Sensitive light scattering probe of enzymatic processes in retinal rod photoreceptor membranes

(vision/guanosine triphosphate-binding protein/phosphodiesterase/protein phosphorylation/rhodopsin)

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ABSTRACT   Light excitation of as little as 0.05% of the rhodopsin in a retinal rod membrane suspension reduces the near-IR optical transmission by 25%. This transmission decrease requires the presence of guanosine triphosphate, is opposite in sign and 25 times larger in amplitude than a GTP-dependent light-scattering signal previously reported in rod outer segment suspensions [Kuhn, H., Bennett, N., Michel-Vallez, M. & Chabre, M. (1981) Proc. Natl. Acad. Sci. USA, 78, 6873–6877], and is kinetically complex. The initial phase of the optical transmission decrease begins after about a 50-ms lag (at 0.05% bleach) and has a first-order time constant of 300–500 ms. The scattering signal returns to the preactinic baseline in a time dependent on the amount of GTP added. A nonhydrolyzable GTP analogue, guanylyl imidodiphosphate, produces a scattering signal that does not return to the preactinic baseline. Adenosine triphosphate strongly inhibits the return of the GTP-dependent transmission decrease to the preactinic baseline. This effect of ATP on the GTP signal apparently requires ATP hydrolysis because it is inhibited by the simultaneous presence of adenylyl imidodiphosphate, a nonhydrolyzable analogue of ATP. The light-scattering signal and the velocity of the activation of a rod outer segment phosphodiesterase saturate when >0.05% of the rhodopsin is bleached and both show nearly identical dependence on light stimulus. It is suggested that these nucleotide-dependent light-scattering signals arise from changes in the state of membrane aggregation that are controlled by enzymatic processes. This hypothesis is supported by the large amplitude of the signals, sedimentation experiments, and a strong membrane concentration dependence. The ATP effects can be rationalized within the above hypothesis as being due to ATP-dependent rhodopsin phosphorylation that adds negative charges to the membrane surface and tends to keep the membranes disaggregated. An additional signal, which increases light transmission, is produced by a second, much more intense flash. The latter signal is interpreted as the result of proton binding by bleached rhodopsin molecules that decreases the negative charge repulsion between the membranes and allows increased aggregation.

Retinal rod outer segments (ROS) contain several proteins, other than the visual pigment rhodopsin, whose activities are modulated by light (1–4). The fact that these proteins are light activated makes it likely that they participate in the process of visual transduction or light adaptation. It is clear that an active form of bleached rhodopsin, R*, catalyzes the binding of GTP to a GTP-binding protein (3, 5–7). Once GTP is bound, the binding protein then activates a phosphodiesterase (PDE) until the bound GTP is hydrolyzed (1, 8). R* catalyzes the binding of GTP to a large number of GTP-binding protein molecules until it is inactivated by either slow thermal processes or faster phosphorylation by rhodopsin kinase (ref. 9; unpublished data).

Kuhn et al. (10) have shown that certain of these light-activated processes in ROS can be followed by light-scattering measurements. Hofmann et al. (11) had previously observed two photoinduced near-IR light-scattering signals in ROS. One of them, the N signal, was a negative change in apparent absorbance and had the same kinetics as the metarhodopsin I–metarhodopsin II transition of photoactivated rhodopsin. The other light-scattering change, the P signal, was a positive change in apparent absorbance with more complex kinetics than the N signal. Treatment with hypotonic salt completely removed the P signal but did not affect the N signal. Kuhn et al. (10) found that the P signal is associated with the binding of the GTP binding protein to R* and, in addition, that there is a second negative change in apparent absorbance in the presence of GTP, which they attribute to release of GTP binding protein from the membrane.

The light-scattering signals promise to be useful in studying enzymatic processes involved in visual transduction, especially since similar signals have been observed in the whole living retina (12). Two major problems, however, need to be overcome to fully exploit the potential of these signals. First, the near-IR light-scattering signals reported before are quite small (parts per thousand in transmittance), making them difficult to use as a probe. Second, the physical origin of the coupling between the biochemical events and the light-scattering signals is obscure. We have found conditions that give ≈25 times larger amplitude light-scattering signals, thus providing a much more sensitive probe of nucleotide-mediated reactions in ROS. In addition, we have formulated a hypothesis for the physical origin of these large-amplitude signals and here we present evidence in support of this hypothesis.

METHODS

Bovine ROS were prepared as described (13) except for use of Tris-buffered saline (Tris/NaCl, 10 mM Tris·HCl/60 mM KCl/30 mM NaCl/2 mM MgCl₂/1 mM fresh dithiothreitol/0.1 mM EDTA, pH 7.0) instead of low-salt Tris and replacement of the continuous sucrose gradient centrifugation with a step float (13K, Sorvall HB-4) from 32.5% sucrose in Tris/NaCl overlaid with Tris/NaCl. ROS were collected (avoiding the sucrose below the band), pelleted, and suspended in light-scattering buffer (10 mM Tris·HCl/100 mM KCl/1 mM MgCl₂/1 mM dithiothreitol, pH 7.3) to a rhodopsin concentration of 5 mg/ml. Sonicated samples were prepared using the 3-mm microprobe of a well-tuned Branson W-140 sonicator at a power level of 4 (actual power input to sample, 1.3 W). During sonication, samples were held in a conical centrifuge tube, under argon, in an ice bath.

The apparatus used for the light-scattering measurements

Abbreviations: ROS, rod outer segments; PDE, phosphodiesterase; Tris/NaCl, Tris-buffered saline; p[NH]ppG and p[NH]ppA, guanylyl and adenylyl imidodiphosphate, respectively.
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was similar to one previously described (13). Two irises were added to limit the cone of light incident on the sample to ±2° and that viewed by the detector to ±3°. Light scattering was monitored as change in transmittance of 730-nm light (isolated with a 10-nm band-pass interference filter). Samples were excited using a 6-ns pulse at 457 nm from a Molectron DL200 dye laser in all experiments except those in which the response was followed as a function of stimulus intensity. In the latter, 570-nm light was used to obtain more uniform penetration of the sample and thus produce more uniform rhodopsin bleaching.

A 2-mm-path-length cell was used in most of the light-scattering measurements. The short path length allowed experiments to be conducted at protein concentrations an order of magnitude higher than those used in previous light scattering studies and hence closer to those found *in vivo*. The short path length also improved overlap between focused actinic and monitoring beams that were used in the nearly coincident (20°) beam geometry. The amount of bleaching caused by the actinic flash was measured by removing neutral density filters and delivering a sufficient number of flashes (with mixing in between) to produce a spectrophotometrically measurable amount of bleaching. The bleaching calibration assumed a constant beam intensity profile. This approximation is accurate to within a factor of 2. For 1-cm-path-length measurements, the actinic beam was not focused to improve beam overlap throughout the cell. All light-scattering measurements were carried out at 20°C.

Light activation of PDE was assayed by monitoring the pH change caused by hydrolysis of cGMP (14, 15) as described (unpublished data). Aliquots of the same ROS suspension (both sonicated and unsonicated) used for light scattering were diluted with light-scattering buffer to 0.5 mg/ml rhodopsin. The solution contained 50 μM GTP, 1 mM ATP, and 1 mM cGMP and was allowed to equilibrate to room temperature in complete darkness. The linear drift in pH associated with "dark" PDE activity was recorded for 1 min so that it could later be subtracted from the light-dependent PDE activity. In these experiments, rhodopsin was stimulated using a 1 msec xenon flash using calibrated neutral density filters to adjust the intensity to the desired levels.

RESULTS

After flash excitation of an ROS membrane suspension under our typical conditions, we found a large light-dependent decrease in transmission in the near infrared (Fig. 1). The signals shown are from mildly sonicated suspensions and are in response to a stimulus intensity above saturation (>0.05% of rhodopsin bleached). Typical maximum amplitudes represent a decrease in transmitted light of ≈25%. The large amplitude change requires the addition of GTP and the duration of the deflection increases with increasing concentration of GTP (Fig. 1). Low concentrations (≥15 μM) of a nonhydrolyzable GTP analogue, guanylyl imidodiphosphate (p[NH]ppG), produce a long-duration light-induced signal of maximum amplitude that does not return to baseline within 20 min.

In the absence of GTP, we observed a small-amplitude signal with characteristics similar to the P signal of Hofmann *et al.* (12) (Fig. 1 *Inset*). The prominent feature of the response under our conditions is the large increase in amplitude and in initial rate of this positive-going signal with the addition of GTP or p[NH]ppG (Fig. 1 and *Inset*). This result is in marked contrast to the observations of Kuhn *et al.* (10), who found a reversal of sign of the signal on addition of GTP and did not see the dramatic increase in signal amplitude. We refer to the transient light-scattering signal we observe in the presence of GTP as the G^+ signal (GTP-dependent positive extinction change) and that observed by Kuhn *et al.* (10) as the G^- signal (GTP-dependent negative extinction change). If a second flash of the same intensity is delivered after the G^+ signal has returned to the baseline, we find a P signal like that observed in the absence of GTP.

The duration of the G^+ signal is consistent with the known kinetics of light-induced GTP hydrolysis. Ascribing the return of the G^+ signal to the preactinic level to GTP hydrolysis, we obtain a value of 3.5 ± 1.1 min^{-1} for the GTP turnover number (see *Discussion*). This result is comparable with previously reported rate constants for the GTPase in bovine rods (5–7, 16).

The G^+ signal saturates at low light intensities comparable with those that saturate the light-stimulated PDE activation. The relative amplitudes of the G^+ light-scattering change and velocities of cGMP hydrolysis in response to light flashes that excite from 0.0001% to 1% of rhodopsin present are shown in Fig. 2. (All data in Fig. 2 are derived from the same sonicated preparation.) The close match of the saturation curves suggests that the G^+ signal originates from phenomena that may be closely related to those responsible for PDE activation.

When we attempted to reproduce the conditions used by previous workers to study near-IR light scattering (10, 11) we observed comparable signals. The main differences in our conditions, compared with those of previous workers, appear to be our use of higher membrane concentrations in a shorter path-length cell and in the degree of disruption of the ROS preparations. In experiments using a 1-cm-path-length cell and order of magnitude lower concentrations of ROS
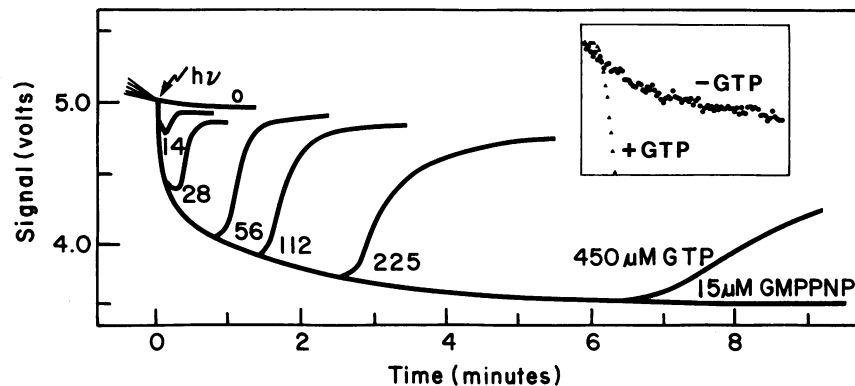

FIG. 1. Effect of actinic light stimulation on near-IR light scattering as a function of GTP concentration. The samples were incubated with the indicated concentration of GTP for 2 min prior to actinic stimulation, which bleached 0.6% of the rhodopsin, at the time shown by the arrow. The preactinic light level gave a signal of 5 V. An increasing downward slope in the preactinic baseline was observed as the GTP concentration was increased. (*Inset*) Early portions of two traces on an expanded scale (height of box, 50 mV; width of box, 600 ms). Signals with 0 and 112 μM GTP are compared. The time of the light stimulus coincides with the left edge.
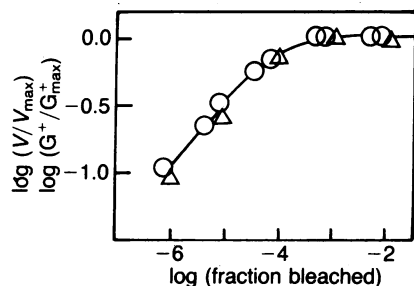
FIG. 2. Comparison of PDE activation (△) and G^+ light-scattering signal (○) as a function of actinic light intensity. Results are plotted as log(PDE velocity/PDE maximum velocity) or as log(G^+ signal amplitude/G^+ maximum signal amplitude) vs. log(fraction bleached).

membranes, which had been passed through a fine syringe needle, we observed the N and P signals reported by Hofmann *et al.* (11) as well as the G^- signal reported by Kuhn *et al.* (10). These signals all have the same sign as well as approximately the same kinetic behavior and relative light-saturation levels as originally reported

The degree of disruption of the ROS plays a role in determining the size of the G^+ signal. As shown in Fig. 3, ROS disrupted by passage through a syringe needle and observed at our typical concentrations yield G^+ signals of much lower amplitude than found under our optimum conditions, and the amplitude of the G^+ signal is strongly dependent on the extent of sonication of the ROS suspension. Initially, as the ROS are disrupted by sonication, the G^+ signal increases. Further sonication, however, attenuates the signal. Note that the photolysis level in Fig. 3 is a factor of 10 above saturation, so the reduced signal amplitude at high optical densities is not merely due to reduced penetration of actinic light.

Disks that had their extrinsic membrane proteins removed by hypotonic washing show no light scattering signal except for the small N signal observed by others under these conditions (11). If the extrinsic membrane proteins are reassociated with the hypotonically washed disk membranes (by addition of 1 mM $MgCl_2$/100 mM KCl), the G^+ signal and all light-scattering signals observed in isolated ROS are restored. Intact disk membranes, produced by dialysis (17) or hypotonic (1:10) shock followed by reassociation of extrinsic membrane proteins, produce signals comparable in size with those obtained in optimally sonicated preparations.

Addition of 2 mM $CaCl_2$ or 2 mM $MgCl_2$ to samples increased the transmitted intensity in the dark by 20% and the amplitude of the G^+ signal by ≈30%. Divalent cations are
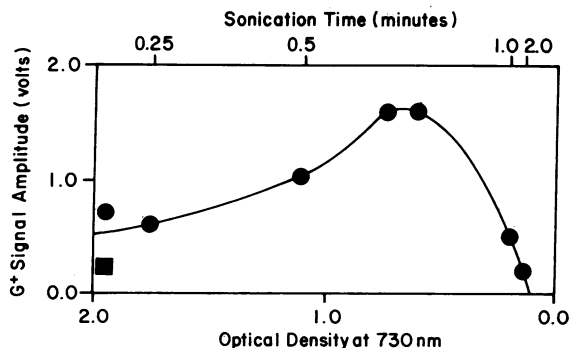

FIG. 3. Amplitude of the G^+ signal as a function of sonication time. Round points represent samples sonicated for the times shown. The optical density at 730 nm was measured. The solid square shows the size of a G^+ signal for a sample that was disrupted by passage through a 20-gauge syringe needle and not sonicated. All samples were 125 μM rhodopsin and 150 μM GTP, and all other conditions were as in Fig. 1.

known to aggregate disk membranes (18). Thus, more-aggregated states increase the optical transmittance of our samples. Because the G^+ signal is so large, we hypothesize that it is due to a change in membrane aggregation. To test this hypothesis, the sedimentation properties of samples containing 750 μM GTP that had been 0.15% bleached shortly before centrifugation were compared with those of similar samples that were unbleached. Both sets of samples contained 125 μM rhodopsin. After 15 s of centrifugation at 6,000 × *g* in an Eppendorf centrifuge, an aliquot was taken from the top of each tube and spectra were recorded in 3% Ammonyx LO to determine rhodopsin content. The pellets and the remainder of the material in the tubes were resuspended in Ammonyx LO, and a second set of spectra were recorded. About 50% more rhodopsin was found in the supernatant fraction of the bleached sample than in the dark control, consistent with the hypothesis that membrane disaggregation is the source of the G^+ signal.

The G^+ signal was strongly modified by the presence of ATP. Light-scattering signals in the presence of ATP alone were small in comparison with the G^+ signal (Fig. 4). Addition of ATP in the presence of GTP, however, resulted in large G^+ signals that did not return to the preactinic baseline. Addition of a low concentration of adenylyl imidodiphosphate (p[NH]ppA), a nonhydrolyzable analogue of ATP, prior to ATP addition counteracted the effect of ATP and restored the reversible nature of the G^+ signal. By itself, p[NH]ppA had little effect on the G^+ signal (data not shown). Addition of GDP and ATP in the absence of GTP produces a transient G^+ signal in contrast to the permanent deflection produced by GTP and ATP.

After a sonicated ROS sample containing GTP and ATP has undergone its full light-induced light-scattering deflection (e.g., at 0.6% rhodopsin bleached), a second, more intense flash (e.g., 6% rhodopsin bleached) causes a light-scattering deflection of opposite sign (Fig. 4). This is not the conventional N signal because the amplitude is nearly 100-fold too large—i.e., nearly as large as the G^+ signal.

DISCUSSION

The light-scattering signals described above exhibit several important features. First, the G^+ signal is very large, shows high light sensitivity, and requires GTP as well as extrinsic membrane proteins. Second, there is a close correspondence between the G^+ signal and PDE activation in terms of response-saturation behavior. Third, the concentration of p[NH]ppG that produces the maximum amplitude G^+ signal is consistent with the K_m of the ROS GTP-binding protein either measured directly (5, 6) or by PDE activation (1, 19). Fourth, the scattering signals show sensitivity to perturbations affecting enzyme activities and membrane charge distributions, such as ATP, p[NH]ppA, or additional light flashes. Fifth, there is a consistency of all measurements to date with a simple model for the mechanism of the G^+ light-scattering signals.

The G^+ light-scattering signal is absolutely dependent on the presence of GTP or a suitable GTP analogue. The G^+ signal returns to the preflash level in a time that increases in proportion to the amount of added GTP (Fig. 1). If we assume the return of the G^+ signal is due to GTP hydrolysis, we can calculate the turnover number for GTP hydrolysis from the G^+ signal duration, Δt, using

$$\text{turnover number} = \frac{([GTP]_{added})}{[\text{GTP binding protein}] \, \Delta t}.$$

The concentration of GTP-binding protein is taken to be 1/10th the concentration of rhodopsin (20), and the flash is assumed to activate all of the GTP-binding protein present. The value obtained for the rate is 3.5 ± 1.1 min^{-1} (average of
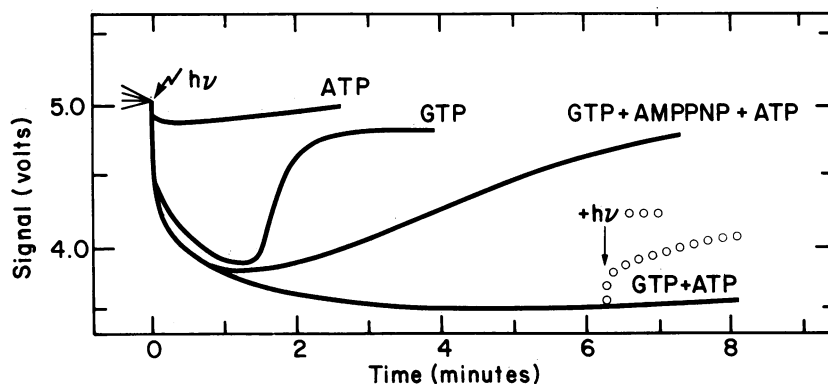
FIG. 4. Effect of ATP and GTP on IR light-scattering signals of ROS membrane suspensions induced by actinic flashes. A stimulus flash bleaching 0.6% of the rhodopsin was applied at the upper left-hand arrow. Signals correspond to 3.2 mM ATP alone (upper curve), 112 μM GTP alone (second curve from top), simultaneous presence of 112 μM GTP and 3.2 mM ATP (lowest curve), and 112 μM GTP/7.5 μM p[NH]ppA (AMPPNP)/3.2 mM ATP (second curve from bottom). A second, more intense flash (6% rhodopsin bleached) partially reversed the ATP-maintained deflection ($\circ\circ\circ\circ\circ$).

six experiments). This corresponds closely to the rate of hydrolysis of GTP found by others for the light-activated GTPase in broken bovine rods. Bennett (7) found a rate of 1–3 min^{-1} for the GTPase, Kuhn (16) found 1 min^{-1}, Godchaux and Zimmerman (5) found 2–4 min^{-1}, and Fung *et al.* (6) found 2 min^{-1} for the bovine enzyme. [It is possible that this rate is faster in more intact systems such as those studied by Biernbaum and Bownds (21) and Robinson and Hagins (22), both of whom found a 3–10 times faster light-dependent GTPase rate in more intact frog rods.] Dark GTPase activities were measured by incubating the sample with GTP for various times prior to light excitation, which decreased the duration of the G$^+$ signal in a linear manner and indicated that the velocity of the dark GTPase is $\approx$25% of the total rate. The nonhydrolyzable GTP analogue, p[NH]ppG, supports the G$^+$ signal with a K_m <10^{-5} M, which is consistent with the reported K_m of GTP-binding protein binding p[NH]ppG (5, 8, 19, 20).

The G$^+$ signal also depends absolutely on the presence of extrinsic membrane proteins. If the extrinsic membrane proteins are washed from the disk membrane by hypotonic conditions (3), the G$^+$ signal is abolished. If extrinsic membrane proteins are partially lost by extensive breakage of the rods during preparation, the amplitude of the G$^+$ signal is attenuated. If the extrinsic membrane proteins are restored to the disk membranes from which they had been removed, the G$^+$ signal (as well as all the other phenomena reported in this paper) is restored.

The G$^+$ signal is enhanced by either 2 mM Ca^{2+} or Mg^{2+} ions, both of which increase aggregation of bovine rod disk membranes (18). When salts aggregate rod disk membranes, the optical transmission increases (with our detection geometry). These facts suggest that the G$^+$ signal arises from temporary disaggregation of membranes. This suggestion is supported by the sedimentation experiments in which there is a GTP- and light-dependent decrease in membrane sedimentation. These experiments are consistent with our interpretation that the G$^+$ signal is due, at least in part, to changes in membrane aggregation.

In most cases, we used comparatively light sonication and found that, although sonication affected the amplitude of the G$^+$ signal, it did not affect the duration. Thus the enzymatic activities responsible for the scattering signal are not disrupted by sonication. In addition, other enzymatic processes known to occur in the ROS (e.g., the light-initiated activation of the PDE- and ATP-dependent quenching of PDE activation) are still observed in the sonicated membranes.

The GTP-binding protein is known to show light-dependent release from the ROS membrane in the presence of GTP

and low salt (e.g., 5 mM Tris·HCl) (3). However, it is unclear whether GTP-binding protein is released from the membrane by light and GTP under the near isotonic salt conditions that we used. Godchaux and Zimmerman (5) have reported that the GTP-binding protein can be released by GTP in 100 mM KCl. Kuhn (3) determined that the α subunit of the GTP-binding protein is released by light in 2 mM ATP/1 mM GTP/5 mM MgCl$_2$/130 mM KCl/20 mM NaCl/8 mM Tris·HCl. Our buffer contains 1 mM MgCl$_2$/100 mM KCl/1 mM dithiothreitol/10 mM Tris·HCl, pH 7.3, and comparison with Kuhn's higher ionic strength conditions suggests that light- and GTP-induced release of GTP binding protein could be significantly enhanced under our salt conditions. Therefore, it is likely that the GTP-binding protein, or at least the α subunit, is released from the disk membrane in the light and in the presence of GTP under our conditions.

Could the release of the GTP-binding protein from the membrane surface cause a change in membrane aggregation? Both the α and β subunits have a net negative charge at neutral pH (20). It has been proposed that the cytoplasmic face of rhodopsin is positively charged (23) with a net charge of +4 to +6 (24). Phosphatidylserine is the only quantitatively significant lipid with a net charge, and evidence has been presented that 8–10 phosphatidylserines per rhodopsin are on the cytoplasmic membrane surface (25). The phosphatidylserine would contribute a negative charge of −8 to −10 to the cytoplasmic membrane surface per rhodopsin. Therefore, the cytoplasmic surface of the rod disk membrane is expected to be net negative with a charge of −2 to −6 per rhodopsin. There are reports that attachment of GTP-binding protein to the membrane involves divalent cations (3, 26). If divalent cations mediate membrane attachment of GTP-binding protein (as they mediate PDE binding), then light-induced GTP-binding protein release could well lead to an increase in negative charge on the membrane surface. This could result in membrane disaggregation that would give rise to the optical transmission decrease of the G$^+$ signal.

An attractive feature of attributing the light-scattering signals to changes in membrane aggregation state is that all our experimental data, including the effects of ATP, p[NH]ppA, and large bleaching flashes, are consistent with a model in which changes in membrane surface charge affect the state of membrane aggregation. In this model, the ATP effect is attributed to phosphorylation of rhodopsin, which would increase the net negative charge of the membrane surface. Phosphorylation would tend to stabilize the disaggregated state that we hypothesize is generated by GTP and light. An alternative possibility that has been considered is that nucle-

oside diphosphate kinase prolongs the signal by regenerating GTP from GDP by using ATP as a phosphate donor. Some nucleoside diphosphate kinase activity is present, but it clearly is not sufficient to sustain an extended G^+ signal. Little light-scattering change is seen in the presence of ATP and the absence of GTP. The facilitation of the ATP effect of GTP is consistent with the observed stimulation of phosphorylation by GTP (unpublished data; 27). This facilitation was interpreted as due to competition of GTP-binding protein and rhodopsin kinase for overlapping binding sites on rhodopsin (unpublished data). In this context, p[NH]ppA could act by inhibiting the rhodopsin kinase. Similar explanations have been offered for certain light-scattering changes in chloroplast thylakoid membranes attributed to membrane protein phosphorylation that disaggregates membrane grana stacks (28, 29).

The signal reversal found with a second, more intense actinic flash is attributed to a decrease in net negative charge on the membrane by light-induced proton uptake in forming metarhodopsin II (30, 31). Proton uptake changes the surface charge of the ROS disk membrane (31) and this phenomenon has been observed to occur in our preparations (unpublished). It is likely that the more intense actinic flash is necessary to observe the reversed signal because each bleached rhodopsin appears to bind at most a single proton. Therefore, a large excess of beached rhodopsin would be required to overcome the opposite charge effect of a small number of multiply phosphorylated rhodopsins produced by the first flash (unpublished data). The large excess of bleached rhodopsins is only slowly phosphorylated, presumably because of limited kinase (unpublished data).

We have not excluded the possibility that vesicle-shape changes contribute to the light-scattering signals reported here. However, the large amplitude of the signals, together with their concentration and salt dependence and the light-dependent sedimentation changes, supports the hypothesis that the G^+ signal is due to disaggregation of the membrane vesicles. It is difficult to imagine any other mechanism (e.g., direct optical effects of surface protein binding) that could cause such large-amplitude signals.

It is likely that the light-scattering signals we have studied are relevant to processes in the intact photoreceptor since Harary *et al.* (12) have observed similar changes in near-IR light scattering in living retina. Furthermore, Chabre and Cavaggioni (32) have observed flash-induced increases in disk membrane spacing disorder in the ROS of living retinas by using time-resolved x-ray diffraction. These changes in membrane spacing disorder reverse on the approximate time scale of the GTP-dependent light-scattering signal and seem consistent with changes in disk–disk interactions. It thus seems that changes in near-IR light-scattering offer a sensitive continuous probe of light- and nucleotide-dependent processes in retinal rod membranes.

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