Articles

Activation of Transducin Guanosine Triphosphatase by Two Proteins of the RGS Family†

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ABSTRACT: RGS proteins (regulators of G protein signaling) constitute a newly appreciated group of negative regulators of G protein signaling. Several members of this group stimulate the guanosine triphosphatase (GTPase) activity of various G protein α-subunits, including the photoreceptor G protein, transducin. In photoreceptor cells transducin GTPase is known to be substantially accelerated by the coordinated action of the γ-subunit of its effector enzyme, cGMP phosphodiesterase (PDE_γ), and another yet unidentified membrane-associated protein factor. Here we test the possibility that this factor belongs to the RGS family of GTPase stimulators. We report a detailed kinetic analysis of transducin GTPase activation by two members of the RGS family, RGS4 and Gα interacting protein (GAIP). RGS4, being at least 5-fold more potent than GAIP, stimulates the rate of transducin GTPase by 2 orders of magnitude. Neither RGS4 nor GAIP requires PDE_γ for activating transducin. Rather, PDE_γ causes a partial reversal of transducin GTPase activation by RGS proteins. The effect of PDE_γ is based on a decreased apparent affinity of RGS for the α-subunit of transducin. Our observations indicate that GTPase activity of transducin can be activated by at least two distinct mechanisms, one based on the action of RGS proteins alone and another involving the cooperative action of the effector enzyme and another protein.

Heterotrimeric G proteins act as transducers, mediating information flow between receptors and effectors in intracellular signal transduction. They become active upon binding GTP as a result of interaction with ligand-occupied receptors and remain active until bound GTP is hydrolyzed by an intrinsic GTPase activity. The active lifetimes of two G proteins, transducin and $G_{q/11}$, are regulated by their effectors, PDE[1] from ROS and phospholipase $C_{\beta 1}$, acting as GTPase activating proteins (*1*, *2*). Therefore, the regulation of G protein GTPase activity appears to be an important mechanism for setting proper signal duration in various signal transduction pathways. Recently, a novel protein family, called RGS for regulators of G protein signaling, has been identified (*3*−*7*). Several members of this family stimulate the GTPase activity of many G proteins, particularly those of the G_i and G_q subfamilies (*8*−*13*). It remains unclear if the RGS proteins also serve as effectors for G proteins or if their primary function is to regulate the lifetime of G proteins in the active state. The latter appears to be true with Sst2p, an RGS protein in *Saccharomyces cerevisiae* (*4*).

The phototransduction cascade, including the light-sensitive receptor (rhodopsin), G protein (transducin), and the effector enzyme (PDE), is one of the favorite models for studying regulation of G protein GTPase activity. The reproducibility of the photoresponse amplitude and duration to identical flashes of light is exceptional (reviewed in ref *14*). This is achieved by precise regulation of the lifetime of each activated component in the phototransduction cascade, including transducin (reviewed in ref *15*). Transducin GTPase activity, which determines the lifetime of activated cGMP phosphodiesterase, is regulated by a cooperative action of PDE_γ and another membrane-associated protein factor that remains to be identified (*16*−*18*). Working together, these proteins are able to accelerate the basal rate of transducin GTPase by over 25-fold to over 1 turnover/s (*17*). Neither of these proteins alone is capable of substantial activation of transducin GTPase. It has recently been reported (*12*) that two members of the RGS family found in the retina, RGSr and RGS4, cause ∼10-fold stimulation of transducin GTPase, even though the rate of maximally activated GTPase in this study was not faster than ∼2 turnovers/min. The authors suggested that RGSr plays a role of transducin GTPase activator in ROS.

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[1] Abbreviations: PDE, rod cyclic guanosine monophosphate phosphodiesterase; PDE_γ, γ-subunit of rod cyclic guanosine monophosphate phosphodiesterase; ROS, rod outer segments; RGS, regulators of G protein signaling; GAIP, G α interacting protein.

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In most published studies RGS proteins were present in excess over substrate G proteins, so no assessment of their real catalytic capacity could be made. Here, for the first time, we present a complete kinetic analysis of transducin GTPase activation by two RGS proteins, RGS4 and GAIP. RGS4, being at least 5-fold more potent than GAIP, stimulates the rate of transducin GTPase by 2 orders of magnitude. The absolute rate of transducin GTPase at saturating RGS4 concentration exceeds 2 turnovers/s. Most importantly, this effect does not require the presence of PDE_γ. To the contrary, PDE_γ apparently interferes with RGS proteins for binding to transducin and partially reverses the RGS-mediated stimulation of transducin GTPase. These data indicate that transducin GTPase can be regulated by at least two different mechanisms, a coordinated action of PDE_γ with a ROS membrane-associated factor or various RGS proteins alone.

EXPERIMENTAL PROCEDURES

Purification of ROS and Various Photoreceptor Membrane Preparations. ROS were purified from frozen retinas (TA & WL Lowson Co., Lincoln, NE) under infrared illumination as described (*19*). Photoreceptor membranes lacking the activity of endogenous GTPase activating factor were obtained by a two-step procedure. First, photoreceptor discs were purified from the ROS in the dark by the protocol described in ref *20*. Briefly, ROS were washed twice with isotonic buffer containing 100 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM Tris-HCl (pH 7.5) and then washed once with distilled water. They were then resuspended in 6% (w/v) Ficoll-400 (Pharmacia-Biotech) and left on ice overnight. Osmotically intact photoreceptor discs were then floated by centrifuging the samples at 27 000 rpm in a SW-28 rotor (Beckman) for 2 h. These disc membranes were free of practically all soluble or peripheral membrane-associated ROS proteins but retained some activity of the endogenous GTPase activating factor. Second, the residual activity of the factor was inactivated by treating discs with 6 M urea for 30 min on ice (*16*). Urea was then removed from the membrane preparation by five consecutive washes of the discs with isotonic buffer. Rhodopsin concentration in these membranes was determined spectrophotometrically (*21*). These membranes were used in all experiments as a source of rhodopsin. They lacked any ability either to hydrolyze GTP or to support activation of transducin GTPase by PDE_γ.

Preparation of Proteins. Transducin was purified from ROS as described in ref *22*. The only modification was that we bleached rhodopsin in the retinas prior to ROS purification. This treatment resulted in an increased yield of transducin since the formation of a tight complex between bleached rhodopsin and transducin prevented its loss from ROS membranes during ROS isolation. Transducin concentration was first estimated by the Bradford assay (*23*) and then the exact concentration of active transducin in each preparation was determined by measuring the maximal amount of rhodopsin-catalyzed GTPγS binding (*24*).

Recombinant PDE_γ was purified by a combination of cation-exchange and reverse-phase chromatography (*25*) from *Escherichia coli* strain BL21 DE3 transformed with an expression plasmid containing a cDNA encoding PDE_γ (*26*). The PDE_γ concentration was determined spectrophotometrically at 280 nm using a molar extinction coefficient of 7100.

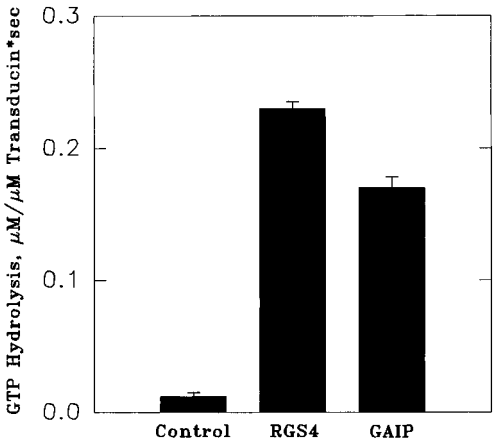

FIGURE 1: Acceleration of transducin GTPase by RGS4 and GAIP. The measurements were performed at 22 °C as described in Experimental Procedures. The reaction mixture contained 1 μM transducin, 40 μM rhodopsin, and 10 μM GTP. It was supplemented with 1 μM RGS4 or 10 μM GAIP. Each bar represents the rate of GTP hydrolysis calculated from five independent measurements. The rates of GTP hydrolysis (mean ± SD) were equal to 0.012 ± 0.003 μM GTP/(μM transducin·s) in the absence of RGS proteins, 0.23 ± 0.005 μM GTP/(μM transducin·s) with RGS4, and 0.17 ± 0.008 μM GTP/(μM transducin·s) with GAIP.

Recombinant RGS4 and GAIP were expressed in *E. coli* and purified as described (*8, 9*). The proportion of active RGS proteins was determined by incubation with a slight molar excess of $G_{i\alpha1}$ in the presence of AlF_4^-, followed by application onto a fast protein liquid chromatography Superdex 200 gel-filtration column (Pharmacia Biotech), as described (*9*). Greater than 90% of the RGS4 protein eluted as a heterodimeric complex with $G_{i\alpha1}$. GAIP is aggregated when examined by gel-filtration chromatography, precluding similar analysis.

GTPase Measurements. Transducin GTPase activity was determined by either a multiple-turnover ([GTP] > [transducin]) or a single-turnover ([GTP] < [transducin]) technique described in detail previously (*17, 27*). Most of the measurements were conducted at room temperature (22−24 °C) in a buffer containing 10 mM Tris-HCl (pH 7.8), 100 mM NaCl, 8 mM $MgCl_2$, and 1 mM dithiothreitol. Some experiments were conducted at 4 °C. The urea-treated photoreceptor discs used as a source of rhodopsin were bleached on ice immediately before the experiments. The reaction was started by mixing 15 μL of the mixture containing all protein and membrane components with 15 μL of [γ-^{32}P]GTP ($\sim10^5$ dpm/sample). The reaction was stopped by the addition of 100 μL of 6% perchloric acid. $^{32}P_i$ formation was measured by modification (*27*) of the method of Godchaux and Zimmerman (*28*).

RESULTS

RGS4 and GAIP Are Strong Accelerators of Transducin GTPase Activity. It was reported earlier that RGS4 and GAIP are efficient activators of the GTPase activity of several G proteins from the G_i and G_q families (*8, 13*). Furthermore, both RGS4 and GAIP (and also RGS1 and RGSr) serve as GTPase activating proteins for transducin (*10, 12*). The data presented in Figure 1 confirm that both RGS4 and GAIP are efficient stimulators of transducin GTPase activity. The addition of 1 μM RGS4 or 10 μM

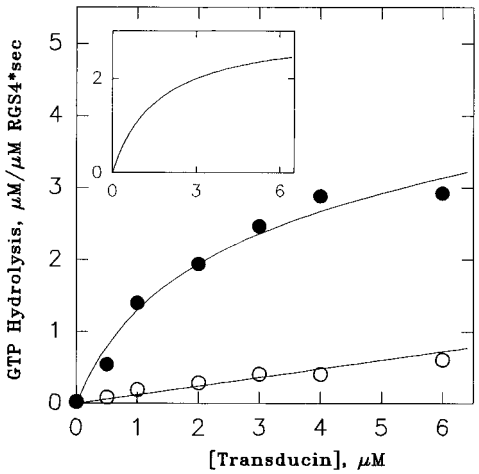

FIGURE 2: Determination of catalytic parameters of transducin GTPase activation by RGS4. Multiple-turnover measurements of transducin GTPase were performed at 22 °C with 50 nM RGS4 (upper curve) or without RGS4 (lower curve). The samples contained 40 μM rhodopsin, 20 μM GTP, and various amounts of transducin. Each point on the graph represents the rate calculated from six independently determined time points. The lower curve was fit by a straight line; the upper curve, by a sum of the same line and a hyperbola. The inset shows the accelerated transducin GTPase obtained after subtraction of the lower curve fit from the upper curve fit. The data are taken from one of two similar experiments.

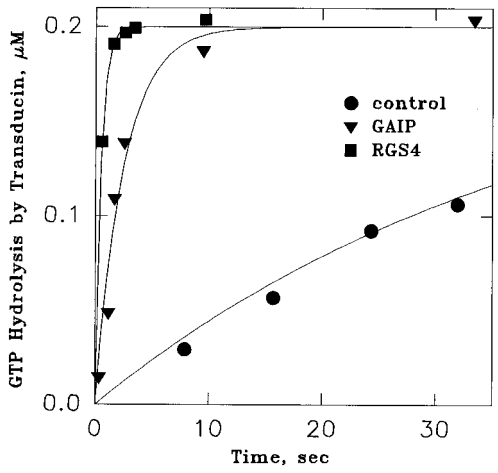

FIGURE 3: Acceleration of transducin GTPase by RGS4 and GAIP under single-turnover conditions. The measurements were performed at 22 °C as described in Experimental Procedures. Reaction mixtures contained 2 μM transducin, 40 μM rhodopsin, and 0.2 μM GTP. The samples were supplemented with 5 μM RGS4 (■) or 30 μM GAIP (▼). No additions were made to the control samples (●). Each point on the graph represents a single time point. Curves were fit as single exponents with the 100% hydrolysis corresponding to 0.2 μM GTP. The data are taken from one of at least two similar experiments.

GAIP to the reaction mixture containing 1 μM transducin and excess of GTP and photoexcited rhodopsin caused ~20-fold activation of the basal rate of transducin GTPase activity (Figure 1). A more detailed kinetic study of transducin GTPase acceleration was performed by two complementary kinetic approaches, Michaelis analysis and single-turnover analysis.

The unique advantage 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 RGS proteins on transducin GTPase under steady-state conditions where the hydrolysis of transducin-bound GTP is immediately followed by a recycling of transducin to the GTP-bound form. This allowed us for the first time to apply Michaelis analysis to the characterization of the receptor-stimulated G protein GTPase activity in the presence of RGS. In the experiment presented in Figure 2 the levels of GTPase activity at various transducin concentrations were measured in the presence or absence of a small catalytic amount of RGS4. The basal GTPase activity in the absence of RGS4 was a linear function of transducin concentration. The "accelerated" GTPase activity (inset), determined after subtraction of the basal GTPase rate (lower curve) from the GTPase rate observed with RGS4 (upper curve), was fitted by a hyperbola. The K_m value determined from these data was 1.7 ± 0.1 μM transducin ($n = 2$). The maximal rate of transducin GTPase turnover by RGS4 was 2.8 ± 0.4 s^{-1}. This rate is almost 2 orders of magnitude faster than previous estimates performed under kinetic conditions not clearly defined (*12*) (we restricted this part of the analysis to RGS4 due to the difficulty in determinations of active GAIP concentration because of its aggregation; see Experimental Procedures).

The second approach used for studying the activation of transducin GTPase by RGS proteins was the single-turnover method described in detail previously (*17, 27*). The principle of this approach is to measure a single synchronized turnover of transducin GTPase after initiating the reaction by adding GTP in an amount less than transducin. The transducin concentrations used in these assays were high enough to ensure that GTP binding to transducin (existing in a preformed complex with bleached rhodopsin) occurs faster than the subsequent GTP hydrolysis. The data shown in Figure 3 indicate that the rate of transducin GTPase at a saturating RGS4 concentration was faster than we could carefully resolve. However, we were able to conclude that the hydrolysis of GTP occurred with a rate constant not less than 2 s^{-1}, in agreement with the conclusion from Figure 2. This is at least an 80-fold acceleration of the basal rate of transducin GTPase observed in the same experiment. At 4 °C this rate is lower and could be carefully resolved. The rate constant was equal to 0.49 ± 0.06 s^{-1} ($n = 2$, data not shown), which corresponded to a (97 ± 13)-fold stimulation of the basal activity of transducin GTPase measured at the same temperature. Another observation from the single-turnover experiments is that the GTPase activating ability of GAIP is substantially lower than that of RGS4. The GTPase turnover rate measured with a saturating concentration of GAIP was only 0.43 ± 0.05 s^{-1} at 22 °C, $n = 2$ (Figure 3, middle curve; see also Figure 6).

PDE_γ Suppresses Transducin GTPase Activation by RGS Proteins. Several recent studies indicate that acceleration of transducin GTPase in ROS is a result of coordinated action of PDE_γ and another not-yet-identified factor tightly associated with the photoreceptor membranes (*17*−*19*). One obvious idea is that this factor is a member of the RGS protein family. In this context it was interesting to test whether PDE_γ is able to further enhance the GTPase accelerating action of RGS proteins as it does with the factor. The use of photoreceptor membranes lacking the activity of endogenous factor substantially simplified our analysis. The data presented in Figure 4 reveal that PDE_γ interacts with RGS in a way completely opposite to its interaction with

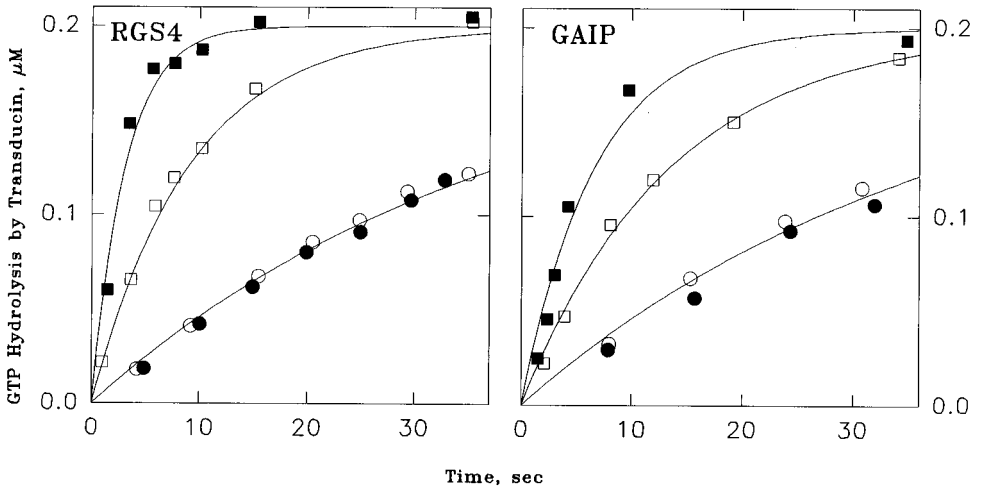

FIGURE 4: Effect of PDE_γ on the RGS-stimulated transducin GTPase activity. The measurements were performed as described in the Figure 3 legend. RGS4 was used at 75 nM, GAIP at 1 μM, and PDE_γ at 10 μM. Squares show the measurements with RGS proteins; circles show control measurements without RGS proteins. Open symbols show the measurements with PDE_γ, closed symbols, without PDE_γ. The data are taken from one of at least three similar experiments.

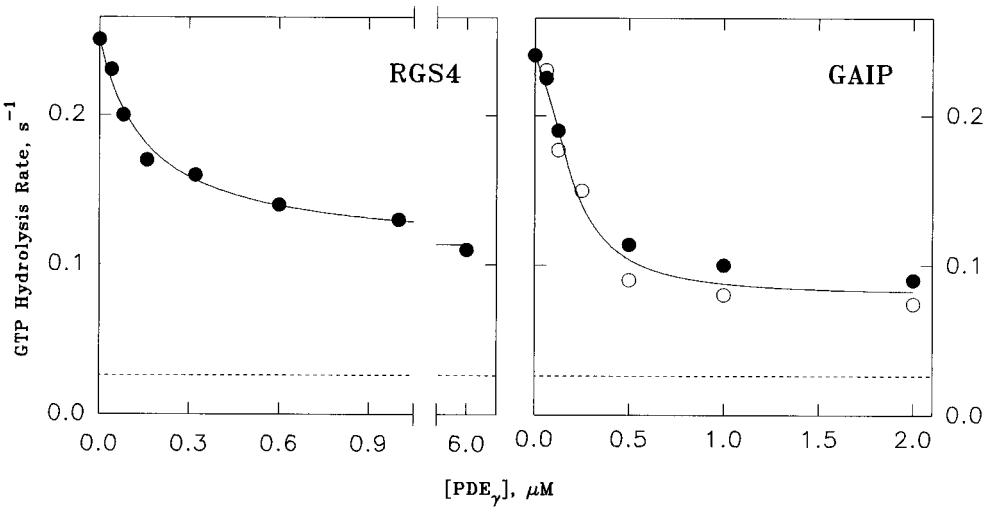

FIGURE 5: Reversal of the RGS-dependent activation of transducin GTPase at various PDE_γ concentrations. Each point on the graph represents the GTPase rate determined from single-turnover measurements (5 points per each PDE_γ concentration; see Figure 3 legend for details). RGS4 concentration, 50 nM; GAIP, 2 μM; GTP, 400 nM; transducin, 2 μM; rhodopsin, 40 μM. Filled symbols indicate the data obtained with the wild-type PDE_γ, open symbols, with the Trp^{70}-Ala PDE_γ mutant. The curves were fit by the Hill equation with the Hill coefficient equal to 1.0 for RGS4 and 1.8 for GAIP. The dashed lines indicate the basal GTPase activity of transducin in the absence of RGS proteins. The data are taken from one of two similar experiments.

the factor. Transducin GTPase acceleration by both RGS4 and GAIP is partially reversed by PDE_γ. The concentration of PDE_γ causing half-saturation of this effect was 0.15 $\pm$ 0.01 μM ($n = 2$) for 50 nM RGS4 and 0.34 $\pm$ 0.05 μM ($n = 2$) for 2 μM GAIP (Figure 5). These numbers are very close to the value of PDE_γ concentration causing half-saturation of transducin GTPase activation in the presence of the endogenous factor (0.28 μM from ref *17*). Interestingly, the Trp^{70}-Ala mutant of PDE_γ, which forms a complex with transducin but does not stimulate its GTPase activity in the presence of the endogenous factor (*26*), caused the same inhibitory effect on the RGS-stimulated transducin GTPase as the wild-type PDE_γ (this mutant was tested only with GAIP).

The data presented in Figure 6 show that the inhibitory effect of PDE_γ is based on the decrease in the apparent affinity of RGS protein for transducin. We performed this part of the analysis only with GAIP since the rates of transducin GTPase at saturating GAIP, but not RGS4, concentrations could be precisely resolved. The dependence of the rate of transducin GTPase on GAIP concentration was measured with and without 1 μM PDE_γ. While the maximal level of transducin GTPase acceleration remained the same (0.43 $\pm$ 0.05 s^{-1} without PDE_γ and 0.43 $\pm$ 0.04 s^{-1} with PDE_γ, $n = 2$), PDE_γ caused an ~5-fold increase in the GAIP concentration required for the half-maximal GTPase stimulation (from 5 $\pm$ 0.8 μM without PDE_γ to 25 $\pm$ 7 μM with PDE_γ).

DISCUSSION

The data reported in this study indicate that two members of the RGS protein family, RGS4 and GAIP, are strong catalytic accelerators of transducin GTPase activity. RGS4 stimulates transducin GTPase to a higher extent than does GAIP and also appears to interact with transducin with higher affinity. At saturating RGS4 concentrations, a slow basal rate of transducin GTPase is accelerated by at least 2 orders of magnitude to the rates of nearly 3 turnovers/s. These observations are consistent with other reported data on G protein GTPase activation by RGS proteins (*8*−*12*). All

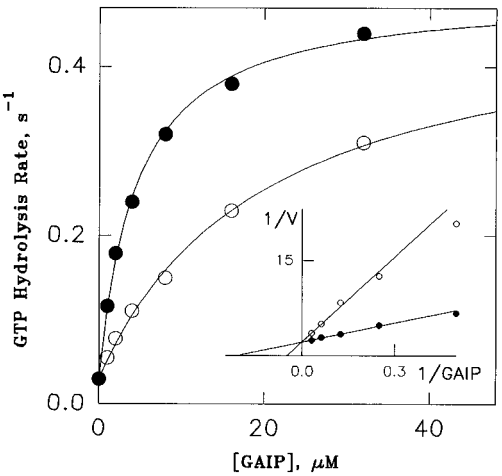

FIGURE 6: PDE$_\gamma$ reduces apparent affinity of GAIP for transducin. Single-turnover measurements were performed at 2 μM transducin, 40 μM rhodopsin, 0.4 μM GTP, and various GAIP concentrations in the presence (○) or absence (●) of 1 μM PDE$_\gamma$. The rate constants for GTP hydrolysis were determined for each GAIP concentration and plotted on the graph. The curves were fit by hyperbolas originating from the values of GTPase activity without GAIP. The inset shows the same data replotted in double-reciprocal coordinates. The data are taken from one of two similar experiments.

RGS proteins tested so far were shown to accelerate GTPase activity of many G proteins from the G$_i$ family, including transducin. However, the mechanism of RGS4 and GAIP action on transducin GTPase appears to be different from the commonly accepted mechanism of transducin GTPase regulation in photoreceptors. There is a consensus opinion in the literature that physiologically fast GTP hydrolysis by transducin in ROS requires a coordinated action of two components, PDE$_\gamma$ and a second protein factor (*16*−*18*). Here we report that PDE$_\gamma$ is not required for transducin GTPase activation by RGS proteins but, to the contrary, serves as an inhibitor of transducin acceleration by these proteins. The data obtained with GAIP (Figure 6) show that PDE$_\gamma$ causes a decrease in the affinity of GAIP for transducin and does not affect the maximal extent of GTPase activation at saturating GAIP concentrations. Interestingly, the reversal of transducin GTPase acceleration by RGS proteins in the presence of PDE$_\gamma$ was only partial at saturating PDE$_\gamma$ concentrations (Figure 5) excluding the possibility that these proteins compete for the same binding site on transducin. These observations are consistent with either a partial overlap of the binding sites for RGS proteins and PDE$_\gamma$ or an allosteric interactions between these sites. The Trp^{70}-Ala mutant of PDE$_\gamma$, which binds to transducin but is unable to stimulate its GTPase, suppresses the stimulation of transducin GTPase by GAIP to the same extent as the wild-type PDE$_\gamma$. This fact indicates that the entire GTPase activation when transducin is in a complex with both RGS and PDE$_\gamma$ is conferred through RGS. The different modes of action of PDE$_\gamma$ in the regulation of transducin GTPase in the presence of the membrane-associated factor and RGS proteins suggest that the GTPase activity of a single G protein can be activated by at least two different mechanisms. Similar observations have been reported for G$_{q\alpha}$, whose GTPase activity can be accelerated either by its effector, phospholipase C$_{\beta1}$ (*2*), or by RGS or GAIP (*13*). Furthermore, RGS4 blocks activation of phospholipase C$_{\beta1}$ by GTPγS-bound G$_{q\alpha}$ (an effect not explained by the GTPase activation) and phospholipase C$_{\beta1}$ inhibits binding of RGS4 to G$_{q\alpha}$. These findings suggest overlapping binding sites for phospholipase C$_{\beta1}$ and RGS on G$_{q\alpha}$ (*13*).

Although no RGS proteins have been localized to the outer segments of photoreceptors so far, it seems important to discuss whether some aspects of photoreceptor physiology might be regulated by retina-specific proteins from the RGS family. This can be addressed in the context of two of the most fundamental properties of photoreceptor cells, their exceptional sensitivity to light and their ability to adapt to various levels of ambient light intensities. High sensitivity of the dark-adapted rods is achieved by high levels of signal amplification at two major steps of the phototransduction cascade. Each photoexcited rhodopsin is thought to be capable of activating several hundred transducin molecules per second and each PDE stimulated by transducin hydrolyzes cGMP with a V_{max}/K_m ratio of at least 5×10^7 s^{-1}M^{-1} (reviewed in ref *29*). Even these impressively large numbers are hardly sufficient to explain the actual dynamics of the photoresponse onset in a dark-adapted rod (*29*). The requirement of PDE for activating transducin GTPase prevents transducin inactivation prior to PDE activation. This requirement can be considered as a mechanism to prevent short-circuiting of the photoresponse at the level of transducin−PDE interaction. The data reported in the present study indicate that RGS proteins, such as RGS4 or GAIP, accelerate GTPase activity of free transducin more efficiently than transducin in a complex with PDE. Therefore, their presence in the dark-adapted photoreceptors may cause a major problem with signal amplification during the photoresponse. The same argument is applicable to RGSr since it has been reported to stimulate transducin GTPase in the absence of PDE (*12*).

The second basic property of photoreceptor cells is their capability to adapt amplitude and duration of photoresponses to broad ranges of light intensities. Several recent reviews emphasize that this property is based on a number of light-dependent molecular mechanisms that make each activated component of the phototransduction cascade work either less efficiently or for a shorter period of time (*15*, *30*). In this respect it is reasonable to propose that RGS proteins might uncouple PDE activation by transducin in light-adapted cells, although to date there is no evidence for such a mechanism. This hypothesis would require that RGS proteins either are transported to the outer segments in response to light or become activated by a light-sensitive mechanism. The goals of future experiments are to establish the nature of the membrane-associated factor and to determine whether RGS family members are expressed in rods and cones and present in their outer segments.

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