased the stimulated enzyme activity. The stimulation caused by PGE_1 appeared to be due, at least in part, to a reversal of an inhibitory action of GTP observed at high concentrations

Fig. 4. Influence of GTP on the effect of PGE_1 on forskolin-stimulated adenylate cyclase

Adenylate cyclase activity was determined in membranes of NG 108-15 cells in the absence and the presence of PGE_1 and GTP at the indicated concentrations. Forskolin ($20 \mu M$) was present under each condition. (a) The results are plotted as a function of the concentrations of PGE_1 (a) and GTP (b) added.

(> $0.1 \mu M$) of this nucleotide in the presence of forskolin. Similarly to GTP, addition of the poorly hydrolysable GTP analogue GTP[S] at very low concentrations (0.01 – $0.1 \mu M$) eliminated the PGE_1 -induced inhibition of adenylate cyclase (Table 1). Furthermore, the inhibition was also mostly decreased in the presence of fluoride (10 mM -KF).

We then studied whether the inhibitory guanine nucleotide-binding regulatory protein G_i might be involved in the observed inhibition of adenylate cyclase

Table 1. Influence of GTP[S] and fluoride on PGE₁-induced inhibition of adenylate cyclase

Adenylate cyclase activity was determined in membranes of NG 108-15 cells in the absence and the presence of PGE₁ (1 μ M) without and with GTP[S] or KF at the indicated concentrations. Forskolin (10 μ M) was present under each condition. The results are means $\pm$ S.E.M. ($n = 3$).

Expt. no.	Addition	Activity (pmol of cyclic AMP/10 min per mg of protein)	
		Control	PGE ₁
I	None	322 $\pm$ 12	207 $\pm$ 8
	GTP[S] (0.01 μ M)	667 $\pm$ 18	675 $\pm$ 15
	GTP[S] (0.1 μ M)	764 $\pm$ 18	758 $\pm$ 12
II	None	297 $\pm$ 10	187 $\pm$ 9
	KF (10 mM)	715 $\pm$ 21	628 $\pm$ 16

Table 2. Influence of *N*-ethylmaleimide on regulation of adenylate cyclase activity in membranes of NG 108-15 cells

Membranes of NG 108-15 cells were pretreated without (Control) and with *N*-ethylmaleimide (35 μ M) for 10 min at 30 °C. This reaction was stopped by addition of dithiothreitol (1 mM). In the membranes pretreated thus, adenylate cyclase activity was determined without and with PGE₁ (1 μ M), GTP (10 μ M) or GTP plus PGE₁. Forskolin (20 μ M) was present under each condition. The results are means $\pm$ S.E.M. ($n = 3$).

Addition	Activity (pmol of cyclic AMP/10 min per mg of protein)	
	Control	<i>N</i> -Ethylmaleimide
None	389 $\pm$ 18	319 $\pm$ 11
PGE ₁	242 $\pm$ 9	212 $\pm$ 7
GTP	467 $\pm$ 21	612 $\pm$ 22
GTP + PGE ₁	672 $\pm$ 22	823 $\pm$ 26

by PGE₁. For this purpose, NG 108-15 cell membranes were treated with *N*-ethylmaleimide (35 μ M for 10 min at 30 °C), which is known to inactivate functionally the G_i-protein (Jakobs *et al.*, 1982, 1986). This treatment increased adenylate cyclase activity measured in the presence of GTP and GTP plus PGE₁. However, treatment of the membranes with the thiol-group-reacting agent did not affect the adenylate cyclase inhibition caused by PGE₁ (Table 2). Furthermore, we used membranes of NG 108-15 cells pretreated with pertussis toxin (50 ng/ml for 16 h). We have shown previously that this treatment abolishes the stimulation of high-affinity GTPase by an opioid-receptor agonist (Grandt *et al.*, 1986). As shown in Table 3, the treatment of the intact cells reversed the inhibition of forskolin-stimulated adenylate cyclase by GTP (10 μ M), to give a 2-fold stimulation. However, the pertussis-toxin treatment had

Table 3. Influence of pertussis toxin on regulation of adenylate cyclase activity by PGE₁ and GTP

Adenylate cyclase activity was determined in membranes of control NG 108-15 cells and of cells pretreated with pertussis toxin (50 ng/ml for 16 h) in the absence and presence of PGE₁ and GTP. Forskolin (10 μ M) was present under each condition. The results are means $\pm$ S.E.M. ($n = 3$).

Addition	Activity (pmol of cyclic AMP/10 min per mg of protein)	
	Control	Pertussis toxin
None	350 $\pm$ 12	308 $\pm$ 13
PGE ₁ (0.1 μ M)	231 $\pm$ 8	198 $\pm$ 7
GTP (10 μ M)	274 $\pm$ 10	602 $\pm$ 15

no effect on the inhibition of the stimulated adenylate cyclase by PGE₁.

DISCUSSION

The data presented here demonstrate that not only is GTP required for stimulation of adenylate cyclase by a stimulatory hormone, but that without GTP a stimulatory hormone can even cause inhibition of adenylate cyclase in membrane preparations. Membranes used for studying regulation of adenylate cyclase by hormones apparently usually contain small amounts of endogenous GTP or GDP, leading to an at least small hormonal activation of adenylate cyclase even without GTP addition. For full expression of the hormone action, addition of GTP is required. The most surprising result obtained in studying regulation of adenylate cyclase in membrane preparations of NG 108-15 cells was that in the absence of GTP the stimulatory hormone PGE₁ induced inhibition of adenylate cyclase. This inhibition, which occurred without apparent lag phase, was observed with the basal unstimulated enzyme as well with enzyme stimulated by the diterpene forskolin.

Mainly two problems have to be discussed here. First, is it the same receptor which induces stimulation and inhibition of adenylate cyclase, or are there two distinct PGE₁ receptor types, one causing adenylate cyclase stimulation and the other one inhibition when activated by the agonist? Although we lack final proof for one or the other hypothesis, we suggest that the two opposing actions of PGE₁ are mediated by one receptor type. This suggestion is mainly based on the similarity in the potency of PGE₁ in causing adenylate cyclase stimulation and inhibition. This similarity was not only observed with the basal adenylate cyclase, which was increased and decreased in its activity by PGE₁ with half-maximal effect at about 0.1 μ M, but also with the forskolin-stimulated enzyme. In the presence of forskolin, which has been shown previously to sensitize adenylate cyclase for the action of stimulatory hormones (Seamon & Daly, 1981), the potency of PGE₁ in both stimulating and inhibiting adenylate cyclase was considerably increased, with half-maximal effect at about 10 nM. Furthermore, if PGE₁ would induce adenylate cyclase inhibition by a distinct inhibitory receptor type, then addition of GTP should not reverse the inhibitory effect of PGE₁. Instead,

at least at the high concentrations of GTP ($10\ \mu\text{M}$), PGE_1 by activating the putative inhibitory receptor should activate the G_i -protein and cause inhibition of adenylate cyclase. However, even under maximal stimulation of adenylate cyclase by forskolin, we never observed inhibition of adenylate cyclase by PGE_1 in the presence of GTP. Thus all these data suggest that the two opposing effects of PGE_1 are mediated by one receptor type.

An even more intriguing problem is how the agonist-activated PGE_1 receptor induces inhibition of adenylate cyclase in the absence of GTP. First, the GTP-independence and the data obtained with agents known to inactivate the G_i -protein (pertussis toxin and *N*-ethylmaleimide) strongly argue against an involvement of the inhibitory G_i -protein in this effect of PGE_1 . Of the adenylate cyclase system components remaining, several possible interactions have to be considered. In the absence of GTP, the agonist-activated PGE_1 receptor may directly interact with the adenylate cyclase catalytic unit and decrease its activity, whereas in the presence of GTP the agonist-occupied receptor leads to an activation of the G_s -protein, which then causes adenylate cyclase stimulation. However, such an interpretation is not corroborated by reconstitution experiments (May *et al.*, 1985). Furthermore, it would suggest that, e.g., in S49 lymphoma cyc^- cells, which lack the G_s -protein (Harris *et al.*, 1985) but contain stimulatory hormone receptors (PGE_1 receptors and β -adrenoceptors), activation of these receptors should cause adenylate cyclase inhibition. However, such an inhibition of basal or forskolin-stimulated adenylate cyclase has not been described so far, either in intact cyc^- cells or in their membrane preparations.

If the agonist-activated receptor does not cause adenylate cyclase inhibition directly, the only known remaining component possibly responsible for this effect is the G_s -protein. A PGE_1 -induced inhibition of adenylate cyclase involving the G_s -protein may be caused by the following sequence of events. In the non-activated state, the adenylate cyclase may be closely associated with G_s or its α -subunit, being responsible for basal adenylate cyclase activity. This association appears to be reinforced by forskolin (Seamon & Daly, 1981). Interaction of PGE_1 with its receptor may cause a dissociation of G_s or its α -subunit from the adenylate cyclase and its association with the agonist-activated receptor. This dissociation should then lead to a decrease in adenylate cyclase activity. Since GTP is not present in sufficient concentrations, the G_s -protein will not re-associate with the cyclase. If GTP is added, G_s will be activated by the hormone-liganded receptor and will re-associate with the cyclase and increase its activity. This hypothesis would suggest that at least some G_s is interacting with the adenylate cyclase even in its basal state. Such a close association of the catalyst with the G_s α -subunit in the absence of activating ligands is supported not only by the comparison of basal and forskolin-stimulated adenylate cyclase activities in wild-type S49 lymphoma cells and their G_s -deficient cyc^- mutants, but also from adenylate cyclase purification experiments (Seamon & Daly, 1981; Arad *et al.*, 1984; Smigel, 1986). It is also feasible that the G_s -protein does not dissociate from the adenylate cyclase

and 'moves' to the agonist-activated receptor, but that the agonist-occupied receptor interacts with the adenylate cyclase- G_s -protein complex. In the absence of GTP, this interaction may lead to an inactivation of the G_s -protein, e.g. by releasing the guanine nucleotide bound to the α -subunit. In the presence of GTP, this inactivation is rapidly reversed, to give an activation with subsequent adenylate cyclase stimulation. As a further possibility, it has to be considered that interaction of PGE_1 with its receptor may lead to a dissociation of the G_s -protein and somehow to the formation of GDP-liganded α_s , acting as a weak inhibitor of adenylate cyclase. Finally, the observed inhibitory effect of PGE_1 may be entirely unrelated to an effect on the G_s -protein and may involve another, not yet identified, component of the signal-transduction system. Under conditions *in vivo*, i.e. in the presence of GTP, such an inhibition of adenylate cyclase by a stimulatory hormone may also occur, but only as a very short-lived intermediate step. Whatever the exact mechanism of guanine nucleotide-independent inhibition of adenylate cyclase by a stimulatory hormone may be, the data presented here corroborate the evidence that the final result of an agonist-activated receptor on adenylate cyclase activity is completely dependent on the permissive function of the guanine nucleotide-binding regulatory protein and its state of activity.

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