Mechanism of Guanine Nucleotide Regulatory Protein-mediated Inhibition of Adenylate Cyclase

STUDIES WITH ISOLATED SUBUNITS OF TRANSGUIDIN IN A RECONSTITUTED SYSTEM*

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Richard A. Cerione§§, Claudia Staniszewski§, Peter Gierschik§, Juan Codina§, Robert L. Somers§, Lutz Birnbaumer[], Allen M. Spiegel[], Marc G. Caron†, and Robert J. Lefkowitz‡**

From the $Howard Hughes Medical Institute, Departments of Medicine, Biochemistry, and Physiology, Duke University Medical Center, Durham, North Carolina 27710, the *National Institutes of Health, Bethesda, Maryland 20205, and the †Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

The retinal nucleotide regulatory protein, transducin, can substitute for the inhibitory guanine nucleotide-binding regulatory protein (N,) in inhibiting adenylate cyclase activity in phospholipid vesicle systems. In the present work we have assessed the roles of the α(αγ) and βγ (βγγ) subunit components in mediating this inhibition. The inclusion of either a preactivated αβγγS (or GTPγS) is guanosine 5'-O-(thiotriphosphate) complex, or the βγγ complex, in phospholipid vesicles containing the pure human erythrocyte catalytic subunit complex, or the GppNHp-stimulated (where GppNHp is guanyl-5'-yl imidodiphosphate) activity (by ~30–60 and 90%, respectively, at 2 mM MgCl2). The inhibitions by both of these subunit species are specific for the N,-stimulated activity with either αβγγS nor βγγ having any direct effect on the intrinsic activity of the catalytic moiety. Increasing the MgCl2 concentration in the assay incubations significantly decreases the inhibitions by both αβγγS and βγγ. Similarly, when the pure hamster lung β-adrenergic receptor is included in the lipid vesicles with N, and C, the levels of inhibition of the GppNHp-stimulated activity by both αβγγS and βγγ are reduced compared to those obtained in vesicles containing just N, and C (but not stimulatory receptor). These inhibitions are reduced still further under conditions where the agonist stimulation of adenylate cyclase activity is maximal, i.e., when stimulating with isoproterenol plus GTP. In these cases the αβγγS inhibitory effects are completely eliminated and the inhibitions observed with holotransducin can be fully accounted for by the βγγ complex. The ability of the βγγ complex to relieve these inhibitions suggests that the receptor may remain coupled to N, (or α, during the activation of the regulatory protein and the stimulation of adenylate cyclase. These results also suggest that under physiological conditions the βγγ subunit complex is primarily responsible for mediating the inhibition of adenylate cyclase activity.

Regulation of adenylate cyclase activity is accomplished through distinct stimulatory and inhibitory pathways. Both of these are initiated by agonist interactions with specific receptor proteins. These interactions enable the stimulatory receptors to promote the activation of a specific nucleotide-binding regulatory protein, designated as N, (1–3) or G, (4), and the inhibitory receptors to activate a distinct nucleotide-binding regulatory protein termed N, (5) or G, (6). These regulatory proteins are heterotrimers of composition αβγ (7) and are structurally distinguishable by their α subunits (α = 42,000 daltons and α = 40,000 daltons) but not by their β (M, ≅ 35,000–36,000) (8, 9) or γ (M, ≅ 5,000–10,000) subunits, which appear to be identical. An analogous type of system operates in vertebrate phototransduction. Here, the absorption of light by the photoreceptor rhodopsin allows it to interact with the heterotrimeric nucleotide regulatory protein transducin (M, αγ = 39,000, β = 35,000, and γ = 9,000 (10, 11)). This interaction activates transducin and thus enables it to stimulate the activity of its biological effector, the cyclic GMP phosphodiesterase (10, 11).

The specific mechanisms by which receptors promote the activation of these regulatory proteins in a lipid milieu, as well as the mechanisms by which the activated regulatory proteins go on to stimulate or inhibit effector enzyme activity, have been the subject of much investigation. A particularly controversial question concerns the mechanism(s) of nucleotide regulatory protein-mediated inhibition of adenylate cyclase activity. Two quite different schemes have been suggested. One of these, based on hydrodynamic studies (7, 12–15), indicates that the activation of the regulatory proteins, in detergent solutions, results in their dissociation into guanine nucleotide and intact βγγ complexes. It can then be predicted that by increasing the levels of βγ in a cellular membrane, as a result of the activation of N, there would be a decrease in hormonal and guanine nucleotide-stimulated cyclic AMP production due to the increased deactivation of N, (i.e., through the reassociation of α with βγ). Support for this scheme has come from studies in platelet membranes and S49 lymphoma wild type membranes, where the addition of βγγ effects a dose-dependent inhibition of N, stimulated adenylate cyclase activity (16, 17).

However, various studies using cyc- membranes suggest that the α subunit is primarily responsible for N,-mediated inhibition of adenylate cyclase. It has been well documented that inhibitory agonists like somatostatin (18), or even guanine nucleotides alone (2, 16, 19–21), can cause significant inhibition of the adenylate cyclase activity in these mutant membranes, which contain neither a functional N, (22, 23)

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‡ Present address: Dept. of Pharmacology, New York State College of Veterinary Medicine, Cornell University, Ithaca, NY 14853.

§ To whom correspondence should be addressed: Box 3821, Dept. of Medicine, Duke University Medical Center, Durham, NC 27710.
Addition of activated resolved cyclase.

Nature (21). These results cannot be explained by a βγ deactivation mechanism but rather suggest some type of direct interaction between N and the catalytic moiety of adenylate cyclase.

Recent achievements in purifying the individual stimulatory and inhibitory components of the adenylate cyclase system (3, 6, 25–29) have enabled us to study the mechanisms by which this enzyme is regulated using reconstituted systems. The advantage of such an approach is that it permits the study of interactions between each of these components in a lipid milieu under well defined conditions. Recently, we succeeded in reconstituting a guanine nucleotide-dependent inhibition of adenylate cyclase activity in phospholipid vesicles (30). An important conclusion of these studies was that the retinal nucleotide regulatory protein, transducin, can specifically substitute for N in mediating this inhibition. This finding offers an advantage for studying the inhibitory pathway of adenylate cyclase since both the α and βγ subunits of the retinal regulatory protein can be readily isolated in high yield and in the complete absence of detergent (31). In the work described here, we have exploited this finding to study the roles of the isolated α and βγ subunits of transducin in mediating inhibition of adenylate cyclase activity in different types of phospholipid vesicle systems.

MATERIALS AND METHODS

Materials—Extracti-Gel D was from Pierce Chemical Co. Octyl-β-D-glucopyranoside (octyl glucoside) was obtained from Calbiochem-Behring, soybean phosphatidylcholine (choline, isoproterenol, and GTP were from Sigma, and Gpp(NH)p was from Boehringer Mannheim. [α-32P]ATP and [3H]-labeled cyanosynephrine were purchased from New England Nuclear. All other materials were obtained from sources previously described (30, 32).

Preparation of the Various Protein Components—N was solubilized from human erythrocyte membranes using sodium cholate and then purified (90%) as previously described (9). N was stored (−90 °C) in 10 mM HEPES (pH 8.0), 1 mM EDTA, 20 mM β-mercaptoethanol, 30% ethylene glycol, 100 mM NaCl, and 3% Lubrol PX (buffer A).

Transducin and rhodopsin were purified (>90%) from bovine retinal rod outer segments as previously published (33, 34). The transducin was stored (−50 °C) in 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA, and 50% (v/v) glycerol (buffer B) while the rhodopsin was stored (−90 °C) in 0.1 M α-methylmannoside, 0.5 M Tris acetate (pH 7.0), 1 mM CaCl2, 1 mM MnCl2, and 10 mM CHAPS (buffer C). The αβγ-GTPγS and αβγ-GDP complexes were purified by Blue Sepharose chromatography from transducin which was initially eluted by GTPγS and GTP, respectively (31). In the latter case, GDP is formed as a result of the GTPase activity of transducin and remains tightly bound to the αβγ subunit (35). The βγ complex was also resolved and purified using Blue Sepharose chromatography. All of the subunit complexes were stored (−90 °C) in 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM DTT, 25% (v/v) glycerol (buffer D); the αβγ-GTPγS and αβγ-GDP preparations also contained 240 and 500 mM KCl, respectively.

The β-adrenergic receptor (βAR) was solubilized from hamster lung or guinea pig lung membranes and purified to apparent homogeneity by a combination of affinity chromatography (Sepharose-alprenolol gel) and high performance liquid chromatography as previously described (27). The receptor preparations were stored

The abbreviations used are: GTPγS, guanosine 5′-O-(thiotriphosphate); Gpp(NH)p, guanyl-5′-yli mitidophosphate; HEPES, 4(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; βAR, β-adrenergic receptor; C, catalytic moiety.

Reconstitution of adenylate cyclase activity in phospholipid vesicles—The reconstitution of adenylate cyclase in the various phospholipid vesicle systems was performed as follows. Soybean phosphatidylcholine (100 µl of a 17 mg/ml sonicated solution) was first incubated with 0.05–0.1 ml of the adenylate cyclase preparation (in buffer A). Typically yielding 2-4% of activity in buffer A) while the rhodopsin was stored (−90 °C) in 0.1 M α-methylmannoside, 0.5 M Tris acetate (pH 7.0), and 25 µl of octyl glucoside (17%) for 20–30 min on ice. At this point, when appropriate, N (0.8 µg in 10 µl of buffer A), rhodopsin (6.5 µg in 15 µl of buffer C), and βAR (10–15 pmol in 40 µl of buffer E) were added to the lipid solutions (incubated 1–3 min at 4 °C). The βAR (40 µl) was preincubated with 5 µM alprenolol (10 µl) and 500 µg of bovine serum albumin (10 µl) prior to its addition to the above mixture. Rhodopsin was included in the vesicle preparations in order to ensure maximum activation of holotransducin (32) (thus, for comparative purposes, rhodopsin was present in all cases as described). In all cases the final volume of the reconstitution incubation was 0.5 ml (i.e. when βAR was not present, an equivalent volume of buffer was added to the incubation).

Upon mixing the lipid solutions with N and the receptor, the mixtures were applied to Extracti-Gel columns (1 ml of gel) at 4 °C which were pretreated with 4 volumes of 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2 mg/ml BSA and then equilibrated with 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol and in some cases 15 mM MgCl2. The eluates from the Extracti-Gel (2 ml in 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol) were applied to Sepharose 6B columns (1 ml of gel) which were then equilibrated with 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol at 4 °C, and then isolated by ultracentrifugation at 4 °C as previously described (30, 32). The resultant protein-lipid pellets were resuspended in 75 mM Tris-HCl (pH 7.8), 1 mM DTT (final volume 0.6–1.3 ml) and either directly assayed for adenylate cyclase activity or treated with holotransducin or its subunit components prior to assay for activity, as described below. The efficiency of reconstitution of βAR (as determined by [3H]iodocyanopindanol binding) typically ranged from 5 to 10%; the efficiency of reconstitution of N, ranged from 20 to 60% (as assessed by [35S]GTPγS binding), and the efficiency of reconstitution of adenylate cyclase ranged from 15 to 40% as assessed by forskolin-stimulated activity (37).

Examination of the Effects of αβγ, βγδ, and Holotransducin on the Reconstituted Adenylate Cyclase Activity—In order to determine the effects of αβγ, βγδ, or holotransducin on the reconstituted adenylate cyclase activity, the resuspended lipid vesicles containing the different components, described in the preceding section, were divided into 0.18–0.35-ml aliquots. Varying amounts of the pure subunit components (0.03–3 µg in 2–20 µl of buffer D), pure holotransducin (0.05–2 µg in 2–15 µl of buffer B), or just buffer were directly added to the vesicle aliquots prior to assay for adenylate cyclase activity (for 30 min at 30 °C as previously described (30)). The efficiency of these components are stored in detergent allows them to be added directly to the lipid vesicles without disrupting the vesicle structure (as indicated by the maintenance of receptor-nucleotide regulatory protein coupling in these vesicles). The rhodopsin-promoted binding effects of GTPγS to the βγδ complex or to the βγδ component, to the extent that stoichiometric amounts of βγγ suggests that 30–75% (typically ~50%) of the protein added properly associates with the lipid vesicles. Throughout these studies the amounts of N, transducin αβγ, βγδ.
Inhibition of Adenylate Cyclase

or rhodopsin are expressed in terms of total protein as determined by the fluorescamine method (3) or by the methods of Lowry (39) or Bradford (40). The molar concentrations of these proteins were determined assuming an M, of 29,000 for the holonucleotide regulatory protein (3), 39,000 for \( \alpha \) (11), 46,000 for \( \beta \gamma \) (11), and 37,000 for rhodopsin (41). The molar concentrations of the \( \beta \)-adrenergic receptor throughout the text are expressed in terms of \([\text{I}^{125}]\text{iodocyanopindolol binding activity (pmol)}\).

RESULTS

Effects of \( \alpha \) and \( \beta \gamma \) on the Adenylate Cyclase Activities in \( N_{1}C \) Vesicles—Previously we reported that the retinal nucleotide regulatory protein, transducin, can substitute for \( N_{1} \) in inhibiting the GppNHp-stimulated adenylate cyclase activity in lipid vesicles containing the pure human erythrocyte N, and a resolved bovine caudate C preparation (30). In order to probe the mechanisms of inhibition of adenylate cyclase we have resolved the \( \alpha \) (\( \alpha \)) and \( \beta \gamma \) (\( \beta \gamma \)) subunit components of transducin by Blue Sepharose chromatography as previously described (31). Fig. 1 shows the SDS gel electrophoresis profiles of these subunit preparations.

Both the \( \alpha \) subunit, which has been preactivated with GTP\( \gamma S \) (\( \alpha \)-GTP\( \gamma S \)), and the \( \beta \gamma \) complex inhibit the GppNHp-stimulated adenylate cyclase activity following their addition to the assay incubations. In the case of the \( \alpha \)-GTP\( \gamma S \) complex (where \[\alpha\] \( \sim \) 100–200 \( \times \) \([N_{1}]) the inhibition at 2 mM MgCl\(_{2}\) is \( \sim \)30% in the experiment shown, although it is frequently as high as 50–60% (49 \( \pm \) 17% S.E., \( n = 6 \), also see Fig. 5 below). At 50 mM MgCl\(_{2}\) this inhibition is markedly reduced (<5%). Similarly, the inhibition by the \( \beta \gamma \) complex (where \([\text{GTP} \gamma \text{S}\] \( \equiv \) 100–200 \( \times \) \([N_{1}]) is much greater at low \([\text{MgCl} \gamma \text{S}\], being 90% (\( \pm \)2% S.E., \( n = 5 \)) at 2 mM MgCl\(_{2}\) compared to about 15% at 50 mM MgCl\(_{2}\). In all cases the \( \beta \gamma \) complex is a much more potent inhibitor than the active \( \alpha \) species.

The inhibitory effects of both subunit components are specific for the \( N_{1} \)-stimulated adenylate cyclase activity. Fig. 3 shows that the direct addition of either the \( \alpha \)-GTP\( \gamma S \) or \( \beta \gamma \) species to phospholipid vesicles containing the resolved caudate adenylate cyclase preparation, alone, has essentially no effect on basal (Mg\( ^{2+} \)-stimulated) or forskolin-stimulated adenylate cyclase activity. These results are completely consistent with our earlier studies where it was observed that neither pure holonucleotide regulatory protein, nor pure holotransducin had any effect on the intrinsic activity of the bovine caudate catalytic unit (30).

The inhibitory effects of the \( \alpha \) subunit are also specific for

![FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of the isolated subunit components of transducin. All lanes show Coomassie Blue-stained protein samples (2 \( \mu l \)) which were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1, the \( \alpha \) subunit of bovine retinal transducin; lane 2, the \( \beta \gamma \) subunit of transducin; lane 3, holotransducin. The \( \gamma \) subunit has migrated off the gels in lanes 2 and 3. The arrows to the left of these lanes show the relative mobility of known molecular weight standards. The \( \alpha \) subunit shown in lane 1 was prepared by eluting the rod outer segment membranes with GTP\( \gamma S \). Identical results are obtained using \( \alpha \) subunits which were prepared by eluting the membranes with GTP (results not shown).](image1)

![FIG. 2. Magnesium dependence of the \( \alpha \)-GTP\( \gamma S \) and \( \beta \gamma \)-mediated inhibitions of the \( N_{1} \)-GppNHp-stimulated adenylate cyclase activity. Resolved C preparation (100 \( \mu l \)) and 0.83 \( \mu g \) of \( N_{1} \) (\( \approx 39 \) pmol) were added to the reconstitution incubations as described under "Materials and Methods." The isolated phospholipid vesicles were resuspended in 1.5 ml of 75 mM Tris-HCl (pH 7.8), 1 mM DTT, and then aliquots (0.35 ml) of these vesicles were incubated with 20 \( \mu l \) of buffer D (\( \bullet \)); \( \alpha \)-GTP\( \gamma S \) (3 \( \mu g \), \( \approx 80 \) pmol) (\( \Delta \)); or \( \beta \gamma \) (3 \( \mu g \), \( \approx 80 \) pmol) (\( \Delta \)) prior to assaying their GppNHp-stimulated adenylate cyclase activity. GppNHp, 0.1 mM. Each data point represents the mean of duplicate determinations from a single experiment which was repeated twice with comparable results.](image2)

![FIG. 3. Effects of \( \alpha \)-GTP\( \gamma S \) and \( \beta \gamma \) on basal and forskolin-stimulated adenylate cyclase activity. Resolved C preparation (100 \( \mu l \)) was added to a reconstitution incubation as described under "Materials and Methods." The isolated phospholipid vesicles were resuspended in 0.6 ml of 75 mM Tris-HCl (pH 7.8), 1 mM DTT, and then aliquots (0.18 ml) of these vesicles were incubated with 20 \( \mu l \) of buffer D, \( \alpha \)-GTP\( \gamma S \) (3 \( \mu g \), \( \approx 80 \) pmol), or \( \beta \gamma \) (3 \( \mu g \), \( \approx 80 \) pmol) prior to assaying their basal and forskolin-stimulated adenylate cyclase activity. Forskolin, 0.1 mM; [MgCl\(_{2}\)] = 2 mM. \( B \), the basal activity measured in the presence of MgCl\(_{2}\) alone. The actual basal activities were 2.9 pmol of cAMP for C alone, 2.6 pmol of cAMP for C + \( \alpha \), GTP\( \gamma S \), and 2.7 pmol of cAMP for C + \( \beta \gamma \). Each data point represents the mean of triplicate determinations from a single experiment which was repeated three times with comparable results. The error bars represent the standard deviation for the triplicate determinations.](image3)
three experiments. The activity is observed in the N/C vesicles alone. However, the addition of aT-GDP now effects significant stimulation (>>10-fold) of the adenylate cyclase activity measured in the presence of GppNHp. These results most likely reflect the ability of aT-GDP to associate with the βγ complex of N, and thus promote the dissociation (and activation) of N, at low [Mg²⁺] (see Discussion).

**Effects of αγ and βγ on the Adenylate Cyclase Activities in βAR/N/C Vesicles**—In order to examine whether the presence of the stimulatory βAR affects the inhibition of adenylate cyclase activity by αγ and βγ, phospholipid vesicle systems containing the pure guinea pig lung βAR, together with N, and C, were constructed. Fig. 5A compares the GppNHp-stimulated adenylate cyclase activities (in the presence and absence of the β-agonist isoproterenol) in βAR/N/C vesicles alone and in these same vesicles following the addition of αγ-GTPγS or βγ. Fig. 5B shows the analogous sets of comparisons, performed in the same experiment, using N/C vesicles. In the case of the βAR/N/C vesicles, there is only a slight stimulation of the GppNHp-stimulated activity (~1.1-fold) by isoproterenol. This is due to the fact that when GppNHp is the activating guanine nucleotide, the agonist advantage is lost, i.e. many of the N, molecules can be activated in the absence of any agonist-receptor-N, interaction (see Discussion). Nevertheless, clear differences are observed in the extents of the inhibitions of the adenylate cyclase activities by both αγ-GTPγS and βγ in the presence and absence of the active subunit species. An inactive αγ subunit can be prepared by initially eluting transducin from rod outer segments with GTP. The hydrolysis of GTP to GDP results in an αγ-GDP complex which can be resolved and purified. The βγ complex has a much higher affinity for αγ-GDP than it does for αγ-GTPγS based on the relative abilities of βγ to promote the ADP-ribosylation by pertussis toxin (42) of these αγ complexes. Fig. 4A shows the results of the addition of αγ-GTPγS to N/C vesicles for conditions where the total [MgCl₂] = 2 mM; [GppNHp] = 0.1 mM. In all cases each data point represents the mean of triplicate determinations. The experimental results shown are representative of three experiments. The *error bars* represent the standard deviation of these determinations.

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2 P. Gierschik and A. M. Spiegel, unpublished results.


\*\*AR. Specifically, in direct comparative studies the \*AR-GTPyS complex effected about a 50% inhibition (52 ± 2%, n = 2) of the GppNHp-stimulated activities in the N/C vesicles while in the presence of \*AR the extents of inhibition of the GppNHp-stimulated activities were reduced to 22% (±3%, n = 2) in the absence of isoproterenol and 16% (±1%, n = 2) in the presence of agonist. Likewise the \*Y complex was a more effective inhibitor of the adenylate cyclase activities in N/C vesicles (94 ± 1%, n = 2) compared to \*AR/N/C vesicles where the inhibitions of GppNHp-stimulated activities were 77% (±2%, n = 2) and 70% (±2%, n = 2) in the absence and presence of agonist, respectively.

These inhibitions can be further reduced under conditions more akin to the true physiological situation where a greater percentage of the N, molecules have been activated via isoproterenol-\*AR complexes, i.e. when assaying the isoproterenol plus GTP-stimulated adenylate cyclase activity. Fig. 6 presents the results of such an experiment where the activities are compared in the presence and absence of the \*Y subunit. In this case the isoproterenol stimulation of the adenylate cyclase activity in \*AR/N,C vesicles, relative to the activity stimulated by GTP alone, is 2.5-fold. Thus the percentage of N, molecules activated via agonist-receptor complexes is significantly increased relative to the conditions shown in Fig. 5A. Under such conditions the basal (GTP alone)-stimulated adenylate cyclase activity is inhibited ~72% upon addition of the \*Y complex, similar to the extents of inhibition by \*yT of the GppNHp-stimulated activities in these vesicles (Fig. 5A). However, the isoproterenol (plus GTP)-stimulated activity is only inhibited by ~45%. The differential inhibition of basal and agonist-stimulated activity in turn results in about a 2-fold increase (from 2.5- to 5.5-fold) in the -fold stimulation by isoproterenol for \*AR/N,C vesicles containing \*Y. This in effect tightens the coupling between stimulatory agonists and adenylate cyclase (43).

The nucleotide regulatory protein-mediated inhibition of isoproterenol (plus GTP)-stimulated adenylate cyclase activity can be completely accounted for by the \*Y subunit. This is illustrated by the results presented in Fig. 7 which show that the inhibition by holotransducin can be fit rather well to the dose-response curve for the effects of \*yT on this activity. Under these conditions the effective activation of N, molecules by isoproterenol-\*AR complexes is such that the addition of \*Y-GTPyS or \*AR-GDP has no effect on the agonist-stimulated activity.

**Discussion**

The specific mechanism(s) by which inhibitory receptors and the inhibitory guanine nucleotide-binding regulatory protein, N, mediate the inhibition of adenylate cyclase activity has been the subject of some controversy over the past few years. In order to examine this mechanism in a well defined system, we developed procedures for reconstituting the guanine nucleotide-dependent inhibition of adenylate cyclase activity in phospholipid vesicles containing pure N, and a resolved C preparation (30) and in lipid vesicles containing the pure \*Y receptor together with N, and C (43). Our previous studies using such systems indicated that transducin, like N, could inhibit the adenylate cyclase activity. Thus, in the present work we have taken advantage of the ready availability of the isolated subunits of transducin to examine the roles of the \* and \*Y components of the regulatory protein in the inhibitory process.

As clearly illustrated by the data presented in Figs. 2 and 5, both the \*yT complex and a preactivated \* species (\*Y-GTPyS) are capable of inhibiting \*N-stimulated adenylate cyclase activity. These inhibitions do not reflect a direct allosteric effect on the intrinsic activity of the catalytic moiety since neither the addition of \*yT nor \*Y-GTPyS has any effect on the activities of lipid vesicles containing C alone (i.e. with no N,). These results are fully consistent with earlier studies using holo-N, or holo-transducin (30).

The inhibition of the \*N-stimulated adenylate cyclase activity by \*yT also supports prior work in various membrane systems where free \*yT from N, was observed to be a potent inhibitor of guanine nucleotide-stimulated activity (16, 17). As outlined earlier, such inhibition is consistent with a scheme where nucleotide regulatory protein activation reflects the dissociation of the heterotrimer into its component \* and \*Y components.
subunits. In this case, the addition of free $\gamma_T$, by shifting the association-dissociation equilibrium of $N_\alpha$ to the inactive heterotrimeric state, would deactivate the system. This is depicted in Scheme 1 where GN represents an activating guanine nucleotide such as GTP or GppNHp. For simplicity only one equilibrium is depicted for the dissociation of $N_\alpha$, the binding of $Mg^{2+}$, and the binding of guanine nucleotide. The fact that $Mg^{2+}$ overcomes inhibition is compatible with this scheme since increasing the divalent metal concentration would shift the equilibrium (in Scheme 1) to the right.

It should be noted that other mechanisms could account for the inhibition of $N_\alpha$-stimulated activity by $\gamma_T$. For example, activation of $N_\alpha$ might reflect a conformational change (but not dissociation) to an intact $N_\alpha$-guanine nucleotide complex. If both the $\alpha_\gamma$ and $\beta_\gamma$ subunits were involved in the binding of $N_\alpha^*$ to $C$ during stimulation, then it would be expected that free $\gamma_T$ could act as a competitive inhibitor of this interaction. However, the stimulatory effects observed when $\alpha_T$-GDP complexes are added to $N_\alpha/C$ vesicles (Fig. 4) are not readily compatible with such a mechanism but rather suggest that $N_\alpha$ activation does directly result in subunit dissociation, as depicted in Scheme 1, at least for cases where $GN = GppNHp$. Specifically, the $\alpha_T$-GDP complex, by virtue of its high affinity for $\gamma_T$, would act to pull the activation equilibrium to the right (in Scheme 1), thereby increasing the levels of active $\alpha_\gamma^*$ and thus causing a net stimulatory response when added to $N_\alpha/C$ vesicles. The fact that this stimulatory effect is greatest under conditions where the levels of $N_\alpha$ activation (and dissociation) are quite low, i.e. when total [MgCl$_2$] < 2 mM, is further compatible with the mechanism presented in Scheme 1. Still, it has yet to be documented that stimulatory receptors, in the presence of hormones and GTP, promote the dissociation of $N_\alpha$ into its subunit components. Thus, at the present time, it is not possible to rule out mechanisms of inhibition by $\gamma_T$ (of hormone plus GTP-stimulated adenylate cyclase activity) which involve a direct interaction between this complex and the catalytic moiety.

The inhibition of the $N_\alpha$-GppNHp-stimulated adenylate cyclase activity, by the preactivated $\alpha_T$-GTP$\gamma$S species, is clearly weaker than that effect by $\gamma_T$. Specifically, under conditions where $\gamma_T$ causes greater than 90% inhibition of the activity, the maximum inhibition obtained with $\alpha_T$-GTP$\gamma$S typically ranges from 30 to 60%. However, the fact that some inhibition is observed with the preactivated $\alpha_T$ species indicates that this subunit must be capable of directly interacting with $C$. This interaction could either occur at a site which overlaps the $N_\alpha$ (or $\alpha_\gamma$) binding site on $C$ or at a distinct site from which the $\alpha_\gamma$ subunit effects an allosteric inhibition of $N_\alpha$ ($\alpha_\gamma$) binding to the catalytic moiety. The latter possibility would be compatible with recent results from the cyc$^*$ variant of S49 lymphoma cells where kinetic evidence indicates that $\alpha_\gamma$ and $\alpha_T$ bind to two distinct sites on adenylate cyclase (21). However, in the case of the cyc$^*$ adenylate cyclase, binding by $\alpha_T$ can apparently exert allosteric effects which inhibit the intrinsic activity of the catalytic moiety. This differs from the results of previous studies using bovine caudate enzyme, as well as those presented here, which indicate that the inhibitory effects of holo-$N_\alpha$, holotrascinucin, and $\alpha_T$ are all confined to the $N_\alpha$-stimulated activity (30). These differences may reflect subtle differences between the catalytic properties of the bovine caudate enzyme and that of the mutant cyc$^*$ cell.

Recently, we reported that the $\beta$-adrenergic receptor can overcome the inhibition of $N_\alpha$-stimulated adenylate cyclase activity by $N_\beta$ (43). The same holds true for the inhibitory effects of transducin. Specifically, the inhibitions by $\alpha_T$-GTP$\gamma$S and $\gamma_T$-GpppNHp-stimulated activity are significantly reduced in $\beta AR/N_\alpha/C$ vesicles compared to $N_\alpha/C$ systems. The fact that the receptor relieves this inhibition, even in the absence of isoproterenol, can be attributed to the "basal" activation of $N_\alpha$ molecules by receptor alone (32, 44). It is interesting that the inhibitions by both subunits are more severely depressed when GTP is used, together with isoproterenol, to stimulate activity compared to when GppNHp is used as the activating guanine nucleotide. This can be explained with the aid of Scheme 2 where $\beta AR = R$, and $H = agonist$. For simplicity the activation of $N_\alpha$ is assumed to directly result in subunit dissociation. In addition, the receptor-promoted activation is depicted as occurring exclusively via agonist-receptor complexes, although some basal activation by receptor alone would in fact also occur (32, 44). Based on the reductions in the fold-stimulation of adenylate cyclase activity by isoproterenol, when GppNHp is the activating guanine nucleotide (Fig. 5A) compared to GTP (Fig. 6), it is likely that in the former case the $N_\alpha$ molecules can be activated either via pathway 1 or pathway 3, while in the latter case pathway 3 predominates. (It should be noted that in these phospholipid vesicle systems as many as 50% of the total $N_\alpha$ molecules which are accessible to guanine nucleotides are not accessible to HR complexes but nevertheless can be spontaneously activated by high concentrations of nonhydrolyzable GTP analogs and $Mg^{2+}$ (32)). As illustrated by the data presented in Figs. 5 and 6, $N_\alpha$ molecules activated via pathway 1 are more susceptible to the inhibitory effects of both $\alpha_T$-GTP$\gamma$S and $\gamma_T$ than are $N_\alpha$ molecules activated via $\beta AR$ (i.e. pathway 3). Thus, it would be expected that a greater percentage of the total adenylate cyclase activity is susceptible to inhibition when GppNHp is the activating guanine nucleotide compared to when GTP is used.

An important outcome of these results is that in $\beta AR/N_\alpha/C$ vesicles the inhibitions by $\alpha_T$-GTP$\gamma$S or $\gamma_T$ are not strictly dependent on the amount of active $N_\alpha$ present (relative to these subunits) but rather on the amount of active $N_\alpha$ which has been formed via the agonist-receptor pathway. Thus, suggests that the $HRO_\alpha$ complex (Scheme 2) must remain intact for a finite period during both the activation process and the stimulation of adenylate cyclase activity. Thus the inhibition by $\gamma_T$ would be reduced if the $\alpha_\gamma$ subunit, when complexed to HR, had a much weaker affinity for $\gamma_T$ compared to $\alpha$ alone. Or, in the event that subunit dissociation

| Scheme 1 | Scheme 2 |
|----------|----------|
| $N_\alpha$ | $N_\alpha$ |
| GN, Mg$^{2+}$ | GN, Mg$^{2+}$ |
| $\beta_8$ | $\beta_8$ |
| $\alpha_\gamma^*$ (Mg$^{2+}$) | $\alpha_\gamma^*$ (Mg$^{2+}$) |
| $\alpha_\gamma^*$ (Mg$^{2+}$) | $\alpha_\gamma^*$ (Mg$^{2+}$) |
| $\gamma_T$ | $\gamma_T$ |
| $\beta_8$ | $\beta_8$ |
| $HR_\alpha$ (Mg$^{2+}$) | $HR_\alpha$ (Mg$^{2+}$) |

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[Diagram of Scheme 1 and Scheme 2]
is not required for activation, HRN* complexes might interact more tightly with C (and thus more effectively compete with free βγT for a binding site on the catalytic moiety) compared to N* alone. Similarly, if HRO*, or (HRN*,*) complexes had an increased affinity for C they would better overcome the inhibitory effects of αγT-GTPγS.

For every case examined, we found the βγT subunit to be a more potent inhibitor of the N*-stimulated adenylate cyclase response as well as effecting a tighter coupling between stimulatory agonists and the effector enzyme by severely depressing basal (GTP alone)-stimulated activity.

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