The Effector Enzyme Regulates the Duration of G Protein Signaling in Vertebrate Photoreceptors by Increasing the Affinity between Transducin and RGS Protein*

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The photoreceptor-specific G protein transducin acts as a molecular switch, stimulating the activity of its downstream effector in its GTP-bound form and inactivating the effector upon GTP hydrolysis. This activity makes the rate of transducin GTPase an essential factor in determining the duration of photoreponse in vertebrate rods and cones. In photoreceptors, the slow intrinsic rate of transducin GTPase is accelerated by the complex of the ninth member of the regulators of G protein signaling family with the long splice variant of type 5 G protein β subunit (RGS9-Gβ5L). However, physiologically rapid GTPase is observed only when transducin forms a complex with its effector, the γ subunit of cGMP phosphodiesterase (PDEγ). In this study, we addressed the mechanism by which PDEγ regulates the rate of transducin GTPase. We found that RGS9-Gβ5L alone has a significant ability to activate transducin GTPase, but its affinity for transducin is low. PDEγ acts by enhancing the affinity between activated transducin and RGS9-Gβ5L by more than 15-fold, which is evident both from kinetic measurements of transducin GTPase rate and from protein binding assays with immobilized transducin. Furthermore, our data indicate that a single RGS9-Gβ5L molecule is capable of accelerating the GTPase activity of ~100 transducin molecules/s. This rate is faster than the rates reported previously for any RGS protein and is sufficient for timely photoreceptor recovery in both rod and cone photoreceptors.

The phototransduction cascade of vertebrate photoreceptor cells is a prototypic G protein-based signal transduction cascade. It is uniquely designed to ensure both a high degree of signal amplification and fast signal termination on the physiological subsecond time scale (reviewed in Refs. 1–3). Vision begins upon light excitation of rhodopsin, which activates many molecules of the photoreceptor-specific G protein, transducin. Activated transducin binds to the inhibitory γ subunits of its effector, PDEγ, increasing its catalytic activity; this leads to a decrease in the intracellular cGMP level, the closure of cGMP-gated ion channels of the photoreceptor plasma membrane, and progression of the photoreponse.

Photoresponse termination requires the timely inactivation of PDE, which occurs when GTP bound to transducin is hydrolyzed to GDP and inorganic phosphate by the GTPase activity of transducin. However, the intrinsic rate of transducin GTPase is much slower than the rate of photoresponse recovery. In the photoreceptor, this problem is solved by the action of a powerful mechanism of transducin GTPase acceleration (reviewed in Ref. 4). Intensive studies over the past decade have indicated that this mechanism is based on a cooperative action between two protein components, the RGS9-Gβ5L complex (5–7) and the transducin target, PDEγ (8–10). In vivo experiments with transgenic mice indicate that both components are essential to ensure photoresponse recovery on a physiological time scale (11, 12). It is now established that the primary catalytic role in activation of GTP hydrolysis belongs to the RGS homology domain of RGS9-Gβ5L. This domain stabilizes the conformation (“transition state”) of the transducin α subunit, which is most favorable for GTP hydrolysis. PDEγ itself does not activate transducin GTPase but rather enhances the activity of RGS9-Gβ5L.

The goal of this study was to elucidate the mechanism by which PDEγ enhances the activation of transducin GTPase by the RGS9-Gβ5L complex. In principle, two possibilities could be considered. First, PDEγ could directly contribute to GTP hydrolysis, for example, by further stabilizing the Gaγ transition state beyond the action of the RGS domain. Second, it could act by increasing the affinity between activated Gaγ and RGS9-Gβ5L. Previous efforts to answer this question using the expressed RGS homology domain of RGS9 yielded contradictory results. McEntaffer et al. (13) reported that PDEγ acts catalytically by increasing the maximal GTPase rate ~2-fold and that it does not change the apparent affinity between the RGS9 homology domain and transducin. To the contrary, Skiba et al. (14) presented evidence that the entire PDEγ effect consists of an ~3-fold increase in the affinity between the RGS9 homology domain and transducin. The reason for this incongruity is unknown. Furthermore, it is unclear whether the RGS9 homology domain could serve as a good model for studying the effects of PDEγ because the degree of transducin GTPase activation by PDEγ observed with native RGS9-Gβ5L within the ROS membranes is at least one order of magnitude higher than the effect observed with the domain alone (10, 9).

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1The abbreviations used are: PDE, type 6 cyclic nucleotide phosphodiesterase from ROS; ROS, rod outer segments; PDEγ, the inhibitory γ subunit of PDE; RGS9, the ninth member of the regulators of G protein signaling family; Gβ5L, the long splice variant of type 5 G protein β subunit; Gaγ, α subunit of transducin; GAP, GTPase-activating protein; GTPγS, guanosine 5’-O-(thiotriphosphate); PAGE, polyacrylamide gel electrophoresis.
This finding implies that the physiologically relevant mechanism of PDEγ action should be addressed with the whole RGS9-ρ5L complex.

In this study, we examined the mechanism by which PDEγ potentiates the GAP activity of the native RGS9-ρ5L complex in bovine photoreceptor membranes. We used a combination of kinetic analysis and direct binding assays and found that the major effect of PDEγ on the activation of transducin GTPase is an increase in the affinity between activated transducin and RGS9-ρ5L by >1 order of magnitude. This work provides the first explanation of how an RGS protein and a G protein effector cooperate in regulating the lifetime of G protein in its active state.

**EXPERIMENTAL PROCEDURES**

**Purification of ROS and Various Photoreceptor Membrane Preparations**—ROS were purified from bovine retinas (TA & W.L. Lawson Co., Lincoln, NE) under infrared illumination as described (15). To obtain membranes lacking most peripheral proteins but retaining an active RGS9-ρ5L complex, ROS were washed under infrared illumination twice with isotonic buffer containing 100 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol and 10 mM Tris-HCl (pH 7.5) and three times by a hypotonic buffer containing 0.5 mM EDTA and 5 mM Tris-HCl (pH 7.5). We will call this preparation "washed membranes" throughout the rest of the text. The amount of residual transducin in "washed membranes" did not exceed 1% of the original endogenous transducin content, as estimated from Coomassie-stained SDS-PAGE gels. Rhodopsin concentration in all membrane preparations was determined spectrophotometrically by measuring the difference in absorbance at 500 nm before and after rhodopsin bleaching (16).

**Preparation of Transducin, PDEγ, and Peptide Encompassing PDEγ Residues 63–87 (PDEγE–63–87)—** Transducin was purified from bovine ROS as described (17). Transducin concentration was first estimated by the Bradford method (18), using bovine serum albumin as the standard, and then the precise concentration of functionally active transducin was measured by the maximal amount of rhodopsin-catalyzed GTP·P·S binding (19). Transducin concentration, as determined with GTP·S, was used in all data analyses. Recombinant bovine wild type PDEγ was expressed in *Escherichia coli* and purified as described in (20). Peptide corresponding to residues 63–87 of PDEγ was synthesized and purified as described (10). The purity and chemical formula of PDEγE–63–87 were confirmed by mass spectrometry and reversed-phase high pressure liquid chromatography. PDEγ and PDEγE–63–87 concentration was determined spectrophotometrically using a molar extinction coefficient ε^280 of 7,100.

**Cloning and Expression of Recombinant Gaα—** Gaα cDNA, preceded by a nucleotide sequence encoding a hexa-histidine amino acid tag, was cut off at HisGα (21) with EcoRI and PstI and inserted into the baculovirus transfer vector pVL1393 digested with EcoRI and PstI. The recombinant baculovirus was generated using a BaculoGold transfection kit (Pharmingen). The resulting recombinant Gaα is N-terminally modified with the His₉ tag and therefore lacks N-terminal myristoyl moiety. Sf9 cells from a 2-liter shaking culture were collected 72 h post-infection. His₉-Gaα was purified from the cytoplasmic fraction using a combination of affinity chromatography on Ni-NTA-agarose (Qiagen) and anion exchange chromatography on MonoQ (Amersham Pharmacia Biotech) essentially as described for Gaα/Gβγ chimerae (22). The concentration of active Gaα able to interact with rhodopsin and Gβγ was determined as the maximal amount of rhodopsin/Gβγ-dependent ATPase binding (19).

**GTPase Measurements—** Transducin GTPase activity was determined by using either a multiple-turnover (GTP) > [transducin]) or single-turnover (GTP < [transducin]) technique as described previously (22). GTPase assays were conducted at room temperature (22–24 °C) in a buffer containing 10 mM Tris-HCl (pH 7.8), 100 mM NaCl, 8 mM MgCl₂, and 1 mM dithiothreitol. Washed membranes, used as the source of both rhodopsin (to activate transducin) and RGS9-ρ5L, were illuminated on ice immediately before the experiments. The reaction was started by adding 10 μl of 32P·GTP at the desired concentration (approximately 10^7 cpm/sample) to 20 μl of washed membranes (30 μg rhodopsin) reconstituted with the proteins of choice. The reaction was stopped by the addition of 100 μl of 6% perchloric acid. 32P, formation was measured with activated charcoal as described (22). All data fitting and statistical analyses were performed with SigmaPlot software, version 6.

**Binding of RGS9-ρ5L Complex to his₉-Gaα Immobilized on Ni-NTA-Agarose—** Washed membranes containing 10 μg of rhodopsin were solubilized in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM dithiothreitol containing 1% lauryl sucrose (Calbiochem). Insoluble material was removed by a 10-min centrifugation at 120,000 × g with a Beckman Airfuge, and the supernatant was mixed with 10 μl of Ni-NTA-agarose beads pre-bound to 5 μl of his₉-Gaα, in 50 μl of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.2% lauryl sucrose. 10 mM NaF, 30 μM AlCl₃, and/or 10 μM PDEγ was added if needed. Samples were incubated on ice for 30 min with occasional shaking. The agarose beads were spun down and washed twice for 5 min with 1 ml of 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 0.25% lauryl sucrose, or the same buffer containing 10 μM NiCl₂. Bound RGS9 was eluted from the resin with the SDS-gel sample buffer, subjected to SDS-PAGE (23), and detected by Western blot analysis using specific antibodies and an ECL substrate kit (Amersham Pharmacia Biotech).

**RESULTS**

The goal of this study was to evaluate the relation between PDEγ and the native RGS9-ρ5L complex in regulating transducin GTPase. We took advantage of the fact that native RGS9-ρ5L is tightly associated with ROS membranes (6), which enabled us to deplete ROS membranes of endogenous PDE, transducin, and other extractable proteins while preserving their entire RGS9-ρ5L content. Previous studies indicate that a significant cooperation between RGS9-ρ5L and PDEγ is observed in experiments utilizing this type of washed ROS membrane preparation (10, 9).

The unique feature of studying components of the phototransduction cascade is that the G protein transducin can be supplied by a practically unlimited amount of its activated receptor, bleached rhodopsin. This makes it possible to study the effects of RGS9-ρ5L on transducin GTPase under steady-state conditions in which hydrolysis of Gα-bound GTP is followed immediately by recycling of transducin to the GTP-bound form (24). As illustrated in Reaction 1 below, this steady-state situation could be analyzed by the Michaelis analysis, where RGS9-ρ5L is considered the enzyme, Gα-GTP the substrate, and Gα-GDP and inorganic phosphate the reaction products (the rate of GTPase reaction under these conditions is not dependent on the concentrations of GTP and bleached rhodopsin to the extent that these concentrations are saturating).

**REACTION 1**

\[
\text{RGS9-ρ5L} + \text{Gα-GTP} \rightleftharpoons \text{RGS9-ρ5L} \times \text{Gα-GTP} \rightarrow \text{RGS9-ρ5L} + \text{Gα-GDP} + \text{P}.
\]

We therefore could assess directly whether PDEγ influences \(k_{\text{cat}}\) or apparent \(K_m\) in this reaction after measuring the rate of the GTPase reaction at a fixed concentration of RGS9-ρ5L and various transducin concentrations. However, before pursuing this analysis we needed to solve two experimental problems outlined below.

First, PDEγ has been documented to inhibit rhodopsin-dependent transducin recycling from the GDP- to the GTP-bound form (25, 26). This is most likely because the sites of transducin interaction with rhodopsin and PDEγ overlap, allowing competition between PDEγ and rhodopsin for binding to transducin (27). As a result, transducin activation is partially blocked (27), allowing the transducin GTPase reaction at a fixed concentration of Gα·GTP in the steady-state Michaelis analysis, when PDEγ is present. We solved this problem by substituting PDEγ by its C-terminal peptide, PDEγ–63–87, which has been shown to represent the GAP activity of PDEγ (10). This peptide essentially lacks the ability to bind to the GDP-bound form of transducin because a different site on PDEγ, PDEγ–24–45, is-
Regulation of Transducin GTPase in Rod Photoreceptors

FIG. 1. PDEγ and PDEγ-(63–87) are equipotent in stimulating transducin GTPase. Time courses of GTP hydrolysis were determined in single-turnover GTPase assays as described under “Experimental Procedures.” Washed ROS membranes containing 20 μM rhodopsin were reconstituted with 2 μM transducin in the presence of 1 μM PDEγ (open circles) or PDEγ-(63–87) (closed circles). In the control experiment, no PDEγ or PDEγ-(63–87) was added (diamonds). The reactions were started by adding 200 nm [γ-32P]GTP. At indicated times the reactions were quenched with perchloric acid. Curves were fit as single exponents with 100% hydrolysis corresponding to 200 nM GTP. The data represent one of two similar experiments.

The next set of experiments illustrated in Fig. 4 provides further proof for our conclusion that PDEγ serves as an “affinity enhancer” between Gαβγ-GTP and RGS9-GβδL. We monitored the binding of RGS9-GβδL to the His6-tagged Gαβγ-DNP immobilized on the Ni-NTA-agarose in the absence or presence of PDEγ. Native RGS9-GβδL was solubilized from washed ROS membranes by the mild, non-ionic detergent lauryl saccharide and

FIG. 2. Anti-RGS9 antibodies block the GAP activity of native membrane-bound RGS9-GβδL. GTP hydrolysis by transducin (5 μM) was determined in the presence of 20 μM washed ROS membranes and 50 μM [γ-32P]GTP either with (filled bars) or without (open bars) PDEγ-(63–87) under multiple-turnover conditions. The reaction mixes were supplemented with different concentrations of sheep anti-RGS9 antibodies (Ab) or total pre-immune sheep IgG as indicated below the bar graph. In the control experiment, anti-RGS9 antibody and IgG were omitted. Reactions were started by the addition of GTP, incubated at room temperature for 20 s, and then quenched with perchloric acid. The data represent one of two similar experiments.
incubated with the Ni-NTA-agarose beads in the presence of the same detergent. The beads were rinsed, and bound proteins were extracted with the SDS-PAGE sample buffer (see “Experimental Procedures”). The amount of RGS9-Gβ5L in the extract was then detected by immunoblotting with anti-RGS9 antibodies. We chose to use full-length PDEγ in this experiment because its higher affinity for transducin than the PDEγ-(63–87) peptide allowed better protein retention upon agarose washes. The leftmost lane shows the amount of RGS9 loaded on the beads. The next two lanes provide a control showing that RGS9-Gβ5L did not bind to inactive GDP-bound Goa both with and without PDEγ. As evident from the last two lanes, transducin activation by AlF4−, which mimics the transition state for GTP hydrolysis most favorable for interacting with RGS proteins (29–31), results in RGS9-Gβ5L binding to transducin, and significant binding was observed only in the presence of PDEγ.

**FIG. 3. Catalytic properties of native RGS9-Gβ5L complex.** The GAP activity of membrane-bound RGS9-Gβ5L was determined in multiple-turnover GTPase assay (A). Washed ROS membranes were reconstituted with various amounts of transducin in the presence (circles) or absence (triangles) of PDEγ-(63–87). In the control experiment, 500 nm anti-RGS9 antibody was added to the reaction mixture to completely block the GAP activity of RGS9-Gβ5L (squares). GTPase reactions were initiated by the addition of 150 μM GTP and stopped after a 20-s incubation. The lower curve (squares) was fit with a straight line (k = 0.029 s−1). Two upper curves (triangles and circles) were fit with the sum of a hyperbola (Y = Vmax/X(Km + X)) and straight line (with the slope of 0.029 s−1 determined from the control with anti-RGS9 antibodies), where Vmax is the rate of GTP hydrolysis at saturation, Km is the apparent Michaelis constant, and X is the transducin concentration. Panel B represents accelerated transducin GTPase only. It was obtained after subtraction of the lower curve fit from panel A from the two upper data sets. The closed symbols and solid lines represent the data from the experiment shown in A, the open symbols and dashed lines represent the data from an experiment carried out under identical conditions but with another set of protein/membrane preparations. In the latter experiment, the data without PDEγ-(63–87) were obtained in duplicate (error bars represent S.E.). The kinetic parameters of accelerated GTP hydrolysis where as follows: Kmic = 3 μM, Vmax = 1.15 μM·s−1, R2 = 0.98 (● and Kmic = 3.3 μM, Vmax = 1.2 μM·s−1, R2 = 0.91 (○) with PDEγ-(63–87); Kmic = 49 μM, Vmax = 0.92 μM·s−1, R2 = 0.99 (▲) and Kmic = 94 μM, Vmax = 1.09 μM·s−1, R2 = 0.97 (▲). Panel C shows the best hyperbolic fits to the data without PDEγ-(63–87) from each experiment of panel B (solid and dashed lines) and the fits to these data constrained by the assumption that Kmic in the presence and absence of PDEγ-(63–87) is the same and equal to 3.5 μM transducin (dotted lines; see “Results” for the explanation for this analysis). Panel D shows the Lineweaver-Burk plots of the data from panel B.

**FIG. 4. Binding of RGS9-Gβ5L to his6-Goα-GDP immobilized on Ni-NTA-agarose.** RGS9-Gβ5L was solubilized from washed ROS membranes with 1% lauryl sucrose. Approximately 10 ng of RGS9-Gβ5L was mixed with 5 μg of his6-Goα-GDP bound to 10 μl of agarose beads in 50 μl of 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.25% lauryl sucrose in the presence or absence of AlF4− and PDEγ, as indicated below the gel picture. Samples were incubated for 30 min on ice. Beads were then washed twice for 5 min with the same buffer containing 20 mM imidazole. RGS9-Gβ5L bound to Goα was detected by Western blotting using anti-RGS9 antibody. The leftmost lane shows the total amount (~10 ng) of RGS9 added to his6-Goα beads. The data represent one of two similar experiments.

**DISCUSSION**

The dual requirement for an RGS protein (RGS9-Gβ5L) and an effector (PDEγ) for the timely inactivation of G protein is a unique feature of the vertebrate phototransduction cascade. It is now established that the major catalytic role in activating transducin GTPase belongs to the RGS homology domain of RGS9 and that PDEγ acts as a facilitator of RGS activity (5, 14). Although PDEγ was historically the first identified protein capable of activating G protein GTPase activity (8), its exact role in this process remained unknown. The data reported in this study indicate that PDEγ acts as an enhancer of the affinity between Goα-GTP and RGS9-Gβ5L. What remains to be elucidated in the future is whether PDEγ acts allosterically by optimizing the conformation of Goα-GTP switch regions, suggested to bind to the RGS9 homology domain or whether it interacts directly with RGS9-Gβ5L.

Our finding that PDEγ enhances the affinity between Goα-GTP and RGS9-Gβ5L provides a solid mechanistic basis for our hypothesis of why both RGS9-Gβ5L and PDEγ are needed for timely transducin inactivation in photoreceptor cells (4, 11). We noted that when a rod photoreceptor is hit by a photon of light, it needs to accomplish two nearly opposite tasks. First, it must transduce the signal from excited rhodopsin to PDE with high efficiency. Second, it has to inactivate the whole cascade within a fraction of a second. If transducin were allowed to be discharged by RGS9-Gβ5L before it formed a complex with PDE, then some transducin molecules would never activate PDE and signal amplification would be diminished. Therefore, the dependence of GTPase activation on transducin association with PDEγ ensures both high efficiency of signal transmission between transducin and PDE and timely photoreponse recovery. It is tempting to speculate that similar mechanisms may be utilized in other G protein-based signal transduction cascades to solve the same general problem of preserving both response sensitivity and time resolution.

Another important finding of this study is that the PDEγ-enhanced activity of RGS9-Gβ5L reaches the rate of ~100 s−1, which is at least one order of magnitude higher than most reported rates for various G proteins (cf. Refs. 24 and 30). However, this rate is on the same order of magnitude as the ~25 s−1 rate constant reported by Mukhopadhyay and Ross (32) for the RGS4-stimulated GTP hydrolysis by Gβγ. It is interesting to consider the RGS9-Gβ5L kcat value of 100 s−1 in the context of phototransduction recovery kinetics. This number sets the upper limit for the rate of PDE inactivation. It appears to be not only sufficient but even excessive when compared with the ~7 s−1 rate of the recovery from single-photon responses of dark-adapted mammalian rods (33, 34). Although the slowest rate-limiting step in the phototransduction recovery may not be transducin GTPase (35), the rate of 100 s−1 is too high when compared with the quenching rates of any phototransduction step derived from physiological experiments (36). This implies...
that transducin GTPase in rods does not work at its maximal rate, either because each RGS9-Gβ5L has to sequentially stimulate the GTPase activity of several transducin molecules and/or because the concentration of activated transducin remains below saturation for RGS9-Gβ5L during the course of the photoresponse.

Remarkably, the GTPase rate of 100 s⁻¹ in is the same range as the photoresponse recovery observed in mammalian cones. For example, the flicker fusion frequency of human cone vision, which reflects the speed of the photoresponse, exceeds 60 s⁻¹ and is ~5-fold higher than in rod vision (37). In this context, Cowan and colleagues (6) reported that bovine cones contain a significantly larger amount of RGS9 than rods and suggested that this difference underlies one of the biochemical mechanisms contributing to faster recovery kinetics of cones. Our finding that RGS9-Gβ5L has the potential to stimulate transducin GTPase at the rate of ~100 s⁻¹ provides a strong support for their idea. Higher RGS9-Gβ5L concentration in cones should result in a higher rate of transducin GTPase and photoresponse recovery by providing a higher frequency of RGS9-Gβ5L encounters with Gα₁-GTP-PDE.

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