Surfaces of Rod Photoreceptor Disk Membranes: Light-activated Enzymes

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ABSTRACT The light-activated GTP-binding protein (GBP) in toad rod outer segments has been located on the cytoplasmic surface (CS) of rod disk membranes by correlating biochemical results with images of quick-frozen, freeze-fractured, and deep-etched rod outer segments. This has been accomplished by selectively removing and replacing the 8–12-nm particles that are found on the CS of disk membranes, exactly in parallel with the GBP. In contrast, the large particles are not correlated with another major disk enzyme, the light-activated cGMP phosphodiesterase. We have been unable to visualize this protein. The surface density of large particles, one particle per eleven rhodopsins in isolated rod outer segments and one particle per nine rhodopsins in intact retina, correlates well with previous biochemical estimates of GBP numbers based on enzyme activity. After the identification of the large particles, we tested the effects of light on the density of particles on the surface of disk membranes in intact retinas. Retinas quick-frozen at various intervals after a bright flash of light show a modest increase (~30%) in particle density by 10 s after the flash but no increase before 1 s. The number of particles on the disk membrane returns to dark levels between 1 and 10 min after the flash. The 1-s latency in the change of particle binding would appear to rule out this process as a mechanism for initiating phototransduction in the rod.

The task of correlating particular protein molecules with the surface features of disk membranes is made tractable by the relative biochemical simplicity of this cell organelle. The major integral membrane protein, rhodopsin, accounts for 70% of the total protein mass in outer segment membranes (6) and is present in about two million copies per disk in frog rods (14). Its location, as suggested in the previous report (21), is reflected in a fine granularity on the intradisk surface. A second integral protein is present in much smaller amounts, about 1,000–3,000 molecules per disk. This protein, referred to as large protein (17), rim protein (17), or ROS 1.2 (16), has been localized by immunocytochemistry to disk rims and incisures. The function of rim protein is unknown, but its possible relation to rim filaments has been discussed in the previous report (21).

Recently, attention has focused on another set of rod outer segment (ROS) proteins which are variably referred to as soluble (6, 7) or, more accurately, peripheral (11, 12). Of this group, those of particular physiological importance are the light sensitive cGMP phosphodiesterase (PDE) and light sensitive GTP-binding protein (GBP). In addition to PDE and GBP, rhodopsin kinase and possibly four other peripheral polypeptides of unknown function have been observed on SDS gels (2, 6, 7, 11, 12, 16). PDE and GBP are involved in the light-dependent regulation of cyclic nucleotide levels in the outer segment.

The very low dark level of PDE activity, in balance with a guanylate cyclase, is thought to regulate the dark concentration of cGMP in the rod to ~70 μmolar (23). Flashes of light which bleach only a few rhodopsin molecules per disk in isolated ROS can increase the PDE activity 30- to 40-fold (24). This may cause the cGMP concentration in the isolated ROS to drop by up to 3% (15). The role of GBP in this process seems to be as a regulator of PDE activity, because GBP (with GTP bound to it) is required for full activity of the PDE (4). Because the light sensitivity of both GBP and PDE is conferred through rhodopsin, it is not surprising that both proteins are, at least transiently, bound to disk membranes (7, 11, 12). There is preliminary evidence from proteolytic digestion studies that both proteins may bind to the membrane through rhodopsin (4, 13). In addition, each of these two peripheral proteins can be selectively removed from disk membranes and subsequently reconstituted (2, 4, 11–13). In this report, we have taken

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advantage of this last biochemical finding to identify the characteristic large particles we observe on the disc membranes. Further, we describe the effect of illumination on this disk surface feature.

MATERIALS AND METHODS

Intact Retinas

We performed all experiments on retinas isolated from the eyes of the toad, *Bufo marinus*. Toads were housed in a tank with running water and were kept under 12-h cycles of light and darkness. We dissected intact retinas under infrared illumination with the aid of an image converter as previously described (21).

Isolated Disk Membranes

Isolated fragments of outer segments were prepared to obtain four different conditions:

(a) Control membranes. These disk membranes contain nearly the same complement of PDE and GBP as that observed in the intact retina. To obtain these membranes, four to six retinas were incubated (0.2 ml per retina) in a solution of 11 mM NaCl, 0.25 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4. This solution is about one-tenth the osmotic pressure of conventional toad Ringer's but contains the control concentration of divalent cations. After a 1-min incubation at room temperature the vials were gently swirled and the pieces of retina withdrawn. Membranes were collected by centrifugation for 2 min at 9,000 *g*. All of these manipulations were carried out in the dark.

(b) Stripped membranes. These disk membranes are completely depleted of both PDE and GBP. To obtain these membranes, a pellet of control membranes was gently resuspended in 5 ml of 5 mM HEPES solution, pH 7.4, at 4°C in the dark. After 2 h, a membrane pellet was obtained by centrifugation for 20 min at 40,000 *g* at 4°C.

(c) GBP-depleted membranes. These membranes had a nearly normal content of PDE. To obtain these membranes, a pellet of control membranes was resuspended in the dark at 4°C in a solution of 11 mM NaCl, 0.25 mM KCl, 10 mM HEPES, and 2 mM EDTA, pH 7.4. This solution was about one-tenth the osmotic pressure of normal toad Ringer's, but was free of any divalent ions. After 2 h, a membrane pellet was produced by centrifugation as described above.

(d) PDE-depleted membranes. These membranes had a nearly normal content of GBP. To obtain these membranes, control membranes were first thoroughly bleached by exposing them to ten successive bright flashes of white light (Strobonar, Honeywell Inc., Los Angeles, CA) and carrying out successive manipulations under room light. The membranes were then suspended in 5 ml of 5 mM TRIS solution, pH 7.4, at 4°C. After 2 h, membranes were collected by centrifugation.

All solutions also contained 1 mM dithiothreitol (DTT) and 0.01 mM phenylmethylsulfonyl fluoride. All membrane pellets were resuspended in a minimal volume (50–100 μl) of the appropriate solution for rapid-freezing.

Membrane Reconstitution

Two separate steps were followed in the reconstitution studies. In the first step, solutions containing the peripheral protein of interest were obtained. These solutions were then mixed with membranes depleted of their peripheral proteins to produce reconstituted membranes.

A solution containing PDE and GBP was obtained by incubating control membranes in 300–400 μl of 5 mM HEPES, pH 7.4, in the dark at 4°C. After 2 h, membranes were collected by centrifugation at 40,000 *g* for 20 min at 4°C. The supernatant contained the peripheral proteins and the membranes were discarded. The supernatant was brought to a final salt concentration of 100 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.4.

GBP for reconstitution was prepared by resuspending control membranes in 300–400 μl of calcium/magnesium free solution containing 11 mM NaCl, 0.25 mM KCl, 10 mM HEPES, and 2 mM EDTA, pH 7.4, at 4°C in the dark. After 2 h, the membrane fraction was discarded and salts were added to the supernatant to final concentrations of 100 mM NaCl, 2 mM $CaCl_2$, 5 mM $MgCl_2$, and 10 mM HEPES, pH 7.4.

PDE for reconstitution was prepared by concentrating the supernatant from the PDE removal procedure described above. The solution was concentrated tenfold using an Amicon ultrafiltration unit with a YM10 filter (Amicon Corp. Scientific Sys. Div., Lexington, MA). Salts were added to the concentrated supernatant to a final concentration of 100 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM TRIS.

In all reconstitution studies, the selected supernatant was incubated with disk membranes fully depleted of their original peripheral proteins. These membranes were prepared as described above under stripped membranes (described in *b* above). They were also thoroughly bleached. For reconstitution, freshly prepared stripped membranes from two retinas were incubated with 300–400 μl of the appropriate supernatant and gently stirred overnight at 4°C. Before rapid-freezing, the reconstituted membranes were diluted in 10 ml of one-tenth isoosmotic Ringer with normal complement of divalent ions. They were then pelleted by centrifugation and resuspended in 50–100 μl of the same solution for freezing.

In the case of the GBP-only reconstitution and the double reconstitution of both PDE and GBP, supernatants were prepared from membranes collected from five toad retinas (~15 nmol rhodopsin) and reconstituted to stripped membranes from two retinas (about a twofold enrichment in the supernatant). The PDE-only supernatant was prepared from 11 toad retinas and reconstituted onto stripped ROS membranes from two retinas (about a fivefold enrichment).

SDS PAGE

Protein weight was estimated by the method of Bradford (5) (Bio-Rad reagent; Bio-Rad Laboratories, Richmond, CA) with bovine gamma globulin as standard. Total rhodopsin concentration of unbleached samples was calculated from the absorbance at 500 nm (A_{500}) measured in a 1% solution of Ammonyx LO (Onyx Chemical Co., Jersey City, NJ) using a Cary 118 spectrophotometer. Samples of selectively depleted and reconstituted membranes were loaded onto 15% SDS slab gels (2) at an estimated concentration of 6 μg rhodopsin per sample. Protein containing supernatants were diluted 1:1 with sample buffer and 20-μl aliquots were run on the gel without further dilution (2–4 μg total protein). Standards used in calibration were, in addition to a mixture of soluble proteins (Bio-Rad Laboratories), purified cattle PDE and GBP kindly provided by M. Applebury and W. Baehr (Purdue University).

PDE Assay

The assay of Zusman (25) as modified by Baehr et al. (1) was used with the following modifications: PEI cellulose plates (Schleicher and Schuell, Inc., Keene, NH) were washed by running once with distilled water and dried immediately before use. Total volume of the reaction mixture was 100 μl, rhodopsin concentration was 0.76 μM, and salts were 10 mM TRIS, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.6 mM $MgCl_2$, 3.0 mM $MgSO_4$, 0.1 mM DTT, 10 μM GTP, and 1.0 mM cGMP (16 mCi/mmol [^{3}H]cGMP or 10 mCi/mmol [^{14}C]cGMP). Radioactive nucleotides were purchased from Amersham/Searle Corp. (Arlington Heights, IL).

Effects of Illumination

To test the effects of illumination on disk surface structure in intact photoreceptors, intact retinas were either kept dark and rapidly frozen or flash illuminated, and then rapid-frozen at various time intervals following the flash. The rapid-freezing machine was operated in complete darkness. A piece of retina was dissected, mounted on a cushion of lung and transferred to the freezing stage under IR illumination. The light source was a 30 ms duration flash of white light. In a spectrophotometer, the flash source was measured to bleach 70%–85% of the rhodopsin content of the retina. A system of electronic or manual triggers (depending on the time interval) was designed to quick-freeze the retina precisely 100 ms, 1 s, 10 s, 1 min, or 10 min after the flash.

Particle Densities in Whole Retinas

One platinum/carbon (Pt/C) replica from each time point was scanned in the electron microscope and virtually all substantial exposures of CS were photographed. The EM negatives were contact reversed and printed at a final magnification of 182,000. A "standard counting area" was defined (a circular field enclosing a total area of 1.15×10^{-2} μm^2) and printed on a transparent overlay. The number of particles falling within the circular field was used to determine the surface density of particles. All regions of CS large enough to accommodate at least one "standard area" were used in the determination and typically, for each time point, 8–10 "standard areas" were counted from five to six different disks. A major limitation in this method was the very small amount of surface area exposed using etched whole retinas.

Quick-freezing and Electron Microscopy

The techniques of quick-freezing, freeze-fracturing, and deep-etching were as reported previously (21). Etching was carried out for 3 min at −95°C. All tissue replicas shown here were produced by rotary shadowing with the Pt/C source at a 24° angle. Replica handling was as described previously. Replicas were observed with either a JEOL 100C or a Philips 400 electron microscope.

RESULTS

The CS of the disk membrane is covered with 8-12-nm particles randomly distributed throughout the surface, but at a lower concentration on the disk rim. In intact retinas in the dark, the particles exist at a density of one particle per nine rhodopsin molecules. The location of these particles, their density, and a consideration of the physiology of the rod outer segment suggests that the particles may be either or both of the important peripheral proteins, PDE or GBP. The work of Kuhn (11, 12) and Baehr et al. (2) in bovine retinas has demonstrated that these peripheral proteins can be selectively removed from disk membranes by careful manipulation of experimental parameters such as light, ionic strength, and chemical composition of the suspending medium. We have extended this work to the toad retina and have studied the structure of membrane surfaces treated to selectively remove or reconstitute either PDE or GBP. In our experimental strategy, the same sample of isolated disk membranes was studied for its biochemical characteristics through SDS PAGE and enzymatic assays and for its structure through techniques of rapid-freezing, etching, and electron microscope observation.

Control and Stripped Membranes

Fig. 1 is a micrograph of an isolated stack of control disk membranes. The plasma membrane has been completely lost. The 12-nm surface particles can be seen to be at much lower concentration on the disk rims. The disk rims, in turn, are connected to each other by filaments described in the previous report (21). The SDS PAGE of the same preparation is also shown in Fig. 1. The major band is identified as rhodopsin with a molecular weight of 37,000. (In the gels of Fig. 1–9, an unlabeled dimer of rhodopsin also appears as a dark band running between GBP and PDE on all disk membrane gels.) Several other bands are visible, some possibly from contaminating membranes, since disk membranes were not purified extensively (typical absorption spectra of these membranes exhibited A_{498}/A_{400} of 0.34–0.37 and A_{280}/A_{498} of 2.7–3.0). By analogy to the molecular identification in cattle retina and as confirmed by our enzymatic assays of depleted membranes (see below), PDE and GBP are identified in the gels in Fig. 1. PDE consists of two high molecular weight polypeptides of ~82,000 and 90,000 and a low molecular weight subunit not shown on these gels. GBP consists of two polypeptides of molecular weights about 39,000 and 37,000 and a low molecular weight subunit which runs with the dye front on these gels and is not shown in Fig. 1–9. The particle density in the isolated membranes (2,700/μm^2) is somewhat less than that in the intact photoreceptor (3,400/μm^2). That is, the isolation procedure for "control membranes" results in a modest loss of material. In contrast, the surface particles can be completely removed through a procedure which also results in the total loss of both PDE and GBP.

Fig. 2 is a micrograph of isolated membranes depleted of both PDE and GBP. The surface particles are completely removed. The surface does not exhibit any distinct structure, but the connecting filaments between rims are still present in some regions. SDS PAGE of this preparation also shown in

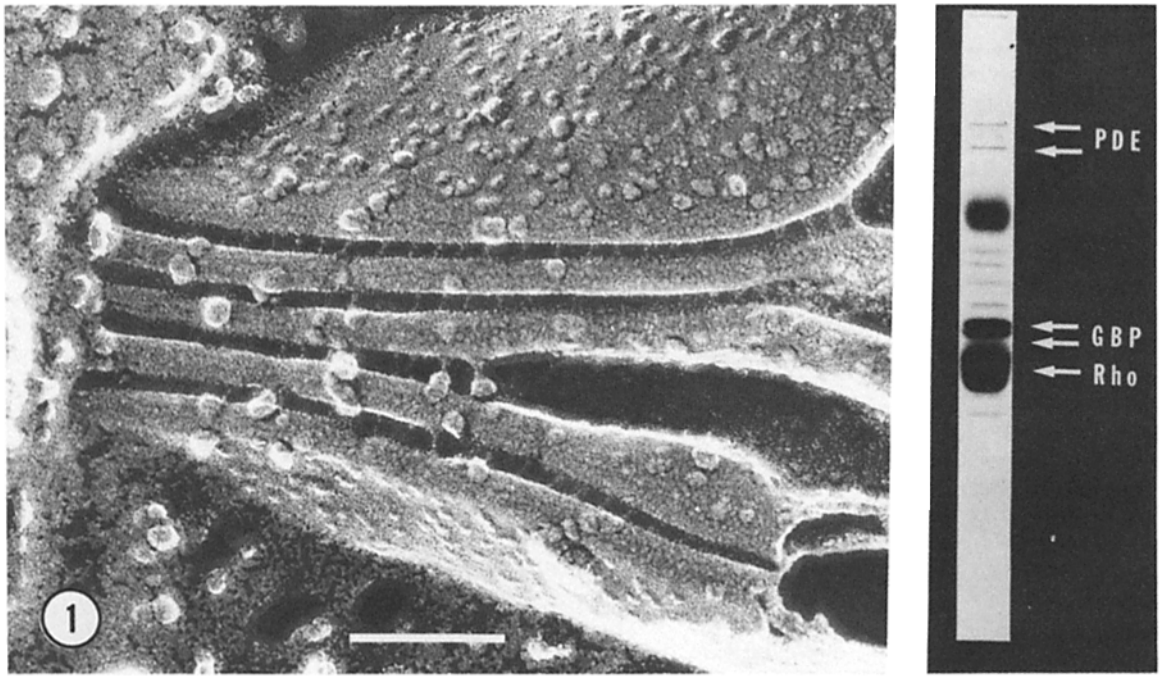


FIGURE 1 The micrographs in Figs. 1–4 are from replicas of fragmented toad ROS some of which were treated to remove specific proteins, then rapidly frozen, fractured at −110°C, deep-etched for 3 min at −95°C, and rotary shadowed at an angle of 24°. All membranes were exposed to fluorescent room lights for several minutes before and during rapid-freezing. Accompanying SDS PAGE profiles display polypeptides present in each preparation. Each gel is labeled to locate rhodopsin (*Rho*), light-activated cGMP phosphodiesterase (*PDE*), and light-activated GTP-binding protein (*GBP*). The unlabeled dark band running between PDE and GBP on all disk membrane gels is a rhodopsin dimer characteristic of this particlar polyacrylamide gel system (2). Fig. 1: Control membranes: isolated, fragmented ROS were prepared in the dark in 0.1 isoosmotic Ringer's with normal amounts of calcium and magnesium. Washed membranes were exposed to fluorescent room light for several minutes before freezing. The accompanying gel shows (*Rho*), GBP, and PDE, as well as rhodopsin dimer (unlabeled), and other less prominent ROS polypeptides. The low molecular weight subunit of GBP runs with the dye front and is not shown on gels of Figs. 1–9. Bar, 0.1 μm. × 200,000.

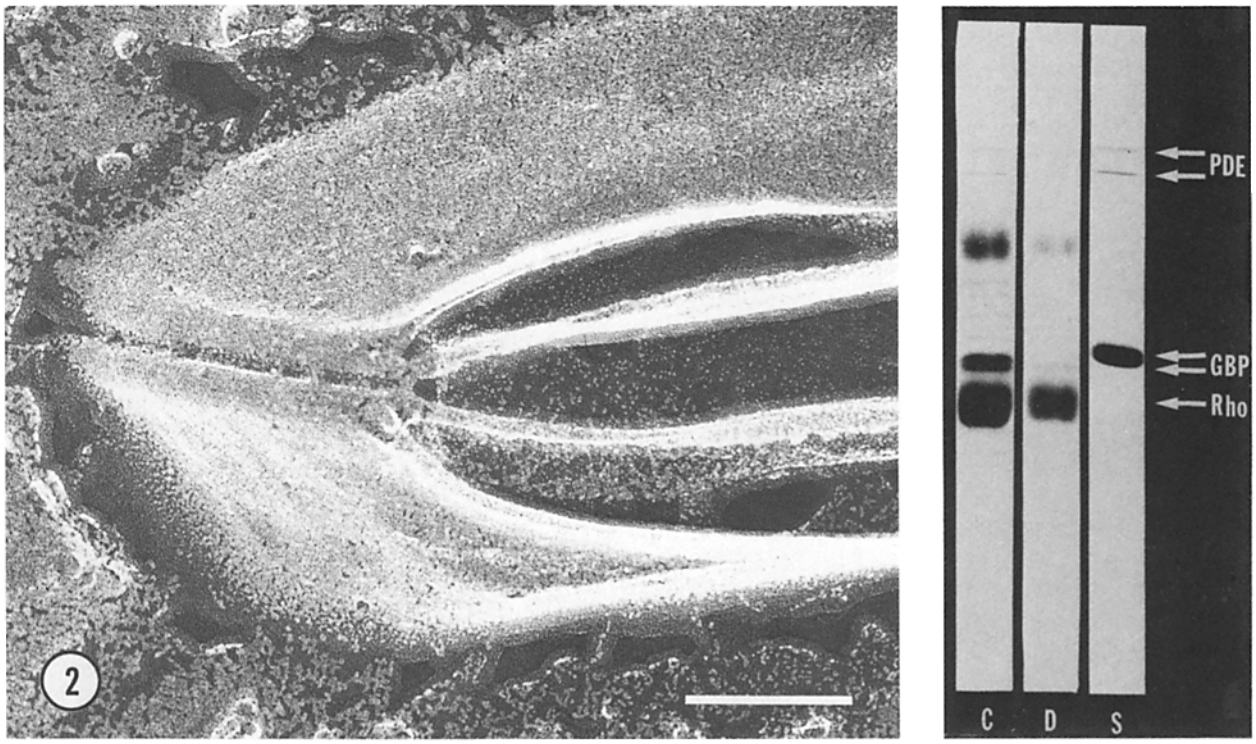


FIGURE 2 Stripped membranes. Isolated, fragmented ROS stripped of both GBP and PDE were prepared by resuspending control membranes in a large volume of 5 mM HEPES at 4°C for 2 h in the dark. These membranes collected for rapid-freezing are stripped of large particles (micrograph) and GBP polypeptides (gel *D*). SDS PAGE also shows polypeptides from control membranes (gel *C*), and polypeptides washed from the membranes and recovered in the supernatant (*S*). Bar, 0.1 μm. × 240,000.

Fig. 2, indicates that the membrane fraction after treatment (Fig. 2, gel *D*) is free of PDE and GBP, whereas the supernatant (Fig. 2, gel *S*) contains both of the peripheral proteins. Rhodopsin, of course, remains with the membrane fraction. The surface particles, therefore, are probably either or both PDE and GBP. Nonidentified peripheral polypeptides which may also be removed from the membrane are unlikely candidates for the surface particles because, apart from PDE and GBP, there exist no other reported peripheral proteins in sufficient concentration to account for the large number of surface particles per disk.

Membranes from Which GBP or PDE Are Selectively Removed

Selective removal and reconstitution experiments allow a more positive identification of the surface particles. Fig. 3 illustrates a micrograph in which GBP is selectively removed. The SDS PAGE of this preparation shows that it still contains PDE and rhodopsin (Fig. 3, gel *D*) but the vast majority of the GBP is in the supernatant phase (Fig. 3, gel *S*). The GBP removal from the membranes, however, was not total as can be observed by the faint band at the GBP molecular weight in the membranous fraction (Fig. 3, gel *D*). Complete, yet selective removal of GBP could not be achieved in our experiments. The surface particles in these membranes are almost completely removed. Only a few can be seen on an otherwise featureless surface. These results suggest that the surface particles must be GBP. Indeed, the following experiment in which PDE is selectively removed confirms this suggestion.

Fig. 4 illustrates a micrograph in which PDE is selectively removed. The membranes (Fig. 4, gel *D*) contain rhodopsin and GBP, but no PDE. PDE is found exclusively in the supernatant. Structurally, the membrane surfaces of PDE depleted membranes are indistinguishable from control membranes (compare with Fig. 1). Surface particles of typical size and density are seen throughout the disk surface, again confirming that the large particles are not PDE.

Reconstituted Membranes

Reconstitution of PDE and GBP onto stripped disk membranes had a slow time course. It was necessary to incubate the membranes overnight at 4°C with a solution containing the peripheral proteins to obtain reconstitution. Over this time course, rod disk membranes lost their characteristic disk shape and became primarily spherical vesicles. Fig. 5 is a micrograph of control membranes and Fig. 6 is a micrograph of membranes stripped of PDE and GBP after overnight incubation at 4°C. The control membranes exhibit the characteristic surface particles, but rims or rim filaments can no longer be distinguished. The stripped membranes, the substrates for reconstitution experiments, exhibit a smooth surface devoid of any particles.

The reconstitution of both PDE and GBP readily restores the stripped membranes to the control density of surface particles. In Fig. 7, a micrograph of such reconstituted membranes is shown along with the SDS PAGE of this preparation. The typical particles are seen at the surface and the SDS PAGE confirms that PDE and GBP are tightly associated with the membranes. Under our experimental conditions, we did not observe complete recovery of surface particle density to that of the control membranes. Reconstituting either protein by itself was difficult to achieve, perhaps because the reassembly requires a specific combination of factors some of which may be missing from the preparations of either protein by itself. However, reconstitution of low levels of GBP to stripped membranes

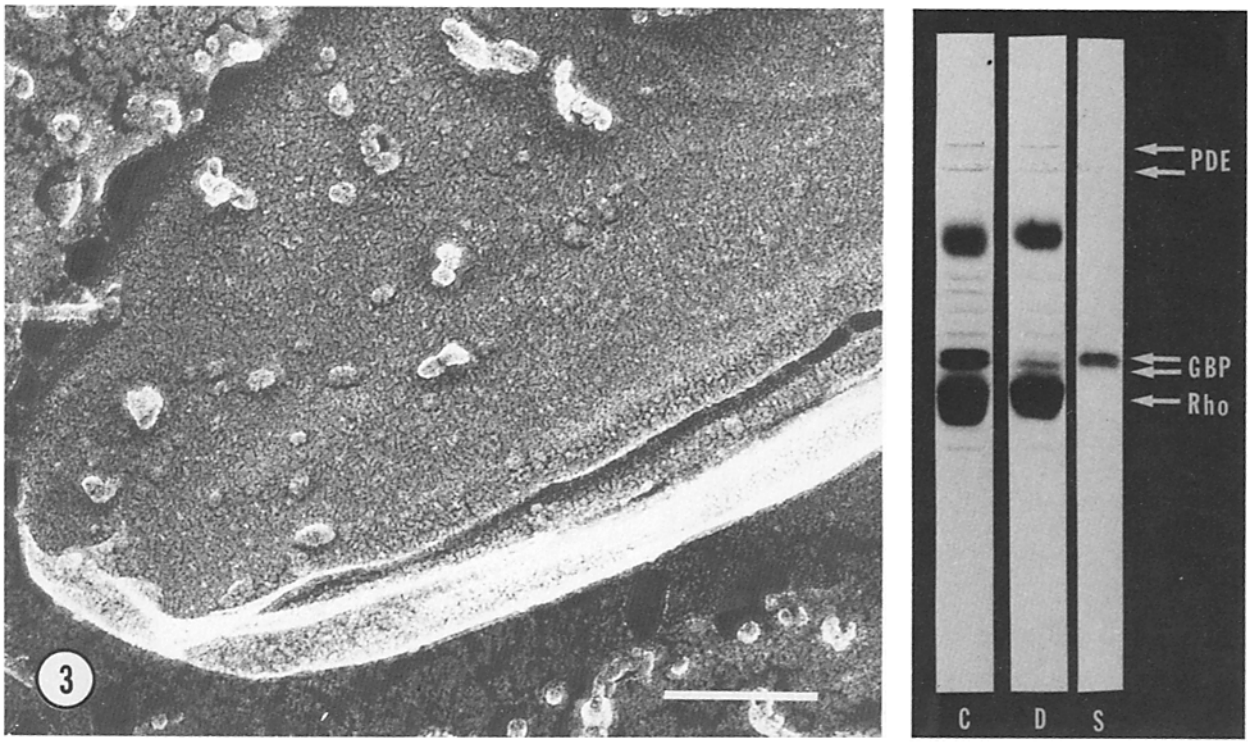


FIGURE 3 GBP-depleted membranes. Isolated fragmented control ROS were selectively depleted by GBP by washing them in the dark in 0.1 isoosmotic Ringer's free of calcium and magnesium with 2 mM added EDTA. These membranes with large particles removed are shown both in the micrograph and the accompanying gel (gel *D*). SDS PAGE also shows control membranes (gel *C*) and GBP polypeptides released into the supernatant wash (gel *S*). As seen in gel *D*, PDE remains with the membrane fraction. Bar, 0.1 μm. × 210,000.

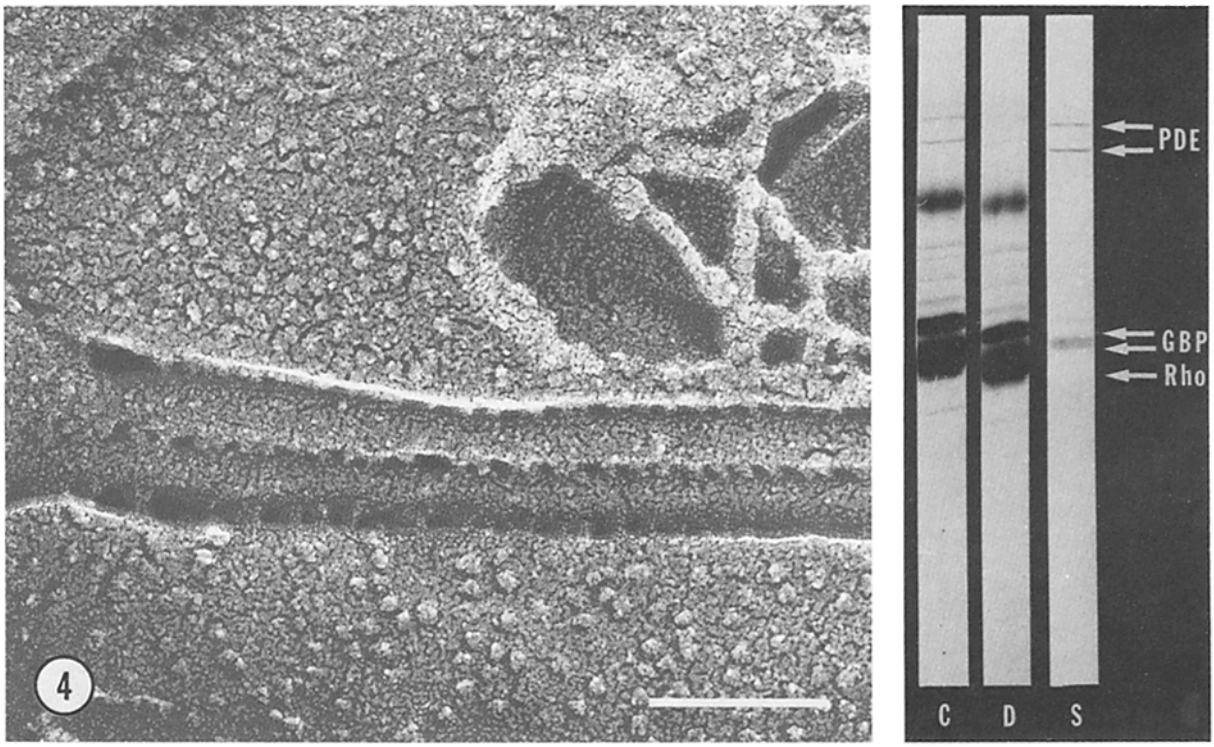
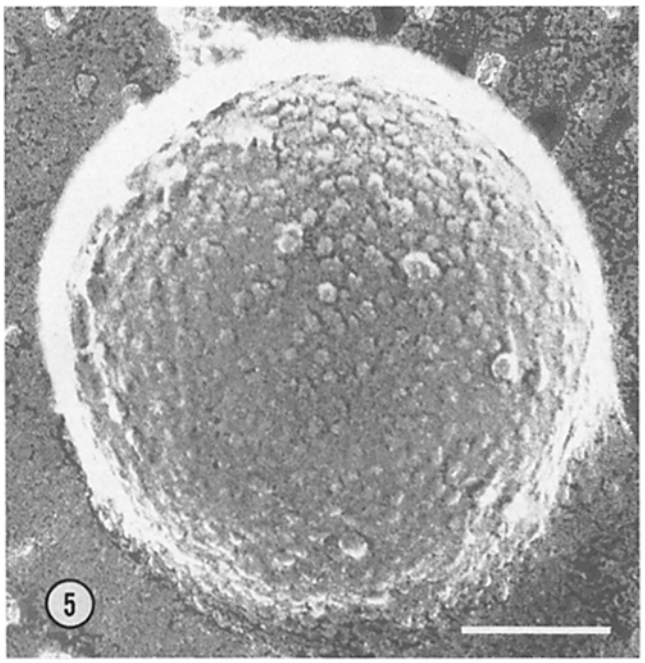


FIGURE 4 PDE-depleted membranes. Isolated fragmented control ROS were selectively depleted of PDE by a two step procedure. First, membranes were fully bleached, then they were washed with 5 mM TRIS at 4°C for 2 h. The PDE-depleted membranes show a normal complement of large particles (micrograph) and large amounts of the GBP polypeptides (gel *D*). PDE could be recovered in the supernatant along with very minor amounts of GBP (gel *S*). Control membranes (gel *C*) are shown for comparison. Bar, 0.1 μm. × 250,000.



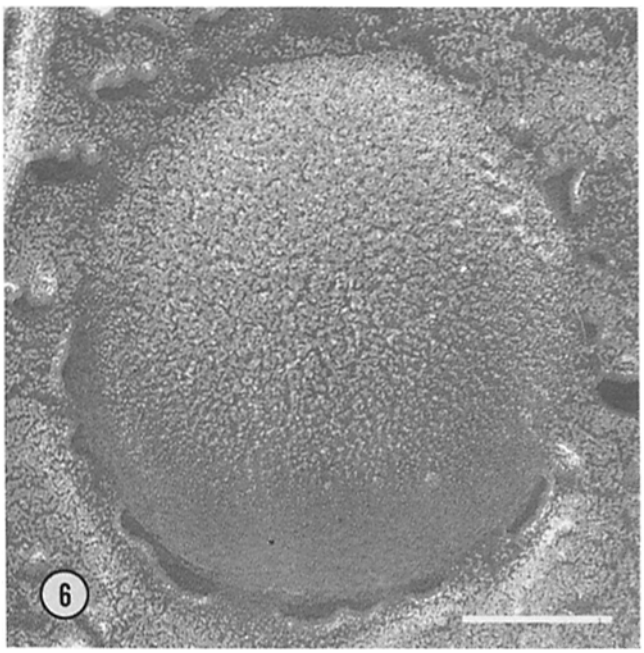


FIGURE 5 The micrographs in Figs. 5–9 are from replicas of fragmented toad ROS which have been held overnight at 4°C with constant stirring before rapid-freezing, fracturing at −110°C, deep-etching for 3 min at −95°C and rotary shadowing at an angle of 24°. Disk membranes held in this way for reconstitution show some stacks of disks, but are primarily in the form of large vesicles. Accompanying SDS gels are labeled, as before, to locate rhodopsin (*rho*), phosphodiesterase (*PDE*), and GTP-binding protein (*GBP*). Fig. 5: Isolated, fragmented control ROS held overnight at 4°C in normal Ringer's are extensively vesiculated, but show a normal complement of large particles. Bar, 0.1 μm. × 240,000. Fig. 6. Isolated, fragmented ROS stripped of PDE and GBP by a low salt wash (5 mM HEPES, 4°C, 2 h), washed, and resuspended in normal Ringer's are vesiculated, but show no large particles after gently stirring overnight at 4°C. Stripped membranes prepared in this way and thoroughly bleached provided the substrate for all subsequent reconstitutions. Bar, 0.1 μm. × 240,000.

branes is accompanied by the reappearance of a small number of large particles as shown in Fig. 8. In this preparation, surface particles of typical dimension are observed, but their density is low. The corresponding SDS PAGE patterns indicate that the membranes contain some GBP, but no PDE. These results strengthen the suggestion that the surface particles are indeed the GBP molecules.

The reconstitution with PDE was even more difficult. Since PDE is present in fewer copies per rhodopsin than is GBP, a partial reconstitution could go undetected in the electron microscope. To minimize this potential limitation, PDE reconstitution was carried out with a tenfold excess of PDE solution to membranes (see Materials and Methods). In addition, reconstituted membranes were assayed for PDE activity in addition to their SDS PAGE patterns. Results of such experiments are shown in Fig. 9. The SDS PAGE demonstrates that PDE was reincorporated onto the disk membrane surface. The PDE enzymatic activity of these membranes tested in the light was similar to that measured in bleached control membranes (~3 mol cGMP/s/mol rhodopsin). Yet, the electron micrographs did not show any of the typical surface particles. The PDE thus appears to have no correlation with any of the large surface particles.

Effects of Light in Intact Photoreceptors

Kuhn (11, 12) and Kuhn and Hargrave (13) have reported that GBP exhibits a transient light-dependent binding onto isolated bovine rod disk membranes in low osmotic strength medium. The time course of this process is unreported. We undertook to determine whether similar light-dependent movements of GBP occur in intact photoreceptors, by following changes in surface density of the large particles on the extradisk surface upon illumination. In addition to determining whether such protein movement occurs in the intact cell, the quick-freeze technique permits a temporal resolution of this movement. An experimental limitation of our electron microscopy, however, was that only the effects of high intensity illumination could be tested because each area of the replicas viewed had to have received a photon.

By this approach, we found that the particle density does change transiently in the intact photoreceptor following bright flash illumination, but only after a relatively long latency. As shown in Fig. 10, the particle density shows little or no change up to 1 s after the flash. An increase is not detectable until 10 s and reaches a peak by 1 min, at which time it is only ~30% above control levels. Thereafter, the particle density declines and returns to dark values by 10 min after the flash.

DISCUSSION

We have determined the molecular identity of the large particles observed on the CS of disk membranes from quick-frozen intact rod photoreceptors in the toad retina. Lacking specific electron microscope markers, such as antibodies, we have instead employed an indirect approach in which we tested the behavior of a morphological feature under conditions which specifically perturbed known protein components of the disk membrane surface. We have taken advantage of the work of Kuhn (11, 12), Kuhn and Hargrave (13), Godchaux and Zimmerman (6, 7), and Baehr et al. (2), who have described procedures to specifically deplete the disk membrane of individual proteins using combinations of light, ionic strength, divalent ions, and GTP. The two proteins to which these

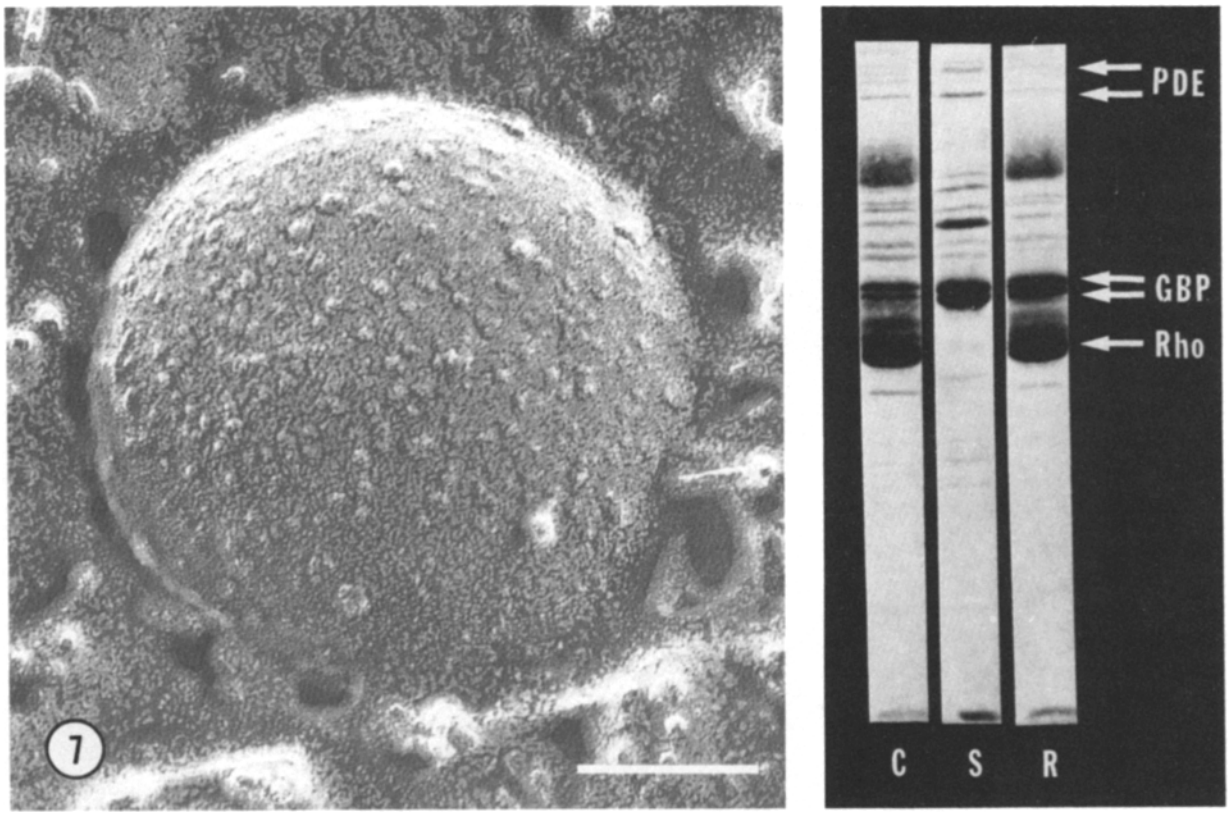


FIGURE 7 PDE and GBP double reconstitution. Restoration of bound GBP and PDE to previously stripped disk membranes was accomplished in two steps. First, a GBP- and PDE-containing supernatant was prepared as for the selective removal experiments (gel *S*). This supernatant was added back to fully bleached stripped membranes, and salt was adjusted to control levels. The membranes-plus-supernatant were gently stirred overnight at 4°C, washed with a large volume of normal Ringer's and membranes were collected by centrifugation. Both proteins bound to the membranes (gel *R*, reconstitution) restoring a fraction of the large particles (micrograph). Control membranes (gel *C*) are shown for comparison. Bar, 0.1 μm. × 240,000.

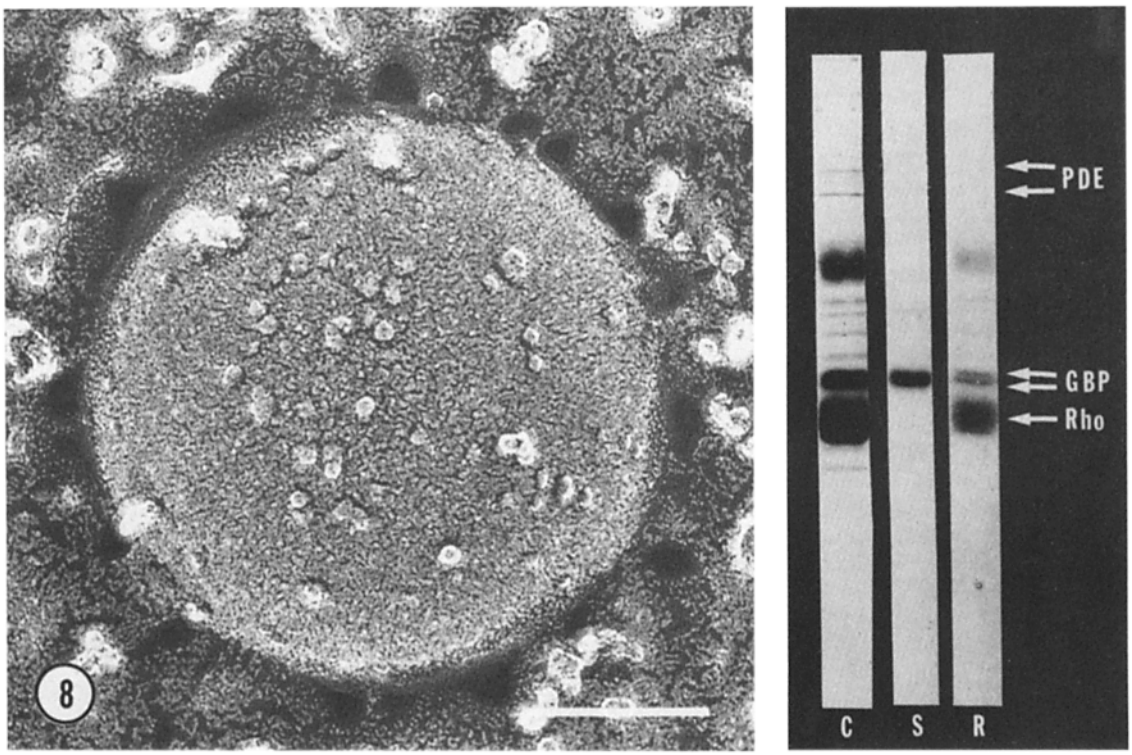


FIGURE 8 GBP reconstitution. A GBP-containing supernatant for reconstitution was prepared as from the "GBP-depleted membranes" above (gel *S*). This supernatant was added to fully bleached membranes previously stripped of PDE and GBP. Salts were adjusted to normal values (with the addition of 5 mM $MgCl_2$) and membranes-plus-supernatant were gently stirred overnight at 4°C. Membranes were washed and collected by centrifugation before rapid freezing. Some GBP was bound to the membranes (gel *R*) and a fraction of the large particles appear on membrane surfaces (micrograph). Bar, 0.1 μm. × 250,000.

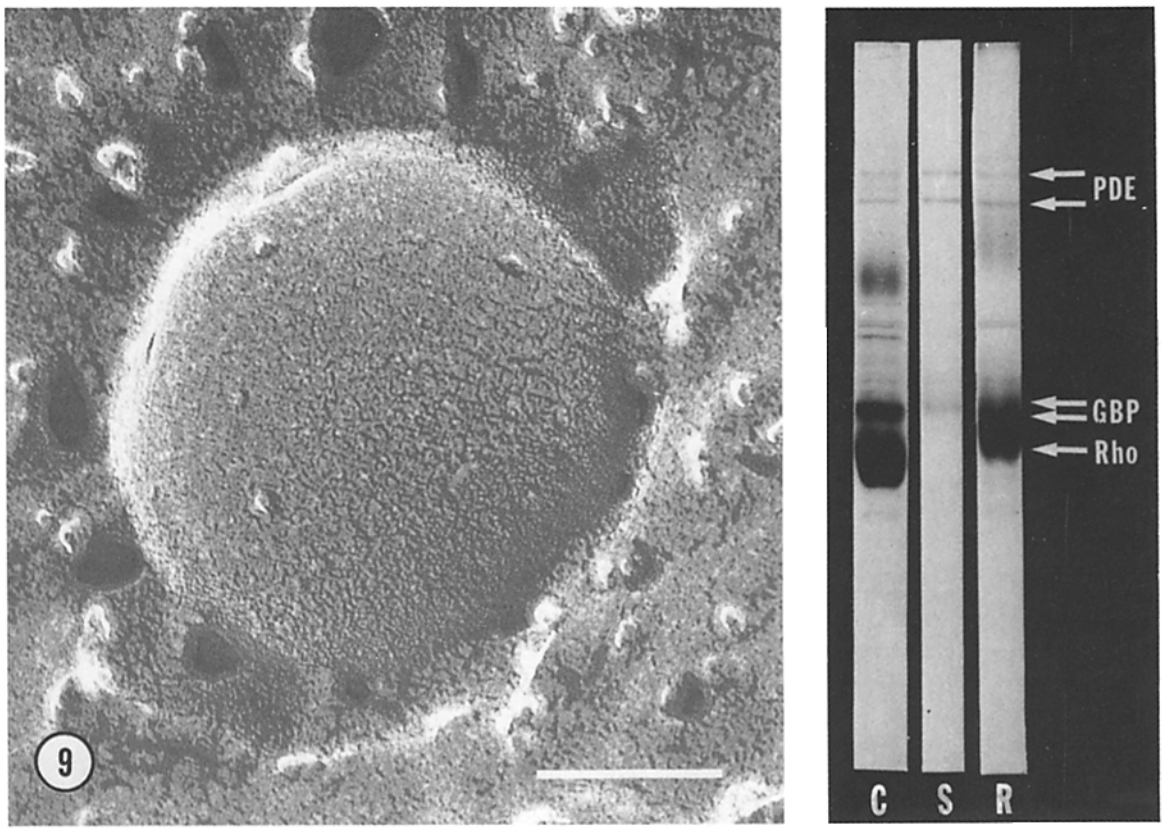


FIGURE 9 PDE reconstitution. A PDE-containing supernatant (gel *S*) was prepared for reconstitution as in "PDE-depleted membranes" above. This supernatant was concentrated tenfold and salts were added back to control values before mixing with bleached, stripped disk membranes. The membranes-plus-supernatant were gently stirred overnight at 4°C, washed and collected by centrifugation. As assayed for activity and from the SDS gel in *R*, at least physiological levels of PDE were bound to the membranes. No large particles were restored to these membranes (micrograph). Bar, 0.1 μm. × 250,000.

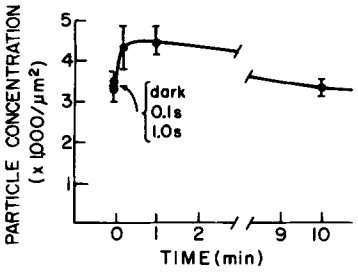


FIGURE 10 Pieces of intact toad retina were rapidly frozen at various intervals (100 ms, 1.0 s, 10 s, and 10 min) after a 50-ms flash which bleached ~80% of the rhodopsin present. Dark adapted, unbleached membranes were rapidly frozen to obtain a control value for the number of large particles present on the cytoplasmic surfaces of disks. Particles were counted as described in the text. Bars represent 95% confidence intervals calculated according to (22). Dark, 100-ms, and 1.0-s values were indistinguishable demonstrating an absolute latency of at least one second for any change in the number of large particles.

techniques are best applied are those in the largest amounts on the disk membrane surface: the light-activated cGMP phosphodiesterase and the GBP. The results of our observations suggest that the large particles are the GBP. The presence or absence of the particles on the disk surface occurs exactly in parallel with the specific depletion or reconstitution of GBP on the disk membrane, but not with that of PDE. Furthermore, the surface density of the particles in the fragmented rod outer segments (1 particle per 11 rhodopsins) is essentially the same as the concentration of GBP determined biochemically. Kuhn (11) has estimated 1 GBP per 13–16 rhodopsins in bovine ROS. The weight estimate of Godchaux and Zimmerman (7) for the subunits of GBP (41,000- and 37,000-mol wt) can be converted to a stoichiometry of 1 GBP per 9 rhodopsins using their values for total protein. Thus, apart from rhodopsin, GBP is the largest single protein component in the ROS and its concentration can fully account for all of the particles observed on the CS.

Biochemical estimates of the amount of PDE present in the rod outer segment are less certain. Baehr et al. (1) place limits on the PDE to rhodopsin ratio of >1:170 and <1:40 and Godchaux and Zimmerman (6, 7) observed a 1:76 stoichiometry (calculated from their raw data). Both estimates are for cattle ROS. None of the large particles in question seem to correlate in their removal or reconstitution behavior with the PDE. This could simply reflect a stoichiometry too low to be observed, though we estimate that random debris deposited on the etched membranes creates a source of uncertainty of about plus or minus 10%. A more likely possibility is that the PDE is represented by membrane particles which are obscured by their size or shape, or that the PDE is localized to some special part of the disk, which is not easily observed with our techniques. This question may be approached in future by immunocytochemistry or reconstitutions in which disks are "overloaded" with PDE.

Having assigned a molecular identity to the large particles, it became appropriate to determine how those molecules are affected by light. A unique advantage of the methods we have used is that the effects of light could be studied in intact retinas. Large particles are observed on the CS of rod disks in slightly higher density in intact retinas than in isolated membranes.

The observed increase in the density of these large particles in intact retina following a bright flash probably reflects an increase in the number of membrane bound GBP molecules. This is in general agreement with biochemical data obtained by others in isolated ROS. Kuhn and Hargrave (13) demonstrated two types of binding to isolated bovine rod disk membranes. They report the binding of an ionic strength dependent (light independent) fraction of GBP molecules. This component is probably represented by the large number of particles bound to the membrane in the dark in the intact rod. In addition, Kuhn (11, 12) shows large changes in GBP binding after exposure to light, if the isolated ROS are subjected to low ionic strength medium. The increased binding after light and reversal of this effect at long times after bleaching (GBP spontaneously unbinds after 70 min, at 20°C) agrees with our observations in intact retina. However, quantitatively we observe a much more modest light effect on binding in intact retinas; a maximum of 1.3-fold increase in intact retina vs. Kuhn's almost 50-fold increase in low salt ROS. In addition, we have resolved temporally both the light-induced binding and its reversal under physiological conditions. We observed a latency of at least 1 s for the light-induced binding and place an upper limit of 10 min (as compared with Kuhn's 70 min) on its return to dark levels.

The physiological significance of these observations is not yet clear. Although the role of PDE and GBP in initiating phototransduction is still controversial (8–10, 18, 19), certainly a latency of one second at saturating light levels would rule out the recruitment of GBP from solution as a factor in initiating the photoresponse. Biochemical events with a long latency and slow time course are much more likely to be related to GBP binding. For instance, Robinson and Hagins (20) reported a light-induced drop in GTP concentration which exhibits a 4-s latency and 10-s halftime in isolated ROS. Biernbaum and Bownds (3) observed a light-induced drop in GTP in isolated ROS with a halftime of 7 s. Since Kuhn (11, 12) and Godchaux and Zimmerman (7) have shown that increasing GTP concentration leads to release of GBP from isolated ROS membranes, it is possible that changes in GTP concentration within the ROS in intact retina may also account for the light-induced change in particle binding reported here.

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