The Regulation of the Cyclic GMP Phosphodiesterase by the GDP-bound Form of the α Subunit of Transducin*

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The functional interactions of the retinal G protein, transducin, with the cyclic GMP phosphodiesterase (PDE) have been examined using the different purified subunit components of transducin and the native and trypsin-treated forms of the effector enzyme. The limited trypsin treatment of the PDE removes the low molecular weight γ subunit (M$_r \approx 14,000$) of the enzyme, yielding a catalytic moiety comprised of the two larger molecular subunits (α, M$_r \approx 85,000$–90,000; β, M$_r \approx 85,000$–90,000), which is insensitive to the addition of either the pure α$_G$-GTPγS species or the pure βγT subunit complex. However, the addition of the pure α$_G$-GDP species to the trypsin-treated PDE (tPDE) results in a significant (90–100%) inhibition of the enzyme activity. This inhibition can be reversed by excess βγT, suggesting that the holotransducin molecule does not (functionally) interact with the tPDE. However, the inhibition by α$_G$-GDP is not reversed by the α$_G$-GTPγS complex, over a range of [α$_G$-GTPγS] which elicits a marked stimulation of the native enzyme activity, suggesting that the activated α$_G$ species does not effectively bind to the tPDE. The α$_G$-GDP complex also is capable of inhibiting the α$_G$-GTPγS-stimulated cyclic GMP hydrolysis by the native PDE. This inhibition can be reversed by excess α$_G$-GTPγS, as well as by βγT, indicating that the binding site for the activated α$_G$ species is in close proximity and/or overlaps the binding site for the α$_G$-GDP complex on the enzyme. Overall, these results are consistent with a scheme where (a) both the small and larger molecular weight subunits of PDE participate in α$_G$-PDE interactions, (b) the activation of PDE by the α$_G$-GTPγS (or α$_G$-GTP) species does not result in the complete dissociation of the γ subunit from the enzyme, and (c) the deactivation of this signal transduction system results from a direct interaction between the α$_G$-GDP species and the catalytic moiety of the effector enzyme.

The phototransduction system from vertebrate retina serves as an excellent model for studying receptor-coupled signal transduction. Each of the components participating in this system has been clearly identified and can be purified from rod outer segments in milligram quantities. These components include the photoreceptor, rhodopsin, and the effector enzyme, the cyclic GMP phosphodiesterase (PDE) (cf. Refs. 1 and 2). Rhodopsin is comprised of a single type of polypeptide chain (M$_r \approx 37,000$) and shares a good deal of structural homology with other G protein-coupled receptors such as the α$_2$- and β-adrenergic receptors and the muscarinic acetylcholine receptors (3–8). The retinal G protein, transducin, also is highly homologous to other members of the G protein family, i.e., the stimulatory and inhibitory G proteins of the adenylate cyclase system (the Gs and Gi proteins, respectively) and the brain G$_m$ (cf. Ref. 9). All of these G proteins are heterotrimeric in structure with their subunits being designated as α, β, γ. In the case of transducin, the molecular weights of the three subunits are as follows: α$_T$ = 39,000, β$_T$ = 36,000, and γ$_T$ = 5,000–10,000 (cf. Ref. 2). Like transducin, the cyclic GMP PDE also is comprised of three types of subunits. The two larger subunits of the enzyme (designated as α, M$_r$ = 85,000–90,000, and β, M$_r$ = 85,000–90,000) make up the catalytic unit which is responsible for the hydrolysis of cyclic GMP, while the smaller molecular weight subunit (γ, M$_r$ = 14,000) (10, 11) is suspected to represent the target for activated transducin (cf. Refs. 12–15).

Over the past few years, a great deal of effort has been devoted toward understanding the general mechanisms which underlie receptor-G protein-coupled signal transduction (cf. Refs. 9, 16, and 17). In all cases, the first step appears to be the activation of the receptor protein, i.e., by the binding of a hormone, or, in the case of the visual system, by the absorption of light by rhodopsin. This promotes the coupling of the receptor to the G protein, which in turn catalyzes the exchange of bound GDP or GTP. In the vision system, it is the binding of GTP which primes transducin for stimulating the activity of the cyclic GMP PDE. This stimulation proceeds until the bound GTP is hydrolyzed to GDP. The GTPase activity serves to deactivate the G protein and to return the system to its starting point.

Despite this general understanding, the details regarding the individual steps in the receptor-stimulated activation-deactivation cycle of the G protein, and the molecular mechanisms by which G proteins interact with, and regulate, effector proteins remain to be delineated. In the vertebrate phototransduction system, it has been commonly proposed that the transducin-mediated stimulation of the PDE activity is the outcome of a direct interaction between an activated α subunit of transducin and the γ subunit of PDE (12–15). However, it is still unclear whether the larger molecular

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weight subunits of the PDE participate in these interactions and whether the αGDP species exerts any regulatory effect on PDE activity, i.e. does the GDP-bound form of αGDP immediately dissociate from the enzyme or does it bind to the effector enzyme and impart some type of regulatory effect? The retinal visual transduction system is especially amenable to probing these types of mechanistic questions because the individual subunit components of transducin (i.e. both the GDP- and GTPyS-bound forms of αGDP and βγT complex), as well as the cyclic GMP PDE, can be easily isolated. In addition, the γ subunit of the enzyme can be removed by limited trypsin treatment yielding a catalytic moiety (i.e. the α and β subunits of the enzyme) which is constitutively active (18, 19). In the studies outlined below, we have examined the abilities of the different forms of the αGDP subunit to functionally couple to both the native and trypsin-treated phosphodiesterase. The results of these studies suggest that the GDP-bound form of αGDP, as well as the active, GTPγS-bound form, is capable of a direct interaction with the PDE. This interaction appears to involve the larger molecular weight subunits of the effector enzyme and may be directly responsible for the deactivation of the signaling system.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dark-adapted bovine retinas were purchased from Hormel Meat Packers, Austin, MN. Trypsin (Type XI), soybean trypsin inhibitor, DEAE-Sepharose, Sephadex G-100, GTP, cyclic GMP, phenylmethanesulfonyl fluoride, HEPES, and EDTA were from Sigma. GTPyS and dithiotreitol were from Boehringer Mannheim. Blue Sepharose was purchased from Pharmacia LKB Biotechnology Inc.

**Purification of Transducin and the Cyclic GMP Phosphodiesterase from Rod Outer Segments—**Rod outer segments (ROS) were purified in an isotonic buffer containing 10 mM HEPES (pH 7.5), 5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 100 mM NaCl, 0.3 mM phenylmethanesulfonyl fluoride and washed several times by brief centrifugation. The ROS were then exposed to room light for 30 min, and the membranes were pelleted (46,000 × g) for 10 min. The pellet was resuspended in a hypotonic buffer containing 10 mM HEPES (pH = 7.5), 1 mM DTT, 0.1 mM EDTA, 0.3 mM phenylmethanesulfonyl fluoride, and then several washes (at least five) were performed by repeated centrifugation and resuspension of the pellet. The supernatants, which contain the cGMP PDE, were pooled for further purification (see below). Holotransducin was eluted from the illuminated ROS membranes by resuspending the pellet in a hypotonic buffer and incubating the membranes (at 0°C) for 30 min in room light. The membranes were pelleted and resuspended in above buffer (supernatant 1). The stoichiometry of the transducin-containing pool was concentrated 10-fold in an Amicon ultrafiltration cell (YM 10 membrane). This procedure yields essentially pure holotransducin (6-10 mg of protein/390 retinas).

The αGDP subunit and the βγT subunit complex of transducin were resolved by Blue Sepharose chromatography (21). The concentrated holotransducin (6-10 mg of protein in 5-10 ml of hypotonic buffer) was applied to a 50-ml Blue Sepharose column previously equilibrated with 10 mM HEPES (pH = 7.5), 6 mM MgCl₂, 1 mM DTT, 25% glycerol (buffer A). The column was washed with 200 ml of the above buffer, and the peak of unbound protein containing βγT was pooled. The bound αGDP was eluted from the Blue Sepharose column with the above buffer supplemented with 0.5 M KC1 (buffer B). It has been reported that the αGDP subunit (22), as well as other G protein-α subunits (cf. Ref. 23), still contain bound GDP following their purification. We have quantitated the amount of bound GDP on αGDP by eluting holotransducin from purified rod outer segments with [α32P]GTP. The eluted [α32P]GTP-labeled αGDP subunit was subjected to Blue Sepharose chromatography, as outlined above, with the [α32P]labeled αGDP subunit being eluted from the resin in high salt (500 mM KC1). This αGDP subunit was subjected to an additional desalting step (Bio-Gel P6 DG) to ensure the removal of any unbound (free) labeled guanine nucleotide. The protein was then lyophilized. The stoichiometry of bound [α32P]GDP (where the GDP is formed by the intrinsic GTPase activity of αGDP (cf. Ref. 22)) per mol of αGDP (as determined by the method of Bradford (cf. Ref. 24)) was measured to be 0.70. The fact that this stoichiometry is less than 1.0 may be due to some of the GDP being inactive, or having lost some of its bound GDP, or it may reflect an overestimate in the total protein present based on the Bradford protein determination. This stoichiometry does agree well with the percentage (50-80%) of purified (unlabeled) αGDP-GTP which is able to bind [γ32P]GTP or [βγ32P]GTP in a light-dependent manner, suggesting that the actual amount of functional active αGDP-GTP present may be overestimated when measured by the Bradford method by at least 20%. Thus, we conclude that the majority of the purified αGDP, which is initially extracted by GTP elution, still contains bound GDP, and it will be referred to as the αGDP-GTP species throughout the text. Both the αGDP- and βγT complexes were concentrated to a final volume of 0.5 mg/ml protein and stored in their solution buffers at -70°C.

The active form of the αGDP subunit (i.e. αGDP-GTP-S) was prepared in an identical manner except that the transducin was eluted from the illuminated ROS by the addition of 100 mM GTPyS (instead of GTP). The subunits were separated by Blue Sepharose chromatography as described above with the purified αGDP-GTPyS complex being stored in buffer B at -70°C.

The cGMP PDE was purified (90%) from the hypotonic wash supernatants essentially as described by Baehr et al. (27) and as outlined in Ref. 28. A DEAE-G-100 column was prepared by layering 1 ml of DEAE-Sepharose over 50 ml of Sephadex G-100. The column was equilibrated with 20 mM HEPES (pH 7.0), 0.5 mM DTT, 0.1 mM EDTA at 4°C. The hypotonic wash fractions were pooled (40 ml, 3-4 mg of protein), and this was applied to the DEAE-G-100 column. The column was washed with 150 ml of the above equilibration buffer, and the PDE was eluted with 0.5 M NaCl in the same buffer. The purified PDE was dialyzed overnight (4°C) in 20 mM HEPES (pH = 7.5), 1 mM MgCl₂, 0.1 M EDTA, 50% glycerol (buffer C). The dialyzed protein (150-200 µg/ml) was stored at -70°C.

The molar concentrations of αGDP-GTP (or αGDP-GTPyS), βγT, and PDE were determined assuming Mᵣ values of 39,000 (cf. Ref. 2), 40,000 (Ref. 2), and 50,000 (Ref. 20), respectively. In all cases, the total protein was measured by the method of Bradford (24).

**Activation of cGMP Phosphodiesterase by Limited Trypsinization—**Purified PDE in buffer C was treated with trypsin (1:1 w/w) for 80 s at 4°C (see "Results" for the time dependence of the proteolytic activation). The proteolysis was stopped by the addition of a 4-fold molar excess of soybean trypsin inhibitor. The resulting PDE was constitutively active and will be referred to below as the trypsin-treated PDE (tPDE). The tPDE was generally prepared in 1-nmol quantities and stored at -70°C in buffer C. Trypsin pretreated with inhibitor had no effect on basal PDE activity at the levels used in these experiments.

**Measurement of cGMP PDE Activity—**The hydrolysis of cGMP by the purified cGMP PDE as measured by the rate of cGMP release using a pH microelectrode as originally described by Yee and Liebman (30). All assays were carried out at room temperature in a final volume of 150 µl and in a buffer containing 10 mM HEPES (pH = 8.0), 3 mM MgCl₂, 60 mM KC1, 1 mM DTT. Generally, all PDE assays contained either intact or trypsin-treated enzyme, and the appropriate components (or buffer controls) were added as indicated in the figure legends. When examining the effects of a particular subunit-component of transducin on PDE activity, the component typically was incubated with the enzyme for 5-10 min on ice prior to their addition to an assay incubation. The components and the PDE were then mixed with the assay mixture for an additional 1-5 min at room temperature to establish a baseline. The assay was always initiated by the addition of cGMP (final concentration = 5 mM) and the pH (in millivols) was measured at one determination per s. At the end of the assay period (~200 s), the buffering capacity (mV/nmol) was titrated by the addition of 0.5 µmol of KOH. The rate of hydrolysis (mV/nmol/s) was determined from the ratio of the slope of the pH record (mV/s) and the buffering capacity of the assay buffer (mV/nmol).

3 P. Guy and R. Cerione, unpublished observations.

4 The addition of A1F₂ and NaCl to the purified αGDP elicits the activation of this subunit, via the formation of an A1F₂-GDP complex (25), as reflected by a stimulation of PDE activity, and by an increase in the intrinsic trypsinophan fluorescence which mimics the fluorescence enhancement induced by active guanine nucleotides, i.e. GTP (26). These effects provide further support for the notion that the pure αGDP species, prepared from GTP-eluted holotransducin, contains tightly bound GDP.
RESULTS

Transducin Interactions with the Trypsin-treated Cyclic GMP Phosphodiesterase—A primary aim of this work was to probe the mechanisms underlying the regulation of the cyclic GMP phosphodiesterase (PDE) by the retinal GTP-binding protein, transducin. Two issues of particular interest were: (a) whether the γ subunit of the cyclic GMP PDE was exclusively responsible for binding the αγ subunit or whether other portions of the enzyme played a role in this coupling, and (b) whether the GDP-bound form of αγ (i.e. the αγ-GDP species), as well as the activated αγ complex (i.e. the αγ-GTP or αγ-GTPγS species), might be capable of coupling to the effector enzyme. As an initial approach toward addressing these issues, we examined the effects of the various purified subunit components of transducin on the trypsin-treated PDE (tPDE). The limited tryptic digestion of the PDE results in a constitutively active enzyme (cf. Refs. 18 and 19), as monitored by measuring the proton release which accompanies the hydrolysis of cyclic GMP (Fig. 1A). The results in Fig. 1B show that only a brief exposure of the purified PDE to trypsin (60–80 s) is necessary to yield an enzyme activity which is typically greater than the activity achieved via the stimulation of native PDE by the αγ-GTPγS complex (see inset to Fig. 2, below). Since these conditions of trypsin digestion appear to yield the removal of the inhibitory γ subunit of the PDE (cf. Ref. 18), it has been generally proposed that the stimulation of PDE activity by an activated transducin molecule reflects the removal of the inhibitory γ subunit of the enzyme by the G protein (cf. Refs. 12–15).

Fig. 2 shows the results obtained when different subunit components of retinal transducin are mixed with the tPDE. The addition of excess amounts of the pure αγ-GTPγS complex to the trypsin-treated enzyme (i.e. αγ-GTPγS ≈ 30 × tPDE) yields no additional stimulation of cyclic GMP hydrolysis and, in fact, shows a slight inhibitory effect (±10% for the data in Fig. 2) under conditions where it clearly stimulates the activity of the native enzyme (inset). These results are in line with the suggestion that the stimulation of the native enzyme activity by an activated αγ-GTPγS complex stems from an interaction between this αγ species and the γ subunit of PDE. Similarly, the addition of excess amounts of the purified retinal βγT subunit complex to the trypsin-treated PDE causes little or no change (±10%) in the levels of hydrolysis of cyclic GMP (Fig. 2). We also have found that the addition of excess βγT to mixtures of native PDE and the pure αγ-GTPγS complex has no effect on the αγ-GTPγS-stimulated hydrolysis of cyclic GMP (i.e. where βγT = 10 × αγ-GTPγS; data not shown). Thus, these results suggest that the βγT complex has little affinity for either the active form of the αγ subunit or for the effector enzyme itself. This is in contrast to the adenylate cyclase system where the βγ complex appears to be capable of inhibiting an α-stimulated enzyme activity (cf. Refs. 31–33) as well as eliciting a direct inhibition of the enzyme (34).

While these results were consistent with previous suggestions regarding transducin interactions with the cyclic GMP PDE, an unexpected finding was obtained when the αγ-GDP complex was added to the incubations containing the tPDE. Specifically, unlike the case for the native PDE, where the addition of αγ-GDP alone or in the presence of stoichiometric amounts of βγT elicits no change in the basal levels of activity (cf. Ref. 28), the addition of excess αγ-GDP to the trypsin-treated enzyme results in a significant decrease in the levels of cyclic GMP hydrolysis (i.e. 80 ± 5% S.E., N = 5, for conditions shown in Fig. 2). This inhibition is specific for the αγ-GDP complex, i.e. no changes in the levels of cyclic GMP hydrolysis are observed when the trypsin-treated PDE is incubated with buffer B alone. The extent of the inhibition is unaffected by the levels of cyclic GMP, over a range of 0.5–5 mM (data not shown), indicating that the inhibition by αγ-GDP is not due to its occupancy of the substrate binding site on the enzyme. As shown by the dose-response profile in Fig. 3, the inhibition of the tPDE activity approaches 100% and typically occurs over a range of levels of αγ-GDP which is similar to the levels of αγ-GTPγS necessary to elicit a stimulation of the native PDE activity (see Fig. 3, inset).4 This

4 While the results shown in the inset to Fig. 3 suggest a slight degree of (positive) cooperativity in the dose response profile for αγ-GTPγS, in other experiments we observe no deviation from a simple hyperbolic profile (data not shown).
suggests that the relative affinities of the αγ-GDP species for the tPDE and the αγ-GTPγS complex for the native enzyme are comparable. Overall, the results presented in Figs. 2 and 3 indicate that the GDP-bound form of the αγ subunit, as well as the GTP- (or GTPγS-) bound form of this subunit, must be capable of a direct interaction with the effector enzyme, and that a site on the effector enzyme other than the γ subunit must be involved in the interactions with the αγ-GDP species.

Fig. 2. The effects of the transducin subunits on the trypsintreated cyclic GMP phosphodiesterase. The trypsin-treated cyclic GMP phosphodiesterase (tPDE) (4 pmol) alone or with the pure αγ-GDP complex (128 pmol), the αγ-GTPγS species (128 pmol), or the βγ subunit complex (330 pmol) was incubated for 5 min at 0 °C prior to adding these components to an assay mixture and measuring the proton release which accompanies cyclic GMP hydrolysis at room temperature. The assay was initiated with 5 mM cyclic GMP. The data shown represent the initial rate for the proton release as determined by titrating the pH changes occurring during the assays with KOH as described under "Experimental Procedures." The activities shown were obtained by subtracting the basal activities measured in the absence of added tPDE (~0.3 nmol of cGMP hydrolyzed per s) and are the average of two determinations (from an experiment that was repeated twice with essentially identical results) with the error bars indicating the range of the determinations. Controls, which were performed with buffers A and B, indicate that these buffers have no effect on tPDE activity. Inset, the stimulation of the cyclic GMP phosphodiesterase by the GTPγS-activated αγ subunit. The native cyclic GMP phosphodiesterase (PDE) (4 pmol) was incubated with the αγ-GTPγS complex (128 pmol) for 5 min at 0 °C prior to adding these components to an assay mixture and measuring proton release at room temperature. The data shown represent the initial rate for the proton release as determined by titrating the pH changes occurring during the assays with KOH. The data bars represent the average of two experiments with the error bars indicating the range of the results from the individual experiments.

Fig. 3. Dose response for the inhibition of the trypsintreated cyclic GMP phosphodiesterase by the αγ-GDP subunit. The trypsin-treated cyclic GMP phosphodiesterase (tPDE) (4 pmol) was incubated with the different levels of the αγ-GDP complex that are indicated in the figure for 5 min at 0 °C. The proton release which accompanies cyclic GMP hydrolysis was measured at room temperature following the addition of 5 mM cyclic GMP. The results shown represent the initial rate for proton release following the addition of cyclic GMP as determined by titrating the pH changes occurring during the assays with KOH as described under "Experimental Procedures." This dose-response profile was performed twice with essentially identical results. Inset, dose response for the stimulation of the native cyclic GMP phosphodiesterase by the αγ-GTPγS complex. The native cyclic GMP phosphodiesterase (4 pmol) was incubated with the different levels of the αγ-GTPγS complex that are indicated on the abscissa for 5 min at 0 °C. Cyclic GMP hydrolysis was measured as described above. The activities shown were obtained by subtracting the total activities from the activities measured with PDE alone (≈0.6 nmol of cyclic GMP hydrolyzed per s).

The βγ complex is able to prevent the inhibition by the αγ-GDP complex of the trypsin-treated enzyme. Specifically, the results presented in Fig. 4 illustrate that when increasing amounts of the pure βγ complex are preincubated with αγ-GDP, prior to the addition of tPDE, an essentially complete (75–100%) restoration of the levels of cyclic GMP hydrolysis occurs (i.e. 88 ± 7% S.E., N = 8, recovery of the activity measured in the absence of αγ-GDP). Thus, the complexation of the βγ complex with αγ-GDP to re-form the intact holo-transducin most likely eliminates αγ-GDP-tPDE interactions.

The ability of the αγ-GTPγS complex to reverse the inhibition by αγ-GDP of the trypsin-treated enzyme activity also was examined. The rationale here was that if under normal physiological conditions, the GBD-bound αγ complex stayed associated with the effector enzyme following GTP hydrolysis (i.e. following the conversion of αγ-GTP to αγ-GDP), then the GTP- (or GTPγS-) and GDP-bound forms of αγ were
likely to share a common binding domain on the effector enzyme. As noted above (cf. Fig. 2), the αGTPγS complex does not elicit a stimulation of cyclic GMP hydrolysis by the trypsin-treated enzyme (Fig. 5, ○), which is unlike the case for the addition of αGTPγS to the native enzyme (cf. Fig. 3, inset). Likewise, under these conditions, there is little effect by αGTPγS on the extent of the inhibition by αGDP of the trypsin-treated enzyme activity (Fig. 5, □). Thus, the active (GTPγS-bound) form of the α subunit is not able to effectively compete with the αGDP species for a site on the PDE molecule. In some cases, at levels of αGTPγS > 50 pmol, a slight inhibition (typically ~10%) of the activity of the trypsin-treated enzyme is observed (cf. Fig. 5, also Fig. 2). This either can reflect the ability of the αGTPγS species to weakly mimic the inhibitory effects elicited by the αGDP complex or an inhibitory effect elicited by a small amount of free α (i.e. GTPγS-free) molecules which are present in the αGTPγS preparation.

**Transducin Interactions with the Native Cyclic GMP Phosphodiesterase**—In order to determine whether the αGDP complex is able to interact with the native PDE, we examined the ability of this αGDP species to inhibit the αGTPγS-stimulated enzyme activity. The results presented in Fig. 6A illustrate that the addition of excess αGDP (65 pmol) to a mixture of αGTPγS (32 pmol) and native PDE (4 pmol) results in about a 50% decrease in the levels of cyclic GMP hydrolysis (i.e. 51 ± 2% S.E., N = 4). As is the case with the trypsin-treated enzyme, the βγ complex is capable of reversing the αGDP-induced inhibition of the native PDE activity with a complete restoration of the activity (93–97% recovery, N = 2) occurring when βγ ~ 5 × αGDP.

Only a partial inhibition of the αGTPγS-stimulated activity by the αGDP complex is obtained under the conditions of these experiments. This may be attributed to a scheme where the binding of the αGTPγS complex to the native PDE makes a binding site accessible (on the activated enzyme) for the αGDP complex (see “Discussion,” below).

**FIG. 4.** Dose response for the βγ-induced recovery from the inhibition by αGDP of the trypsin-treated cyclic GMP phosphodiesterase. The αGDP complex (~65 pmol) was preincubated with the different amounts of the βγ complex indicated in the figure for 20 min at 0 °C and then added to assay incubations containing the trypsin-treated PDE (4 pmol). The assays were initiated by the addition of 5 mM GMP, and the relative activities of the cyclic GMP phosphodiesterase were determined by measuring the proton release accompanying cyclic GMP hydrolysis at room temperature. In these assays, the maximum activities ~1.45 nmol of cyclic GMP hydrolyzed per s, and the (basal) activities in the absence of added PDE were ~0.3 nmol per s. In the absence of added βγ, the inhibition by αGDP was ~65%.

The inhibition by αGDP can be reversed by increasing the amounts of αGTPγS present in the assay incubation. For example, when αGDP = 2 × αGTPγS, there is a 40–60% inhibition of the αGTPγS-stimulated enzyme activity (e.g. compare the open and closed circles corresponding to an abscissa value of 32 pmol of αGTPγS in Fig. 6B: also cf. Fig. 6A), while when αGTPγS = 2–3 × αGDP, the inhibition by αGTPγS is essentially eliminated (~5%, N = 2, e.g. compare the open and closed circles corresponding to an abscissa value of 192 pmol of αGTPγS in Fig. 6B). Although maximal stimulation by αGTPγS of the native PDE activity can be achieved for the conditions described in Fig. 6B, the levels of αGTPγS necessary for half-maximal stimulation are typically higher than those obtained in the absence of αGDP, by about a factor of 2. Overall, these results suggest that the αGDP and αGTPγS complexes cannot be bound, simultaneously, to an activated PDE molecule (see below).

**FIG. 5.** Effects of the αGTPγS complex on the inhibition of the trypsin-treated cyclic GMP phosphodiesterase by the αGDP species. The trypsin-treated cyclic GMP phosphodiesterase (tPDE) (4 pmol) was preincubated with the different amounts of αGTPγS indicated in the figure, in the absence (■) and presence (□) of αGDP (~38 pmol) for 5 min at 0 °C. The relative activities of the tPDE were determined by measuring the proton release accompanying cyclic GMP hydrolysis at room temperature. The data points represent the average of two experiments; the range for the different determinations was ±10%. In these experiments, the average maximal activity was ~4 nmol of cyclic GMP hydrolyzed per s (the basal activity, i.e. in the absence of added PDE, was ~0.5 nmol of cyclic GMP hydrolyzed per s).

**DISCUSSION**

Currently, little is known regarding the molecular details characterizing the coupling of GTP-binding proteins to biological effectors (i.e. enzymes or ion channels). The retinal phototransduction system offers an excellent model for probing these mechanisms since most of the key components of this signal pathway can be purified rather easily and in relatively high abundance. Previous schemes depicting the interactions between transducin and the cyclic GMP PDE...
were based, for the most part, on the findings that a free $\alpha_T$ subunit which contains a tightly bound, active guanine nucleotide (such as GTPγS or GppNHp) is sufficient to stimulate cyclic GMP hydrolysis by the effector enzyme (cf. Refs. 15 and 35). The fact that the $\betaY$ subunit complex, either alone or in the presence of activated $\alphaT$ subunits, has no effect on PDE activity is consistent with the suggestions that G protein-effector enzyme coupling is specific for the $\alphaT$ subunit.

Since the limited trypsin treatment of the PDE results in an enzyme which lacks the low molecular weight $\gamma$ subunit and which is constitutively active, it has been proposed that the interaction of the active form of $\alphaT$ with PDE occurs at the $\gamma$ subunit of the effector enzyme (12-15). This interaction was thought to release the inhibitory subunit from the catalytic subunit(s) of the PDE and thus enhance the hydrolysis of the substrate, cyclic GMP. Recently, it has been suggested that there are at least two forms of $\gamma$ subunits (15). The concentrations of transducin typically used for in vitro assays ($\leq1 \muM$) were proposed to be sufficient to remove one, but not both, of the inhibitory $\gamma$ subunits from the PDE molecules (cf. Ref. 15). However, trypsin treatment would effectively eliminate both inhibitory subunits and generate a fully active enzyme. Taken together, these various notions are seemingly consistent with the results presented here which illustrate that the active $\alpha_T$-GTPγS species does not enhance the activity of the tPDE and that the trypsin-treated enzyme is typically more active than the $\alpha_T$-GTPγS-stimulated enzyme. However, they would not predict the finding that the GDP-bound form of the $\alphaT$ subunit markedly inhibits the activity of the trypsin-treated enzyme. Rather, the ability of the $\alpha_T$-GDP complex to inhibit the tPDE suggests some type of a direct interaction between this $\alphaT$ subunit and the catalytic moiety of PDE (i.e. with the $\alpha$ and/or $\beta$ subunits of this enzyme).

Overall, the results presented here can be incorporated into a working model for the interactions of the GTP- (or GTPγS-) and GDP-bound forms of $\alphaT$ with the effector enzyme (Fig. 7). For simplicity, the PDE molecule is shown to contain only a single lower molecular weight, $\gamma$ subunit. However, the presence of two types of $\gamma$ subunits on the PDE would not change the general features of this model.

$$\alpha_T GTP + \gamma-(\alpha-\beta) \rightarrow \gamma-(\alpha-\beta) \rightarrow \alpha_T GTP$$

1. **Inactive PDE**
2. **Active PDE**
3. **GDP**
4. **RHO**
5. **HT**

**Fig. 7. Scheme depicting the interactions of the $\alpha_T$-GTPγS complex and the $\alpha$-GDP species with the native cyclic GMP phosphodiesterase.** The cyclic GMP phosphodiesterase (PDE) is suggested to be comprised of a single type of $\gamma$ subunit, although recent reports suggest that more than one type of $\gamma$ subunit may exist (15). RHO = light-activated rhodopsin. HT = holotrancducin. The initial interaction of $\alpha_T$-GTP with the cyclic GMP PDE is suggested to occur exclusively on the $\gamma$ subunit of PDE. The interaction of $\alpha_T$-GDP with PDE is suggested to occur on a larger molecular weight subunit(s); the specific location of the $\alpha_T$-GDP binding site on the enzyme has not been identified.
suggest that the initial interaction between an active αγ-GTP species, as well as an αγ-GTPyS complex, and native PDE would occur at the γ subunit of the enzyme (step 1 in Fig. 7). This interaction is not suggested to elicit the complete release of the γ subunit from the enzyme, for reasons that will be described below, but rather shifts its position in a manner that increases the accessibility of the catalytic site and thereby stimulates the production of GMP via cyclic GMP hydrolysis. In the case of the αγ-GTP complex, the hydrolysis of the bound GTP to GDP would alter the conformation of αγ and increase its affinity for a binding domain on a larger molecular weight subunit(s) of the enzyme (step 2). This binding interaction between the αγ-GTP complex and the enzyme does not block substrate (cyclic GMP) binding but rather must induce a change in the conformation of the enzyme, resulting in an immediate inhibition of its catalytic activity. The binding site for the αγ-GDP complex on the larger molecular weight subunit(s) is likely to be immediately adjacent to the binding site of the αγ-GTP or αγ-GTPyS species on the γ subunit of the enzyme. Such a location may explain the recent observations that an activated αγ-GppNHp complex can be cross-linked to the larger molecular weight subunits of the native PDE (cf. Ref. 36). Thus, the initial binding of an αγ-GTPyS complex to the PDE would prevent the binding of the αγ-GTP complex to the catalytic moiety, such that at high concentrations of αγ-GTPyS the inhibition by αγ-GDP is eliminated (cf. Fig. 6B). This also requires that the αγ-GTPyS species, following its initial interaction with the γ subunit of the effector enzyme, does not become physically separated from the larger molecular weight subunits of the PDE. Otherwise, the bound αγ-GTPyS complex could not prevent the binding of αγ-GDP to the catalytic moiety. The results of steady state kinetic studies, monitoring the inhibition of the native PDE by the purified γ subunit, also suggest that the stimulation of the effector enzyme by activated transducin does not result in the complete removal of the γ subunit from the enzyme complex (cf. Ref. 37). However, in the trypsin-treated enzyme, the binding domain for the αγ-GTPyS species (i.e. the γ subunit) is proteolytically removed, and thus the αγ-GTPyS complex does not effectively interfere with the inhibitory effects of the αγ-GDP species (cf. Fig. 5).

We find that the addition of the βγ subunit complex can prevent the inhibition of PDE activity by the αγ-GDP species. This then provides a potential mechanism for the signaling system to return to its resting state. Specifically, the reassociation of the αγ-GDP species with βγ would form holotransducin (step 3 in Fig. 7) which does not inhibit the enzyme activity. Most likely, this reflects the inability of the holotransducin to bind to the enzyme, thereby enabling the G protein to recouple to light-activated rhodopsin (step 4) and returning the PDE to its initial basal state to await another stimulatory signal from an active αγ subunit.

It should be noted that many of the results reported in this study also would be consistent with a model where both the αγ-GTP (or αγ-GTPyS) species and the αγ-GDP complex bind to a single, common site on a larger molecular weight subunit(s) of PDE. In this case, the αγ-GTP (or αγ-GTPyS) would bind with higher affinity to the native (heterotrimERIC) PDE, stimulating enzyme activity, while the αγ-GDP would bind more tightly to the activated form of PDE and inhibit activity. However, our results, when viewed within the context of this scheme, would imply that a significant percentage (if not all) of the αγ-GTP (or αγ-GTPyS) species dissociates from the activated effector enzyme under conditions where the αγ species can initially bind to the native enzyme. Such a scheme seems unsatisfactory since the dissociation of the αγ-GTP (or αγ-GTPyS) from the activated enzyme would then enable the PDE to return to its basal, inactive state, even before the occurrence of the GTPase activity on the αγ subunit. In addition, if the αγ-GTP (or αγ-GTPyS) species dissociates from the activated, native PDE, it is difficult to reconcile how the αγ-GTPyS species effectively protects against the inhibition by the αγ-GDP complex of the native enzyme activity.

Clearly, the key feature of the model shown in Fig. 7 (and an apparent requirement of any scheme proposed to explain these findings) is that the αγ-GTP species is directly responsible for the deactivation step in the visual transduction pathway through its ability to interact with the effector enzyme. Future studies will be directed toward characterizing the specific domains involved in this interaction, as well as the nature of the putative inhibitory-conformational change which is induced in the PDE by this αγ species. It also will be of interest to re-examine the molecular mechanisms underlying the interaction of active αγ complexes with the effector enzyme. Particularly relevant to this issue is whether the photoreceptor, by remaining associated with the active αγ subunit at least until it couples to the PDE, might enhance the stimulation of the effector enzyme activity. We have, in fact, recently reported that an antibody which binds to the COOH-terminal of the αγ subunit (i.e. at the COOH-terminal) accentuates the stimulation of cyclic GMP hydrolysis by the αγ-GTPyS complex (28). It therefore will be interesting to examine whether the photoreceptor might function in a similar role and if either the photoreceptor or the COOH-specific antibody against αγ will alter the affinities of the active α subunit for the effector enzyme or in any way affect the interplay between the GTP- and GDP-bound forms of αγ in regulating PDE activity.

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