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Photolyzed rhodopsin catalyzes the exchange of GTP for bound GDP in retinal rod outer segments

(visual transduction/bovine retinas/amplification/light-activated GTPase)

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ABSTRACT **We have studied the binding of guanyl nucleotides to retinal rod outer segment membranes to determine how light activates a cyclic GMP phosphodiesterase and a GTPase. We found that rod outer segment membranes contain tightly bound radioactive GDP after incubation in the dark with [^{3}H]GDP or [α-^{32}P]GTP. Reconstituted membranes containing only rhodopsin and phospholipid bind almost no GDP. More than 80% of the radioactive GDP bound to rod outer segment membranes could be released by subsequent illumination. At low light levels, the rate and extent of GDP release were markedly enhanced by the presence of GTP or p[NH]ppG, a nonhydrolyzable analog of GTP. The kinetics of binding of p[NH]ppG paralleled the kinetics of release of bound GDP, indicating that p[NH]ppG was exchanged for bound GDP. The maximal amount of bound p[NH]ppG was 1 per 30 rhodopsins when photolyzed membranes were incubated with 10 μM nucleotide. Under these conditions, p[NH]ppG binding was half-maximal when only 1 in 90,000 rhodopsins was photolyzed. This corresponds to the catalyzed exchange of 500 p[NH]ppG for bound GDP per photolyzed rhodopsin. We propose a light-activated GTP–GDP amplification cycle involving a guanyl nucleotide binding protein with GTPase activity (E). The essence of this cycle is that photolyzed rhodopsin catalyzes the formation of E·GTP from E·GDP (the major species in the dark) by nucleotide exchange. The formation of several hundred E·GTP per photolyzed rhodopsin may be the first stage of amplification in visual excitation.**

Rod outer segments (ROS) contain two light-activated enzymes: a phosphodiesterase specific for cyclic GMP (1–3) and a GTPase (4, 5). In freshly detached ROS, the photolysis of one rhodopsin molecule leads to the hydrolysis of about 10^4 cyclic GMP molecules within 100 msec (6). In disc membrane suspensions, one photolyzed rhodopsin leads to the hydrolysis of 4×10^5 cyclic GMP per sec (7). For a turnover number of 800 sec^{-1} (8), it was calculated that one photolyzed rhodopsin activates some 500 phosphodiesterase molecules (7, 9). Electrophysiological studies have shown that cyclic GMP depolarizes the ROS plasma membrane within milliseconds after being injected intracellularly and that it increases the latency of the light-induced hyperpolarization (10, 11). These findings suggest that the photoactivation of the phosphodiesterase may be an important step in visual excitation. How then does one photolyzed rhodopsin switch on many phosphodiesterase molecules? The requirement of the phosphodiesterase for GTP in addition to photolyzed rhodopsin points to the GTPase as a likely link in the activation sequence (12). ROS contain a peripheral membrane protein consisting of 37,000- and 41,000-dalton subunits that binds guanyl nucleotides and has GTPase activity in the presence of bleached membranes (13). We report here studies of the effect of light on the binding of GTP (or an analog) and GDP to ROS membranes. The striking finding is that one photolyzed rhodopsin molecule catalyzes the exchange of several hundred GTP for tightly bound GDP. We propose that this catalyzed exchange is the first stage of amplification in visual excitation.

MATERIALS AND METHODS

Materials. [α-^{32}P]GTP, [^{3}H]GTP, and [^{3}H]GDP were purchased from New England Nuclear. [^{14}C]GMP, [^{14}C]ATP, [^{14}C]ADP, and [^{3}H]p[NH]ppG were from Amersham. All other chemicals and nucleotides were either from Sigma or from P-L Biochemicals. Frozen bovine retinas were obtained from American Stores Packing (Lincoln, NE).

ROS Membrane Preparations. Bovine ROS membranes were isolated in buffer solution containing 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.5) at 4°C under dim red light (Kodak safelight filter no. 2). In this procedure, 100 thawed retinas were stirred gently in a beaker containing 30 ml of 45% (wt/vol) sucrose in buffer for 3 min. The suspension was then diluted with an additional 100 ml of 45% sucrose and centrifuged at $27,000 \times g$ for 20 min. The supernatant was collected and diluted with an equal volume of buffer and recentrifuged at $40,000 \times g$ for 15 min. The resulting crude ROS membranes were further purified by flotation centrifugation in 35% sucrose in buffer as described (14), except that all subsequent homogenization steps were omitted. Purified ROS membranes were washed twice with buffer and stored frozen at −70°C. ROS membranes isolated by this procedure routinely gave A_{278}-to-A_{498} ratios between 2.3 and 2.6. The rhodopsin concentration was determined in 0.1 M tridecyltrimethylammonium bromide by using a molar absorptivity at 498 nm of 42,600 M^{-1} cm^{-1} (14).

Determination of Membrane-Bound Nucleotides. The binding of radioactive nucleotides to ROS membranes (1.5 μM in rhodopsin) was assayed by filtration through a 0.45-μm type HA Millipore filter which retained the ROS membranes. The filter was washed five times with 3 ml of buffer and assayed for radioactivity.

ROS membranes containing tightly bound [α-^{32}P]GDP were routinely prepared by incubating ROS membranes (100 μM in rhodopsin) in buffer solution containing 0.5 μM [α-^{32}P]GTP. After 90 min of incubation at 4°C, the sample was diluted 1:10 with ice-cold buffer solution and centrifuged at $45,000 \times g$ for 15 min in order to remove most of the unbound radioactivity. The membrane pellet was resuspended to a final rhodopsin concentration of 3 μM in buffer solution and aliquots were subjected to different degrees of photolysis. The photolyzed membranes were immediately added to 5 vol of buffer con-

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Abbreviations: ROS, rod outer segments; R*, photolyzed rhodopsin; E, guanyl nucleotide binding protein with GTPase activity; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate.

taining a nucleotide, filtered, and then assayed for radioactivity. Membrane-bound radioactive nucleotides were extracted (15) and analyzed by thin-layer chromatography on polyethyleneimine-cellulose with 1 M LiCl as developing solvent.

Conditions for Photolysis. Photolysis was carried out in an apparatus consisting of an array of calibrated neutral density filters and a Corning 3-71 color filter. Samples of ROS membranes containing various amounts of photolyzed rhodopsin were obtained by exposing 3 μM rhodopsin to attenuated light. A 5-sec exposure with a neutral density filter (OD = 1) in the 0.8-cm path gave 8% photolysis of rhodopsin as measured by the change in A_{498} of detergent-solubilized ROS membranes. Lower degrees of photolysis were obtained by using shorter exposure times and by introducing an additional neutral density filter (OD = 1 or 2).

RESULTS

Release of Bound GDP by Light and GTP. ROS membranes incubated with [α-^{32}P]- or [^{3}H]GTP or [^{3}H]GDP incorporate radioactive nucleotide. Incorporation reached a limiting level after a few minutes of incubation. Thin-layer chromatography showed that the incorporated nucleotide is GDP. Brief sonication did not release the radioactive GDP, which indicates that the nucleotide was bound to a membrane component rather than sequestered within the internal aqueous space of the ROS membrane vesicles. The binding sites for GDP are evidently not on rhodopsin because reconstituted membranes containing only purified rhodopsin and egg yolk phosphatidylcholine did not bind GDP. However, the binding of GDP was restored by the addition of a purified ROS peripheral membrane protein to these reconstituted membranes (unpublished results). The extent of binding depended on the degree of photolysis and the concentration of unbound nucleotide, as described below. The incorporation was carried out in the dark in one series of experiments to enable us to determine the subsequent effect of very low light levels on the release of the labeled GDP. Incubation of ROS membranes (100 μM in rhodopsin) with 0.5 μM [α-^{32}P]GTP at 4°C in the dark for 90 min resulted in the incorporation of about 1.3 mmol of [α-^{32}P]GDP per mol of rhodopsin. Only a small amount of nucleotide was bound in the dark because incorporation depends on the catalytic action of photolyzed rhodopsin. Five- to 10-fold more nucleotide was bound when the membranes were photolyzed (see below). About 80% of the radioactive GDP bound after incubation in the dark was released with a half-time of less than 1 min after photolysis of 60% of the rhodopsin, whereas nearly all of the GDP remained bound in the dark (Fig. 1). The photoactivated release of bound nucleotide is specific for GDP. The small amount of bound GMP, ATP, or ADP was not released by light. Gel filtration chromatography on Sephadex G-15 showed that the released GDP is free in solution rather than bound to a soluble protein in the filtrate.

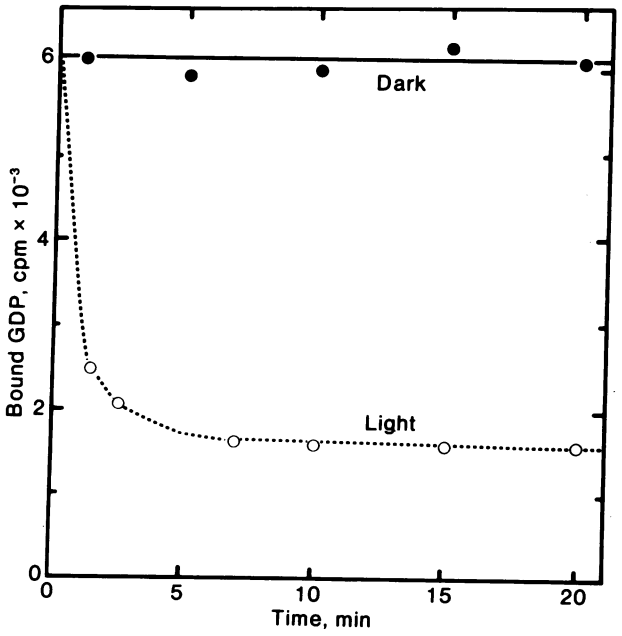

FIG. 1. Kinetics of light-induced release of [α-^{32}P]GDP from ROS membranes at 22°C. About 60% of the rhodopsin was photolyzed at zero time. Almost no GDP was released in the dark. The concentration of bound [α-^{32}P]GDP in the membrane was 1.25 mmol per mol of rhodopsin before photolysis and that of unbound GDP was 5 nM.

The extent and rate of release of GDP were markedly decreased by using a highly attenuated flash that photolyzed only 0.06% of the rhodopsin and by lowering the temperature to 4°C. Under these conditions, more than 90% of the radioactive GDP was associated with the membranes after 15 min (Fig. 2). However, if GTP was present, most of the bound GDP was released within a few minutes. The rate of release was about 2-fold faster in 1.5 μM GTP ($t_{1/2} \approx 1$ min) than in 0.5 μM GTP ($t_{1/2} \approx 2$ min). In the absence of photolyzed rhodopsin, these levels of GTP did not induce appreciable release of bound GDP within 15 min (Fig. 2). Thus, *GTP markedly enhances the effect of light in releasing bound GDP.*

The effectiveness of various nucleotides in enhancing the photorelease of bound radioactive GDP was investigated. GTP, p[NH]ppG, and GDP were most effective, whereas GMP, ATP,

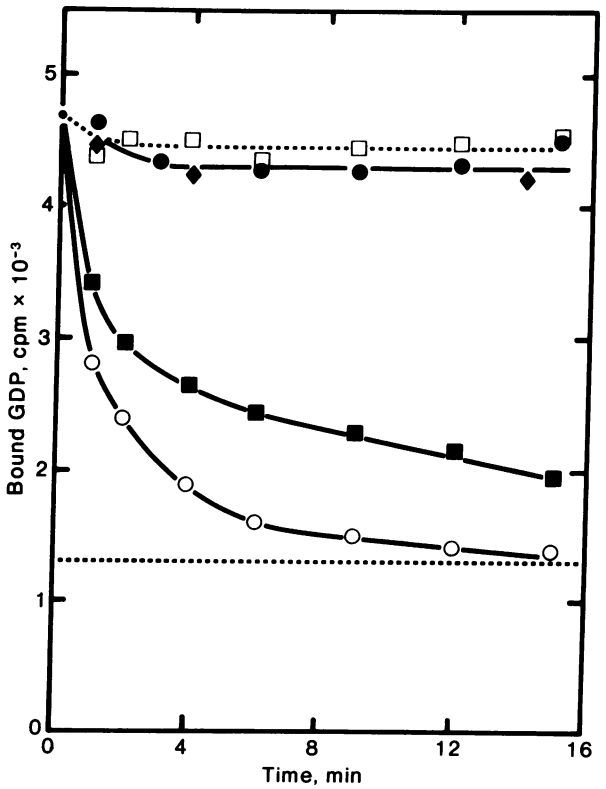

FIG. 2. GTP-enhanced photorelease of bound GDP from ROS membranes at 4°C. The amount of GDP retained by membranes containing 0.06% photolyzed rhodopsin in the absence (□) or presence of 0.5 μM (■) or 1.5 μM (○) GTP was measured as a function of time. The controls were ROS membranes incubated in the dark with 0.5 μM (●) or 1.5 μM (♦) GTP. The point at zero time corresponds to 1.04 mmol of [α-^{32}P]GDP bound per mol of rhodopsin. The residual level of bound GDP 15 min after 100% photolysis in the presence of GTP is denoted by the lower dashed line.

ADP, AMP, CTP, and UTP had almost no effect. The rate of release depended on the concentration of GTP, p[NH]ppG, or GDP, which raises the possibility that the binding protein contains more than one nucleotide binding site.

The dependence of the release of bound GDP on the concentration of photolyzed rhodopsin was measured. In the absence of GTP, release of bound GDP was half-maximal when 1 in 70 rhodopsins was photolyzed (Fig. 3). The light-dependence of the release of GDP in the presence of 0.1 or 1 μM GTP was then determined. The effect of GTP is to markedly enhance the release of GDP at very low light levels. Release was half-maximal when 1 in 4300 rhodopsins was photolyzed in 0.1 μM GTP (Fig. 4). In 1 μM GTP, even less photolyzed rhodopsin was required to release bound GDP; release was half-maximal when only 1 in 15,000 rhodopsins was photolyzed.

Photorelease of Bound GDP Occurs by Exchange with GTP. The mechanism by which GTP enhances the photorelease of bound GDP was studied by comparing the kinetics of binding of p[NH]ppG with the kinetics of release of GDP. ROS membranes containing a nearly maximal amount of bound radioactive GDP were prepared by incubating membranes (15 μM in rhodopsin) containing 0.1% photolyzed rhodopsin with 2 μM [α-^{32}P]GTP. Under these conditions, 12 mmol of radioactive GDP was bound per mol of rhodopsin, 10-fold more than was bound by dark membranes. p[NH]ppG, the nonhydrolyzable analog of GTP, was used subsequently instead of GTP in this experiment to simplify the analysis. The percentage of GDP released paralleled the percentage of p[NH]ppG that became bound to the membranes (Fig. 4). This finding indicates that *p[NH]ppG exchanged for bound GDP in the presence of photolyzed rhodopsin.* A detailed study of the effect of light on the exchange of guanyl nucleotides will be published elsewhere. It is important to note here that our dark ROS membranes contain endogenous bound GDP, which exchanges very slowly unless the membranes are photolyzed. Hence, dark ROS membranes take up much less radioactive guanyl nucleotide than do membranes containing a small amount of photolyzed rhodopsin.

Exchange of GTP for GDP Is Highly Amplified. The binding of p[NH]ppG to ROS membranes containing different amounts of photolyzed rhodopsin was studied to ascertain the roles of GTP and photolyzed rhodopsin in the exchange process. The amount of bound nucleotide depended on the concentration of p[NH]ppG in the incubation mixture and on the degree of photolysis (Fig. 5). A 30-min incubation with 10 μM

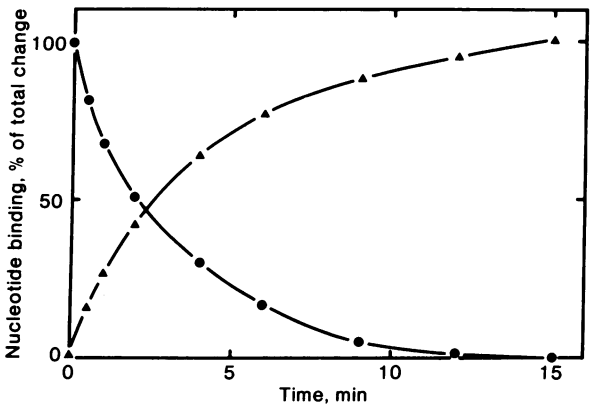

FIG. 4. Comparison of binding kinetics of [^{3}H]p[NH]ppG (▲) and release of bound [α-^{32}P]GDP (●) from ROS membranes containing 0.1% photolyzed rhodopsin at 22°C. The ordinate gives the percentage of p[NH]ppG incorporated relative to the amount bound at the end of a 15-min incubation or the percentage of GDP retained above the background value at 15 min. These membranes initially contained 11.8 mmol of [α-^{32}P]GDP per mol of rhodopsin. After 15 min, the membranes contained 16.9 mmol of p[NH]ppG and 3.4 mmol of GDP per mol of rhodopsin.

p[NH]ppG led to a maximal incorporation of 1 ± 0.2 p[NH]ppG molecule per 32 rhodopsin molecules. Binding above the dark background level was half-maximal when the degree of photolysis was 0.14%, 0.014%, and 0.0011% for 0.1, 1, and 10 μM p[NH]ppG, respectively. Incubation with 10 μM p[NH]ppG led to binding above the dark background level of 5.5 mmol of p[NH]ppG per mol of rhodopsin when the concentration of photolyzed rhodopsin was 0.011 mmol per mol of rhodopsin. *Hence, one photolyzed rhodopsin molecule catalyzed the binding of 500 p[NH]ppG molecules. This high degree of amplification was achieved without hydrolysis of the GTP analog.* A 1-μm-diameter disc contains 35,000 rhodopsin molecules, and so about 1100 p[NH]ppG molecules can become bound (or 1 p[NH]ppG molecule per 32 rhodopsin molecules). The observed binding of 500 p[NH]ppG molecules indicates that one photolyzed rhodopsin can catalyze the exchange of about half of the nucleotide molecules bound to that disc.

Dark membranes incorporated 2.7, 6.8, and 17.2 mmol of p[NH]ppG per mol of rhodopsin after a 30-min incubation with 0.1, 1.0, and 10 μM p[NH]ppG, respectively. This degree of incorporation could come from the presence of a very small level of photolyzed rhodopsin in our membrane preparations.

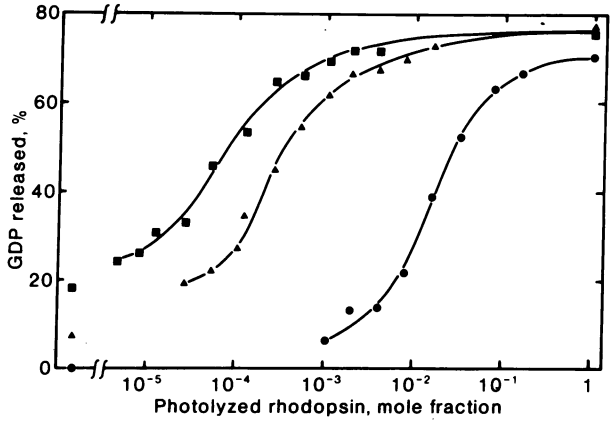

FIG. 3. Dependence of release of bound GDP on mole fraction of photolyzed rhodopsin in 0 (●), 0.1 (▲), and 1 (■) μM GTP at 4°C. The extent of GDP released in the dark is denoted by the individual symbols near the origin of the x-axis. ROS membranes initially contained 1.04 mmol of bound [α-^{32}P]GDP per mol of rhodopsin.

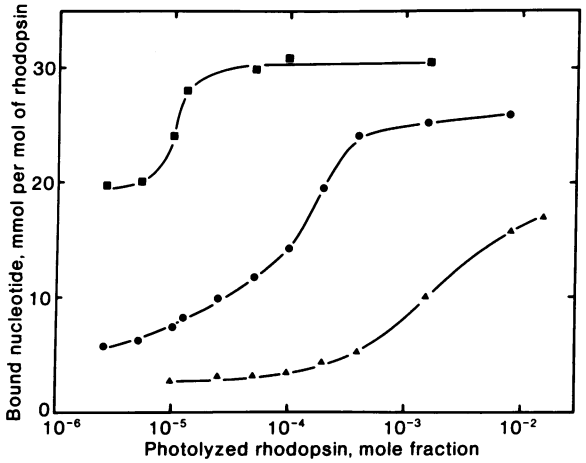

FIG. 5. Dependence of binding of p[NH]ppG to ROS membranes on mole fraction of photolyzed rhodopsin at 22°C. ▲, 0.1 μM p[NH]ppG; ●, 1 μM p[NH]ppG; ■, 10 μM p[NH]ppG.

The 10 μM p[NH]ppG binding curve in Fig. 5 suggests that there may be two populations of vesicles, one-third with no photolyzed rhodopsins and two-thirds with at least one photolyzed rhodopsin per disc. For a Poisson distribution, this ratio shows that the discs contained an average of 1.11 photolyzed rhodopsins. If a disc has 35,000 rhodopsins, this corresponds to a degree of photolysis of 3.2×10^{-5}. The level of opsin in our membrane preparations is much higher than this value, yet these opsins did not catalyze nucleotide exchange. Intact ROS must be able to switch off the capacity of photolyzed rhodopsin to catalyze this reaction, as is known for other light-activated events (9, 16).

DISCUSSION

How does a single photolyzed rhodopsin lead to the exchange of several hundred GTP for bound GDP? The observed amplification is large and so photolyzed rhodopsin (R*) must act catalytically to achieve this exchange. Our experimental observations can be interpreted in terms of a light-activated amplification cycle (Fig. 6).

(*i*) E·GDP, the major species in the dark, binds R* to form R*·E·GDP. We postulate that R* catalyzes GTP–GDP exchange by forming a complex with E·GDP because this is the simplest mechanism consistent with our experimental findings. Amplified GTP–GDP exchange occurred in the presence of gramicidin A or alamethicin, which shows that the transmembrane potential does not mediate this process. E·GDP and R* probably form a R*·E·GDP complex by encountering each other through diffusion in the plane of the disc membrane, which is known to be highly fluid (17).

(*ii*) GTP exchanges for bound GDP. This exchange could occur in two different ways. One possibility is that GDP dissociates from R*·E·GDP to give R*·E, which then binds GTP. Alternatively, GTP may bind to a second nucleotide binding site, which would release GDP from the first site.

(*iii*) R*·E·GTP dissociates to E·GTP and R*, which then binds another E·GDP. This recycling of R* is revealed by the finding that one photolyzed rhodopsin catalyzes the exchange of hundreds of bound nucleotides. It is evident that recycling does not require hydrolysis because a high degree of amplification was observed with p[NH]ppG.

(*iv*) Finally, E·GTP is hydrolyzed to E·GDP. This step accounts for the finding that incubation of ROS membranes with [α-^{32}P]GTP leads to the incorporation of [α-^{32}P]GDP.

In the dark, the binding protein is almost entirely in the E·GDP state. The formation of E·GTP

$$\text{E·GDP} + \text{GTP} \rightarrow \text{E·GTP} + \text{GDP} \qquad [1]$$

is thermodynamically feasible because the equilibrium constant of this reaction is of the order of unity (to be published) and the concentrations of GDP and GTP in ROS in the dark are both about 2 mM (18). However, reaction 1 does not proceed to an appreciable extent in the dark because of a large activation barrier. The role of photolyzed rhodopsin is to decrease this barrier, and so the level of E·GTP is determined primarily by the concentration of R*. The binding protein is restored to the dark state by the hydrolysis of E·GTP, which is also a thermodynamically favorable reaction:

$$\text{E·GTP} + \text{H}_2\text{O} \rightarrow \text{E·GDP} + \text{P}_\text{i}. \qquad [2]$$

Is E·GTP formed rapidly enough for it to be an intermediate in visual excitation, which occurs in times of milliseconds to a few seconds (19, 20)? In the presence of 0.5 μM GTP, the half-time for formation of E·GTP is several minutes. However, the rate is much faster at higher levels of GTP. At 2 mM GTP, the physiological level (18), E·GTP is formed in less than 4 sec (unpublished results), and so it may be a kinetically competent intermediate.

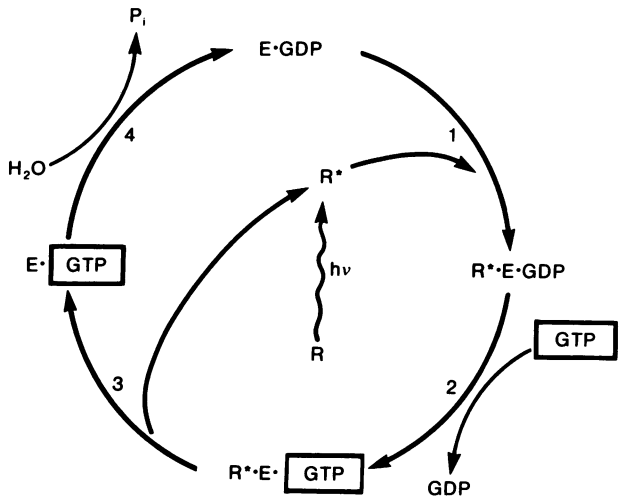

FIG. 6. Proposed light-activated amplification cycle.

Our experimental findings can be related to the work of other laboratories suggesting that cyclic GMP may be a transmitter in visual excitation in rod cells (6, 7, 10, 11). One molecule of photolyzed rhodopsin leads to the hydrolysis of 5×10^5 molecules of cyclic GMP per second (7). This amplification is probably achieved in two stages (9): the first is the activation of several hundred phosphodiesterases and the second is the hydrolysis of nearly a thousand cyclic GMPs per second by each activated phosphodiesterase molecule. It is known that GTP (or a nonhydrolyzable analog) is needed in addition to light for the activation of the phosphodiesterase and that light activates a GTPase in ROS membranes (1). We think it likely that *E·GTP is the activator of the phosphodiesterase. The formation of several hundred E·GTP per photolyzed rhodopsin may be the first stage of amplification in visual excitation.*

Many similarities between the photoactivated cyclic GMP phosphodiesterase in ROS and the catecholamine-activated adenylate cyclase in the plasma membrane of erythrocytes and other cells have been noted (12). GTP and hormone are needed for activation of adenylate cyclase (16, 21), just as GTP and photolyzed rhodopsin are required for activation of the phosphodiesterase. Both enzymes exhibit persistent activation in the presence of a nonhydrolyzable analog of GTP. Moreover, a GTPase is likely to couple the receptor with adenylate cyclase (22) or cyclic GMP phosphodiesterase (12) in the activation pathway. Our experiments reveal an even deeper homology between these signal-transducing systems. The exchange of bound GDP for GTP that is catalyzed by photolyzed rhodopsin is strikingly similar to the catecholamine-induced displacement of bound GDP by GTP (15). We have observed a gain of 500 in the formation of E·GTP catalyzed by photolyzed rhodopsin. It will be interesting to see whether a comparable degree of amplification occurs in the β-adrenergic receptor system and whether the receptor-catalyzed formation of E·GTP is the first stage of amplification in other sensory systems.

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