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Inhibition of hormonally regulated adenylate cyclase by the $\beta\gamma$ subunit of transducin

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Transducin (T), the GTP-binding protein of the retina activates the cGMP phosphodiesterase system, and presents analogies with the proteins G_s and G_i which respectively mediate adenylate cyclase activation and inhibition by hormone receptors. These proteins are all comprised of an α subunit carrying the GTP-binding site and a $\beta\gamma$ subunit made of two peptides. The β peptide (35 kd) appears similar in the three proteins. We demonstrate here that purified $T_{\beta\gamma}$ inhibits adenylate cyclase from human platelet membranes. This inhibition was observed when adenylate cyclase was stimulated by GTP, prostaglandin E_1 (PGE_1), NaF and forskolin, but not when stimulated by GTP(γ)S. In the presence of GTP and forskolin, the $T_{\beta\gamma}$-induced maximal inhibition was not additive with the α_2-receptor-induced adenylate cyclase inhibition mediated by G_i. Both inhibitions were suppressed at high Mg^{2+} concentrations, which are also known to dissociate $T_{\beta\gamma}$ from $T_{\alpha\text{-GDP}}$. This suggests that these adenylate cyclase inhibitions are due to the formation of inactive complexes of $G_{s\alpha\text{-GDP}}$ with $T_{\beta\gamma}$ or $G_{i\beta\gamma}$. $T_{\beta\gamma}$-induced inhibition did not require detergent and could be suppressed by simple washing. $T_{\beta\gamma}$ effects are dependent on its concentration rather than on its total amount. This suggests that $T_{\beta\gamma}$ can operate in solution with no integration into the membrane. Similar inhibitory effects of $T_{\beta\gamma}$ are observed on adenylate cyclase from anterior pituitary and lymphoma S49 cell lines.

Key words: adenylate cyclase inhibition/β subunit of GTP-binding proteins/hormone receptors/Mg^{2+}/transducin

Introduction

The transduction process of extracellular signals into intracellular messages is of basic importance for cell function. Thus, it is not surprising that essentially the same mechanisms have been retained by nature during evolution to perform this function. It has become clear that molecular mechanisms involved in stimulation and inhibition of the adenylate cyclase and those implied in visual transduction in retinal rods are very similar (Fung *et al.*, 1981; Manning and Gilman, 1983; Chabre *et al.*, 1984). All these systems are made up of three components: a receptor, a GTP-binding protein and an effector. In the visual system the receptor is rhodopsin, which captures photons (the signal). The GTP-binding protein is called transducin (T) and is composed of three subunits, T_α, T_β (35 kd) and T_γ (6 kd) (Kühn, 1980). The effector is a cyclic GMP phosphodiesterase (Fung *et al.*, 1981; Uchida *et al.*, 1981). In hormonally-regulated adenylate cyclase systems, the hormone (signal) is recognized by a very specific receptor. There are two distinct GTP-binding proteins, one for mediating enzyme stimulation (N_s or G_s) the other for mediating enzyme inhibition (N_i or G_i) (Rodbell, 1980). Both G_s and G_i appeared to contain the same β subunit (35 kd) (G_β) and a different α subunit respectively termed $G_{s\alpha}$ (42 kd) and $G_{i\alpha}$ (39–40 kd) (Gilman, 1984a; Hildebrandt *et al.*, 1984a). The β subunit of the hormonal G proteins is tightly associated to a γ subunit (6–10 kd) as has been shown for transducin (Gilman, 1984a; Hildebrandt *et al.*, 1984a). In the three transducing processes, the β subunits appear to be very similar (Manning and Gilman, 1983). However, it is likely that the γ subunit of G proteins is different from the γ subunit of transducin, since the cDNA that encodes the γ subunit of bovine transducin does not hybridize with brain, heart or liver mRNA (Van Dop *et al.*, 1984). Since transducin represents 20% of rod cell proteins, purification of $T_{\beta\gamma}$ is more easily accomplished than with $G_{\beta\gamma}$. In the hormone systems, stimulation of adenylate cyclase activity occurs with the dissociation of G_s into $G_{\beta\gamma}$ and GTP-bound G_α. This dissociation depends on the presence of GTP and Mg^{2+} (Northup *et al.*, 1982; Iyengar and Birnbaumer, 1982). The GTP carrying G_α is the active subunit, while $G_{\beta\gamma}$ may be inhibitory (Northup *et al.*, 1982). More recently, it was shown that when resolved subunits of G_i were incubated with platelet membranes, the inhibitory activity was found to reside largely with $G_{\beta\gamma}$ (Katada *et al.*, 1984a,1984b).

The aim of the present study was to investigate the biochemical effects of $T_{\beta\gamma}$ on several membrane-bound adenylate cyclase systems and to compare its effects with those of $G_{\beta\gamma}$, already reported (Katada *et al.*, 1984a,1984b).

Results

$T_{\beta\gamma}$ inhibits human platelet membrane adenylate cyclase

We first measured the effect of $T_{\beta\gamma}$ on human platelet membrane adenylate cyclase under experimental conditions identical to those reported by Katada *et al.* (1984a) to study the effects of $G_{\beta\gamma}$ on the same system. We particularly respected the pre-incubation period (15 min) of the subunit with membranes, the presence of a small amount of detergent (Lubrol 0.005%) and forskolin. Under these conditions $T_{\beta\gamma}$ induced a dose-dependent and saturable inhibition (Figure 1A). A 50–70% inhibition could be obtained with an EC_{50} (concentration of $T_{\beta\gamma}$ giving half maximal inhibition) of 0.2 μg/ml. Human platelet membranes are known to contain α_2 adrenergic receptors mediating an adenylate cyclase inhibition (Jakobs *et al.*, 1981; Aktories and Jakobs, 1981). In this system the inhibition is not dependent on the presence of Na^+, and appears to result in an increase in the concentration of Mg^{2+} required to stimulate the adenylate cyclase system (Bockaert *et al.*, 1984). Adenylate cyclase inhibition is accompanied by the activation of a GTPase and is unobservable in the presence of non-hydrolysable GTP analogs (Jakobs and Schultz, 1983). When added together, norepinephrine (NE) and $T_{\beta\gamma}$ produced a non-additive inhibition which was slightly greater than that produced by each ligand alone (Figure 1A). The temperature-denatured $T_{\beta\gamma}$ subunit was inactive (Figure 1A).

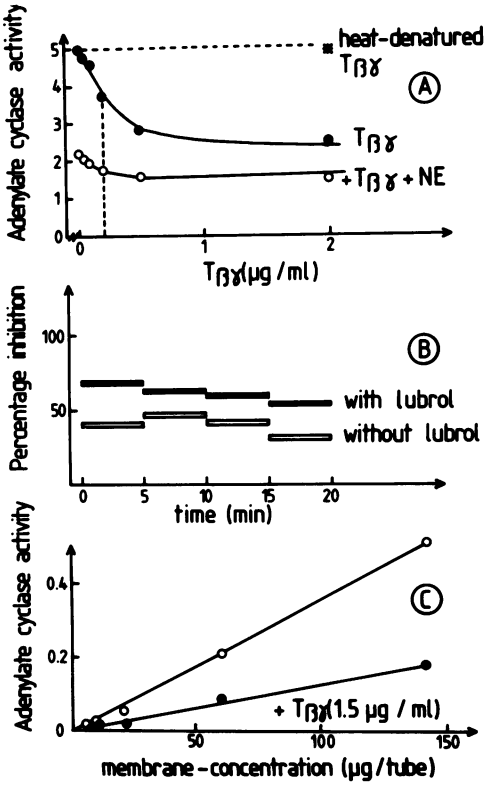

Fig. 1. General characteristics of $T_{\beta\gamma}$-induced inhibition of human platelet membrane adenylate cyclase. **(A)** Concentration-dependent effect of $T_{\beta\gamma}$ on adenylate cyclase activities (nmol cAMP/mg protein/15 min). $T_{\beta\gamma}$ was heated for 15 min at 90°C prior to assay (*). **(B)** Kinetics of $T_{\beta\gamma}$-induced inhibition in the presence and absence of Lubrol: membranes were pre-incubated for 0, 5, 10, 15 and 20 min before addition of solution D plus [^{3}H]cAMP and [α-^{32}P]ATP. The reaction was then continued for 5 min. Horizontal bars represent the percentage of $T_{\beta\gamma}$-induced inhibition compared with controls during these 5 min measurement periods. $T_{\beta\gamma}$ stock solution (0.5 mg/ml) and the same volume of solution A alone for controls were diluted in solution C with or without Lubrol PX (closed bars or open bars, respectively). $T_{\beta\gamma}$ final concentration was 2 µg/ml control adenylate cyclase activity. In the presence of Lubrol, they were 1.68, 2, 1.3, 0.85 and 0.60 nmol/5 min/mg protein during the five periods tested. Control adenylate cyclase activities in the absence of Lubrol, were 1.74, 2.42, 1.93, 1.43 and 1.29 during the five periods tested. **(C)** Dependence of adenylate cyclase activity on the concentration of platelet membranes in the absence (○) and in the presence (●) of $T_{\beta\gamma}$ (1.5 µg/ml). Adenylate cyclase activities are in nmol cAMP/mg protein/15 min.

Pre-incubation with sodium fluoride (NaF), which is a general activator of the adenylate cyclase system probably because it dissociates G_s and generates an active $G_{s\alpha}$ subunit (Sternweiss and Gilman, 1982), increased the $T_{\beta\gamma}$ inhibition (Table I). Pre-incubation with prostaglandins E_1 (PGE_1) a stimulatory hormone of the adenylate cyclase system, decreased the percentage of inhibition (Table I).

Similarly, the α_2 receptor-mediated inhibition is lower in the presence of PGE_1 than in its absence (Bockaert *et al.*, 1984). Forskolin is a potent activator of the adenylate cyclase catalytic unit systems, and in its presence, the $T_{\beta\gamma}$ inhibition was greater (Table I). GTP(γ)S irreversibly dissociates the G_s subunits and thus induces an irreversible activation of the system (Gilman, 1984b). In the presence of GTP(γ)S, inhibitory hormones are unable to produce their effect on adenylate cyclase (Jakobs *et al.*, 1981). A similar result was obtained with $T_{\beta\gamma}$ which did not inhibit a GTP(γ)S pre-activated adenylate cyclase (Table I).

Table I. Effect of various adenylate cyclase activators on $T_{\beta\gamma}$-induced inhibition of platelet membrane adenylate cyclase

Addition during pre-incubation period	Control	In presence of the $\beta\gamma$ subunit (1 µg/ml)	% Inhibition
GTP (10^{-5} M)	52	32	38
+ PGE_1 (10^{-5} M)	2722	2111	22
+ NaF (10^{-2} M)	364	175	52
+ Forskolin (10^{-5} M)	4836	2040	58
GTP(γ)S (10^{-5} M)	522	477	9

Adenylate cyclase activities (pmol/mg protein/15 min). Membranes were pre-incubated for 15 min at 30°C in solution B which, in this particular experiment, received guanyl nucleotides, PGE_1, NaF and forskolin, as indicated in Table I. After 10 min, $T_{\beta\gamma}$ or the control solution were added. After 15 min, the measurement of adenylate cyclase activity was started by addition of solution D without GTP or forskolin.

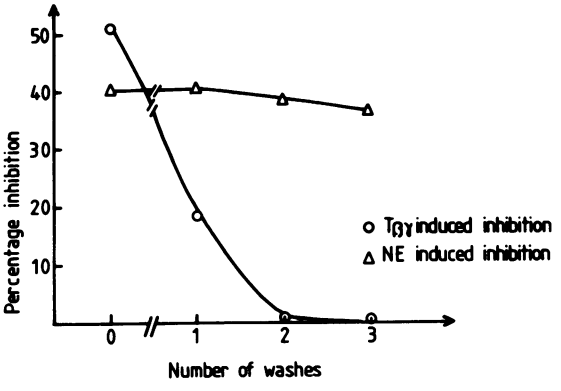

Fig. 2. Reversibility of $T_{\beta\gamma}$ inhibition of platelet membrane adenylate cyclase. $T_{\beta\gamma}$ stock solution (0.5 mg/ml) and an equivalent volume of solution A were diluted in solution C to prepare solution T_1 (5 µg/ml of $T_{\beta\gamma}$) and C_1, respectively. 350 µl of T_1 or C_1 were incubated with 700 µl of membranes (3500 µg) and 700 µl of solution B. After 10 min at 30°C, ¼ of this preparation was taken up and adenylate cyclase activities were determined by mixing 50 µl aliquots with 40 µl of solution D (plus [^{3}H]cAMP and [α-^{32}P]ATP) and 10 µl of H_2O or NE (10^{-3} M final) (wash 0). The remaining preparation was centrifuged 10 min at 30 000 *g*. The pellet was resuspended in ¾ of the initial volume [composed of 262 µl of solution C_1, 525 µl of solution B and 525 µl of membrane suspension buffer (see Materials and methods)], the principle being to keep the concentrations of membranes and other compounds constant. After a 10 min incubation period at 30°C, adenylate cyclase activities were determined (wash 1). Two more washes were performed using the same protocol. Results are expressed in percent inhibition as compared with the activity obtained without $T_{\beta\gamma}$ (incubation without $T_{\beta\gamma}$). Adenylate cyclase activities were 3.28, 1.86, 1.15 and 1.06 nmol cAMP/15 min/mg protein after 0, 1, 2 and 3 washes respectively.

The membrane integration of $T_{\beta\gamma}$ is not required to obtain adenylate cyclase inhibition

The time-course of $T_{\beta\gamma}$ inhibition was tested by measuring the adenylate cyclase activity over 5-min periods, from 0 to 15 min after addition of $T_{\beta\gamma}$ to platelet membranes. $T_{\beta\gamma}$ inhibited adenylate cyclase with no observable lag (Figure 1B). When the same experiment was done in the absence of Lubrol, the inhibition was slightly lower, but was optimal during the first 5-min period (Figure 1B). At a $T_{\beta\gamma}$ concentration just necessary to obtain a maximal inhibition (1.3 µg/ml), the adenylate cyclase inhibition (60%) was identical when tested in the presence of increasing membrane concentrations, from 6 to 147 µg (even in the presence of 350 µg, the inhibition was 58%, although the adenylate cyclase activity was no longer linear) (Figure 1C).

Platelet membranes were pre-incubated with or without $T_{\beta\gamma}$

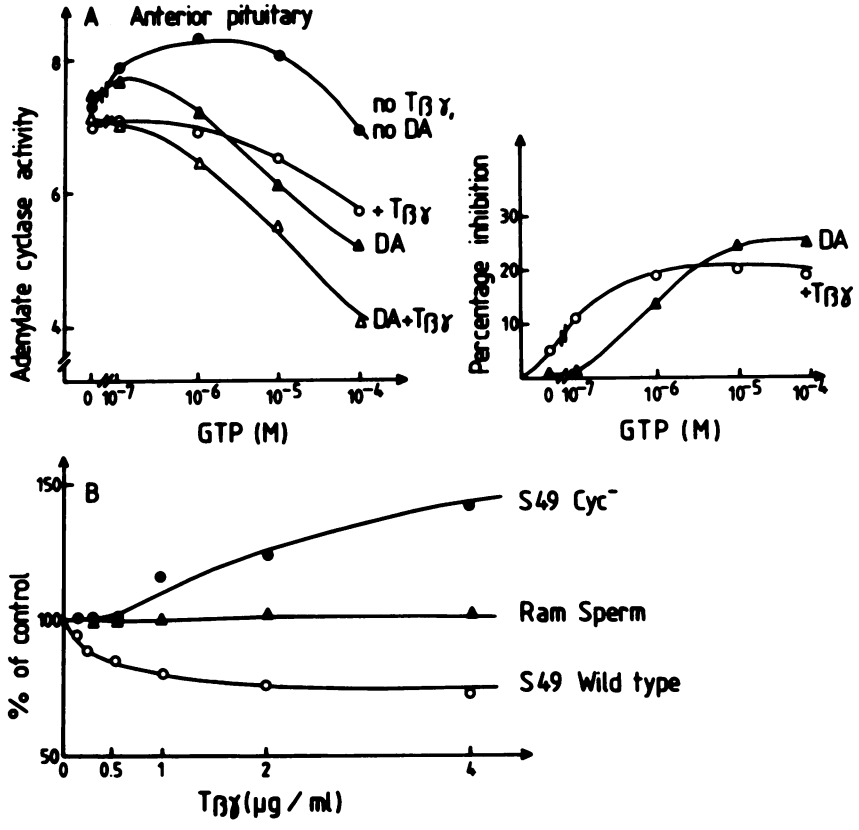

Fig. 3. Inhibition by $T_{\beta\gamma}$ of other membrane-bound adenylate cyclases. **(A)** Anterior pituitary adenylate cyclase. Membranes (20–25 µg in 10 µl) were pre-incubated at 30°C with or without 1.5 µg $T_{\beta\gamma}$ in 10 µl of solution C without Lubrol PX and 70 µl of an incubation medium containing (in 100 µl final), 50 mM Hepes (pH 7.4), 0.15 mM ATP, 1 mM EDTA, 2.5 mM $MgCl_2$, 5 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.05 mg bovine serum albumin, 0.1 mM RO-1724 and different concentrations of GTP, with or without dopamine (10 µM). After 10 min 10 µl of a solution, containing 0.001 µCi [^{3}H]cAMP, 1 µCi [α-^{32}P]ATP and 100 µM forskolin, was added. The reaction was stopped after 10 min. Adenylate cyclase activity is given in nmol cAMP/mg protein/10 min. The percentage of inhibition (compared with control) obtained in the presence of $T_{\beta\gamma}$ or dopamine (DA) is given as a function of GTP concentrations (right part). **(B)** A concentration-dependent effect of $T_{\beta\gamma}$ on different membrane-bound adenylate cyclases. Adenylate cyclase activities were determined as described in Materials and methods and expressed as a percentage of activities obtained without $T_{\beta\gamma}$. They were 452, 6815 and 150 pmol cAMP/15 min/mg protein in S49 lymphoma cyc$^-$, wild-type and in ram sperm membranes respectively.

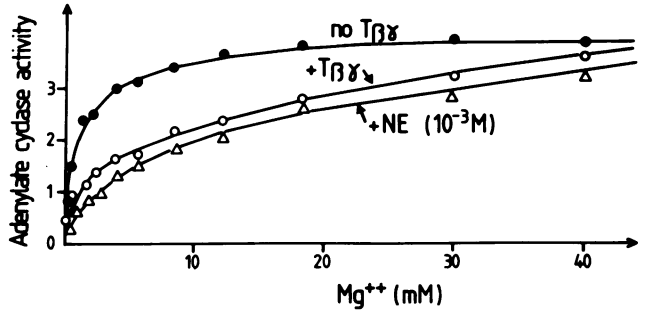

Fig. 4. Mg^{2+} dose-activation curves in the absence and in the presence of $T_{\beta\gamma}$ or NE. Adenylate cyclase activities are given in nmol/15 min/mg protein. Solution B contained various Mg^{2+} concentrations and solution D did not contain Mg^{2+}. Free Mg^{2+} concentrations are given in abscissae and calculated as previously described (Bockaert *et al.*, 1984). The $T_{\beta\gamma}$ and NE concentrations were 2 µg/ml and 10^{-3} M, respectively.

for 10 min in medium A including 0.005% Lubrol. An aliquot was taken to determine the adenylate cyclase activity and the NE inhibition of this enzyme. Membranes were centrifuged and resuspended in a volume of medium A, calculated to keep the same platelet membrane concentration. After a 10 min pre-incubation, an aliquot was taken to measure the same activities. This washing procedure was carried out three times. After the first centrifugation, the $T_{\beta\gamma}$ inhibition was reduced from 50 to 18% and was absent after two washings (Figure 2). In contrast, the NE-induced inhibition was unaffected by this washing procedure (Figure 2).

$T_{\beta\gamma}$ also inhibits other membrane-bound adenylate cyclases

In anterior pituitary membranes, we have shown that dopamine produced a GTP-dependent adenylate cyclase inhibition (Bockaert and Sebben-Perez, 1983; Enjalbert and Bockaert, 1983). In this system, $T_{\beta\gamma}$ also inhibited the enzyme in a GTP-dependent manner (Figure 3A). Similar inhibition was also obtained in S49 wild-type membranes with an EC_{50} similar to that found with platelet membranes (0.4 µg/ml). On the contrary, $T_{\beta\gamma}$ was stimulatory in S49 cyc$^-$ membranes, a membrane in which the $G_{s\alpha}$ subunit is believed to be absent (for review, see Johnson *et al.*, 1980). Note that EC_{50} for the inhibition in the wild-type was six times lower than the EC_{50} for the stimulation in the mutant (Figure 3B). $T_{\beta\gamma}$ had no effect in ram sperm membranes, a system devoid of G_s (Stengel and Hanoune, 1981) (Figure 3B).

$T_{\beta\gamma}$ inhibition as the function of Mg^{2+} concentration

In platelet membranes and under basal adenylate cyclase conditions, the Mg^{2+} dose-activation curve was hyperbolic. As previously described (Bockaert *et al.*, 1984), in the presence of norepinephrine, the Mg^{2+} dose-activation curve was more complex and shifted to the right (Figure 4). A very similar Mg^{2+}

dose-activation curve was obtained with $T_{\beta\gamma}$. In the absence and in the presence of $T_{\beta\gamma}$ or NE, the maximal adenylate cyclase activities obtained were identical. In other words, $T_{\beta\gamma}$ and NE did not inhibit the adenylate cyclase at high Mg^{2+} concentrations (Figure 4).

Discussion

Inhibition of membrane-bound adenylate cyclase by the $T_{\beta\gamma}$ subunit of transducin mimics that obtained with $G_{\beta\gamma}$. It is probably mediated by the same process as hormonal inhibition, that is, an interaction with G_s. The functional similarity between $T_{\beta\gamma}$ and $G_{\beta\gamma}$ action is supported by several lines of evidence. (i) The inhibition of platelet adenylate cyclase activity by both proteins was dose dependent and saturable. The maximal inhibition and the EC_{50} obtained with $T_{\beta\gamma}$ (Figure 1A) or $G_{\beta\gamma}$ (Katada *et al.*, 1984a,1984b) were almost identical. Moreover, the EC_{50} of $T_{\beta\gamma}$ (6 nM) is close to that reported by Northup *et al.* (1983) for the enhancement of the rate of deactivation of $G_{s\alpha}$ by $G_{\beta\gamma}$ (2 nM). (ii) As previously observed for $G_{\beta\gamma}$ (Katada *et al.*, 1984a), the inhibition by $T_{\beta\gamma}$ of platelet adenylate cyclase activity was less potent in the presence of a stimulatory hormone such as PGE_1, than in the presence of NaF and forskolin (Table I). (iii) The inhibition by $T_{\beta\gamma}$ of membrane-bound adenylate cyclase from S49 wild-type cells (Figure 3) was similar to that observed with $G_{\beta\gamma}$ (Katada *et al.*, 1984b). (iv) On S49 cyc^- cells (Figure 3), $T_{\beta\gamma}$ produced the same surprising effect as did $G_{\beta\gamma}$ (Katada *et al.*, 1984b): no effect at a low dose (<0.5 μg/ml) but stimulation at a higher dose (>1 μg/ml).

The inhibitory action of added $T_{\beta\gamma}$ (Figure 1A) as well as that of $G_{\beta\gamma}$ (Katada *et al.*, 1984a,1984b) was not additive with the hormonally-induced cyclase inhibition. The three types of inhibition reached the same saturable level close to 60% at millimolar Mg^{2+} concentrations. Moreover, they all depend on Mg^{2+} in the same way, the inhibition being progressively reduced in the presence of higher Mg^{2+} concentrations and almost totally suppressed when Mg^{2+} concentrations reached a few 10^{-2} M. These results suggest that a common Mg^{2+}-sensitive mechanism underlies the three types of inhibition (for detailed analysis of Mg^{2+} effects, see Bockaert *et al.*, 1984).

The substitution of GTP by GTP(γ)S suppresses the action of $T_{\beta\gamma}$ (Table I) or $G_{\beta\gamma}$ (Katada *et al.*, 1984a). This indicates that the inhibitory effect of the $\beta\gamma$ subunits of either T or G is mediated through an interaction with a guanine nucleotide-bearing subunit, probably the α subunit of G_s (Katada *et al.*, 1984a,1984b). Clearly, the inhibitory action of the $\beta\gamma$ subunits requires a system in which the hydrolysis of GTP occurs. This suggests that they interact with $G_{s\alpha}$ in its GDP-binding state, as will be discussed. The fact that $T_{\beta\gamma}$ does not directly act on the catalytic unit of the cyclase, but rather indirectly through an interaction with G_s, is further supported by the observation that $T_{\beta\gamma}$ does not inhibit the ram sperm adenylate cyclase, a membrane-bound enzyme deprived of G_s. We have no clear explanation for the stimulatory action of $T_{\beta\gamma}$ in S49 cyc^-. It is possible that the α subunit of G_i present in S49 cyc^- membranes produces a slight inhibition of the adenylate cyclase (as reported by Katada *et al.*, 1984b), which could be buffered by $T_{\beta\gamma}$. However, any explanations should wait until we know exactly the molecular deficiency of the six proteins of S49 cyc^-. So far, we only know that S49 cyc^- has no ADP ribosylated and functional $G_{s\alpha}$.

The $T_{\beta\gamma}$ subunit and probably the endogenous $G_{\beta\gamma}$ interacts with $G_{s\alpha}$ through a cytoplasmic site

We have shown here that $T_{\beta\gamma}$ produces inhibition of the adenylate cyclase with no need for integration into the membrane (Figures 1B, 1C, 2 and 3A). The fact that $T_{\beta\gamma}$ concentration, rather than the absolute amount of $T_{\beta\gamma}$, was the limiting factor, indicates that the $T_{\beta\gamma}$ pool interacting with the cyclase was in equilibrium with the soluble pool of $T_{\beta\gamma}$. A simple sedimentation and washing of the membranes pre-incubated with $T_{\beta\gamma}$ suppressed the inhibition. In contrast, the ability for hormones to inhibit adenylate cyclase was not modified by this treatment: the endogenous $G_{\beta\gamma}$ does not seem to become free in the cytoplasm at any stage.

The $T_{\beta\gamma}$ subunit of transducin in rod cells is a loosely bound peripheral protein which can be solubilized by a slight modification of the medium (Kühn, 1980). On the contrary, hormonal $G_{\beta\gamma}$ proteins do not seem to be solubilizable without detergent. They could either be integral membrane proteins, or very tightly bound peripheral proteins. Yet, $T_{\beta\gamma}$ produces the same effect as $G_{\beta\gamma}$ qualitatively and quantitatively. The β peptides of both proteins appear to be almost identical by their amino acid composition (Manning and Gilman, 1983). Both $T_{\beta\gamma}$ and $G_{\beta\gamma}$ proteins would thus interact with $G_{s\alpha}$ through cytoplasmic sites. A hydrophobic anchor, too short to be detected on the amino acid composition, might exist in the $G_{\beta\gamma}$ proteins and not in $T_{\beta\gamma}$. It is possible that this anchor resides on the γ subunit since T_γ and G_γ are claimed to be different (Van Dop *et al.*, 1984). Another tempting hypothesis would be that $G_{\beta\gamma}$ may acquire an affinity for the membrane by a post-translational addition of a fatty acid, as is the case for the p21 protein, product of the *ras* oncogene (Sefton *et al.*, 1982). Actually, the p21 protein has been shown to share some characteristics with the GTP-binding proteins of retinal as well as of hormonal systems (McGrath *et al.*, 1984; Gilman, 1984b). The similarities between $G_{\beta\gamma}$ and $T_{\beta\gamma}$ have also been recently demonstrated by Kanaho *et al.*, (1984), who show that $G_{\beta\gamma}$ could replace $T_{\beta\gamma}$ by reconstituting the rhodopsin-stimulated GTPase activity of T_α.

The mechanism of $T_{\beta\gamma}$ (and of endogenous $G_{\beta\gamma}$) inhibition could involve their association with the $G_{s\alpha}$ subunit in its GDP-binding state

It is now well documented, in the hormonal system as well as in the retinal one, that in the GTP-binding state, the α subunits spontaneously dissociate from the corresponding $\beta\gamma$ subunits. In the rod cell, $T_{\beta\gamma}$ only binds to $T_{\alpha\text{-GDP}}$ (T_α charged with GDP) to form a monomeric $T_{\alpha\text{-GDP}}$-$T_{\beta\gamma}$ complex: it does not interact with $T_{\alpha\text{-GTP}}$ (T_α charged with GTP).

As shown by Fung (1983), $T_{\beta\gamma}$ does not seem to be involved in the regulation of the hydrolytic activity of $T_{\alpha\text{-GTP}}$. It is only required to bind T_α to the receptor and to catalyze the GDP-GTP exchange. It is tempting to speculate that when added to the hormonal system $T_{\beta\gamma}$ would also interact with $G_{s\alpha\text{-GDP}}$ ($G_{s\alpha}$ charged with GDP). The absence of inhibitory effects when the non-hydrolyzable analogs of GTP were used, is also in favor of this hypothesis. It has been recently observed that high Mg^{2+} concentrations dissociate the T_α-$T_{\beta\gamma}$ complex even in its GDP binding state (Deterre *et al.*, 1984): high Mg^{2+} concentrations shift the equilibrium $T_{\alpha\text{-GDP}}$-$T_{\beta\gamma}$ $T_{\alpha\text{-GDP}}$ + $T_{\beta\gamma}$ to the right, and therefore prevents association of added $T_{\beta\gamma}$ to $T_{\alpha\text{-GDP}}$. Similarly, it could also prevent the interaction of $T_{\beta\gamma}$ with $G_{s\alpha\text{-GDP}}$. This would provide a straightforward explanation for the suppression of the inhibitory effect of $T_{\beta\gamma}$, $G_{\beta\gamma}$ and the inhibitory hormone by high magnesium concentrations.

Finally, why does the interaction of $T_{\beta\gamma}$ (or $G_{\beta\gamma}$) with $G_{s\alpha\text{-GDP}}$ result in an inhibition of adenylate cyclase? At least two possibilities exist. The first one could be that reassociation between

$G_{s\alpha\text{-GDP}}$ and $T_{\beta\gamma}$ decreased the possibility of GDP/GTP exchange (deactivation of G_s) in hormonal systems (Northup *et al.*, 1983; Bockaert, 1985), whereas on the contrary, this reassociation is absolutely necessary for GDP/GTP exchange in the visual system (for review, see Kühn, 1984). The second explanation would be that after GTP hydrolysis, $G_{s\alpha\text{-GDP}}$ still activates the cyclase, at least partially (see Kimura and Shimada, 1983; Hildebrandt *et al.*, 1984b), as long as it is not reassociated with $G_{\beta\gamma}$. In other words, the GTPase step would be necessary but not sufficient to turn off the activation triggered by light or by hormones: the reassociation of $G_{s\alpha\text{-GDP}}$ with the $G_{\beta\gamma}$ subunit would be the final turn-off signal.

Materials and methods

Solution A (solution in which $T_{\beta\gamma}$ was kept) consisted of 12 mM of 4-(2-hydroxyethyl)1-piperazine ethanesulfonate (Hepes) (pH 7), 0.6 mM dithiothreitol, 70 mM $MgCl_2$ and 40% v/v glycerol. Solution B (pre-incubation solution) was 150 mM Hepes (pH 8), 0.75 mM ATP, 6 mM $MgCl_2$, 20 mM creatine phosphate, 0.5 mg/ml creatine-kinase, 0.3 mg/ml bovine serum albumin. Solution C (dilution buffer of $T_{\beta\gamma}$) was 50 mM Hepes (pH 8), 1 mM EDTA, 1 mM dithiothreitol, 0.05% Lubrol PX (Sigma). Solution D (incubation solution) was 125 mM Hepes (pH 8), 2.5 mM EDTA, 5 mM $MgCl_2$, 10 mM creatine phosphate, 0.25 mg/ml creatine-kinase, 0.25 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidione (RO 20-1724), 25 μM GTP and 25 μM forskolin.

Pure $T_{\beta\gamma}$ was prepared as described by Deterre *et al.* (1984). Briefly, transducin was solubilized by GTPγS upon illumination of ROS membranes (Kühn, 1980). It was then loaded onto an ion-exchange chromatography column (Polyanion SI, Pharmacia FPLC system) and eluted by an $MgCl_2$ gradient. $T_{\beta\gamma}$ was eluted before $T_{\alpha\text{-GTP}(\gamma)S}$. The $T_{\beta\gamma}$ preparation used for all these experiments (0.5 mg/ml) was kept frozen (−80°C) in solution A. Platelet-rich plasma in sodium citrate (0.5%) was obtained from the Montpellier 'Centre de Transfusion Sanguine'. Membranes were prepared according to Insel *et al.* (1982) except that the last membrane suspension was done in 25 mM Hepes (pH 8), 1 mM EDTA, and 0.1 mM dithiothreitol at a protein concentration of 20−25 mg/ml prior to storage at −80°C.

Rat anterior pituitary, ram sperm and S49 lymphoma membranes were prepared as previously described (Bockaert *et al.*, 1984; Stengel and Hanoune, 1981; Jakobs and Schultz, 1983). Ram sperm and S49 lymphoma membranes were kept frozen at −80°C in 1 mM $NaHCO_3$ and 20 mM Hepes (pH 7.5) plus 3 mM EDTA respectively. Rat anterior pituitary membranes were used fresh in 1 mM Tris maleate (pH 7.2), 1 mM EGTA and 300 mM sucrose.

Assays

Except where otherwise stated, membranes (40−50 μg protein in 20 μl) were pre-incubated at 30°C with 20 μl solution B, 10 μl of a given dilution of $T_{\beta\gamma}$ (or 10 μl of the equivalent dilution of solution A as control) and 10 μl of water with or without a specific compound to be tested. $T_{\beta\gamma}$ and solution A were diluted with solution C. After 15 min, 40 μl of solution D, plus 0.001 μCi [^{3}H]cAMP and 0.5−1.0 μCi [α-^{32}P]ATP was added. When present, NE was added in solution D. The reaction was continued for 15 min at 30°C, then stopped and [^{32}P]cAMP was quantified as previously described (Bockaert *et al.*, 1976).

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