Light Activation of One Rhodopsin Molecule Causes the Phosphorylation of Hundreds of Others

A REACTION OBSERVED IN ELECTROPERMEABILIZED FROG ROD OUTER SEGMENTS EXPOSED TO DIM ILLUMINATION*

(Received for publication, March 9, 1990)

Brad M. Binder†, Michael S. Biernbaum§, and M. Deric Bownds¶

From †The Neuroscience Training Program, §Laboratory of Molecular Biology, and ¶Department of Zoology, University of Wisconsin, Madison, Wisconsin 53706

A rhodopsin phosphorylation reaction that occurs with high-gain is observed if measurements are made in electropermeabilized frog rod outer segments (ROS) stimulated by a dim flash of light in the operating range of the photoreceptor. Flashes of light exciting 1000 or fewer of the $3 \times 10^6$ rhodopsins present/ROS result in the incorporation of 1400 phosphates from ATP into the rhodopsin pool for each excited rhodopsin (Rho*). This amplification decreases with increasing light intensity, falling most sharply after each disk has absorbed one photon. The high-gain reaction is lost if the ROS are broken into vesicles by shearing, leaving a low-gain rhodopsin phosphorylation characterized in previous studies using brighter illumination. The high-gain but not the low-gain phosphorylation appears to be regulated by G-protein and by calcium levels in the range over which intracellular calcium changes when rod photoreceptors are illuminated. Kinetic measurements made on the phosphorylation observed at higher light intensities shows that it initially occurs rapidly enough for a role in terminating the photoresponse. The high-gain phosphorylation observed at lower light intensities may play a global role in regulating light-adaptation of the rod photoreceptor, and its existence suggests that a search for a similar high-gain modification in systems using the homologous β-adrenergic or muscarinic acetylcholine receptors might be rewarding.

When vertebrate rod photoreceptors are illuminated, photoisomerized rhodopsin (Rho*)† serially activates many G, (transducin) molecules which in turn stimulate cGMP hydrolysis by activating cGMP-phosphodiesterase (PDE). The consequent reduction in levels of cGMP causes the closure of plasma membrane channels that are gated by this ligand. Illumination also causes phosphorylation of rhodopsin by a kinase (Bownds et al., 1972; Kuhn and Dreyer, 1972; Frank et al., 1973) with an upper limit of nine phosphorylation sites/phorotivated rhodopsin (Wilden and Kuhn, 1982; Aton et al., 1984). The prevailing idea has been that illumination does not activate rhodopsin kinase but that light exerts its effect on rhodopsin to make its phosphorylation sites available (Kuhn et al., 1973, Frank et al., 1973, Frank and Duszyn, 1975; Weller et al., 1975). It has been shown that unactivated G, bound to activated rhodopsin inhibits rhodopsin phosphorylation (Kelleher and Johnson, 1988). However, the original report of rhodopsin phosphorylation in frog (Bownds et al., 1972) as well as later studies from several labs (Bownds et al., 1974; Miller and Paulsen, 1975; Miller et al., 1977; Sitaramayya and Liebman, 1983b; Aton, 1986) suggest that non-excited rhodopsins can also be phosphorylated. In addition, recent studies raise the possibility that bleaching of rhodopsin leads to activation of rhodopsin kinase (Fowles et al., 1998; Palczewski et al., 1989). One purpose of this study has been to determine how many rhodopsin molecules that have not absorbed a photon can be phosphorylated. We have examined this question in electropermeabilized preparations of ROS, which are known to retain more of their protein compliment than cells disrupted by other methods (Gray-Keller et al., 1990). The preparation has been previously used to study other protein phosphorylation reactions in rods (Rinder et al., 1988). After finding a high-gain rhodopsin phosphorylation reaction with dim flashes of light we have proceeded to characterize its calcium sensitivity and relation to G, activation.

Another purpose of this work has been to evaluate the prevailing hypothesis regarding the role of rhodopsin phosphorylation: that it decreases the affinity between rhodopsin and G, and thus is capable of causing the rod photoresponse to turn off (Sitaramayya et al., 1977; Shichi et al., 1984; Arshavsky et al., 1985; Aton and Litman, 1984; Miller and Dratz, 1984; Miller et al., 1986; Wilden et al., 1986; Sitaramayya and Liebman, 1983a; Sitaramayya, 1986). It may require as little as 1 or 2 phosphates/Rho* in the presence of arrestin to maximally quench the interaction between rhodopsin and G, (Bennett and Sitaramayya, 1988). The hypothesis requires that a bleached rhodopsin acquire at least 1 phosphate group by 1 s after illumination. While one report has suggested that rhodopsin can be phosphorylated within several s after illumination (Sitaramayya and Liebman, 1983b) and another has shown that by 15 s after illumination there can be an increase of approximately 2 phosphates/Rho* (Miller and Dratz, 1984), the stoichiometry has not been measured at early enough time points after illumination to determine if it is sufficient to be an underlying mechanism of response termination. We find that rhodopsin phosphoryls-
tion at high light levels is fast enough and of sufficient gain at early times to play this role.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP was obtained from Du Pont-New England Nuclear. Percoll was bought from Pharmacia LKB Biotechnology Inc., Piscataway, N.J. [πH 7.5](Rana catesbiana) or Rana grylio) as described previously (Biernbaum and Bownds, 1985). Briefly, retinas were isolated from dark-adapted frogs, gently shaken in 5% Percoll-Ringer’s solution, and supernatant withdrawn. The retinas were shredded with fine forceps and particles allowed to settle. The supernatant from this process was pooled with the supernatant from shaking the retinas, layered on Percoll-Ringer’s solution (the same ionic constituents with 75 mM potassium isethionate, 15 sodium isethionate), and centrifuged at 10,000 rpm for 5 min. The lowest of the three bands typically contained soluble and peripheral proteins were separated from integral membrane proteins by centrifugation (Hamm and Bownds, 1986). All samples were quenched with 200 μl of 10% trichloroacetic acid in the dark or 4 min after calibrated illumination when maximum phosphorylation had occurred (see “Results” below). They were then removed, cut into 0.5-cm sections, solubilized, and radioactivity measured as above. The pH gradient in the gel was determined by running a nonradioactive sample in parallel with the radioactive samples, slicing as above, and soaking each section in distilled water for 1 h before determining the pH with a pH electrode. The gradient of the gel using these methods was from approximately pH 4.0 to 6.5.

**Protein Phosphorylation**—Intact rod ROS were diluted with Percoll-Ringer’s solution and gently centrifuged for 20 s. The pellet was resuspended in intracellular medium and for most experiments cells were electrophoreinized in a chamber 6 mm on a side as described previously (Binder et al., 1989) with three 5000 V/cm pulses, each of which took 2 ms to decay 50% of the initial discharge. This yielded leaky cells as determined by their inability to exclude the fluorescent dye dyesulfonyl cyanine (Yoshikami et al., 1974). All manipulations were carried out in infrared illumination, and observed by using an infrared image converter.

**Results**

High-gain Rhodopsin Phosphorylation Is Observed at Low Light Intensities—Previous studies from this lab have indicated that the gain of light-induced rhodopsin phosphorylation, the number of phosphate groups incorporated into opsin protein for each rhodopsin bleached by light, can increase as lower levels of light are used (Bownds et al., 1972, 1974; Miller et al., 1977). To determine the maximum amplification that can occur, we examined the reaction at low light levels in electrophoreinized ROS. This preparation was used because it retains 3-fold more protein than ROS broken by shearing and is the most intact preparation currently available that will permit the access of [γ-32P]ATP used to monitor the reaction (Gray-Keller et al., 1990). Fig. 1A shows the stoichiometry of phosphate incorporation into the rhodopsin pool as a function of rhodopsin bleached, observed 4 min after a calibrated flash is delivered. At the lowest intensities tested (<1000 Rho*), ~1400 (±450) phosphates are incorporated into the rhodopsin pool per Rho*. Light intensities bleaching less than 600 rhodopsin molecules could not be examined because the light-induced phosphorylation could not be distinguished from background phosphorylation that occurs in
High-gain Rhodopsin Phosphorylation at Dim Illumination

Fig. 1. High stoichiometry of rhodopsin phosphorylation and total incorporation of phosphate into the ROS. A, maximum incorporation of phosphate into the rhodopsin pool per Rho* (gain); and B, total incorporation of phosphate per ROS are shown as a function of flash intensity. In A, the solid line is drawn from a binomial probability equation assuming a maximum gain of 1600 phosphates/Rho* and 2000 independent targets. The dashed line is from the same equation assuming 4000 independent targets (see Gray-Keller et al., 1990). In B, the solid line is drawn by hand and the dashed line is a conversion of the solid line in A to absolute signal rather than gain. Phosphorylation is monitored with $[^{32}\text{P}]$ATP in electroporinized ROS incubated in intracellular assay medium as described under "Experimental Procedures." All samples in this and the following three figures were allowed to incubate 4 min after illumination, this being the time at which maximum phosphorylation has occurred. The data in this and the following figures are the mean (±S.D.) from at least three experiments. In this figure the plotted points for light intensities greater than 1000 Rho* represent data from more than 15 separate experiments.

The gain of the light-induced phosphorylation drops sharply as flash intensities are increased: 372 P/Rho* at 10$^4$ Rho*/ROS, 68 P/Rho* at 3.3 × 10$^4$ Rho*/ROS, and 34 P/Rho* at 10$^6$ Rho*/ROS. The solid line through the data in Fig. 1A is generated using the binomial probability equation described in the previous paper (Gray-Keller et al., 1990) assuming 2000 independent targets and a maximum gain of 1600. The window of light intensities generating from 10$^3$ to 10$^4$ Rho*, over which each of the approximately 2000 disks within the ROS absorbs a photon, would appear to be a crucial one. Above this range high gains for both rhodopsin phosphorylation and G activation (Gray-Keller et al., 1990) are diminished (see "Discussion").

If the data are plotted to indicate total phosphate incorporation per ROS as a function of rhodopsin bleaching, as in Fig. 1B, the reaction appears to be approximately linear with light intensity at levels generating between 600 and 10$^4$ Rho*, but the effectiveness of flash illumination then diminishes as more rhodopsin is bleached with a maximum signal occurring when ~3 × 10$^4$ Rho*/ROS are generated. This is approximately the number of rhodopsin kinase molecules in an ROS (Kuhn, 1978; Hamm and Bownds, 1986) suggesting the maximum extent of phosphorylation may be limited by the amount of kinase present (see "Discussion"). The binomial equation used in Fig. 1A fits these data (dashed line in Fig. 1B) at dim, but not at bright lights, suggesting two processes may be occurring (see below). The phosphorylation signal measurable above background is half-maximal when approximately 4 × 10$^3$ Rho*/ROS are generated, i.e. when approximately 40 nM Rho* with respect to the internal volume of the ROS is generated. The $K_m$ of purified rhodopsin kinase for bleached rhodopsin measured in dilute suspension is 4 μM (Palczewski et al., 1988). Even when 100% of the rhodopsin is bleached over 30 s in the presence of 2 mM ATP only a small fraction of the total rhodopsin pool receives at least 1 phosphate group (approximately 33 mmol/mol Rho present); a fraction that is similar to the maximum number of G activated and bound to the membrane (~20 mmol/mol Rho). However, if continuous bright white illumination supporting photoreversal of rhodopsin bleaching is used for longer times (30–60 min), we observe incorporation similar to that reported by others (cf. Shichi and Sommese, 1978; Shichi and Williams, 1979; Aton et al., 1984; Aton, 1986).

Because the high-gain phosphorylation is an unexpected result, we have considered four potential sources of error for the measurements: 1) inaccurate measurement of photons absorbed, 2) inaccurate measurement of ATP specific activity, 3) phosphorylation of a protein other than rhodopsin that co-migrates with rhodopsin on our gel system; and 4) individual experimental error.

These were addressed as follows: 1) measurements were repeated with three different light sources that have been carefully calibrated both by directly measuring the amount of rhodopsin bleached and by measuring the electrical photocresponse of intact cells (cf. Nicol et al., 1987). All three light sources give similar results. 2) The specific activity of ATP released by acid quenching of the reaction mixtures was determined by HPLC. An acid-extractable pool of ATP (100 μM with respect to the internal volume of the ROS) remains bound to the ROS after quenching. If both the bound (unlabeled) and soluble pools are used equally, this could lead to approximately a 20% underestimate of phosphate incorporation in the experiments of Fig. 1. If the bound pool of ATP is used preferentially, this would lead to an even larger underestimate of phosphate incorporation. This seems unlikely, because if a reaction mixture containing permeabilized ROS and ATP is sedimented, the specific activity of ATP in supernatant and ROS pellet is the same. 3) To insure that rhodopsin was the substrate for light-sensitive phosphorylation rather than a contaminating protein co-migrating with rhodopsin on the gels, three controls were performed. (a) We separated soluble, peripheral, and integral proteins by the methods of Hamm and Bownds (1986) after exposure to $[^{32}\text{P}]$ATP and light and found that only the rhodopsin bands in the integral membrane fractions phosphorylated in a light-dependent manner. (b) Rhodopsin was polymerized by heat so that it migrated as a tight band near the origin of the gel,
rather than near the middle of the gel, thus eliminating contamination from proteins that migrate near the middle of the gel. The high-gain phosphorylation was still seen in the rhodopsin pool. (c) We used a modified gel system (Matesic and Liebman, 1987) to separate rhodopsin from a 39-kDa “rhodopsin-like” integral membrane protein. With this system we still see the high-gain phosphorylation in rhodopsin. Some phosphorylation of the 39-kDa protein is noted (data not shown) but it is small (not more than 5% of the total signal) and cannot account for the high-gain phosphorylation in rhodopsin. 4) The high-gain phosphorylation has been documented in over 30 experiments by one of the co-authors (Binder). A second co-author (Biernbaum) has carried out independent experiments and confirmed the same high gains.

The experiments of Fig. 1 demonstrate that many unbleached rhodopsin molecules are phosphorylated for each Rho* formed, but do not specify how many phosphate groups are placed on each. Rhodopsin contains 9 residues that can be phosphorylated, but under the extreme conditions required to phosphorylate all of these, disrupted preparation, high light levels, millimolar ATP levels and extended incubation times, only 1–2 phosphates/rhodopsin are incorporated within 4 min (Wilden and Kuhn, 1982; Aton et al., 1984). Thus it seems unlikely that in the minimally disrupted preparation used here, illuminated with dim light, that more than 1–2 phosphates would be incorporated by each dark rhodopsin molecule. We have compared high- and low-gain phosphorylation using isoelectric focusing, which resolves rhodopsins containing more phosphate groups at more acid pH values (Kuhn and McDowell, 1977; Aton et al., 1984; Aton, 1986). We used a stimulating intensity 10 fold brighter than the lowest shown in Fig. 1 (1.2 × 10^6 Rho*/ROS) to insure detection and resolution of multiply phosphorylated species and compared this with stimulating intensities of 1.2 × 10^6 and 3 × 10^6 Rho*/ROS. With increasing illumination the phosphorylated species of rhodopsin shift to more acidic values, suggesting that phosphorylated rhodopsin molecules acquire more phosphate groups. We have not quantitated the number of phosphate groups on a given rhodopsin because the small amounts of material available here have precluded mass and phosphate analyses of the sort carried out by Aton et al. (1984) and the pl values for rhodopsins from the frog are different than those from cow (Fong et al., 1985). We are currently pursuing this issue, as well as examining whether the two major rhodopsin components in the frog ROS preparations (Fong et al., 1985) are differentially phosphorylated.

Disrupting Rod Structure Abolishes High-gain Rhodopsin Phosphorylation—To examine the dependence of high-gain phosphorylation on soluble proteins and rod structure, we compared rhodopsin phosphorylation in electropermeabilized ROS disrupted with a 26-gauge needle which yields vesicles of ROS membranes. The sheared preparation loses a much larger amount of its protein compliment than electropermeabilized ROS (Gray-Keller et al., 1990). Levels of dark phosphorylation are similar in both preparations, but the amplified phosphorylation observed with dim lights is lost in the sheared preparation (Table I). Measurable phosphorylation still occurs but with diminished gain, so that the concentration of ROS must be increased from 8 to 25 μM to permit its detection. If disruption has resulted in the dilution of a limiting soluble component, one might expect more concentrated suspensions of ROS vesicles to show higher gain phosphorylation, but this is not observed (Table I) at the highest intensity tested. We have not been able to measure a gain above ~35 phosphates/Rho* in the sheared cells (Table I), in agreement with a previous report from this lab using unpurified and sheared ROS (Miller et al., 1977). We thus think it likely that structural integrity of the ROS is required to correctly position reactants for high-gain phosphorylation, as it is for maximal gain of G activation (Gray-Keller et al., 1990), and that breaking the 60-μm-long structures into vesicles removes the high-gain chemistry. The requirement for structure applies only to times immediately after illumination, for if electro-permeabilized ROS are disrupted into vesicles within 3 s after illumination, the high-gain phosphorylation is still observed (data not shown).

Decreasing Calcium Levels over the Same Range as the Light-induced Calcium Decrease Observed in Living Cells Inhibits High-gain Phosphorylation—Calcium has been proposed as a regulator of adaptation in rods (Bownds, 1980; Koch and Stryer, 1988; Matthews et al., 1988; Nakatani and Yau, 1988). The low-gain rhodopsin phosphorylation observed at brighter light intensities is not significantly altered as calcium levels are changed between 10^-5 and 10^-6 M (Fig. 2, bottom curve). (This is different from the result obtained in studies on less purified preparations of sheared ROS (Her-molin et al., 1982.) However, the high-gain rhodopsin phosphorylation observed at lower light intensities is calcium-sensitive (Fig. 2, top two curves). The most striking effect is observed with the dimmest lights tested (bleaching 3800 Rho*/ROS). Lowering calcium from 200 to 100 nM, the range over which calcium falls when intact photoreceptors are illuminated (Ratto et al., 1988), causes a 70% drop in the gain of the phosphorylation, from 400 to 117 phosphates/Rho*. Be low 100 nM calcium, the high-gain phosphorylation of rhodopsin is restored. We have determined that the time-course of phosphorylation remains unchanged at these calcium levels and that phosphatase activity is not expressed in electropermeabilized rods. This suggests that these calcium effects are due to a change in the maximum gain of the reaction and not

**TABLE I**

| Phosphates/Rho* | Electropermeabilized ROS | Sheared ROS |
|-----------------|--------------------------|-------------|
| 1.2 × 10^6 Rho* | 372 ± 91^a               | NS^b        |
| 3.3 × 10^6 Rho* | 60 ± 51                  | NS          |
| 1.2 × 10^6 Rho* | 34 ± 24                  | 16 ± 10     |

^a All data are the mean ± standard deviation from at least three experiments. ^b No significant increase over dark signal is measured.
an alteration of its time course.

Perturbing G, Activation Inhibits High-gain Rhodopsin Phosphorylation—The fact that both rhodopsin phosphorylation and G, activation have high gains over the same range of light intensities led us to question whether there might be a link between the reactions. The experiments of Figs. 1 and 2 were done in the presence of ATP and GTP to permit normal G, function. Several studies have shown the low-gain rhodopsin phosphorylation and G, activation to be antagonistic; phosphorylation of rhodopsin leads to less activation of G, (cf. Miller and Dratz, 1984; Bennett and Sitaramayya, 1988) and binding of G, to rhodopsin can inhibit phosphorylation (Kelleher and Johnson, 1988). One might expect then, that enhancing the activation of G, after light activation, which can be accomplished by replacing GTP with the non-hydrolyzable analog GTPyS (Gray-Keller et al., 1990), would enhance high-gain rhodopsin phosphorylation. The opposite result is observed. Fig. 3 demonstrates that replacement of exogenous GTP with GTPyS depresses high-gain phosphorylation more than 80%. Elution of G, from the membrane is not changed when exogenous GTP is replaced with GTPyS (Gray-Keller et al., 1990). Replacement of GTP with GDPβS, which reduces activation of G-protein (Robinson et al., 1986; Rybin, 1986), decreases rhodopsin phosphorylation approximately 50%. The low-gain rhodopsin phosphorylation is not influenced by either nucleotide. These compounds, of course, might have effects not specific to G, chemistry that are responsible for their inhibition of high-gain phosphorylation.

We attempted to see if high-gain phosphorylation was altered by monoclonal antibody 4A (kindly provided by Heidi E. Hamm, University of Illinois) which is known to block activation of C-protein in sheared cells (Hamm and Dowda, 1984). However, this molecule did not gain access sufficient to measurably block the G, activation observed in electropermeabilized cells.

To further probe the high-gain phosphorylation and possible links to G, activation, we have also examined the effects of adding cGMP and varying ATP concentration. cGMP has been shown to alter the nucleotide specificity of Rho*—activated G, (Robinson et al., 1986) and ATP is thought to shorten the interaction between Rho* and G, (Liebman and Pugh, 1980). Addition of 200 μM 8-bromo-cGMP, a hydrolysis-resistant analog of cGMP, has no effect on the high-gain phosphorylation (Fig. 3). This, plus the observation that neither protein kinase C nor cyclic AMP-dependent protein kinase appear to phosphorylate rhodopsin (Binder et al., 1989)

---

**Fig. 3.** Effects of GTPγS, GDPβS, and 8-Br-cGMP levels on rhodopsin phosphorylation. The gain of rhodopsin phosphorylation at a flash intensity of 1.2 x 10^6 Rho*/ROS is shown under control conditions (from Fig. 1) and after adding 30 μM GTPyS, 0 μM GTPγS, or 200 μM 8-Br-cGMP.

---

**Fig. 4.** Lowering [ATP] reduces the gain of rhodopsin phosphorylation. The effects of lowering the concentration of added ATP from 200 μM (control) to 5 μM are shown. Numbers next to each set of bar graphs denotes the number of Rho*/ROS. Data for control conditions are taken from Fig. 1. Note that the scale for the change in phosphates per Rho* are different for the left and right sets of bar graphs.

**Fig. 5.** Illumination causes a rapid increase in the phosphate content of rhodopsin. A, in the left panel is shown the number of phosphate groups incorporated into the rhodopsin pool per Rho* within the first 6 s after a 20-ms flash of light bleaching 1.2 x 10^6 Rho*/ROS. B, the right panel extends this data for 5 min after the flash. The line in the left panel is from a linear regression analysis of the data.
The rate drops to 0.1 phosphate/Rho*/s after approximately 10–15 s. Maximum phosphorylation occurs 2–4 min after a flash of this intensity (Fig. 5B). Thus it appears that each Rhox can receive at least one phosphate group on the time scale of PDE activity-inactivation, compatible with a causal role for the phosphorylation in terminating the response at this light intensity. It is interesting to note that the rate of rhodopsin phosphorylation drops 3-fold when only 6% of the maximum incorporation of phosphate is achieved. The rapid phase of phosphorylation is not altered when ATP levels are reduced from ~200 to 3 μM; that is, under conditions when the high-gain but not low-gain phosphorylation is inhibited, the slow phase, but not the rapid phase, is inhibited (see "Discussion"). An apparent dephosphorylation is noted after 4 min in Fig. 5B; however, dephosphorylation in individual experiments was either incomplete or absent in this preparation of electropermeabilized cells. A paper to follow describes a light-inhibited dephosphorylation observed only in intact cells and not in permeabilized cells.

**DISCUSSION**

The main finding of this paper, shown in Fig. 1, is a high-gain rhodopsin phosphorylation observed when less than \(3 \times 10^6\) of the \(3 \times 10^6\) rhodopsin molecules in an ROS are excited by light and only if the macroscopic structure of the ROS is maintained intact. Breakage of the 60-μm-long structures into vesicles abolishes the reaction. We do not know whether the maximum gain observed, ~1400 phosphates/Rho*, is the real maximum, because we were not able to measure light-induced phosphate incorporation above dark levels using flashes forming less than 600 Rho*/ROS. Also, the magnitude of high-phosphate incorporation above dark levels using flashes forming less than 600 Rho*/ROS. Also, the magnitude of high-phosphate incorporation above dark levels using flashes forming less than 600 Rho*/ROS.

We are inclined to treat this as a distinctively different (low-gain) reaction because it is much less sensitive than the high-gain rhodopsin phosphorylation observed when less than 3 × 10^6 rhodopsin molecules in an ROS are excited by light and only if the macroscopic structure of the ROS is maintained intact. Breakage of the 60-μm-long structures into vesicles abolishes the reaction. We do not know whether the maximum gain observed, ~1400 phosphates/Rho*, is the real maximum, because we were not able to measure light-induced phosphate incorporation above dark levels using flashes forming less than 600 Rho*/ROS. Also, the magnitude of high-phosphate incorporation above dark levels using flashes forming less than 600 Rho*/ROS. Also, the magnitude of high-phosphate incorporation above dark levels using flashes forming less than 600 Rho*/ROS.

It is interesting that disruption of electropermeabilized ROS into vesicles several s after illumination does not destroy high gain phosphorylation, even though the disruption is occurring early in the time course of the phosphorylation reaction which reaches its maximum after 4 min. This indicates that the stoichiometry of the reaction is set before phosphate incorporation actually occurs, possibly by light-induced rapid activation or binding of a kinase or regulator, a reaction that does not occur if the intact ROS is broken into vesicles.

**Models for Rhodopsin Phosphorylation—** One model that might explain early setting of the final stoichiometry would be that a bleached rhodopsin molecule causes the rhodopsin kinase molecules in its domain to be converted to a more active form, with each "activated" kinase rapidly and preferentially attaching to a bleached rhodopsin molecule if it is found, otherwise to an unbleached rhodopsin. After a slower phosphorylation of the opsin substrate, the kinase becomes inactive.

In support of this is the observation that calculations (Gray-Keller et al., 1990) occur over the range of illumination in which single disks are receiving their first photon absorption, but with a difference that may prove to be significant. A binomial probability equation makes the best description of high-gain rhodopsin phosphorylation if 2000 targets/Ros are assumed, high-gain G activation is best fit by assuming 4000 targets (Gray-Keller et al., 1990). It is possible that this is reflecting the fact that the G activation draws on components adsorbed to a single disk surface, of which there are approximately 4000, while rhodopsin phosphorylation involves the binding of rhodopsin kinase and thus its removal from the interdiskal spaces, of which there are approximately 2000. There is enough scatter in the data of Fig. 1 (this paper) and Fig. 4 of Gray-Keller et al. (1990) to suggest, but not prove, this point.

These compartment models are consistent with the abolition of high-gain rhodopsin phosphorylation and G activation that is observed upon mechanical disruption of ROS. The decrease in G activation occurs because disruption mimics the effect of light by releasing G, with bound nucleotide into the medium. Disruption does not activate rhodopsin phosphorylation because it causes loss of reactants or compartments required for the high-gain reaction.

It is interesting that disruption of electropermeabilized ROS into vesicles several s after illumination does not destroy high gain phosphorylation, even though the disruption is occurring early in the time course of the phosphorylation reaction which reaches its maximum after 4 min. This indicates that the stoichiometry of the reaction is set before phosphate incorporation actually occurs, possibly by light-induced rapid activation or binding of a kinase or regulator, a reaction that does not occur if the intact ROS is broken into vesicles.

**Models for Rhodopsin Phosphorylation—** One model that might explain early setting of the final stoichiometry would be that a bleached rhodopsin molecule causes the rhodopsin kinase molecules in its domain to be converted to a more active form, with each "activated" kinase rapidly and preferentially attaching to a bleached rhodopsin molecule if it is found, otherwise to an unbleached rhodopsin. After a slower phosphorylation of the opsin substrate, the kinase becomes inactive. In this context, Aton (1986) reports that up to 50% of the phosphorylated opsin in cattle is non-photoactivated, and tentative evidence for the existence of a light-activated kinase has been obtained in two recent reports (Bowles et al., 1988; Palczewski et al., 1989). This model might also provide an explanation for the biphasic nature of phosphate incorporation shown in Fig. 5, with the rapid and slow phases corresponding to phosphorylation of bleached and unbleached rhodopsin, respectively. The idea that there are two separate substrate pools is consistent with the observed effect of lowering ATP levels from 200 to 3 μM: the initial rapid phase of phosphorylation as shown in Fig. 5 (presumably reflecting the more favorable interaction of kinase with bleached rhodopsin) is not altered, but the slower phosphorylation measured after 4 min (kinase acting on unbleached rhodopsin) is decreased by 60%. It would be desirable to confirm this model by documenting that the biphasic kinetics of Fig. 5 are also observed at the most dim light levels used, but the rapid phase is obscured by experimental noise.

A second model for high-gain rhodopsin phosphorylation proceeds from what has been the conventional view: that the reaction occurs only when a rhodopsin molecule excited by light exposes its C-terminal serine and threonine residues to action of a rhodopsin kinase that is continuously present and active. High-gain phosphorylation might occur because a single excited rhodopsin molecule, in an ROS that has absorbed 4000, while rhodopsin phosphorylation involves the binding of rhodopsin kinase and thus its removal from the interdiskal spaces, of which there are approximately 2000. There is enough scatter in the data of Fig. 1 (this paper) and Fig. 4 of Gray-Keller et al. (1990) to suggest, but not prove, this point.

These compartment models are consistent with the abolition of high-gain rhodopsin phosphorylation and G activation that is observed upon mechanical disruption of ROS. The decrease in G activation occurs because disruption mimics the effect of light by releasing G, with bound nucleotide into the medium. Disruption does not activate rhodopsin phosphorylation because it causes loss of reactants or compartments required for the high-gain reaction.

**Models for Rhodopsin Phosphorylation—** One model that might explain early setting of the final stoichiometry would be that a bleached rhodopsin molecule causes the rhodopsin kinase molecules in its domain to be converted to a more active form, with each "activated" kinase rapidly and preferentially attaching to a bleached rhodopsin molecule if it is found, otherwise to an unbleached rhodopsin. After a slower phosphorylation of the opsin substrate, the kinase becomes inactive. In this context, Aton (1986) reports that up to 50% of the phosphorylated opsin in cattle is non-photoactivated, and tentative evidence for the existence of a light-activated kinase has been obtained in two recent reports (Bowles et al., 1988; Palczewski et al., 1989). This model might also provide an explanation for the biphasic nature of phosphate incorporation shown in Fig. 5, with the rapid and slow phases corresponding to phosphorylation of bleached and unbleached rhodopsin, respectively. The idea that there are two separate substrate pools is consistent with the observed effect of lowering ATP levels from 200 to 3 μM: the initial rapid phase of phosphorylation as shown in Fig. 5 (presumably reflecting the more favorable interaction of kinase with bleached rhodopsin) is not altered, but the slower phosphorylation measured after 4 min (kinase acting on unbleached rhodopsin) is decreased by 60%. It would be desirable to confirm this model by documenting that the biphasic kinetics of Fig. 5 are also observed at the most dim light levels used, but the rapid phase is obscured by experimental noise.

A second model for high-gain rhodopsin phosphorylation proceeds from what has been the conventional view: that the reaction occurs only when a rhodopsin molecule excited by light exposes its C-terminal serine and threonine residues to action of a rhodopsin kinase that is continuously present and active. High-gain phosphorylation might occur because a single excited rhodopsin molecule, in an ROS that has absorbed less than 10^10 photons, in some manner catalyzes the conversion of several hundred other molecules into substrate for the known rhodopsin kinase (cf. Aton, 1988). This would presumably require energy input and/or accessory proteins.

Generation of rhodopsins as kinase substrate might also imply their conversion into catalysts of G activation. This would add an additional step to the known transduction cascade for very low light intensities: one photon bleaches one rhodopsin, which then transforms several hundred other dark rhodopsins into the excited form that activates G. These excited rhodopsins are then more slowly inactivated by phosphorylation. The rate of 1 G/Rho*/ms, determined by Vuong et al. (1984) by extrapolating from measurements made at higher light intensities, would be an overestimate because more rhodopsins than the one that absorbed a photon would be activating G. There is at present no experimental evidence for this model.

Measurements of Gray-Keller et al. (1990)
were not performed at early enough times after flash illumination to evaluate the point. We are currently using isoelectric focusing to determine whether the rhodopsin phosphorylated in the high-gain reaction is bleached or unbleached.

For either of these models, G; must be considered as a potential mediator. The data show that any perturbation of G; activation, suppressing its activation (with GDP/35) or preventing its inactivation (with GTPγS) inhibits the high-gain phosphorylation by 50-90%. This, plus the observation that high-gain G; activation and rhodopsin phosphorylation are observed at light levels bleaching less than ~10^-11 Rhö*/ROS (Gray-Keller et al., 1990), suggest that the high-gain rhodopsin phosphorylation may require activation and inactivation (via hydrolysis of bound GTP) of G;

Calcium also appears to play a modulatory role. The observation is that a 60% decrease in high-gain phosphorylation occurs as calcium is lowered over the same narrow window of concentration (200-100 nM) that occurs in the living cells upon illumination (Ratto et al., 1988). It has been suggested that a calcium decrease desensitizes the photoresponse (Bownds, 1980; Yau and Nakatani, 1985), either by inhibiting the excitation pathway linking rhodopsin bleaching to PDE activation and a cGMP decrease (Kawamura and Bownds, 1961), or by activating guanylate cyclase (cf. Koch and Stryer, 1988). The data here suggest that one should consider whether the calcium decrease might depress light sensitivity in part through its inhibition of high-gain rhodopsin phosphorylation.

Roles for Rhodopsin Phosphorylation—It is the observation of a rapid phase at brighter light intensities, which within seconds incorporates 1–2 phosphates for each Rhö*, that provides support for the conventional model for the function of rhodopsin phosphorylation: that it reduces the interaction between Rhö* and G; which leads to rapid inactivation of PDE (Sitaramayya et al., 1977; Sitaramayya and Liebman, 1983a; Sheihi et al., 1984; Aton and Litman, 1984; Miller and Dratz, 1984; Arshavsky et al., 1985; Miller et al., 1986; Wilden et al., 1986; Sitaramayya, 1986).

The stoichiometries obtained in this and the previous paper at dimmer lights suggest a possible link between maximal levels of rhodopsin phosphorylation, light-activated G; that remains bound to nucleotide and ROS membrane, and the amount of PDE inhibitory γ subunit present. We observe a maximum incorporation, following brief illumination that bleaches 100% of the rhodopsin present, of 0.04 mol of phosphate/mol Rhö*. This corresponds to 1.2 x 10^6 phosphate groups/ROS, and since isoelectric focusing data indicate that most of the rhodopsin containing phosphate is multiply phosphorylated, this probably reflects actual modification of 2–4 x 10^5 Rhö*/ROS. This is approximately the number of G; molecules that remain bound to membrane and nucleotide following saturating illumination (20% of the total G; in the ROS, or 2 x 10^14 molecules/ROS) and also the number of PDE binding sites for G; (corresponding to the number of γ subunits of PDE). This suggests a very simple stoichiometry for these membrane associated reactions: number of phosphorylated Rhö = number of G; activated and retained by electropermeabilized ROS = number of γ subunits of PDE. This raises the possibility of regulation of activation-inactivation by functional ensembles of these enzymes. Further isoelectric focusing studies, now in progress, will be required to precisely quantify the number of phosphorylated rhodopsin molecules and confirm this suggested stoichiometry.

It is possible that high-gain phosphorylation, in which light excitation of one receptor molecule causes the covalent modification of many others, may play a global role in the photoreceptor. An intriguing parallel is found in the bacterial chemotaxis system: methylation of nonactivated receptor molecules can lead to general adaptation of the bacteria (Hazelbauer et al., 1989). It is not unreasonable to suggest further that different residues on opsin might be altered by high- and low-gain phosphorylation (cf. McDowell et al., 1986), analogous to the situation observed in the β-adrenergic system (Hausdorff et al., 1989), and that they have different meanings. Numerous examples of different sites of phosphorylation on the same enzyme having different regulatory significance have been noted (Cohen, 1982). Multiple dark rhodopsins eventually phosphorylated by the bleaching of one rhodopsin might play a modulatory role in setting the time course of G; PDE or guanylate cyclase activation. The work of Goldberg's laboratory (Dawis et al., 1988) provides strong evidence that a functional ensemble of all these components is regulated by light to cause large increases in flux through the cGMP pathway that may play a role in adaptation (Rownds and Thomson, 1988; Kondo and Miller, 1988). A further link of rhodopsin phosphorylation and the adaptation state of the cell is our recent finding of a rhodopsin phosphatase activity, observed only in intact cells (not in permeabilized cells), whose expression is inhibited by levels of background illumination that light adapt the photoreceptor. Thus there is a correlation between the persistence of rhodopsin phosphorylation and the adaptation state of the rod photoreceptor. A final point is that the discovery of a high-gain phosphorylation at low light stimulus levels in the rhodopsin receptor system suggests that it might be worthwhile to look for a similar phenomenon in systems utilizing the homologous β-adrenergic and muscarinic acetylcholine receptors.

Acknowledgments—We thank Peter Calvert, John Kirsch, and Shymin Huang for their technical assistance with the IIPCLC analyses.

REFERENCES

Arshavsky, V. Y., Dizhoor, A. M., Shestakova, I. K., and Phillipov, P. P. (1985) FEBS Lett. 181, 264-266
Aton, B. R. (1986) Biochemistry 25, 677-680
Aron, B., and Litman, B. J. (1984) Exp. Eye Res. 38, 547-559
Aton, B. R., Litman, B. J., and Jackson, M. L. (1984) J. Gen. Physiol. 85, 83-105
Bennett, N., and Sitaramayya, A. (1988) Biochemistry 27, 1710-1715
Berovic, J. L., DeBlasi, A., Stuning, W. C., Caron, M. G., and Lefkowitz, R. J. (1989) Science 246, 235-240
Bierbaum, M. S., and Bownds, M. D. (1985) J. Gen. Physiol. 85, 83-105
Binder, B. M., Brewer, E., and Bownds, M. D. (1989) J. Biol. Chem. 264, 8857-8864
Bownds, M. D. (1980) Photochem. Photobiol. 32, 487-490
Bownds, M. D., and Thompson, D. (1988) Proceedings of the Yamada Conference (Hara, T., ed) Vol. 21, pp. 241-246. Yamada Science Foundation, Osaka
Bownds, M. D., Gordon-Walker, A., Gaide-Huguenin, A.-C., and Robinson, W. (1971) J. Gen. Physiol. 58, 925-937
Bownds, M. D., Dawes, J., Miller, J., and Stahlman, M. (1972) Nature New Biol. 237, 125-127
Bownds, D., Brodie, A., Robinson, W. E., Palmer, D., Palmer, J., Miller, J., and Shedlovsky, A. (1974) Exp. Eye Res. 18, 253-269
Chader, G. J., Fisher, R. T., O'Brien, P. J., and Krishna, G. (1976) Endocrinology 93, 1385-1390
Cohen, P. (1982) Nature 296, 613-620
Cote, R. H., Nicol, G. D., Burke, S. A., and Bownds, M. D. (1986) J. Biol. Chem. 261, 12905-12975
Davies, S. M., Graeff, R. M., Heyman, R. A., Walseath, T. F., and Goldberg, N. D. (1988) J. Biol. Chem. 263, 8771-8785
Fong, S.-L., Landers, R. A., and Bridges, C. D. B. (1985) Vision Res. 15, 1615-1620
Frank, R. N., and Buzney, S. M. (1975) Biochemistry 14, 5110-5117

High-gain Rhodopsin Phosphorylation at Dim Illumination 15339
High-gain Rhodopsin Phosphorylation at Dim Illumination

Frank, R. N., Cavanagh, H. D., and Kenyon, K. R. (1973) *J. Biol. Chem.* 248, 596-608

Gray-Keller, M. P., Biernbaum, M. S., and Bownds, M. D. (1990) *J. Biol. Chem.* 265, 15323-15332

Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. (1989) *J. Biol. Chem.* 264, 12657-12665

Hamm, H. E., and Bownds, M. D. (1984) *J. Gen. Physiol.* 84, 265-280

Hamm, H. E., and Bownds, M. D. (1986) *Biochemistry* 25, 4512-4523

Hazelbauer, G. L., Park, C., and Nowlin, D. M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 1448-1452

Hermolin, J., Karell, M. A., Hamm, H. E., and Bownds, M. D. (1982) *J. Gen. Physiol.* 79, 633-655

Huang, H. V., Molday, R. S., and Dreyer, W. J. (1973) *FEBS Lett.* 37, 285-289

Kawamura, S., and Bownds, M. D. (1981) *J. Gen. Physiol.* 77, 571-591

Kelleher, D. J., and Johnson, G. L. (1988) *Mol. Pharmacol.* 34, 452-460

Koch, K. W., and Stryer, L. (1988) *Nature* 334, 64-66

Kondo, H., and Miller, W. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 1399-1403

Kuhn, H. (1978) *Biochemistry* 17, 4389-4395

Kuhn, H., and Dreyer, W. J. (1972) *FEBS Lett.* 20, 1-6

Kuhn, H., and McDowell, J. H. (1977) *Biophys. Struct. Mech.* 3, 199-203

Kuhn, H., Cook, J. H., and Dreyer, W. J. (1973) *Biochemistry* 12, 2490-2502

Liebman, P. A., and Pugh, E. N., Jr. (1980) *Nature* 287, 734-736

Matesic, D., and Liebman, P. A. (1987) *Nature* 326, 600-603

Matthews, H. R., Murphey, R. W., Fain, G. L., and Lamb, T. L. (1988) *Nature* 334, 67-69

McDowell, J. H., Curtis, D. R., Baker, U. A., and Hargrave, P. A. (1988) *Invest. Ophthalmol. Vis. Sci.* 26, 291

Miller, J. L., and Dratz, E. A. (1984) *Vision Res.* 24, 1509-1521

Miller, J. A., and Paulsen, R. (1975) *J. Biol. Chem.* 250, 4427-4432

Miller, J. A., Paulsen, R., and Bownds, M. D. (1977) *Biochemistry* 16, 2633-2639

Miller, J. L., Fox, D. A., and Litman, B. J. (1986) *Biochemistry* 25, 4983-4988

Nakatani, K., and Yau, K.-W. (1988) *Nature* 334, 69-71

Nicol, G. D., Kaupp, U. B., and Bownds, M. D. (1987) *J. Gen. Physiol.* 89, 297-319

Palczewski, K., McDowell, J. H., and Hargrave, P. A. (1988) *J. Biol. Chem.* 263, 14067-14073

Palczewski, K., Arendt, A., McDowell, J. H., and Hargrave, P. A. (1989) *Biochemistry* 28, 8764-8770

Ratto, G. M., Payne, R., Owen, W. G., and Tsien, R. Y. (1988) *J. Neurosci.* 8, 3240-3246

Robinson, P. R., Radeke, M. J., Cote, R. H., and Bownds, M. D. (1986) *J. Biol. Chem.* 261, 313-318

Rybin, V. O. (1988) *Biochimica et Biophysica Acta* 911, 1035-1041

Shichi, H., and Somers, R. L. (1978) *J. Biol. Chem.* 253, 7040-7046

Shichi, H., and Williams, T. C. (1979) *J. Supramol. Struct.* 12, 419-424

Shichi, H., Yamamoto, K., and Somers, R. L. (1984) *Vision Res.* 24, 1523-1531

Sitaramayya, A. (1986) *Biochemistry* 25, 5460-5468

Sitaramayya, A., and Liebman, P. A. (1985a) *J. Biol. Chem.* 258, 1205-1209

Sitaramayya, A., and Liebman, P. A. (1985b) *J. Biol. Chem.* 258, 12106-12109

Sitaramayya, A., Virmaux, N., and Mandel, P. (1977) *Neurochem. Res.* 2, 1-10

Tsien, R. Y., and Rink, T. J. (1980) *Biochim. Biophys. Acta* 600, 623-638

Tsien, R. Y., and Rink, T. J. (1981) *J. Neurosci. Methods* 4, 73-86

Vuong, T. M., Chabre, M., and Stryer, L. (1984) *Nature* 311, 659-661

Weller, M., Virmaux, N., and Mandel, P. (1975) *Proc. Natl. Acad. Sci. U. S. A.* 72, 391-395

Wilden, U., and Kuhn, H. (1982) *Biochemistry* 21, 3014-3022

Wilden, U., Hall, S. W., and Kuhn, H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 1174-1176

Yau, K.-W., and Nakatani, K. (1985) *Nature* 313, 579-582

Yoshikami, S., Robinson, W. E., and Hagins, W. A. (1974) *Science* 185, 1176-1179