Light-dependent Binding of G-Protein to Outer Segment Membranes of Toad Photoreceptors

NANCY J. MANGINI, DAVID R. PEPPERBERG, and WOLFGANG BAEHR

From the Department of Ophthalmology, Lions of Illinois Eye Research Institute, University of Illinois College of Medicine, Chicago, Illinois 60612, and the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

ABSTRACT Light-dependent changes in the binding of G-protein were analyzed in outer segment disk membranes obtained from photoreceptors of the toad (Bufo marinus) retina. Isolated, intact retinas, incubated in oxygenated Ringer's solution at 23 ± 1°C, were subjected to various conditions of illumination and then incubated in darkness for specified periods. The retinas were then chilled (0-4°C) and the receptor outer segments (ROS) were isolated. Binding of the α- and β-subunits of G-protein to the ROS membranes was analyzed by quantitating $G_\alpha$ and $G_\beta$ extracted from the membranes with hypotonic medium lacking GTP vs. hypotonic medium containing GTP (H and HG extracts, respectively). For retinas illuminated and then immediately chilled for analysis, the extent of G binding (relative abundance of $G_{\alpha,\beta}$ in the HG extract) increased with the extent of bleaching of the visual pigment. Near-maximal binding was observed after bleaches of ≥30%. With an increasing period of incubation in darkness after ~70% bleaching, the extent of binding declined gradually to low levels characteristic of unbleached retinas. The period required for half-completion of the decline was ~10 s. A gradual decline in G binding, from a rapidly developing peak value, was also observed with an increasing period of exposure to intense light. Viewed in the context of previous electrophysiological data, our results indicate that sustained bleaching desensitization of the rods does not depend upon a persisting state of "tight binding" (immobilization) of G-protein by bleached visual pigment.

INTRODUCTION

After exposure to intense light, rod photoreceptors of the isolated vertebrate retina attain a stable state characterized by markedly reduced sensitivity to test flashes. Previous studies have revealed a strong dependence of the sustained desensitization upon the extent of visual pigment bleaching; the persisting net decrease in sensitivity far exceeds the loss expected on the basis of reduced...
efficiency of quantum capture (Grabowski and Pak, 1975; Pepperberg et al., 1978; Bäckström and Hemilä, 1979; Clack et al., 1982; also see Baylor and Lamb, 1982; Cornwall et al., 1983). These and other studies indicate that bleached visual pigment (B) has a direct desensitizing effect on phototransduction in dark-adapting rods, resembling in certain respects the desensitization sustained by a relatively small quantity of photoactivated rhodopsin (R*) (for a review, see Pepperberg, 1984).

Two recent studies provide evidence for the involvement of a GTP-utilizing reaction in the process of bleaching desensitization. (a) In rods of the isolated toad retina previously desensitized by 30% bleaching, superfusion with p(CH₂)ppG [guanosine 5'-[β,γ-methylene]triphosphate, a hydrolysis-resistant analogue of GTP] promotes increases in the amplitude and duration of the intracellularly recorded flash response; these changes oppose the ordinarily permanent changes caused by pigment bleaching (Clack et al., 1982). (b) When applied to the isolated retina of the skate after major (≥87%) bleaching, p(CH₂)ppG induces a lowering of the threshold for the extracellularly recorded PIII response (Clack and Pepperberg, 1984). Extensive bleaching thus appears to cause a GTP-dependent reaction to become limiting for the operation of the phototransduction process.

Abundant data indicate the involvement in phototransduction of guanine nucleotide-binding protein (G-protein), a multisubunit, peripherally bound membrane protein that mediates the photic activation of cGMP phosphodiesterase (PDE) in the rod outer segment (ROS) (for reviews, see Miller, 1981; Stieve, 1986). In ROS membrane preparations containing GTP at a saturating level, the activation (GTP-charging) of G by a weak flash (≪1% bleach) proceeds with a gain of ~10³ (Fung and Stryer, 1980; Vuong et al., 1984; Bennett and Dupont, 1985; Hofmann and Reichert, 1985). However, the removal of GTP blocks the formation of activated G (G*). In GTP-depleted ROS membranes, strong irradiation induces a long-lived condition of relatively tight binding of G to the membranes (Kühn, 1980a, b; Baehr et al., 1982). This condition is thought to reflect the “trapping” of G by R* in a complex (R* · G) that immediately precedes the GTP-charging step. After bleaches (≥10%) that generate a molar level of R* approaching or exceeding that of G, GTP-depleted preparations form little additional R* · G upon flash stimulation (Kühn et al., 1981; Bennett, 1982). The reduced gain of the rhodopsin/G system [i.e., low quantity of d(R* · G) generated by dR*] presumably reflects immobilization of a major portion of the G by previously formed R*, and thus a decrease in the G available for interaction with dR*.

Together, the data just reviewed reveal an interesting parallel between electrophysiological sensitivity and the flash-generated interaction between visual pigment and G-protein. Both bleaching desensitization of the rod and the persisting “tight binding” of G in GTP-depleted ROS membranes depend on the presence of a bleaching product (B or R*). Both phenomena furthermore appear

1 Throughout this article, we use the term “gain” and the prefix “d” to describe the incremental effects of a weak flash. For the case just cited, dR* → dG* gain is ~10³.
to be regulated by a reaction utilizing GTP. Might bleaching desensitization directly reflect the immobilization of a large fraction of G by bleached pigment, and a consequent reduced gain of the \( dR^* \rightarrow dG^* \) reaction?

The question just asked relates closely to another unresolved issue, that of the GTP level prevailing at the site of the R*-G complex within bleached, intact photoreceptors. In both isolated ROS and in outer segment–inner segment (OS-IS) preparations, light induces a decrease in the bulk level of GTP (Bignetti et al., 1978; Biernbaum and Bownds, 1979, 1985; Robinson and Hagins, 1979; Salceda et al., 1982; also see Dawis et al., 1986). For example, in OS-IS preparations, 2% bleaching causes the bulk GTP level to fall to \( \sim 60\% \) of the dark control level; this decrease is transient, and GTP recovery parallels a recovery in electrophysiological sensitivity (Biernbaum and Bownds, 1985). However, since current technology does not afford an in vivo probe of local utilization of GTP by the R*-G complex, it remains unclear whether the GTP level in the bulk cytosol matches the GTP level in the microenvironment of R*-G. In vitro evidence for an influence of GTP level on the lifetime of R* (Hofmann et al., 1983; Hofmann, 1985) and the likely dependence of the R*-G interaction on other cellular components (e.g., 48-kD protein, opsin kinase, ATP, and PDE; cf. Kuhn, 1983) emphasize the possible involvement of multiple factors on the extent and time course of the R*-G interaction in vivo.

The present study was undertaken to elucidate the effects of light on the capacity for interaction between G-protein and visual pigment. Our specific aim was to determine whether conditions yielding sustained bleaching desensitization also lead to a persisting state of high affinity of G for bleached pigment. Our experiments have employed the isolated retina of the toad (Bufo marinus) and have involved (a) light/dark incubation of the intact retina under physiological conditions, and (b) subsequent chilling of the retina, isolation of the ROS, and analysis of the extractability of the \( \alpha \)- and \( \beta \)-subunits of G from the ROS membranes. For retinas incubated less than \( \sim 10^3 \) s after major (\( \sim 70\% \)) bleaching, we find that the ROS membranes exhibit a substantially elevated extent of G binding, by comparison with membranes obtained from unbleached retinas. The lingering capacity for G binding may relate closely to the mechanism regulating sensitivity in bleached rods (see Discussion). However, the condition of “tight binding” is not permanent; thus, G appears not to be immobilized in sustained bleaching desensitization. Some of our results were presented at the 1984 meeting of the Association for Research in Vision and Ophthalmology (Pepperberg et al., 1984).

**METHODS**

**Materials**

Electrophoresis-grade chemicals and protein standards used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories (Richmond, CA). Enzyme-grade sucrose (Schwarz-Mann, Spring Valley, NY) was used to prepare step gradients. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). CX-10 immersible ultrafiltration units (Millipore Corp., Bedford,
MA) or Centricon-10 microconcentrators (Amicon Corp., Danvers, MA) were used to concentrate extracts of washed ROS. Bovine eyes were obtained from Lincoln Meat Packing Co., Chicago, IL.

Toads (Bufo marinus) were obtained from Lemberger-Kons Scientific Co. (German-town, WI) and maintained in an aquarium on a diurnal cycle of 12 h light/12 h dark. Before the experiments, animals were dark-adapted for 3–12 h and killed by double pithing. Dissections and subsequent procedures were carried out under dim red light, at an ambient temperature of 23 ± 1°C. Eyes were enucleated and hemisected, and the eyecup was immersed in oxygenated Ringer’s solution (110 mM NaCl, 2.5 mM KCl, 0.86 mM CaCl₂, 1.6 mM MgCl₂, 5.6 mM glucose, and 3.0 mM HEPES, adjusted with NaOH to pH 7.8 [Brown and Pinto, 1974]). Retinas were isolated as previously described (Perlman et al., 1982).

Adapting Irradiations

Retinas were positioned photoreceptor side up in a 35- × 10-mm plastic dish (two retinas per dish) containing 2 ml of oxygenated Ringer’s solution and were light-adapted using either 504-nm or white light. The 504-nm irradiations employed a photostimulator similar to that previously described (Clack and Pepperberg, 1982; Perlman et al., 1982). Briefly, light from a tungsten-halogen source was spectrally shaped by passage through a 504-nm interference filter (Schott Optical Works, Inc., Duryea, PA), attenuated by Schott neutral density filters, and focused to provide full-field illumination of the retina from above. For irradiations with white light, retinas were exposed to room light (100-W incandescent bulbs housed in diffusing globes). Incident intensities of the unattenuated 504-nm beam and the diffuse white light were measured with a calibrated photodiode (PIN-5DP, United Detector Technology, Inc., Santa Monica, CA) positioned in the plane of the retina. The intensity of the unattenuated 504-nm light was ~6.8 μW·cm⁻², equivalent to ~1.7 × 10⁵ incident quanta·μm⁻²·s⁻¹ (q·μm⁻²·s⁻¹); that of the diffuse white light was ~230 μW·cm⁻². As measured in separate spectrophotometric experiments (see below), 15 min exposure to the standard, unattenuated 504-nm light and 10 min exposure to the white light induced approximately equal bleaches of ~70%.

Transretinal Spectrophotometry

Absorption spectra were obtained using a recording spectrophotometer (model 320, Perkin-Elmer Corp., Oakbrook, IL) modified for the analysis of a horizontally positioned retina (cf. Perlman et al., 1982). After measurement of the absorbance spectrum of the unilluminated retina, the preparation was irradiated (15 min exposure to 504-nm light or 10 min exposure to diffuse white light) and then reanalyzed for absorbance at 10-min intervals. At 525 nm, the stabilized change in absorbance induced by the irradiation was compared with the overall change exhibited upon subsequent exhaustive bleaching in the presence of hydroxylamine (Perlman et al., 1982).

Purification of ROS

After defined periods of light/dark incubation of the intact retina, retinas were chilled and isolation of the ROS was begun. Unless otherwise noted, all procedures were carried out under dim red light. All centrifugations and washes of the membrane suspensions were carried out at 0–4°C. (Previous studies have shown that the binding of G to R* occurs rapidly even at 0°C. By contrast, the release of G in GTP-depleted preparations is highly temperature dependent; at low temperatures [0–4°C], the release is essentially blocked [Kühn, 1983; also see text accompanying Fig. 5].) Retinas (two per sample) were transferred to a 12- × 75-mm capped plastic tube containing 1 ml of chilled, buffered
sucrose (26% wt/vol) (shake solution). The ROS were broken off by manually shaking the tube for ~90 s. Large pieces of retina were removed and the remaining suspension was layered onto a discontinuous gradient of sucrose contained in an 11-× 60-mm centrifuge tube (cf. Fig. 2). All sucrose solutions (including the shake solution) contained 20 mM Tris, adjusted with HCl to pH 7.5, 110 mM NaCl, 2 mM MgCl₂, 0.01–0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM dithiothreitol (DTT). The gradients were centrifuged for 30 min at 35,000 rpm (~160,000 g at r(max; SW60 rotor) in an ultracentrifuge (L8-70, Beckman Instruments, Inc., Fullerton, CA). Two rhodopsin-containing bands resulted from this centrifugation: "ROS I" (at the interface of the 29 and 35% sucrose solutions) and "ROS II" (at the interface of the 35 and 40% sucrose solutions), together yielding ~170 μg rhodopsin per retina.² For the analysis of binding of G, the ROS I and ROS II bands were routinely pooled.

Bovine eyes obtained within 30 min of animal slaughter were transported on ice to the laboratory; retinas were removed and ROS were isolated and purified as described above.

**Washing of ROS Membranes**

Soluble and peripherally bound polypeptides were extracted from the ROS membranes by sequential washing with isotonic buffer (I), hypotonic buffer (H), and hypotonic buffer containing 40–80 μM GTP (HG). I buffer consisted of Ringer's solution supplemented with 0.01 mM PMSF and 0.1 mM DTT; H buffer contained 10 mM Tris, pH 7.6, 1 mM EDTA, 0.01 mM PMSF, and 0.1 mM DTT. The procedures were as follows. (a) Isotonic washes (two washes, 2 ml/wash): the membrane suspension was diluted with 1–2 vol of isotonic buffer and centrifuged at 25,000 rpm for 10 min (Beckman SW-60 rotor). The resulting supernatant was decanted and stored at 4°C; the pellet was resuspended in 2 ml of isotonic buffer by repeated passage through a disposable pipette. This suspension, and all those obtained subsequently, were centrifuged at 20,000 rpm for 10 min. The two I supernatants were pooled and stored at 4°C. (b) Hypotonic washes (two washes, 2 ml/wash): the ROS pellet was resuspended in low ionic strength buffer (H). The suspension was incubated for 10 min at 4°C and centrifuged, and the cycle was repeated. The two H supernatants were pooled and stored. (c) Hypotonic washes with 40–80 μM GTP added: the membranes were washed with HG buffer, as described above, and the supernatants were pooled and stored. The final pellet (G-depleted ROS) was resuspended in 200 μl of a solution containing 50% glycerol, 10 mM Tris (pH 7.6), and 1 mM DTT, and stored at ~20°C. G-depleted ROS from both dark- and light-adapted retinas were analyzed by SDS-PAGE to assay for the completeness of the extraction procedure; the results indicated that these washed membranes contained ≤10% of the Gα,β,γ originally present.

**Concentration of Supernatants**

I extracts were concentrated ~10-fold by ultrafiltration. The H and HG extracts were concentrated ~20-fold by evaporation in a vacuum (Savant Speed Vac concentrator, model SVC-100H, Savant Instruments, Inc., Hicksville, NY) or by ultrafiltration. In all experiments involving the quantitation of "percent G bound" (see below), H and HG extracts obtained from a pair of (identically treated) retinas were concentrated to equal final volumes; for the subsequent electrophoresis step, we used equal aliquots of the concentrated H and HG extracts.

² The frog eye contains ~10 nmol of opsin (Bridges, 1976). Assuming 10 nmol opsin/retina in toad, and a molecular weight 35 kD for opsin, 170 μg of opsin represents ~50% of the total complement. We have assumed that the opsin-containing membranes recovered are representative of the whole population of photoreceptors.
Electrophoresis and Densitometric Analysis

Extracted proteins were separated by SDS-PAGE, using low cross-linked gels, as described previously by Baehr et al. (1979, 1982). Resolved bands were analyzed by densitometry of Coomassie-stained gels. We assumed equal efficiency of staining for all proteins analyzed densitometrically. Control experiments, using bovine serum albumin as a standard, indicated that staining intensity vs. protein content was linear up to ∼10 µg protein in a single band. For the gels used to analyze G binding, the maximum protein content in a single band was ≤ 8 µg.

Determination of Percent G Bound

The relative amounts of Gα and Gβ in the H and HG extracts (see above) were quantitated in the Coomassie-stained gels by integrating the areas under the Gα and Gβ densitometric peaks (arbitrary densitometric units). The contribution of the ∼7-kD (γ) subunit of G was minimal and so was discounted. In general, a given extract (H or HG) contained roughly equimolar amounts of Gα and Gβ (see Results). The parameter “percent G bound” was calculated as \[ \frac{(G_{\text{HG}})}{(G_{\text{H}} + G_{\text{HG}})} \times 100\% \]. In this expression, Gα and Gβ represent the combined values determined for Gα and Gβ in the respective H and HG gel lanes. For brevity, we henceforth refer to the combined levels of these subunits as Gαβ. Since H and HG aliquots were equivalent volumes, “percent G bound” defines the relative abundance of Gα and Gβ contained in the H and HG extracts. It should be emphasized that our assay quantitates the binding of G after chilling, disruption, and extraction of initially intact photoreceptors. The parameter “percent G bound” directly measures the capacity for the R*.G interaction in hypotonically washed membranes and provides an upper limit for the extent of tightly bound G in the intact cell (cf. Discussion).

Immunoblotting

Polyclonal antibodies made against toad 48-kD protein (Mangini et al., 1984) were used to examine the identity of the homologous polypeptide in bovine ROS. Toad and bovine ROS proteins separated by SDS-PAGE (as described above) were electrophoretically transferred to nitrocellulose (Burnette, 1981). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit second antibodies (Boehringer-Mannheim, Indianapolis, IN) were used to visualize cross-reacting polypeptides.

Results

Mobilities of Toad and Bovine Polypeptides

Identification of major toad ROS proteins was based on a comparison with identified bovine ROS proteins as analyzed by SDS-PAGE (Baehr et al., 1979, 1982). Fig. 1 illustrates the general correspondence of the major polypeptides present in the respective samples. The high molecular weight subunits of toad phosphodiesterase (Mr = 92 and 85 kD) appear as a well-resolved doublet, although the mobilities differ slightly from those of bovine PDE (Mr = 88 and 84 kD). The polypeptide identified as “48 kD” in the toad sample was characterized on the basis of its light-modified behavior (Kühn, 1978; Mangini et al., 1984; Pfister et al., 1985). In the low cross-linked gels used here, this protein migrates with an apparent molecular weight of ∼51 kD. The homologous band in the bovine sample (Mr = 54 kD in our gel system) was identified based on its cross-reactivity with polyclonal antibodies raised against the toad 48-kD protein (Fig. 1B).
As is the case for bovine ROS (Baehr et al., 1982), the α- and β-subunits of G are prominent components of toad ROS. Previous work has shown that the relative mobilities of Gα and Gβ differ in low cross-linked (Baehr et al., 1982) vs. conventional (Laemmli, 1970) gels. As Fig. 1 illustrates, the doublet in toad ROS consisting of Gα and Gβ corresponds closely to that of the Gα and Gβ doublet of the bovine sample. Excision of the toad Gα and Gβ bands from the present gel system and rerunning of the proteins in adjacent lanes of a Laemmli gel (data not illustrated) established the identification shown in Fig. 1.
Sedimentation Behavior of Toad ROS

After purification on a sucrose step gradient, toad ROS were recovered in two distinct bands (Fig. 2). Light microscopy revealed that the upper band (ROS I) consisted primarily of fragmented ROS and stacks of disks; particles in the lower band (ROS II) were significantly larger. Fig. 2 also compares the lengths of particles contained in the initial suspension with those of the ROS I and ROS II fractions. The average length of the ROS II particles was similar to that of particles in the initial suspension; ROS I particles were significantly smaller than those in the initial suspension. With respect to a given fraction, particles obtained from both dark- and light-adapted retinas were similar in length.

The distribution of G between the ROS fractions and the extent to which light alters this distribution were of particular interest. Fig. 3 illustrates the abundance of the Gβ subunit, relative to opsin, in ROS I and ROS II fractions obtained...
from four unbleached and eight illuminated preparations. The relative abundance of $G_o$ was similar in ROS I and ROS II obtained from dark-adapted retinas. For reasons possibly relating to the light-modified behavior of $G$, the illumination of retinas led to a significant difference in the relative amount of $G_o$ recovered in ROS I vs. ROS II. However, for both dark- and light-adapted conditions, the total recovery of $G_o$ (RI + RI1) was similar. Among the preparations described in Fig. 3, two unbleached and four bleached preparations exhibited a separation of the $G_o$ and opsin monomer bands sufficient to afford similar densitometric analysis for $G_o$. For dark-adapted ROS I and ROS II, the mean relative densitometric units for $G_o$ were 0.17 and 0.22, respectively; the values for light-adapted ROS I and ROS II were 0.13 and 0.22, respectively. This distribution did not differ substantially from that of $G_o$. For routine analysis of $G$ binding for a given sample, we pooled the ROS I and ROS II fractions.

From the data of Fig. 3, we obtained an estimate for the overall quantity of $G$ recovered with both the ROS I and II fractions. For both dark- and light-adapted preparations, the analysis yielded a densitometric $G_o$/opsin ratio of ~1:7; this corresponds to a molar $G_o$/opsin ratio of 1:8, assuming $M_o$ $G_o = 59$ kD and $M_o$ opsin = 35 kD (Baehr et al., 1982). These ratios are approximate, since the analysis was based on Coomassie staining of proteins. Furthermore, these ratios are based on the quantity of $G$ recovered with the ROS fractions; from analysis of solubilized proteins in the centrifugation tube, we estimate that isolation and purification was complete.
centrifugation of the ROS eluted ~50% of the G initially present in the photoreceptors (data not shown). Roof and co-workers (Roof et al., 1982; Roof and Heuser, 1982) recently reported a G/opsin ratio in toad rods of ~1:10, based on quantitation of disk membrane particles identified as G-protein.

Percent G Bound Before and After Light Adaptation

G-protein is peripherally bound to dark-adapted ROS membranes under moderate ionic strength conditions (isotonic) and can be eluted by low ionic strength (hypotonic) medium (Baehr et al., 1979). Bleaching in the absence of GTP, however, causes G-protein to bind tightly to ROS membranes. The resulting complex is stable for many minutes at room temperature (Kühn, 1980a; Emeis et al., 1982) and cannot be readily disrupted by unsupplemented hypotonic medium. However, the addition of GTP to the hypotonic medium disrupts this complex and elutes the subunits of G (Godchaux and Zimmerman, 1979; Kühn, 1980a, b). We have used this property of G-protein binding to examine the effects of light and dark adaptation of the intact, isolated retina.

Fig. 4 shows the results obtained when retinas maintained in darkness (A) or subjected to defined illumination (B and C) were analyzed for the binding properties of Gₐβ. The representative gel data in each section of the figure show polypeptides contained in isotonic (I), hypotonic (H), and hypotonic plus GTP (HG) extracts of the ROS (see Methods). On the basis of densitometry of such gels, we quantitated the amount of Gₐ and Gₐβ eluted by the various washes. The histograms in the upper portion of the figure show the relative distribution of Gₐβ among the I, H, and HG extracts.

For ROS isolated from unbleached retinas, a substantial portion of G (>50%) was eluted by washing the membranes with I medium (Fig. 4A). Subsequent washing with H and HG media eluted successively smaller portions of Gₐ and Gₐβ. The portion of Gₐβ eluted by the final HG wash represented ≤10% of the total extractable G. The illumination of retinas altered the elution profile of Gₐβ. Irradiation for 10 min with white light (~70% bleach) dramatically reduced the proportion of G contained in the I and H extracts and increased the fraction present in the HG extract (Fig. 4B). 15 min irradiation with 504-nm light, which also bleached ~70% of the visual pigment, caused qualitatively similar but less extensive changes in the relative abundance of Gₐβ among the I, H, and HG extracts (Fig. 4C).

Dependence of G Binding on Conditions of Light and Dark Adaptation of the Retina

Variation with period of incubation. As is the case for other isolated retina preparations, the bleaching of rods in the isolated toad retina leads to a permanent loss of electrophysiological sensitivity (sustained bleaching desensitization). The principal aim of this study was to examine the aftereffects of substantial bleaching on the binding of G, and to determine, specifically, whether irradiation causes a permanent increase in binding. To quantitate the light-induced change in extractability of G (cf. Fig. 4), we employed an empirical parameter, “percent G bound,” which reflects the proportion of Gₐβ exhibiting “tight binding” to the
ROS membranes (quantity extracted by the HG medium, normalized to that cumulatively extracted by the H and HG media; see Methods).

The standard irradiation used in the dark-adaptation experiments consisted of a 15-min exposure to 504-nm light (incident intensity at the plane of the retina, \( \sim 1.7 \times 10^3 \text{ Wcm}^{-2} \text{ s}^{-1} \)) that cumulatively bleached \( \sim 70\% \) of the rhodopsin initially present. Intracellular recordings from rods of the isolated toad retina

**FIGURE 4.** Quantitation of \( G_a \) (open bars) and \( G_d \) (stippled bars) in extracts obtained from dark- and light-adapted retinas. Histograms A–C indicate data obtained from retinas maintained in darkness (A), illuminated for 10 min with white light (B), and illuminated for 15 min with 504-nm light (C). The labels I, H, and HG identify, respectively, supernatants obtained on extraction with isotonic medium, hypotonic medium, and hypotonic medium containing 40–80 \( \mu \text{M} \) GTP. For each condition (A, B, or C), the amount of \( G_a \) or \( G_d \) contained in a given extract was normalized to the cumulative amount of that subunit eluted by the I, H, and HG washes. Each histogram illustrates the mean of data obtained in multiple experiments; the error bars indicate SEM values. Shown below each panel are gel data obtained in a single representative experiment of the indicated type. For each condition, aliquots applied to the H and HG lanes represent, on a volumetric basis, equal fractions of the respective extract. Aliquots applied to the I lanes represent 5–10\% of the total isotonic extract.
have shown that 70% bleaching leads to a sustained threshold elevation (i.e., sustained desensitization) of \( \approx 3 \) log units (Leibovic, K. N., and J. E. Dowling, personal communication). Selection of the 15-min duration for the standard exposure was based on two considerations. (a) Metarhodopsin II (MII), a bleaching intermediate, is known to exhibit high reactivity with G in vitro (Emeis et al., 1982; Bennett et al., 1982; Hofmann et al., 1983). On the possibility that the presence (and decay) of MII might complicate the interpretation of the bleaching aftereffect, we wished to minimize the amount of MII present at the conclusion of the adapting exposure. This factor motivated the use of the longest workable duration of the exposure. (b) A recent study employing the isolated retina of bullfrog indicated that bleaching exposures of up to 20 min in duration (cumulative bleaching, \( \geq 67\% \)) yield rod desensitizations that are stable for several hours (Perlman et al., 1982). This result, and the results of related studies (e.g., Pepperberg et al., 1978), suggested the suitability of 15 min irradiation as a condition for the present G-binding study.

Fig. 5 shows results obtained when retinas subjected to the standard irradiation delivering \( \approx 1.5 \times 10^8 \) quanta/cm\(^2\) were incubated in darkness (23 ± 1°C) for defined periods before initiating the biochemical analysis. This irradiation caused a substantial increase in percent G bound, from 16 ± 6% (mean ± SEM for unbleached retinas; solid triangle in Fig. 5) to 55 ± 5% (light-adapted retinas; solid circle, 0 min incubation in darkness). With increasing time in darkness, percent G bound gradually declined. At 60 min, the level did not differ significantly from that measured for unbleached retinas. In another set of experiments, retinas were analyzed for percent G bound at various times after a 10-min exposure to intense white light that also bleached \( \approx 70\% \) of the rhodopsin (data not illustrated). The results obtained were qualitatively similar to those observed for green light; however, for a dark-adaptational period of \(< 60\) min, percent G bound was on the average greater for retinas exposed to the white light. Initially, after white light irradiation, percent G bound was 86 ± 3% (mean ± SEM); after 30 and 60 min of dark incubation, percent G bound was 68 ± 4% and 17 ± 1%, respectively. That is, after both irradiations used to establish a condition of \( \approx 70\% \) bleaching, there developed a recovery to a condition of "loosely bound G," i.e., a relatively low quantity of G\(_{\alpha,\beta}\) in the HG extract. In each case, the period of dark adaptation required for half-completion of the recovery was \( \approx 10^3 \) s.

In the series of experiments just summarized, retinas were chilled after a defined period of dark adaptation. This initial step was followed by isolation of ROS and subsequent extraction of G. Previous studies (Kühn, 1980a, b) involving illumination of ROS membranes have shown that chilling stabilizes the condition of tight binding of G. However, the question arose as to whether the observed decline in binding is characteristic of the retina preparation itself (at termination of the incubation), or whether processes operating during the work-up procedure contribute significantly to the decline. To answer this question, we did the following experiment. Retinas exposed to the standard bleaching irradiation were immediately chilled and the ROS were isolated. Before continuing with the extraction of G, the ROS sample was incubated in darkness at 4°C for 60 min. Subsequent analysis of percent G bound yielded a value of 70%, a value far
greater than that exhibited by intact, bleached retinas dark-adapted at 23 ± 1°C for 60 min. This finding indicates that the gradual decline in percent G bound (Fig. 5) does not result merely from processes occurring after isolation and chilling of the ROS.

The transience of binding observed after a standard bleach led us to examine whether continuous light can preserve a condition of tight binding. The inset in Fig. 5 (open circles) describes the time course of G binding for retinas exposed to 504-nm light for periods ranging from 5 to 90 min. For these experiments,
involved 5 min exposure to this more intense light, followed by incubation in darkness. The results were similar to those obtained from the continuously illuminated retinas. Increasing the intensity of the light presented for 5 min appeared to accelerate the decline in G binding (open squares, Fig. 5, inset).

Taken together, the data of Fig. 5 indicate that under conditions of extensive and permanent bleaching, the light-induced increase in percent G bound is transient. The decline from an initial peak of binding occurs independently of the presence vs. the absence of maintained light.

**Effect of varying bleaching energy.** Retinas were bleached to various degrees by exposure to steady 504-nm light; the duration of the exposure was typically 15 min. Immediately after the bleach, retinas were chilled and the ROS were isolated. Fig. 6 shows the results obtained for percent G bound over a range of adapting illuminations. Also shown on the abscissa in Fig. 6 are results from separate experiments in which retinas were analyzed spectrophotometrically before vs. after illumination. These spectrophotometric data indicate the corre-
spondence between the cumulative energy of the irradiation (quanta per square micron) and the extent of bleaching. Together, the G binding and the spectrophotometric data show that a substantial increase in percent G bound occurred with increasing irradiation. Near-maximal binding was observed when ≥30% of the rhodopsin was bleached.

**DISCUSSION**

**Light-dependent Changes in Binding of G_{α,β}**

In this study, we have quantitated the binding of G-protein in preparations obtained from illuminated, intact retinas. The experiments have specifically addressed the dependence of binding on the extent and time course of irradiation, factors whose effects on receptor sensitivity have been analyzed extensively in previous studies. Our principal observations are as follows. (a) Increasing the energy of irradiation of the intact retina increases the proportion of G, exhibiting tight binding in the isolated, washed ROS. (b) With an increasing period of exposure to intense light, or with an increasing period of darkness after ~70% bleaching, the proportion of tightly bound G declines from an initial maximum. Both conditions yield a similar time course of decline, and both lead ultimately to a condition of minimal binding resembling that of unilluminated preparations.

The data of Fig. 6 present an interesting comparison with bleaching data obtained from GTP-depleted ROS membranes. We observed that retinas receiving ~2.4 × 10^7 quanta/µm^2 showed no significant elevation of percent G bound (Fig. 6, solid circle with smallest abscissa value); from a comparison of this irradiation with that yielding ~29% bleaching (5.7 × 10^7 quanta/µm^2), we estimate that the smaller irradiation induced a bleach of ~10%. By contrast, in GTP-depleted membranes, bleaches of ~10% cause virtually complete “tight binding” of the G (Kühn et al., 1981; Bennett, 1982). The minimal value of percent G bound observed after ~10% bleaching, the absence of complete G binding immediately after extensive bleaching, and the transience of binding (Fig. 5) may relate to the presence of GTP within intact photoreceptors, a possibility discussed further below.

**Interpretation of Percent G Bound, and Implications for Bleaching Desensitization**

The qualitative effect of light on the extractability of G resembles that described in earlier studies involving the illumination of isolated, GTP-depleted ROS (Kühn, 1980a; Baehr et al., 1982; Pfister et al., 1983). This similarity clearly suggests that, as in the previously studied system, the “tight binding” of G evident in ROS obtained from illuminated retinas reflects a direct association of G with visual pigment on the disk membranes. That is, the empirical parameter “percent G bound” (Figs. 5 and 6) quantitates G trapped by a reactive form of the pigment (R* or B) in our chilled, isolated ROS.

However, interpretation of the physiological significance of percent G bound requires consideration of possible changes in G binding that could occur during, or be induced by, the assay procedure. Previous studies and the present experiments (text accompanying Fig. 5) indicate that tightly bound G is relatively stable
at 4°C in washed (GTP-depleted) ROS. Our finding that percent G bound reaches ~80% for certain conditions of illumination of the retina (Fig. 5, inset) is consistent with a stability (preservation) of tightly bound G under the conditions of our assay. Thus, it is likely that the assay procedure preserves most or all of the tightly bound G that may have been present within the intact rods at the time of completion of the incubation at 23 ± 1°C. However, indications that G-GDP (or a form devoid of nucleotide ligand) is the tightly bound form of G (Fung and Stryer, 1980; Stieve, 1986), and that other, relatively soluble proteins may displace G from (compete with G at) the reactive site of the pigment (Kühn, 1983; Kühn et al., 1984), suggest two processes by which tightly bound G might form during preparation of the ROS membranes for analysis. (a) In illuminated preparations, hydrolysis of the GTP ligand of presumably loosely bound G* (G* → G by GTPase reaction) could occur after incubation of the intact retina but before treatment of the ROS membranes with HG medium, i.e., in isolated ROS deficient in GTP. In the presence of activated pigment (R* or B), the conversion of G* to G could support the formation of tightly bound G. (b) Although light promotes an increased association of the 48-kD protein with ROS membranes (Kühn et al., 1984; Wilden et al., 1986), isotonic washing readily extracts the 48-kD protein from ROS obtained even from bleached retinas. Rhodopsin kinase is similarly extractable by isotonic medium (Kühn, 1978). By dispersing the 48-kD protein or rhodopsin kinase, early steps in the assay procedure could unmask the G-binding site of the visual pigment and support the subsequent formation of tightly bound G.

The above considerations suggest that chilling, isolation, and washing of the ROS membranes do not diminish, but may augment, the level of tightly bound G prevailing within the intact rods on completion of the incubation at 23 ± 1°C. Accordingly, we interpret “percent G bound” to indicate an approximate upper limit for G binding in situ. That is, percent G bound quantitates the capacity for G binding by reactive visual pigment; this capacity is fully expressed in isolated, washed (GTP-depleted) ROS membranes at 4°C. On this interpretation, the data of Fig. 5 (solid circles) indicate that early in the period of dark adaptation after 70% bleaching, disk membranes of the intact rods possess a high capacity for G binding. The percent of G bound in situ at this time may be smaller than the corresponding value for the in vitro parameter “percent G bound.” However, for periods of dark adaptation ≥60 min after a 70% bleach, the proportion of G tightly bound within the intact rods cannot be significantly greater than the very low values for percent G bound evident in Fig. 5. As emphasized in the Introduction, bleaches of this magnitude in the isolated retina cause a pronounced desensitization of the rods that is sustained indefinitely, i.e., for periods of at least several hours. The line of reasoning just presented indicates, by contrast, that such bleaching does not induce a permanent tight binding of G in situ. We are thus led to the principal conclusion of the present study: that sustained bleaching desensitization does not depend upon the immobilization of a major fraction of G by bleached pigment.

This conclusion leaves open the interesting alternative possibility that the desensitization persists because G is in the loosely bound, GTP-charged state. That is, bleached pigment could depress sensitivity at the dR* → dG* step by
sustaining in darkness a high level of G* (G-GTP), thereby depleting the level of G available for activation by dR* (Pepperberg, 1984; Pepperberg and Clack, 1984). The notion that bleached photoreceptors of the isolated retina contain a permanently elevated level of G-GTP is consistent with the observation of elevated PDE* in previously bleached ROS preparations (Keirns et al., 1975), and with results obtained from an invertebrate species (Musca domestica), linking photoreceptor GTPase activity with the metarhodopsin state of the visual pigment (Blumenfeld et al., 1985; also see Stieve, 1986). Such a possibility also accounts for the observed sensitizing activity of p(CH2)ppG in bleached retinas (Clack et al., 1982; Clack and Pepperberg, 1984). It supposes that in the post-bleach condition, a maintained elevation of G* causes G (and perhaps GTP) to become limiting for the operation of the dR* → dG* reaction; p(CH2)ppG supports the formation of relatively long-lived dG* [incremental quantity of G charged with p(CH2)ppG], thereby increasing the effective dR* → dG* gain.

A final note concerns the time course of decline of "percent G bound," which after major bleaching required a period of ~10^3 s for halving of the initially exhibited increase (Fig. 5). Regardless of whether G is "tightly bound" in situ, this observation implies a time scale of at least ~10^3 s for complete "stabilization" of the visual pigment/G/PDE system in situ. After a brief irradiation that bleaches a major fraction of the visual pigment, rods of the isolated retina typically require a period of ~10^3 s to attain the stable, desensitized state (see, e.g., Grabowski and Pak, 1975). The processes measured by our assay may relate to those governing the approach to the steady state of bleaching desensitization.

We thank Dr. Meredith L. Applebury for helpful discussions during the course of this study.

This research was supported by grants EY-05494, EY-00257, and EY-04801 from the National Institutes of Health. D.R.P. is a Robert E. McCormick Scholar of Research to Prevent Blindness, Inc.

Original version received 22 February 1986 and accepted version received 10 June 1986.

REFERENCES


Bennett, N., M. Michel-Villaz, and H. Kühn. 1982. Light-induced interaction between rhodop-


