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Light Inhibition of Bovine Retinal Rod Guanylyl Cyclase Mediated by $\beta\gamma$-Transducin†

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ABSTRACT: Photoreceptor guanylyl cyclase (ROS-GC), converting GTP into cGMP and pyrophosphate, is a key enzyme in the regulation of the visual transduction cascade. ROS-GC requires GC-activating proteins (GCAPs) and low free [Ca] for full activity. We found that when choline or potassium were the major cations present, light caused a 70% inhibition of stimulated ROS-GC in native unstripped membranes. In the presence of sodium ions, however, no inhibition was observed. ROS-GC activity of ROS membranes, stripped of transducin and other components, was not affected by light when reconstituted with GCAP1 only. However, when stripped ROS membranes were reconstituted with both GCAP1 and either transducin ($T_{\alpha\beta\gamma}$) or the $T_{\beta\gamma}$-subunits, the inhibition of ROS-GC by light was restored. The T_{α}-subunit alone was ineffective. These results suggest that under saturating light conditions, ROS-GC may be regulated by $T_{\beta\gamma}$ and cations, providing a possible mechanism of desensitization and light adaptation.

The molecular components of the phototransduction cascade in vertebrate retinal rod photoreceptors are now well established. Absorption of a photon by the photopigment rhodopsin leads to the activation of the heterotrimeric G-protein transducin, resulting in the activation of phosphodiesterase and hydrolysis of the second messenger cGMP [for reviews, see (*1*, *2*)]. cGMP is synthesized in the outer segments of retinal rod photoreceptors by a particulate guanylyl cyclase (ROS-GC).[1] ROS-GC is activated by guanylyl cyclase-activating proteins (GCAPs) at low free [Ca] and is inhibited when [Ca] is increased in the dark (*3*). Calcium regulation of ROS-GC is thought to play a major role in the process of light adaptation, and in recovery from photobleaching. The calcium sensitivity of the different components of the visual transduction cascade has been reviewed recently (*4*). Two retina-specific GCs (GC1 and GC2) have been cloned (*5*−*7*), and three GCAPs that confer calcium sensitivity to mammalian GCs have been identified

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[1] Abbreviations: ROS, rod outer segment(s); GCAP, guanylyl cyclase-activating protein; GC, guanylyl cyclase; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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(GCAP1, GCAP2, and GCAP3) (*3, 8*−*11*). When expressed in cell lines, GC1 is activated by GCAP1 or GCAP2, while GC2 is largely insensitive to GCAP1 (*12*). The specific localization of these GCs to different cell types in the retina is not completely clear. Bovine ROS-GC has been suggested to be an autophosphorylating protein kinase (*13*). GCAP-dependent ROS-GC activity in saponin-permeabilized intact ROS was found to be modulated by endogenous PKC and PKA activities which affected maximal velocity and calcium sensitivity, respectively (*14, 15*).

Potential regulation of ROS-GC by light has received little attention, in part for technical reasons. Most ROS-GC assays measure the formation of cGMP, and the overwhelming activity of the light-activated phosphodiesterase in ROS preparations precluded accurate measurements of ROS-GC in light. Since GC catalyzes the conversion of GTP into cGMP and pyrophosphate, we developed a colorimetric GC assay based on measurement of pyrophosphate rather than cGMP (*14*). The sensitivity and accuracy of this assay, which monitors the formation of pyrophosphate in real-time, were shown to be comparable to other assay systems. Using this real-time assay, we have now addressed the question of potential regulation of ROS-GC by light. Here, we report that light inhibits ROS-GC in native ROS membranes in a sodium-dependent fashion. We also show, using a reconstituted system of stripped ROS membranes and purified GCAP1, that addition of holo-transducin confers a light-dependent and sodium-sensitive inhibition of ROS-GC. Light-independent inhibition of ROS-GC was finally achieved with purified T$\beta\gamma$ subunits while the Tα subunit was ineffective.

METHODS

Preparation of Components. Ca^{2+}-depleted bovine retinal rod outer segments with an intact plasma membrane were isolated on mixed sucrose−Ficoll gradients as described (*16*). All procedures with rod outer segments or rod outer segment membranes were carried out in darkness or dim red light unless otherwise indicated. Intact Ca^{2+}-depleted ROS were further purified by additional sucrose gradient centrifugation (20−40% w/w sucrose in Hepes), washed with 600 mM sucrose, 20 mM Hepes (pH 7.4), and stored in this medium at a final rhodopsin concentration of 200 μM. All media throughout purification and washing contained 100 μM EDTA. Stripped ROS membranes were prepared by osmotic lysis of ROS. After freeze−thawing the suspension of intact ROS, ROS membranes were washed 3 times by dilution with 40 volumes of ice-cold 10 mM Hepes and 1 mM EGTA (pH 7.4), followed after 2 min by addition of 25 mM tetramethylammonium chloride and centrifugation at 12000*g*. Residual transducin was removed from the membranes by the second wash step carried out in the presence of 100 μM GTP. EGTA was omitted from the final wash step. Transducin was purified as described by Kühn (*17*). In brief, ROS membranes were bleached, and soluble/peripheral proteins, except for transducin, were removed by hypotonic lysis in 20 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 1 mM dithiothreitol, followed by sedimentation of the membranes. Transducin binds tightly to bleached rhodopsin in the absence of GTP and can be eluted subsequently from the membranes by addition of 100 μM GTP. Scans of gels stained with Coomassie blue suggest that the transducin preparation used was >95% pure. T_α and $T_{\beta\gamma}$ were purified as described by Bigay and Chabre (*18*). Scans of gels stained with Coomassie blue showed that the only significant contamination in T_α was about 10% $T_{\beta\gamma}$, while the only significant contamination in $T_{\beta\gamma}$ was about 10%T_α. GCAP1 and GCAP1-His6 were purified as described by Rudnicka-Nawrot al. (*19*).

Guanylyl Cyclase (GC) Assay. GC catalyzes the conversion of GTP into equal amounts of cGMP and pyrophosphate. Production of pyrophosphate was measured with a colorimetric pyrophosphate kit (Sigma, St. Louis, MO) as described previously (*14, 15*). With this assay, we measured GC activity either in intact ROS immediately following permeabilization of the plasma membrane by saponin (0.025%) or in stripped ROS membranes reconstituted with GCAP1 or GCAP1 plus transducin without the use of saponin. ROS-GC in saponin-permeabilized intact ROS was measured under conditions as described before with two modifications (as indicated between parentheses). In brief, ROS-GC activity was measured in a cuvette containing 2 mL of 50 mM KCl, 75 mM of an isotonic mixture of NaCl and choline chloride (as indicated, and replacing 75 mM NaCl), 1 mM potassium citrate, 1.5 mM $MnCl_2$ (replacing 30 mM NaF to inhibit pyrophosphatase activity endogenous to ROS), 100 μM Pefabloc SC, 400 μM BAPTA, and components of the pyrophosphate detection kit (Sigma, St. Louis) [in final concentration: 15 mM imidazole hydrochloride (pH 7.4), 0.03 mM EDTA, 0.066 mM $MnCl_2$, 0.006 mM $CoCl_2$, 2 mM $MgCl_2$, 0.26 mM β-NADH, 4 mM fructose 6-phosphate, 1 mg of BSA, 1 mg of sugar stabilizer, 0.33 unit of PP-dependent fructose-6-phosphate kinase, 4.95 units of aldolase (EC 4.1.2.13), 3.33 units of glycerophosphate dehydrogenase (EC 1.1.1.8), and 33.3 units of triosephosphate isomerase (EC 5.3.1.1)]. Stripped ROS membranes were reconstituted for 10 min at 25 °C in a volume of 30 μL containing 100 μM rhodopsin, 3 μM GCAP1, and either 8 μM transducin ($T_{\alpha\beta\gamma}$), 1 μM $T_{\beta\gamma}$, or 1.7 μM T_α; in all cases, transducin or transducin subunits were added first followed by the addition of GCAP1. After 1 min, the suspension was diluted to a final volume of 2 mL, and ROS-GC was assayed with the pyrophosphate kit (as detailed above) in the presence of 4 mM $MgCl_2$, and NaCl as indicated. Throughout all experiments, 400 μM BAPTA was added to the cuvette to buffer free calcium concentration to below 10 nM, while ROS-GC was initiated by the addition of 500 μM GTP. Addition of GTP caused a small, fast, and transient signal due to pyrophosphate contamination of GTP; after this rapid signal subsided, ROS-GC rates were linear for at least 20 min and the slope was taken as a measure of ROS-GC activity. ROS-GC activity was measured in samples kept in darkness or in samples bleached for 5 s with white light until photochemical equilibrium was established (bleaching 80% of rhodopsin present). ROS-GC assays were initially carried out with an SLM DW2C dual-wavelength spectrophotometer in a temperature-controlled cuvette house at 25 °C. Experiments were later carried out employing a Cary50 with alternate measurements at 340 and 410 nm, respectively, every 300 ms.

RESULTS

Prevention of ROS-GC Inhibition by Sodium Ions. This study was aimed at examining the possible effect of light on ROS-GC activity measured in bovine ROS. This study was made possible by the availability of a simple real-time

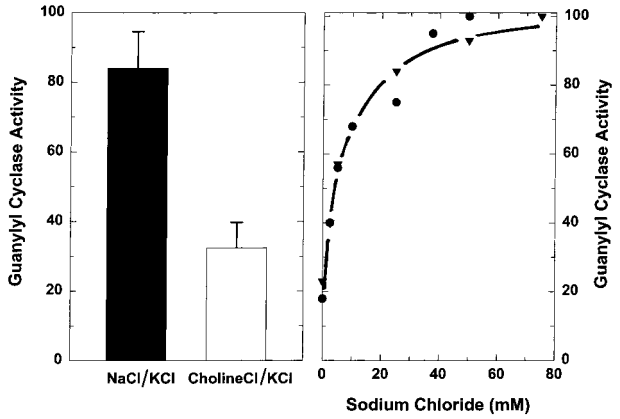

FIGURE 1: Effect of light and sodium on ROS-GC activity in intact ROS. ROS-GC activity was measured as described under Methods in intact calcium-depleted ROS, immediately following membrane permeabilization with 0.025% saponin. ROS-GC activity was measured in fully bleached ROS, and ROS-GC activity was normalized with respect to the activity in unbleached control samples. Left panel: ROS-GC activity in bleached ROS in KCl/choline chloride (50 mM/50 mM) medium was compared to that in KCl/NaCl (50 mM/50 mM) medium. Average values and standard deviations from seven experiments with different ROS preparations are presented. Right panel: ROS-GC activity in bleached ROS was measured as a function of sodium chloride concentration (isotonic replacement of choline chloride by sodium chloride) in the presence of a constant concentration of 50 mM KCl. ROS-GC activity in bleached membranes was normalized with respect to ROS-GC activity in unbleached membranes (symbols represent results from experiments with different ROS preparations).

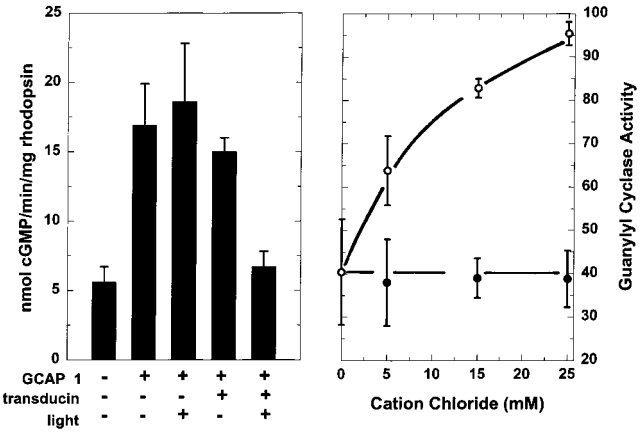

FIGURE 2: Effect of light and sodium on ROS-GC activity in stripped ROS membranes reconstituted with GCAP1 and transducin. Reconstitution protocol and subsequent measurement of ROS-GC activity were as described under Methods. Left panel: ROS-GC activity in stripped ROS membranes was measured after reconstitution with GCAP1 or GCAP1 plus transducin as indicated. Average values with standard deviations are presented for five to seven experiments (except for the last column that represents the average and range from two experiments). Right panel: stripped ROS membranes were bleached and reconstituted with both GCAP1 and transducin. ROS-GC activity was measured as a function of the concentration of sodium chloride (open circles) or potassium chloride (filled circles). The data illustrated represent average values ± standard deviations of four to eight experiments. ROS-GC activity in bleached reconstituted membranes was normalized to that observed in unbleached membrane reconstituted with GCAP1.

colorimetric assay to measure pyrophosphate which avoids the complications associated with measuring the formation of cGMP in the presence of light-activated phosphodiesterase. GCAP-dependent ROS-GC activity was measured in the presence of the Ca^{2+} chelator BAPTA (free [Ca] <10 nM). In a first set of experiments, we measured the effect of bleaching of rhodopsin on ROS-GC activity in ROS with a full complement of proteins (unstripped membranes). Bovine ROS with an intact plasma membrane were purified on sucrose−Ficoll gradients, and access to the cytosol was then obtained by permeabilization of the ROS plasma membrane with 0.025% saponin immediately before the ROS-GC assay. Under these conditions, ROS-GC was significantly inhibited by bleaching of the visual pigment rhodopsin when choline and potassium were the major monovalent cations present, but not when sodium was the major monovalent cation present (Figure 1). ROS-GC activity observed when ROS were bleached in potassium/choline medium, on average, was reduced to 32% compared with ROS-GC activity in the unbleached control (Figure 1, left panel). In contrast, ROS-GC activity observed when ROS were bleached in potassium/sodium medium, on average, was reduced to only 84% when compared with that in the unbleached control. Cations present in the medium (potassium, sodium, or choline) had no effect on the pyrophosphatase assay or the ROS-GC activity in unbleached ROS membranes (*15*). These observations suggest that bleaching of rhodopsin results in inhibition of ROS-GC activity in the absence of sodium ions, and that the inhibition can be prevented by the presence of sodium ions. This interpretation is supported by the effect of sodium/choline mixtures on ROS-GC activity in bleached ROS as illustrated in Figure 1 (right panel). A significant prevention of the inhibition of ROS-GC activity by bleaching was already observed in the presence of 50 mM KCl, 5 mM NaCl, and 70 mM choline chloride, while a half-maximal effect was observed at about 50 mM KCl, 10 mM NaCl, and 65 mM choline chloride.

Transducin Mediates Bleaching of Rhodopsin to ROS-GC. Hydroxylamine treatment of bleached ROS membranes causes a rapid decay of the active species metarhodopsin II and prevents binding of transducin to bleached rhodopsin (*20*). We therefore treated bleached ROS prior to the ROS-GC assay with hydroxylamine and observed that ROS-GC activity was restored to the level observed for the unbleached control (results not illustrated). This suggested that transducin could perhaps mediate the effect of bleaching rhodopsin on ROS-GC activity. To address this question, we have used washed ROS membranes stripped from peripheral membrane-associated proteins such as GCAP and transducin. Stripped ROS membranes were subsequently reconstituted with purified GCAP1 or GCAP1 plus transducin, and ROS-GC activity was assessed. In the pyrophosphate assay system, the GCAP1 dependence of ROS-GC activity was very similar to that described before (*8*). Addition of GCAP1 alone (Figure 2, left panel) resulted in a more than 3-fold increase in GC activity; and neither basal ROS-GC activity (no GCAP1 or transducin present) nor GCAP1-dependent ROS-GC activity (no transducin present) was affected by bleaching. Addition of both GCAP1 and transducin to unbleached membranes increased ROS-GC activity to the same level as observed with GCAP1 alone. Addition of both GCAP1 and transducin to bleached membranes, however, prevented the increase in ROS-GC activity observed when GCAP1 was added alone. These results suggest that addition of transducin imparts light sensitivity to GCAP1-dependent ROS-GC activity.

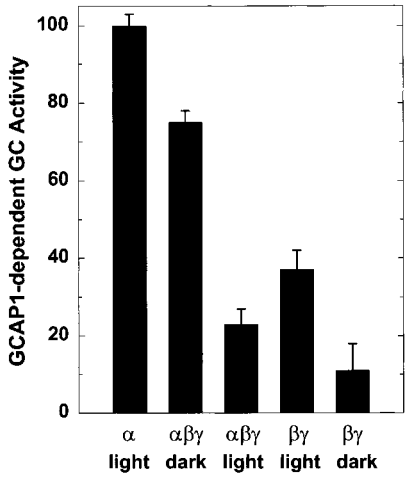

FIGURE 3: $T_{\beta\gamma}$ inhibits GCAP1-dependent ROS-GC activity. The effect of purified T_α and $T_{\beta\gamma}$ on GCAP1-dependent ROS-GC activity was examined in a reconstituted system as described in the legend of Figure 2. The data tabulated represent average values ± standard deviations of three to seven experiments. The ROS-GC activities are normalized with respect to the ROS-GC activity observed with ROS membranes reconstituted with GCAP1 alone.

Inhibition of ROS-GC activity was prevented by sodium ions in ROS with a full complement of soluble/peripheral proteins (Figure 1). Hence, we examined the ability of sodium ions to prevent the inhibition of ROS-GC activity by bleaching in the preparation of stripped membranes reconstituted with both transducin and GCAP1. Quite similar to the observations in saponin-permeabilized intact ROS with a full complement of ROS proteins present (Figure 1), sodium ions in the range between 5 and 25 mM progressively reduced the inhibition of ROS-GC activity by bleaching in stripped membranes reconstituted with both GCAP1 and transducin, whereas potassium ions had no effect (Figure 2, right panel). We conclude that upon bleaching, transducin, or one of its subunits, prevents or reverses GCAP stimulation of GC depending on the cations present. Sodium ions prevent inhibition while other cations such as choline or potassium favor inhibition.

Transducin βγ-Subunits Modulate ROS-GC in the Absence of Light. Transducin consists of three subunits, $\alpha\beta\gamma$, which dissociate in part upon photobleaching of rhodopsin. We therefore next addressed the question whether the effects of transducin were mediated by T_α or by $T_{\beta\gamma}$ (Figure 3). In the absence of GCAP1, neither transducin nor $T_{\beta\gamma}$ affected ROS-GC activity. In the presence of GCAP1, addition of T_α (1.7 μM) to bleached membranes did not lead to inhibition of GCAP1-dependent ROS-GC activity. In contrast, addition of $T_{\beta\gamma}$ (1 μM) caused inhibition in both bleached and unbleached membranes. In a control experiment, recombination of the purified subunits of transducin led to the phenotype observed for heterotrimeric transducin as shown in Figure 2, verifying the biological activity and integrity of the individual transducin subunits. As light causes the dissociation of T_α from $T_{\beta\gamma}$, one would predict that with $T_{\beta\gamma}$ alone, light is no longer required to cause inhibition of GCAP1-dependent ROS-GC activity, and this was indeed observed (Figure 3).

DISCUSSION

In this study we have addressed the question of potential regulation by light of ROS-GC in bovine ROS. Bleaching of rhodopsin resulted in a 60−70% inhibition of ROS-GC which could be prevented in a rather cation-selective way by sodium ions. We were able to address the light dependence of ROS-GC by using a GC assay that measures pyrophosphate rather than cGMP, circumventing interference by the potent light-activated phosphodiesterase. No inhibition of ROS-GC by light was observed when ROS membranes stripped of peripheral and soluble proteins were reconstituted with GCAP1 alone to stimulate full ROS-GC activity (*8*). The minimum requirement for the reconstitution of light inhibition of ROS-GC was addition of GCAP1 plus transducin. Since light causes the dissociation of $T_{\beta\gamma}$ from T_α, we investigated the potential roles of both on ROS-GC. We found that only $T_{\beta\gamma}$ appears to interact with the GCAP1−ROS-GC complex as inferred from its ability to inhibit ROS-GC activity; addition of $T_{\beta\gamma}$ but not T_α to the GCAP1−ROS-GC complex resulted in inhibition of ROS-GC independent of light (Figure 3). The effect of $T_{\beta\gamma}$ in darkness was more pronounced compared with the effect of $T_{\beta\gamma}$ on ROS-GC activity in bleached membranes (compare the last two bars in Figure 3), and this may be due to the fact that bleached rhodopsin has been shown to interact with $T_{\beta\gamma}$ (*21*) and lower somewhat the availability of $T_{\beta\gamma}$ to interact with ROS-GC. T_α could prevent inhibition of ROS-GC by $T_{\beta\gamma}$ in darkness, suggesting that T_α could prevent the interaction between $T_{\beta\gamma}$ and its effector (i.e., the GCAP1−ROS-GC complex) as has been observed for other heterotrimeric G-proteins (*22*). Chen et al. (*23*) have identified a potential consensus motif (**Q**XX**ER**) in proteins that interact with the $\beta\gamma$-subunits of G-proteins. The retina-specific GC's contain either a **Q**XX-**EK** or a **Q**XX**DR** motif in a highly conserved portion of the C-terminal cytoplasmic domain, suggesting a possible site of interaction with $T_{\beta\gamma}$. A possible model for the action of transducin on ROS-GC is illustrated in Figure 4. The results of this study suggest that ROS-GC is the second target to be acted on by transducin in addition to the well-established activation of cGMP phosphodiesterase, and the first target to be directly regulated by $T_{\beta\gamma}$.

Inhibition of ROS-GC by light required relatively high bleaching levels (1% or greater, data not illustrated). It is unlikely that light regulation of ROS-GC is important under the low light levels at which rod photoreceptors function. Rather, we believe the effects observed here may involve down-regulation of ROS-GC under light levels that saturate rods (and cones) during bright daylight illumination. Perhaps the most intriguing aspect of the light inhibition of ROS-GC is that its prevention, by increasing sodium concentration in the suspension medium, was half-maximal at about 10 mM. Little is known with certainty about the cytoplasmic sodium concentration in the outer segments of intact and functioning rod photoreceptors. The total sodium content of frog ROS in the intact retina in bright light was found to be about 40% of that in the dark-adapted retina (*24*). The high sodium content in the dark is most likely due to the sustained inward sodium current via the cGMP-gated channels, while the sodium−potassium pump located in the rod inner segment cannot keep up. The sodium content in mammalian ROS in darkness may even be higher than that of frog ROS in view of the increased surface/volume ratio. This suggests the following scenario concerning the potential physiological significance of the sodium-dependent inhibition of ROS-GC by light. In darkness, sodium concentration in ROS is

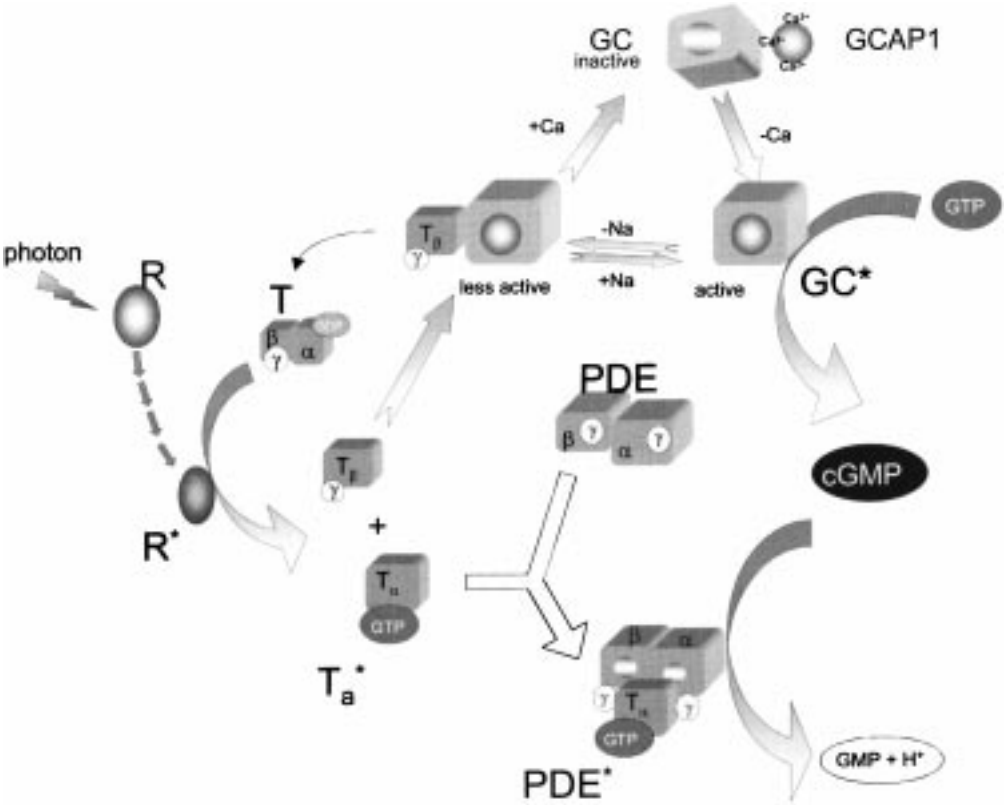

FIGURE 4: Putative model of GC regulation by $T_{\beta\gamma}$ in ROS. Rhodopsin (R) is bleached by light to form R*. R* (metaII) catalyzes the GDP/GTP exchange on T_α and dissociates $T_{\alpha\beta\gamma}$ into T_α-GTP (T_α*) and $T_{\beta\gamma}$. The role of T_α* is to activate PDE, an essential step in the phototransduction cascade. As shown in this paper, a possible role for $T_{\beta\gamma}$ is to interact with GC which is highly active in the presence of GCAP1 and low [Ca]. When [Na] is reduced due to the reduction of the dark current after photobleaching, GC is inhibited. When [Na] and [Ca] increase during the recovery phase, GC inhibition is reversed, and $T_{\beta\gamma}$ is enabled to recombine with T_α to form $T_{\alpha\beta\gamma}$. A similar pathway is envisioned in cones.

relatively high (perhaps as high as 20 mM or higher). Bleaching of rhodopsin results in a rapid drop in cytoplasmic calcium due to the closure of cGMP-gated channels and the continuous extrusion of Ca^{2+} by the light-insensitive Na−Ca+K exchanger. This in turn leads to an increase in ROS-GC activity, accelerating cGMP synthesis and reopening of the channels. The high sodium levels prevailing in darkness would prevent a rapid onset of ROS-GC inhibition by light. Upon sustained bright illumination, rod cells saturate, and, in time, the Na−K pump will lower cytosolic sodium to values below 10 mM, inhibition of ROS-GC by $T_{\beta\gamma}$ would be initiated, and a futile cycle of cGMP synthesis and immediate hydrolysis by fully active phosphodiesterase would be diminished. We previously described the effects of PKC-mediated phosphorylation on the maximal velocity of ROS-GC and suggested that this could contribute to a diminished ROS-GC activity in sustained bright light. Perhaps, multiple regulatory features of ROS-GC in retinal rods contribute to down-regulate ROS-GC and minimize a potentially wasteful and futile cycle of cGMP synthesis and hydrolysis when rods are saturated under sustained bright light.

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