Contribution of the Guanosinetriphosphatase Activity of G-Protein to Termination of Light-Activated Guanosine Cyclic 3′,5′-Phosphate Hydrolysis in Retinal Rod Outer Segments†

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ABSTRACT: Light activation of GTP binding to G-protein and its eventual hydrolysis are hypothesized to lead to activation and inactivation of cGMP phosphodiesterase (PDE) in vertebrate rod disk membranes (RDM). However, the reported GTPase rate of 3 per minute is too slow to account for the observed rapid inactivation of PDE. Our investigations on GTPase activity showed that RDM isolated in the dark have considerable dark GTPase activity, which is enhanced by light. In dark and light, the enzyme exhibits biphasic substrate dependence with two K_m's for GTP of 2–3 and 40–80 μM at 22 °C and <1 and 10–25 μM at 37 °C. The K_m's were not influenced by light. On the basis of G-protein content of the RDM, the V_{max}'s for the two activities at 37 °C in light are 4–5 and 20–30 GTPs hydrolyzed per minute per G-protein. RDM washed free of soluble and peripheral proteins do not have measurable GTPase activity in the dark or light. Purified G-protein alone also did not turn over GTP, apparently because bleached rhodopsin is required for it to bind GTP. Reconstitution of washed membranes with purified G-protein restores both the low- and high-K_m GTPase activities. Inactivation of G-protein as measured by PDE turnoff and dissociation signal recovery is found to be faster at higher than lower [GTP], consistent with the observation that the higher GTPase activity associated with the higher K_m also resides in the G-protein. In reconstituted RDM containing excess amounts of rhodopsin kinase, a condition under which rapid inactivation of bleached rhodopsin leaves PDE activity entirely dependent upon activated G-protein, PDE activated by dim light flash turned off rapidly, with a time constant of 4.7 s. These observations show that the GTPase activity of G-protein is close to being adequate to inactivate light-activated G-protein.

In mammalian rod photoreceptors, the dark current flowing into the outer segment is suppressed transiently in response to light stimulus. After a saturating light flash at 36 °C, the dark current ceases in a few milliseconds and recovers with a time constant of about a second or less (Baylor et al., 1984; Penn & Hagins, 1972). In the last few years, the biochemical reactions leading to the reduction in dark current have been elucidated, and it is generally accepted that light activates hydrolysis of cyclic GMP, leading to a decrease in cyclic GMP dependent cation conductance into the rod [for a recent review, see Pugh and Cobbs (1986)]. The sequence of reactions leading to the hydrolysis of cGMP is as follows: photolysis of rhodopsin to metarhodopsin II (R*), activation of several hundred copies of a GTP binding protein (G-protein, transducin, G_t) by R*, and activation of cyclic GMP phosphodiesterase (PDE) by the activated G-protein (G*, G·GTP) (Godchaux & Zimmerman, 1979; Fung & Stryer, 1980; Uchida et al., 1981; Fung et al., 1981; Liebman & Pugh, 1982; Cook et al., 1985). Recovery of the dark current flow into the outer segment should result from termination of cyclic GMP hydrolysis, reestablishing the level of cGMP via synthesis by guanylate cyclase and restoring the cGMP-dependent conductance. To terminate the light-activated cGMP hydrolysis, R*s, G·GTPs, and the activated PDEs have all to be inactivated. None of these processes should take longer than the recovery time observed for rods. It was hypothesized that R*s are inactivated by phosphorylation (Liebman & Pugh, 1980), following which the G*s are inactivated by the hydrolysis of bound GTP to GDP by the GTPase activity endogenous to the G-protein (Wheeler et al., 1977; Fung, 1983). PDE was presumed to be inactive in the absence of G*. Recent reports have shown that R* is multiply phosphorylated (Wilden & Kuhn, 1982), that phosphorylation inactivates R* (Sitaramayya et al., 1977; Shichi et al., 1984; Miller & Dratz, 1984), that the kinetics of phosphorylation are compatible with a role for this reaction in the termination of PDE activity (Sitaramayya & Liebman, 1983b), and that a 48-kilodalton (kDa) protein (S-antigen, arrestin) (Zuckerman et al., 1985) can speed up the inactivation of partially phosphorylated R* (Wilden et al., 1986; Bennett & Sitaramayya, 1988). However, the GTPase activity is reported to have a rate of 3 per minute or lower (Kuhn, 1980; Yamanaka et al., 1985; Fung, 1983), too slow to account for the rapid termination of PDE activity observed in vitro (Sitaramayya & Liebman, 1983b) or inferred from the recovery phase of rod light response (Baylor et al., 1984; Penn & Hagins, 1972). In view of the hypothesized significance of GTPase activity in turning off the cGMP cascade, we reinvestigated this activity in rod disk membranes (RDM). Some preliminary observations have been presented at Biophysical Society and ARVO meetings (Sitaramayya & Casadevall, 1987a,b).

Materials and Methods

Preparation of RDM. To measure GTPase activity in the unactivated RDM, the membranes were prepared under in-

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frared light with the aid of an image convertor. For the purposes of this study, an unactivated preparation is defined as one in which the PDE activity in the dark is not enhanced by the addition of GTP. RDM with little or no GTP-enhanced dark PDE activity were obtained when we followed the isolation procedure described below. Bovine eyes were removed within 30 min after the animals were killed and placed in a light-tight container without ice. The container was left at room temperature for an additional 3 h. The rest of the procedure was done under infrared light. Retinas were removed and placed in 45% sucrose (w/v) in MOPS buffer [20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT), pH 8.0], 10 retinas in 5 mL in 50-mL capacity centrifuge tubes, and vortexed at maximum speed for 1 min. The suspension was mixed with additional sucrose solution to a volume of about 40 mL, and 0.8 mL of 1 M NH_2OH was added. The suspension was vortexed for about 30 s, overlaid with about 2 mL of MOPS buffer, and centrifuged for 30 min at 27000*g*. The processing of retinas to this point was done with buffers at room temperature. The centrifugations and the rest of the procedure were at 0–4 °C. The crude RDM were collected from the sucrose–buffer interface, diluted in 2 volumes of MOPS buffer, vortexed for a few seconds, and centrifuged for 15 min at 27000*g*. The pellet was suspended in 38% (w/v) sucrose in MOPS buffer, overlaid with 2 mL of MOPS buffer, and centrifuged for 30 min at 27000*g*. The floating RDM were collected, diluted, and spun down as before. The RDM in the final pellet were suspended in 0.5 mL per five retinas and usually contained about 120 μM rhodopsin.

Preparation of Stripped RDM (sRDM). RDM stripped of soluble and peripheral proteins were prepared as described earlier (Sitaramayya, 1986). Briefly, RDM were extracted several times with a hypotonic buffer containing 1 mM EDTA, treated with 5 M urea for 30 min (Shichi et al., 1983), and washed again with hypotonic buffer, and finally with MOPS buffer. sRDM were suspended in MOPS buffer, divided into small aliquots, and stored at −30 °C. These membranes retained no measurable rhodopsin kinase or GTPase activity and served as a source of rhodopsin in reconstitution studies. The protein composition of an sRDM preparation is shown in Figure 7.

Isolation of Phosphodiesterase and G-Protein. PDE and G-protein were isolated as described by Baehr et al. (1979, 1982), dialyzed against MOPS buffer containing 50% glycerol, and stored at −30 °C. Figure 7 shows representative preparations of these proteins resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained by Coomassie Blue.

Preparation of Rhodopsin Kinase. A partially pure preparation of rhodopsin kinase was extracted from bovine RDM using procedures described earlier (Sitaramayya, 1986). The protein composition of a kinase preparation is shown in Figure 7.

GTPase Assay. GTPase activity was assayed in a total volume of 75 μL of MOPS buffer essentially as described by Wheeler and Bitensky (1977). The reaction mixture contained RDM or a reconstitution of proteins isolated from RDM. The concentration of rhodopsin when RDM were assayed was held at 2 μM or lower unless otherwise specified. In reconstitutions, rhodopsin concentration was held at 2 or 4 μM with G-protein concentration between 0.06 and 0.3 μM. The amount of PDE, kinase, and ATP, when added to assays, was varied. Dark GTPase activity was measured under infrared light. Light-activated activity was determined in room light on preparations that were held in the dark and exposed to room light for 30 s before initiation of the assay. In both cases, the reaction was initiated by the addition of [γ-^{32}P]GTP. A 20-μL aliquot was withdrawn at 1, 2, and 3 min after the addition of GTP and mixed with 1 mL of activated charcoal in 10% trichloroacetic acid and 5 mM sodium phosphate. The suspension was held on ice for 10 min, charcoal was spun down, and ^{32}P in the supernatant was measured by liquid scintillation counting. At 22 °C, GTPase activity was linear with time for at least 3 min at all substrate concentrations tested (0.5–300 μM). At 37 °C, aliquots were withdrawn at 15, 30, and 45 s for substrate additions below 5 μM in order to ensure that the activity is linear with time. In some earlier experiments, light-stimulated activity was determined in assays to which RDM and GTP were added in the dark, and the reaction was initiated with a bright light flash (13% of rhodopsin bleached). From a parallel assay mixture, an aliquot was withdrawn at the time the light flash went off and served as the zero time control. These experiments revealed that there was considerable GTP hydrolysis between the addition of GTP to the assay and the light flash which led us to investigate the properties of the dark activity as shown under Results. These assays also showed that there was no significant difference in the activities of bright flash-activated RDM and the RDM assayed in room light and therefore the light-stimulated activities were routinely measured in room light.

PDE Assay. Cyclic GMP phosphodiesterase (PDE) activity was measured in RDM or the reconstituted system by the pH recording method as described by Liebman and Evanczuk (1982).

Estimating the Active Fraction of Purified G-Protein and PDE. Reconstituted preparations containing rhodopsin, G-protein, and PDE mixed in the same ratio as in RDM did not express as much light-activated PDE activity as RDM (Sitaramayya et al., 1986). Activities obtained with different batches of proteins varied substantially, and on average, the reconstituted system was about a third as active as RDM. It was not possible to test if sRDM used as the source of rhodopsin were less effective compared to rhodopsin in RDM. In order to test the possibility that not all the purified PDE and G-protein are enzymatically active, the following method was used. In testing for PDE, 0.06 μM PDE was light activated in the presence of 4 μM rhodopsin, 250 μM GTP, and varied amounts of purified G-protein. The PDE activity approached saturation at a [G-protein] of about 2 μM. The maximum PDE activity obtainable was calculated from Lineweaver–Burk plot. By comparison of this V_{max} with the activity obtained for the same preparation of PDE on trypsin activation, the fraction of PDE activatable by G-protein is calculated. [It was reported earlier that at very high concentrations of G-protein, PDE in a light-activated reconstituted system can express activity as high as or close to that obtained with trypsin activation of the same PDE (Sitaramayya et al., 1986).] For example, in one experiment done at 22 °C, the trypsin-activated activity for 0.06 μM PDE was 31.2 μM cGMP hydrolyzed s^{-1}, while the V_{max} for light-activated activity was 26.3 μM. Therefore, 84% of PDE in this preparation was activatable by G-protein.

In testing for the activity of G-protein using the same principle, G-protein reconstituted with sRDM (0.3 μM with 4 μM rhodopsin) was light activated by a bright flash in the presence of 250 μM GTP and increasing amounts of purified PDE. Just as in the case of PDE, the V_{max} was compared with trysin-activated activity for PDE. For example, in one ex-

periment, the V_{max} was 66.7 μM cGMP hydrolyzed s^{-1}. The trypsin-activated activity of the PDE employed in this experiment was 513 μM cGMP s^{-1} (μM PDE)$^{-1}$. Therefore, the 0.3 μM G-protein in the reconstitution was able to activate 66.7/513 or 0.13 μM PDE, or only 0.13/0.3 or 43% of the G-protein is able or available to activate the PDE.

In using the above procedures to estimate the active fraction of purified G-protein and PDE, it was assumed that the active PDE is a 1:1 complex between the two proteins (Sitaramayya et al., 1986). There has been no experimental evidence so far to suggest otherwise. Earlier reports have also suggested that inactive G-proteins do not form unproductive complexes with PDEs and therefore do not remove PDEs from the pool available for activation (Fung et al., 1981; Sitaramayya et al., 1986). It should be emphasized here that since G-protein has multiple functional domains—sites involved in binding to membrane, to GTP, and to PDE and sites involved in hydrolyzing GTP—molecules ineffective in activating PDE do not necessarily fail to bind and hydrolyze GTP. In fact, as seen under Results, RDM and reconstituted preparations with little or no dark PDE activity have considerable dark GTPase activity. The measurement of the active fraction of G-protein as done here is only to underscore that a fraction of purified protein is denatured.

Protein Measurement. Protein concentration in purified preparations was measured by the method of Sedmak and Grossberg (1977) using bovine serum albumin as standard. The G-protein content of RDM was measured by electrophoresis, Coomassie Blue staining, and densitometry of the stained bands as described earlier (Sitaramayya et al., 1986) using purified G-protein as standard. Rhodopsin concentration in RDM and sRDM was measured by spectrophotometry as in earlier reports (Sitaramayya et al., 1986).

Dissociation Signal. Kuhn et al. (1981) reported that bleaching RDM in the absence of GTP results in increased light scattering by RDM. This response was referred to as the "binding signal" since it was thought to result from the binding of G-protein to bleached rhodopsin. Bleaching RDM in the presence of GTP decreases light scattering by the membranes, a response referred to as the "dissociation signal", and is hypothesized to result from the dissociation of G-protein–GTP from bleached rhodopsin. This decrease relaxes with the exhaustion of GTP (Bennett, 1982; Bennett & Dupont, 1985). Measurements of the dissociation signal reported here are done as described by Kuhn et al. (1981), at 708 nm using a modified Durrum D117 spectrophotometer.

Activation and Inactivation of G-Protein. Activation and inactivation are loosely defined in this paper. Activation is equated with increased GTPase activity and ability to activate PDE. The decay of PDE activity in light-activated preparations is presumed to result from hydrolysis of G-protein-bound GTP and is defined as inactivation.

Materials. Fresh bovine eyes were purchased from MO-PAC, Souderton, PA. [γ-^{32}P]GTP was from ICN. Chemicals were obtained from Sigma.

Results

Substrate Dependence of GTPase Activity. When RDM were assayed for GTP hydrolyzing activity at 22 °C, in the dark or in room light, the activity increased with [GTP] beyond 300 μM and was higher at all substrate concentrations when the assays were done in room light. The Lineweaver–Burk plots for all dark and light-activated membranes were biphasic, suggesting that there were two enzyme activities hydrolyzing GTP (Figure 1). The half-saturating GTP concentrations for the two activities were 2–3 and 40–80 μM, and these

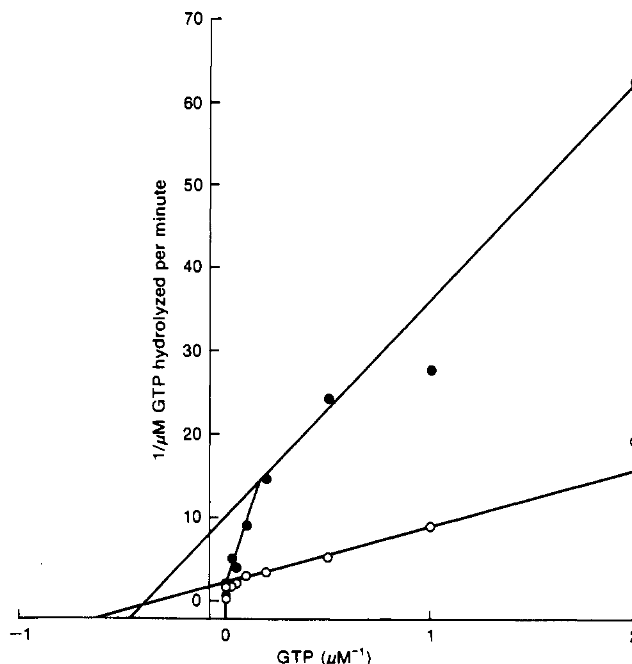

FIGURE 1: Lineweaver–Burk plots of substrate dependence of GTPase activity. GTPase activity was measured in 75 μL of MOPS buffer at 22 °C in the dark (●) or in room light (○). GTP concentration was varied between 0.5 and 300 μM. The concentration of G-protein in the assays was 0.16 μM.

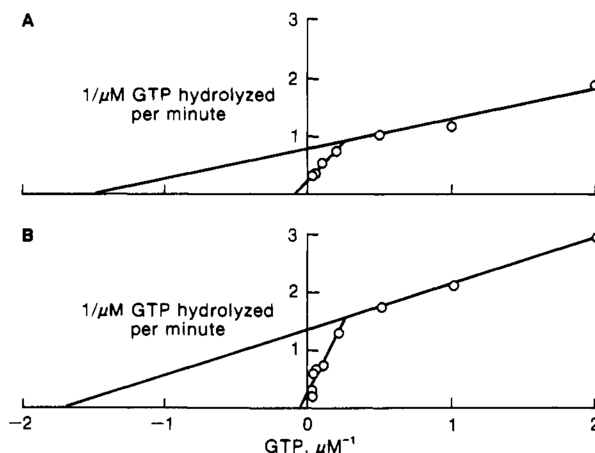

FIGURE 2: Kinetics of GTPase activity in RDM and in reconstituted systems. Assays were done at 37 °C in 75 μL of MOPS buffer. Concentration of G-protein in the assays was 0.16 μM for RDM (B) and 0.3 μM for reconstitutions (A). Rhodopsin concentration was 3.7 μM in assays with RDM and 4.0 μM in reconstitutions. Both assays were done in room light. Lineweaver–Burk plots of data obtained at 0.5–300 μM GTP as substrate are shown.

activities are hereafter referred to as low- and high-K_m activities, respectively. The half-saturating concentration for GTP for either activity was not significantly different between light and dark measurements.

The difference between the V_{max}'s in light and dark experiments was about two GTPs hydrolyzed per minute per G-protein for the low-K_m activity and about seven GTPs for the high-K_m activity (0.6 and 4.4, respectively, for the two activities in the dark and 2.6 and 11.8 in light). These values were based on the determination of G-protein content of the RDM used in these assays. At 37 °C, the K_m's for the two activities were 0.5–0.6 and 10–25 μM in assays done in room light (Figure 2B). The respective V_{max} values were 4–5 and 20–30 GTPs hydrolyzed per minute per G-protein. The K_m's were not determined at 37 °C in the dark. The activity measured in the dark at 500 μM GTP varied considerably

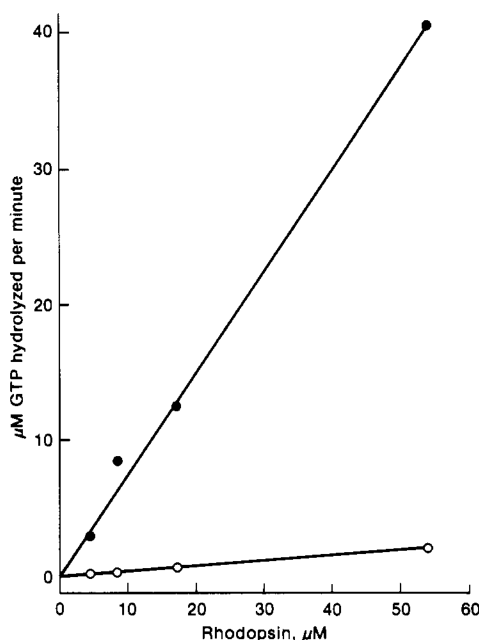

FIGURE 3: GTPase activity at various membrane concentrations. GTPase activity was measured at 22 °C in RDM at 30 μM (○) or 1 mM (●) GTP. The membrane concentration in assays was represented by rhodopsin which was varied between 4.5 and 54 μM. The assays at the two substrate concentrations were done on different preparations of RDM, and the G-protein content of these preparations was not measured.

between preparations, ranging from 2 to 12 GTPs hydrolyzed per minute per G-protein.

Linearity with Membrane Concentration. The GTPase activity in RDM was linear with rhodopsin concentration upto 54 μM. The linearity was evident whether assays were done at lower (30 μM) or higher (1 mM) GTP concentration (Figure 3).

GTPase Activity of Reconstituted G-Protein. In order to verify if the two GTPase activities are due to G-protein or caused by two different enzymes, purified G-protein was reconstituted with urea-washed sRDM, and the GTPase activities were measured at different GTP concentrations in room light at 37 °C. The Lineweaver–Burk plot of the activities showed a biphasic response, with the two activities having K_m's of 0.6 and 10 μM, respectively (Figure 2A). The V_{max}'s for the two activities were 4 and 13 GTPs per minute per G-protein. G-Protein or sRDM by themselves had no measurable activity (G-protein hydrolyzes bound GTP to GDP but cannot bind another GTP in the absence of bleached rhodopsin). The number of GTPs hydrolyzed per minute per G-protein, as assayed at 300 μM GTP at 37 °C, varied widely between preparations of G-protein, ranging from 1.5 to 15.

Reconstitution of purified G-protein with sRDM in the dark also elicited considerable GTPase activity. The dark and light GTPase activities had no correlation with the dark and light-activated PDE activities obtained when these reconstituted preparations were supplemented with purified PDE and the PDE activity measured under the same conditions in which GTPase activity was measured. For example, the reconstituted preparation shown in Figure 6 (upper trace) had negligible PDE activity in the dark and high light-activated activity, but, for the same preparation, dark GTPase activity was 31% of the total or in-light activity.

Influence of GTPase Activity on the Dissociation Signal. Activation of G-protein by R* in the presence of GTP was shown to produce a dissociation signal (Kuhn et al., 1981; Bennett, 1982, 1986). The signal relaxes when R*s decay spontaneously (several minutes at 22 °C) and G·GTPs hydrolyze to G·GDPs. If the decay of R* is speeded up considerably with the use of NH_2OH (Hofmann et al., 1983), the rate of recovery of the dissociation signal would then mostly reflect the rate of hydrolysis of GTP bound to G-protein. If G-protein has a higher GTPase rate at higher [GTP], it should then be possible to observe a difference in the decay rate of the dissociation signal between lower and higher [GTP] in the presence of NH_2OH.

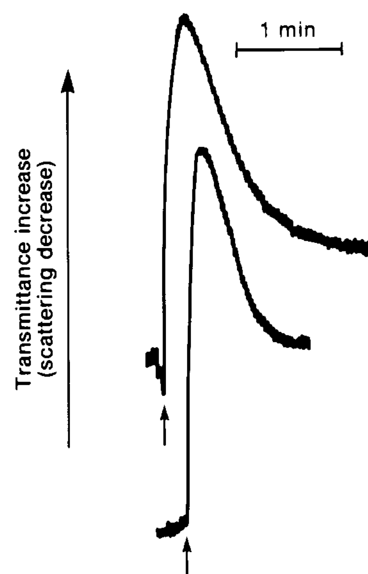

FIGURE 4: Influence of the GTP concentration on the dissociation signal in a reconstituted system in the presence of NH_2OH. Measurements were made at 22 °C in 1 mL of MOPS buffer containing 10 mM NH_2OH, 5 μM rhodopsin in sRDM, 1 μM G-protein, and 20 μM (upper trace) or 500 μM (lower trace) GTP. The light flash (↑) bleached 5×10^{-4} fraction of rhodopsin.

In the reconstitution experiment shown in Figure 4, rhodopsin, G-protein, GTP, and 20 mM NH_2OH were mixed and light activated at a 5×10^{-4} bleach at 22 °C. Rhodopsin kinase was not added, and therefore kinase-mediated GTP-dependent influences were avoided (Sitaramayya, 1986). Under these conditions, as seen in Figure 4, the dissociation signal recovered slower at 20 μM GTP (upper trace, half-time of about 35 s) than at 500 μM GTP (half-time of about 27 s, lower trace), suggesting that the two GTPase activities were indeed due to G-protein. While the influence of the two activities on the recovery of the dissociation signal is apparent from Figure 4, the rates of recovery do not strictly correspond to the GTPase rates since the inactivation of R* by NH_2OH takes about 5 s under the experimental conditions. Also, given the differences in the V_{max}'s for the two GTPase activities (2 and 7 GTPs per minute), one would expect a difference of about 20 s between the recovery rates of the dissociation signals at the two GTP concentrations. However, the observed difference is only about 8 s. In a similar experiment on RDM, the dissociation signal recovered faster at 500 μM GTP than at 20 μM, though again, the difference is less than expected. One possible reason is that the recovery of the dissociation signal also depends upon yet unidentified events that follow GTP hydrolysis. Nevertheless, both in reconstituted membranes and in RDM, the results are in the direction predicted on the basis of the GTPase data.

GTPase Activity and Its Influence on PDE Turnoff in Reconstituted Preparations. (1) In the Absence of Kinase. It has been reported that in the presence of ATP or GTP, rhodopsin kinase phosphorylates (Bownds et al., 1972; Kuhn & Dreyer, 1972; Frank et al., 1973) and inactivates R* (Si-

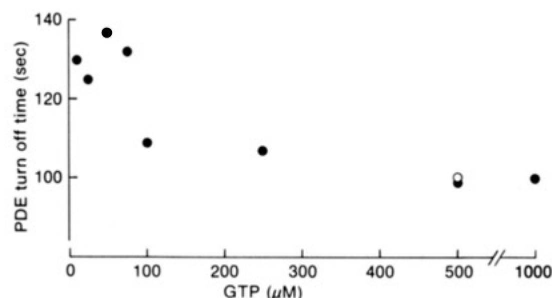

FIGURE 5: Influence of GTP concentration on the termination of PDE activation in reconstituted systems. PDE assays were done at 37 °C in 0.5 mL of reconstituted system in MOPS buffer containing 4 μM rhodopsin (sRDM), 0.3 μM G-protein, 0.06 μM PDE, a varied concentration of GTP, and 5 mM cGMP. PDE was activated by a light flash that bleached 5 × 10^{-5} fraction of rhodopsin in the assays. When included (O), [ATP] was at 500 μM. Turnoff time in seconds was derived by dividing the [cGMP] in micromolar hydrolyzed by the time PDE activity was fully terminated by the initial velocity of PDE in micromolar per second.

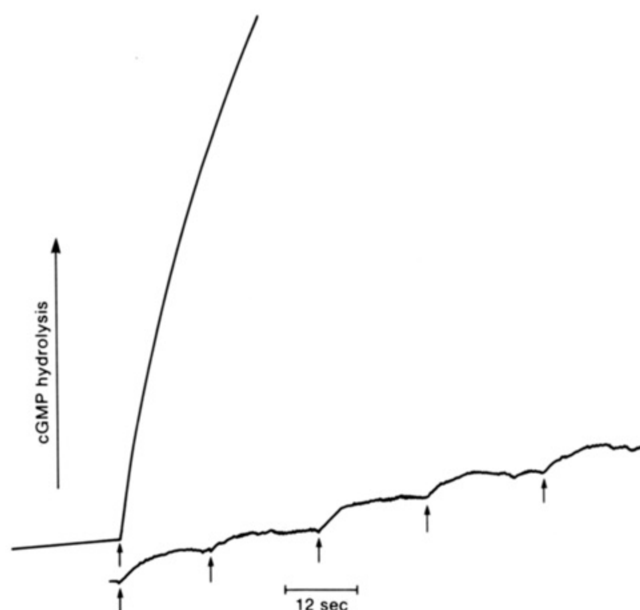

FIGURE 6: Influence of excessive amounts of rhodopsin kinase on the light-activated PDE activity in reconstituted preparations. PDE assays were done at 37 °C in 0.5-mL reconstitutions containing 4 μM rhodopsin (sRDM), 0.3 μM G-protein, 0.06 μM PDE, 5 mM cGMP, 1 mM each of ATP and GTP, and 138 μg of partially purified rhodopsin kinase in which the putative kinase protein (65 kilodaltons) is about 15–20%. The reactions were initiated by light flashes (↑) that bleached 5% of rhodopsin in the upper trace and 5 × 10^{-5} fraction of rhodopsin in the lower trace.

taramayya et al., 1977; Aton & Litman, 1984; Miller & Dratz, 1984; Arshavsky et al., 1985). In the absence of kinase, R* decays spontaneously but at a much slower rate (Sitaramayya & Liebman, 1983a). Once R* decayed, the PDE activity should last only until the G-proteins activated during the lifetime of R* have also been inactivated. The rationale for this experiment was that without the influence of kinase, the observed PDE turnoff should be faster at higher [GTP] than at lower [GTP] because the GTP hydrolysis rate (G-protein inactivation) was greater at higher [GTP].

As seen in Figure 5, at 37 °C, the time constant for the decay of PDE activity (micromolar cGMP hydrolyzed by the time the activity was fully terminated divided by the initial rate in micromolar per second) was about 130 s at a lower concentration of GTP (131 ± 5 s for [GTP] between 10 and 75 μM) and decreased to about 100 s at a higher concentration (102 ± 4 s for [GTP] between 250 and 1000 μM). The faster rate has GTP dependence of the nature of the high-K_m GTPase activity in that it saturated between 250 and 500 μM GTP as would be expected of an activity with a K_m of 10–25 μM. It was shown earlier that the half-maximal effect of rhodopsin kinase on the PDE turnoff was at 1400 μM GTP in the absence of ATP (Liebman & Pugh, 1980). Also, in the experiment shown here, supplementing the reconstitution with 500 μM ATP did not influence the turnoff, showing that the reconstitution was free of kinase and kinase-mediated influence on the PDE turnoff.

(2) *In the Presence of Kinase.* When the reconstitution mixture was supplemented with partially purified rhodopsin kinase and ATP, PDE activity initiated by a dim light flash was turned off rapidly. As the kinase level in the assay was raised, the turnoff time decreased (Sitaramayya, 1986). It can, however, be argued that as the kinase level is increased, at a certain concentration of kinase there would be no further reduction in turnoff time with higher kinase because the time it takes to inactivate R* is insignificant compared to the inactivation rate of G-protein. The rate of PDE decay at that stage can represent the true GTPase rate if GTPase activity indeed inactivates G-protein.

As seen in Figure 6, at high concentrations of kinase, the PDE activity initiated by a dim light flash was terminated with a time constant of 4.7 ± 0.5 s for the five flashes shown (in another similar experiment done with a different batch of proteins, a turnoff time constant of 2.7 ± 0.8 s was measured; $n = 3$ flashes). Kinase concentrations up to the one used in Figure 6 continued to shorten the PDE turnoff time. Higher concentrations of kinase could not be tested due to limitations imposed by the noise in the measurements, and therefore the delay in turning off PDE observed in Figure 6 was not entirely due to GTPase activity.

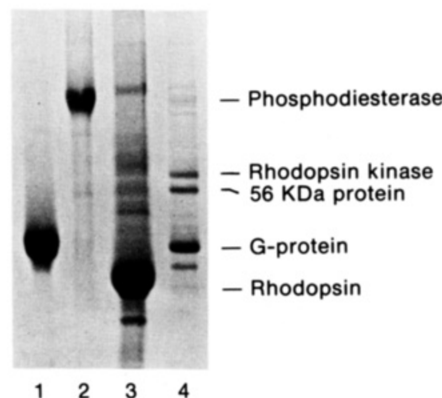

FIGURE 7: Protein preparations used in studies reported here. The experiments reported in Figures 2A and 4–6 were done on different batches of purified preparations. This figure shows a Coomassie Blue stained electrophoretogram of proteins from one batch. Lane 1, G-protein; lane 2, PDE; lane 3, sRDM; lane 4, kinase preparation.

Influence of Putative Regulators of GTPase Activity. For reconstitution of GTPase activity, only sRDM and G-protein were required while the assays in which the turnoff time for PDE was determined also contained kinase, cyclic GMP, ATP, and PDE. It was possible that one or more of these components increased the GTPase rate, resulting in the observed rapid PDE turnoff (Figure 6). To test for their influence, GTPase reconstitutions were individually supplemented with these putative regulators at concentrations at which they were present in the PDE turnoff measurements as shown in Figure 6. It was observed that kinase, ATP, PDE, and cyclic GMP had no significant influence on GTPase activity at 10 or 250 μM GTP as substrate (data not shown).

Discussion

Rod response to a near-saturating light flash, suppression, and recovery of dark current flowing into the outer segment last 1–2 s (Baylor et al., 1984; Penn & Hagins, 1972). The recovery results from termination of cGMP hydrolysis, synthesis of cGMP, and restoration of cGMP-gated conductance into rod. However, these three processes need not be strictly successive. There could be considerable overlap in time. For example guanylate cyclase activity could be triggered with a brief delay after PDE activation, and the synthesis of cGMP could excede hydrolysis even while the termination of hydrolysis is in progress. Should that be the case, termination of cGMP hydrolysis following light activation may be slower than the recovery of the electrical response of the cell. However, it has not yet been possible to measure, in vivo, the activation and inactivation of PDE alone.

In vitro, the fastest turnoff time for PDE activity in RDM was reported to be about 4 s at 38 °C (Sitaramayya & Liebman, 1983b). Since this observation was not affected by guanylate cyclase or the cGMP-dependent cation conductance, the individual activated enzymes in the cascade, R*, G·GTP, and PDE, should all be inactivated no slower than the observed turnoff time. The inactivation of R* was reported to be rapid, completed within 2 s at very small fractional bleach levels (Sitaramayya & Liebman, 1983b), suggesting that the turnoff time was probably rate limited by the GTPase activity of G-protein.

Wheeler and Bitensky (1977) and Wheeler et al. (1977) characterized GTPase activity in frog rod disk membranes. They found substantial dark activity, with two GTPase activities having K_m's of 1 and 90 μM GTP at 37 °C. Assuming that most of the protein in RDM is rhodopsin and that there is 1 G-protein per 13 rhodopsins (Sitaramayya et al., 1986), the V_{max}'s reported for these 2 activities, respectively, are 0.25 and 6 GTPs per minute per G-protein. Only the lower K_m activity was reported to be light sensitive. Similar results were also reported by Vandenberg and Montal (1984) for squid photoreceptor membranes. Observations reported here differ from these studies only in that in bovine RDM both the activities are light sensitive and the K_m's for GTP are <1 and 10–25 μM, respectively, for the two activities. Bennett and Dupont (1985), measuring [^{14}C]GTP binding to bovine RDM, also found two binding affinities with K_d's of 0.1 and 20 μM, respectively.

In our RDM preparations, the V_{max} for light-sensitive activity of the higher K_m component was about 15–20 GTPs per minute per G-protein, or 3–4 s/GTP, which is not very far from being acceptable as the mechanism of inactivation of G-protein in the recovery phase of light response in vertebrate rods. The reported rates for GTPase activity in bovine RDM have generally been around 3 GTPs or less per minute (Kuhn, 1980; Yamanaka et al., 1985; Fung, 1983), close to what we observed for the low-K_m activity, and in all cases, the reason for the low rate is that the assays were done at low substrate concentration (4 μM or less).

Since there was a substantial dark GTPase activity in RDM, we measured (unpublished observations) activities of guanylate cyclase and phosphoprotein phosphatase, two enzymes likely to be present in RDM and whose activity could cause overestimation of GTPase activity as measured in our experiments. The cyclase activity in RDM was about 40–120 pmol of GTP utilized min^{-1} (nmol of rhodopsin)$^{-1}$ in room light at 37 °C. Considering that the rhodopsin:G-protein ratio in RDM is about 13 (Sitaramayya et al., 1986), only about 1 GTP per minute per G-protein was attributable to the cyclase activity. No phosphoprotein phosphatase activity could be measured in the RDM used in these experiments. These observations appear to suggest that the procedure employed to measure GTPase activity is valid.

The high-K_m GTPase rate comes close to being meaningful as the mechanism of G-protein inactivation, but that could be valid only if the activity indeed belonged to G-protein and not to another GTP-hydrolyzing enzyme in the rod disk membranes. We found that urea-washed sRDM or purified G-protein alone does not turn over GTP and that when mixed together in light the preparations elicit both the low- and high-K_m GTPase activities. In addition, the two activities of G-protein can be demonstrated to have a predictable influence on the dissociation signal (Figure 4) and PDE turnoff (Figure 5). These results show that both GTPase activities reside in G-protein. Rybin and Gureeva (1985) have reconstituted bovine sRDM with purified G-protein and found identical results. In fact, these authors suggested that their results reflect the presence of more than one binding site for guanine nucleotides in the G-protein molecule. While the amino acid sequence analyses seem to suggest that there may only be one binding site for GTP on the α-subunit of G-protein (Hurley et al., 1984; Medynski et al., 1985; Yatsunami & Khorana, 1985; Dever et al., 1987), the possibility of an additional site of lower affinity being on the other subunits or in a pocket in the folded conformation of G-protein cannot be excluded. In fact, Kohnken and McConnell (1985) have shown that both α- and β-subunits of G-protein have GTP binding sites. Also, it is possible that the low-affinity site is not strictly a GTP binding site. Robinson et al. (1986) showed that cGMP influences GTP binding to G-protein, which suggests a possibility that cGMP binds to a site on this protein.

The high K_m for GTPase activity suggests that the activity may be saturated only at several hundred micromolar GTP, a concentration that was considered possibly nonphysiological by Wheeler et al. (1977), but Robinson and Hagins (1979) demonstrated that carefully isolated frog rod outer segments have GTP levels close to 2 mM. Therefore, the high-K_m GTPase activity is unlikely to be limited by [GTP] in the cell. It should also be pointed out that in experiments where rapid PDE turnoff was reported in RDM, [GTP] was at 250 μM (Sitaramayya & Liebman, 1983b), a concentration at which the high-K_m GTPase activity would be saturated. We suggest that GTP functions as an effector at higher concentrations. In experiments not shown here, we found, like other investigators did earlier (Wheeler & Bitensky, 1977), that the K_m for GTP for light activation of PDE (bright flash, 37 °C) is submicromolar like the low K_m for the GTPase activity. In both cases, the result suggests that binding of GTP to G-protein saturates with a K_m of about 1 μM or lower. At higher concentrations of GTP, it is possible that a second guanine nucleotide binding site is occupied by GTP, and under these conditions, the rate of hydrolysis of GTP bound to the high-affinity site is greatly enhanced. Another possible interpretation based upon the observations of Bennett and Dupont (1985) is that G-protein has only one binding site for GTP but that there are two conformational states of G-protein, one with a lower affinity for GTP than the other.

Kuhn (1981) reported that upon GTP binding, G-protein dissociates into G-α·GTP and G-β·γ and these subunits are released into solution from the membrane though the G-α·GTP is released to a greater extent than the G-β·γ. If the G-α·GTP has a GTPase rate different from that of the undissociated protein, it would complicate the interpretation of our results. In fact, Fung (1983) observed that G-α·GTP has a lower

GTPase activity which was enhanced by G-β·γ. He also reported that it takes only 1 β·γ subunit per 10 G-α's to achieve maximal rate of GTPase. Since the G-β·γ:G-α ratio in solution as observed by Kuhn is at least 0.25, it is unlikely that GTPase activity of G-α·GTP alone would be a significant fraction of the total activity.

The observations that sRDM and purified G-protein do not hydrolyze GTP and that when mixed together they elicit both dark and light-activated GTPase activities suggest two notable points: that the GTPase activity is due to G-protein and that a fraction of G-protein lost the requirement to be activated by bleached rhodopsin in order to elicit GTPase activity. Since both RDM and the reconstituted system have dark GTPase activity that does not correlate with dark PDE activity, it appears possible that a fraction of G-proteins fails to couple with PDE or may not be targeted at PDE.

While the results reported here and those found by Rybin and Gureeva (1985) show that the high-K_m GTPase activity makes it possible to inactivate G-protein much more rapidly than hitherto believed possible, there is an apparent discrepancy in that in the reconstituted system the PDE turnoff was very rapid (4.7 s) even when the high-K_m GTPase activity of the very G-protein used in such experiments can account for hydrolysis of only 6-7 GTPs per minute. We believe that the reason for the difference is in the fact that in measuring the GTPase rate we are measuring the concentration of GTP hydrolyzed in the reaction and dividing that by the concentration of G-proteins added to the assay, ignoring what fraction of them are actually enzymatically active. However, in measuring the PDE turnoff time, we are looking at the exponential decay of activated proteins. If all molecules of the purified G-protein are active, it would be possible to measure rates at least as high as those found in RDM from which the protein is isolated. Alternately, whatever the fraction of active G-protein, if we can instantaneously activate them in the presence of 100 μM or higher GTP, inactivate the activator, and follow the decay of G·GTPs, it should be possible to measure rates similar to the turnoff rate measured for PDE in Figure 6. However, such measurements are not feasible with the radioisotope assay utilized in this study. In analyzing the fraction of active protein in G-protein preparations, we found that individual preparation varied very much from each other even though they were all prepared exactly under the same conditions. The active protein fraction in our preparations, measured as that capable of activating PDE, was between 20% and 50%. In variation with the observations of Miller et al. (1987), incubating G-protein with sRDM for longer periods of time before GTPase assay did not increase the hydrolysis rate. Also, the reconstituted system could not be missing a soluble component (Dratz et al., 1987) since the activity in RDM itself is linear with membrane concentration and did not appear to depend upon soluble cofactors. Reviewing the reports from other laboratories, we found that the G-protein preparations used in those studies were probably also not fully active. The GTPase rate in a study by Cerione et al. (1987) was 0.2 GTPs per minute per reconstituted G-protein. Higashijima et al. (1987) found that only half the α-subunits of G-protein in their experiments were binding guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS). Wensel and Stryer (1986) report that 5 μM Ta (α-subunit of G-protein) with bound GTPγS was required to elicit 35% of maximum activity from 0.95 nM PDE (>5000-fold excess) whereas such activation was achieved in RDM at a G-protein:PDE ratio of 5 (Sitaramayya et al., 1986). The properties of the preparations of G-protein or its subunits in our experiments and those of these authors suggest that the properties either of the isolated proteins or of their subunits differ significantly from the native protein or that the isolated protein is denatured to some extent during purification from RDM. It is also possible that part of the G-protein activity is already inactivated during the isolation of RDM which can account for the high variability in the light-activated PDE activity in RDM preparations. Robinson and Hagins (1979) observed that the GTPase activity in intact frog outer segments could be 20 times higher than in isolated RDM. Since G-protein has many functional sites, intramolecular binding sites for the subunits and binding sites to rhodopsin, to GTP, and to PDE, loss of the function of one or more of these sites is possible during the preparation of RDM or G-protein, considering the high susceptibility of the protein to oxidation. The G-protein routinely isolated in our laboratory has, on an average, 0.94 ± 0.37 (n = 3) mol of guanine nucleotide bound/mol, but GTP binding activity may not necessarily estimate or predict the ability to hydrolyze GTP. In fact, Hingorani and Ho (1987) found that modifications that did not afffect nucleotide binding diminished the GTPase activity as well as PDE-activating ability of G-protein.

In conclusion, the observations reported here suggest that GTPase activity in bovine RDM is higher than hitherto reported, may be much higher in rods, and comes close to being considered as a viable mechanism of inactivation of light-activated G-protein.

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