Light Stimulates Phosphorylation of Two Large Membrane Proteins in Frog Photoreceptors†

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ABSTRACT: By photoactivating rhodopsin, light indirectly initiates a series of biochemical reactions within photoreceptors as part of the visual process. I herein report that one of the light-stimulated reactions in bullfrog photoreceptors is the phosphorylation of two previously unreported proteins (220 and 240 kDa). Their phosphorylation by endogenous kinase(s) is readily observed in freshly isolated, fragmented rods. On subcellular fractionation, the labeled proteins copurify with the membranes of the outer segments, from which they cannot be extracted with low ionic strength. They appear to be integral membrane proteins of the disk or plasma membranes. Their light-induced phosphorylation is also observed in intact receptors when excised frog retinas are incubated under in vivo conditions with $^{32}PO_4$. Thus, appropriate kinase(s) is (are) present within outer segments and presumably is (are) the one(s) responsible for phosphorylation in fragmented cells. In the presence of adenosine 5′-(γ-[^{35}S]thiotriphosphate) ([^{35}S]ATP-γ-S), light can also stimulate thiophosphorylation, leading to preferential labeling of the 220-kDa protein. On the basis of four criteria (electrophoretic mobility, membrane location, binding of concanavalin A, and mobility shifts with SH oxidation), the 220-kDa protein appears to correspond to the membrane protein previously identified at the rims of rod disks [Papermaster, D. S., Schneider, B. G., Zorn, M. A., & Kraehenbuhl, J. P. (1978) *J. Cell Biol. 78*, 415–425]. Identity of the other substrate protein is unknown. When fragmented cells are illuminated with a flash of 1-ms duration, the half-time for phosphorylation is about 1 min with ATP at 0.1 mM. If the intensity of the flash is varied, half-maximal saturation occurs with the photoactivation of about 2% of rhodopsin. cGMP reduces incorporation of ^{32}P into both proteins by 50% as cGMP is increased from ≤0.1 to 50 μM. In kinetics, light sensitivity, and cGMP dependence, phosphorylation of the proteins closely resembles phosphorylation of rhodopsin. This suggests that the three kinase reactions are closely linked and that phosphorylation of the large membrane proteins may participate in the visual process.

Rhodopsin is the major protein to be phosphorylated within vertebrate rods (Bownds et al., 1972; Kuhn & Dreyer, 1972; Frank et al., 1973). Its reaction is light stimulated. Light must transform rhodopsin into a photoactivated state before its corresponding kinase can recognize it as a substrate (Weller et al., 1975; Frank & Buzney, 1975; Kuhn, 1978; Paulsen & Bentrop, 1983). Rhodopsin kinase is unusual in two respects: it apparently exists only in an active state, and it is highly specific for rhodopsin, remaining unreactive toward other proteins (Weller et al., 1975; Shichi & Somers, 1978). The physiological function of rhodopsin phosphorylation may be related to the regulation of cGMP hydrolysis (Sitaramayya & Liebman, 1983a). Due to its relatively slow kinetics, the reaction has been generally thought to reflect a step in the slow processes of adaptation. However, recent work indicates that its kinetics increases with dim illuminations (Sitaramayya & Liebman, 1983b), suggesting that it may also participate in receptor excitation.

Light absorption by rhodopsin is also responsible for the phosphorylation and dephosphorylation of several small polypeptides (Polans et al., 1979; Biernbaum et al., 1983), whose functions have not yet been clarified. To further investigate kinase events involving minor proteins, I began a study of light-induced thiophosphorylation in vertebrate rods. For most kinases ATP-γ-S[1] readily replaces ATP as the substrate donor, leading to thiophosphorylation. Thiophosphate groups normally resist hydrolysis by phosphatases, and so the thionucleotide is now routinely used to study kinase reactions in isolation [e.g., Cassidy et al. (1979) and Cassel & Glaser (1982)]. In the course of my investigation, I found that light stimulated the thiophosphorylation of not only rhodopsin but also a large polypeptide or protein, with a molecular mass of 220 kDa (Szuts, 1983). This paper is an extension of that observation, showing that the light reaction also occurs with ATP, the physiological nucleotide donor. Light also phosphorylates a second large protein at 240 kDa.

The reaction involving the 220-kDa protein is of physiological interest because this phosphoprotein appears to be related to, if not identical with, the "large intrinsic membrane protein" at the circumference of the flattened disks of rods (Papermaster et al., 1978). The cytoarchitecture of the disks has received considerable attention in the past. One of their interesting features is that a cytoplasmic gap of only 110 Å (Cohen, 1968; Leeson, 1971) separates the plasma membrane of the cell from the disk rims. Such and other anatomical considerations suggest that the rim protein is "a potential candidate for participation in the interactions between the photoexcited disk and the hyperpolarized outer segment plasma membrane" [p 424 in Papermaster et al. (1978)]. Like rhodopsin, rim protein is an integral membrane molecule that

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[1] Abbreviations: kDa, kilodaltons; ROS, rod outer segment(s); ATP-γ-S, adenosine 5-(γ-thiotriphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydoxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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transverses the disc membrane, bearing sugar groups on the side facing the intradiskal lumen (Molday & Molday, 1979). However, the rim protein is not a visual pigment: it is about 5 times larger in molecular mass, lacks retinal (Molday & Molday, 1979), and does not cross-react with anti-opsin (Papermaster et al., 1976).

Materials and Methods

Materials. Live bullfrogs, *Rana catesbeiana*, were purchased from Acadian Biological Supply Co. (Rayne, LA). ATP, GTP, cGMP, ATP-γ-S, (isobutylmethyl)xanthine, phenylmethanesulfonyl fluoride, phosphocreatine, and creatine kinase, were obtained from Boehringer-Mannheim. Calbiochem-Behring was the source of filamin, dithiothreitol, and Pronase, while Sigma Chemical Co. was the supplier of concanavalin A labeled with fluoresceinyl isothiocyanate (FITC-Con A). Myosin was obtained from Bethesda Research Laboratories, oxidized glutathione was from Nutritional Biochemicals Corp., and 2-mercaptoethanol along with protein standards for gel electrophoresis was from Bio-Rad Laboratories. Radioactively labeled reagents, $NaH^{32}PO_4$, [α-^{32}P]-ATP, [γ-^{32}P]ATP, [^{3}H]ATP, [^{35}S]ATP-α-S, and [^{35}S]ATP-γ-S, were all purchased from New England Nuclear. Spectrin from human erthrocytes was a gift of Dr. Lynne Coluccio.

Preparation of Crude Rod Outer Segment (ROS) Suspension. The entire procedure was performed in the dark under infrared illumination (Szuts, 1980). Frogs were dark adapted for at least 2 h prior to dissection. ROS were isolated by gently shaking the retinas in about 1 mL of Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.5 mM $MgSO_4$, 10 mM Hepes, adjusted to pH 7.4 with 4.4 mM NaOH). The suspension was filtered through a nylon mesh (37-μm opening), and 1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, 1 mM EGTA (buffered to neutrality), and 10 mM KF were added to the filtrate, which contained ROS. The suspension was vortexed to ensure that the plasma membrane of outer segments became leaky and was immediately used for phosphorylation assays. Usually a pair of retinas was prepared per experiment.

Phosphorylation and Thiophosphorylation Assay of Crude ROS Fragments. All assays were performed at room temperature in the dark under infrared illumination. Outer segment suspensions were divided into a control fraction and a test fraction (each about 0.5 mL), and each was combined with a mixture of ATP and [γ^{32}P]ATP. Typical final concentrations were 0.1 mM ATP, 10 μCi/ml [γ-^{32}P]ATP, and 15 μM rhodopsin. In a few designated experiments, 60 mM phosphocreatine and 1 mg/mL creatine kinase were added to the reaction mixtures to regenerate ATP. Light-induced phosphorylation by endogenous kinases was initiated with a flash that illuminated the test sample 10 s after admixture with nucleotides. The phosphorylation reactions were allowed to proceed in the dark for up to 30 min following the 1 ms long flash. Reactions were quenched with addition of Laemmli's sample buffer (Laemmli, 1970), which was modified to contain 10% sucrose, 3% $NaDodSO_4$, 2.5 mM Tris, 19.2 mM glycine, and 0.01% bromophenol blue.

Quenching blocked the kinase reactions within 10 s of solution delivery. This was determined in experiments in which radioactive labeling was assayed as the time interval between flash and solution delivery was varied from −30 s (where quenching preceded stimulus) to 30 s. Thiophosphate was used as the label in these tests because thiophosphorylation is a faster reaction than phosphorylation and because corresponding measurement accuracy is greater (compare Figures 5 and 6).

2-Mercaptoethanol (3%) was added to the solubilized samples, which were kept for several hours at room temperature before electrophoresis on $NaDodSO_4$/polyacrylamide slab gels (3% in stack, 6% in resolving phase) as described by Laemmli (1970). Samples were not boiled prior to electrophoreis, because boiling leads to increasingly larger aggregates of rhodopsin, which mask the banding pattern of other proteins. Extensive boiling caused the loss of not only rhodopsin from the gel but also the proteins of interest at 220 and 240 kDa. The dye front was routinely run off the gels to improve resolution of the larger proteins. Gels were stained with Coomassie blue (Weber & Osborn, 1969), dried either onto paper or between clear cellophane, and autoradiographed with Kodak X-Omat film. Because radioactive incorporation among proteins varied over 3 orders of magnitude, several autoradiograms were prepared per gel. Either in the wet state or in the cellophane-dried form, the gels were scanned with a Zenieh laser densitometer to measure protein content of each band. A known quantity of myosin, which was coelectrophoresed in parallel with the samples, served as reference. Selectivity of the Coomassie stain was assumed to be the same for the proteins of interest. The molecular mass of the large proteins was estimated by interpolation with myosin (200 kDa), spectrin (220 and 240 kDa), and filamin (250 kDa) as reference standards. The relative incorporation of ^{32}P between lanes within an autoradigram was measured by laser densitometry, while the absolute incorporation was quantified with a liquid scintillation counter by cutting out and solubilizing the bands of one of the lanes (Mahin & Lofberg, 1966).

For thiophosphorylation, ATP-γ-S and [^{35}S]ATP-γ-S (30–50 μCi/mL) were substrate donors in the reaction mixtures. Stained gels were soaked in Enlightening (New England Nuclear) prior to drying and fluorography.

Control experiments showed negligible, if any, loss of ^{32}P or ^{35}S from labeled proteins during gel electrophoresis and fixation. Electrophoresis under acid conditions, pH 2 (Fairbanks & Avruch, 1972), did not yield higher levels of incorporated radioactivity into proteins of interest.

Phosphorylation Assay with Excised Retinas. Retinas were separately incubated in the dark at room temperature in about 1 mL of oxygenated Ringer's solution, to which was added 10 mM glucose and $NaH^{32}PO_4$ (final concentration 0.1 mCi/mL and 0.28 mM for phosphate). After 30 min of incubation, one of the retains was illuminated by a flash. At 2.5 min after stimulus, both the test and the unilluminated control retinas were separately mixed with 1 mL of 10% trichloroacetic acid and kept on ice for 15 min. Retinas were rinsed with a medium containing 5% trichloroacetic acid, 1 mM Na_3PO_4, and 1 mM ATP, and their outer segments were brushed off into a fresh solution of the same medium. The crude ROS suspension was filtered through nylon mesh, pelleted, and resuspended with Ringer's solution (resuspension and pelleting repeated twice) before solubilization for gel electrophoresis.

Light Stimulus. An electronic flash unit (Sunpak Auto 322) was used to illuminate samples. Flash duration was about 1 ms. A Schott KG3 heat filter, a Wratten No. 25 cut-off filter, and a glass diffuser were interposed between sample and flash unit so that ROS were evenly illuminated with red light. Maximal absorption by rhodopsin occurred at 620 nm, where self-screening by the visual pigment is negligible. Calculated absorbance at 620 nm was about 0.01 for the test tubes used. With this optical arrangement, maximum intensity from the electronic unit photoactivated 20% of rhodopsin per flash. Wratten neutral density filters were interposed to further reduce the level of photoactivation. To increase it, the Wratten

No. 25 filter was removed from the light path.

Subcellular Fractionation and Extraction of ROS Membranes. Light-induced phosphorylation of crude ROS from four retinas was allowed to proceed for 15 min after flash stimulus. The solutions were cooled, with the rest of the procedure being performed at 0–4 °C under dim red illumination. A total of 1.2 mL of the ROS suspension was mixed with 8.7 ml of 37% w/w sucrose (dissolved in 1 mM EGTA, 10 mM KF, pH 7.2) to yield 33% w/w sucrose. This was transferred to a centrifuge tube, overlayed with 20% w/w sucrose (dissolved in 1 mM EGTA, 10 mM KF, pH 7.2), and centrifuged. Outer segments floating at interface were collected in 1 mL, mixed with 10 mL of 20% w/w sucrose, and pelleted by centrifugation. The pellet of ROS was resuspended in 1 mL of Kuhn's medium of low ionic strength (5 mM Tris-HCl, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.4; Kuhn, 1982). Aliquots were set aside for rhodopsin assay and gel electrophoresis, while the rest was further diluted with 10 mL of Kuhn's medium, to which now 100 μM GTP was also added. The solution was kept in the dark for 1 h to optimize extraction of peripheral membrane proteins before cell membranes were again centrifuged into a pellet. The latter was resuspended in 0.5 mL of 1 mM dithiothreitol and 1 mM EGTA (titrated to pH 8.1 with NaOH; Shinozawa et al., 1980), and after sampling for rhodopsin and gel electrophoresis, the suspension was further diluted with 10 mL of the same medium. Again, extraction was performed for 1 h in the dark. After the final centrifugation, the pellet of ROS membranes was resuspended in Ringer's solution.

Labeling with FITC-Con A. The procedure of Molday & Molday (1979) was followed.

Rhodopsin Assay. For all experiments, rhodopsin content was assayed spectroscopically by bleaching difference in the presence of 10 mM hydroxylamine, with a molar extinction coefficient of 42 000 M^{-1} cm^{-1} at 502 nm.

Results

Conditions for Observing Light-Induced Reaction. Figure 1 illustrates the effect of light on protein phosphorylation in crude ROS preparations. As described by others (Bownds et al., 1972; Kuhn & Dreyer, 1972; Frank et al., 1973), rhodopsin is the major protein to be phosphorylated. But in addition, light also stimulates labeling of two other proteins, which migrate with an apparent mass of 220 and 240 kDa in this gel system. Radioactivity in each of these bands is about 1000 times less than that in rhodopsin (R_1). As seen in Figure 1, rhodopsin readily forms multimers, which under certain conditions could mask the radioactivity of all proteins above 40 kDa. In these experiments it was crucial to minimize rhodopsin polymerization. Although no exhaustive study has been performed on this subject, solubilization conditions cited under Materials and Methods were found to be adequate. Staining with Coomassie blue reveals no multimers above the dimer in Figure 1, while the corresponding autoradiogram shows the presence of pentamers and possibly even hexamers. This is because radiography is more sensitive than dye staining and also because the film was overdeveloped with respect to rhodopsin so that the small amount of ^{32}P in the proteins of interest could be detected. The latter are unlikely to be polymerized forms of rhodopsin: (a) mild controlled heating of solubilized samples prior to electrophoresis increased the concentration of dimers and trimers without appreciably altering the labeling of large proteins, and (b) solubilization conditions that reduced rhodopsin polymerization did not alter labeling of the 220-kDa protein. Moreover, polymerization appears to be a sequential process, for which the concentration

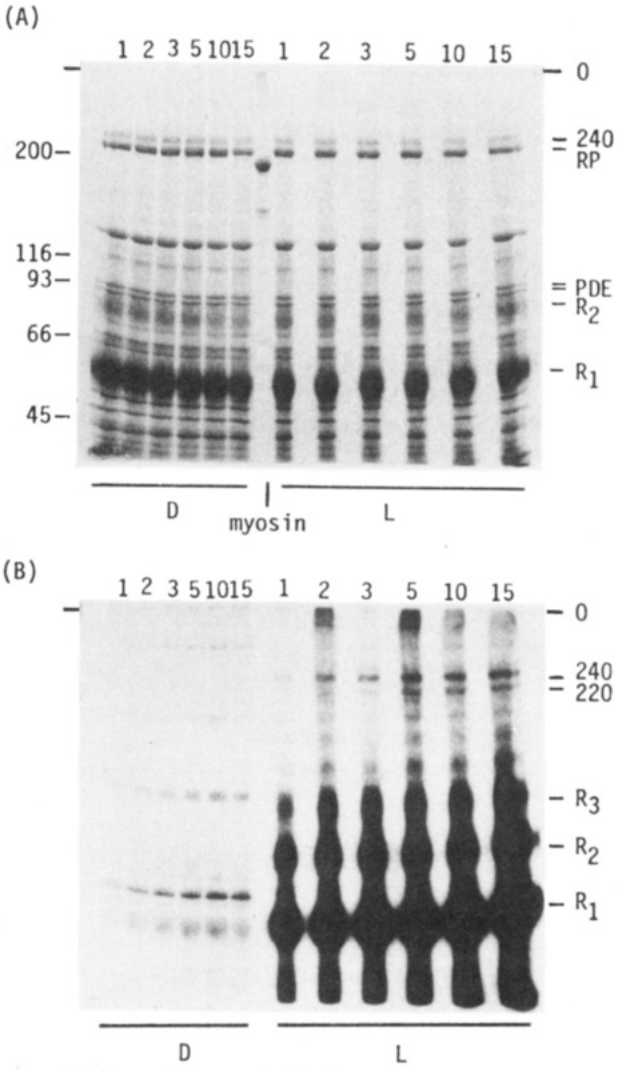

FIGURE 1: Electrophoregram (A) and corresponding autoradiogram (B) from a representative experiment designed to measure kinetics of light-induced phosphorylation in a suspension of crude outer segments. Suspension was divided into control (D for dark) and test (L for light) fractions. The latter was exposed to a flash that photoactivated 20% of rhodopsin. At increasing intervals, aliquots were removed, and their reaction was quenched at times indicated above each lane in minutes. Intervals for control (D), were measured from time of mixing. Rhodopsin was 20.1 μg/lane. RP refers to rim protein. PDE marks location of the major subunits of phosphodiesterase, at 92 and 93 kDa. Rhodopsin and its multimers are identified as R_1, R_2, and R_3, with apparent mass of R_1 and R_2 being 57 and 82 kDa, respectively. Size markers are in kilodaltons.

of the higher multimers progressively decreases. Formation of a large aggregate at 220 kDa, for example, would require a more abundant precursor at about 180 kDa, which was unobserved.

Labeling of the two large proteins is readily observed with crude preparations. Incorporated radioactivity was greatly reduced with purified fragmented outer segments (presumably due to loss of soluble kinase) or with intact outer segments (presumably due to decreased yield of receptors). All phosphorylation experiments were performed with fresh preparations. Freezing, followed by subsequent thawing, reduced the extent of labeling of the large proteins by 60% when compared to control.

Labeled bands on the autoradiogram correspond to Coomassie blue stained bands on the gel. One of these at 220 kDa represents the large intrinsic membrane protein (herein called rim protein) that Papermaster and co-workers found at the

outer margins and incisures of frog disks (Papermaster et al., 1978). Identification of the 220-kDa band as the rim protein is based on previous reports. The rim protein forms the major Coomassie blue staining band above 150 kDa, even with unpurified outer segments (Papermaster et al., 1976). Molday & Molday (1978) reported its molecular mass from frog to be about 260 kDa, which is in close agreement with my value of 220 kDa given that they used a 9% gel that leads to higher estimates. The protein at 220 kDa was found to bind concanavalin A, also in agreement with Molday and Molday. And finally, the 220-kDa protein behaved as an integral membrane protein on subcellular fractionation of rod cells. Additional tests confirmed that the labeled bands at 220 and 240 kDa are proteins. Pronase digestion of the already labeled preparation abolished the radioactive bands on subsequent electrophoresis, while a single extraction with 3 mL of chloroform/methanol/12 M HCl (200/100/1) did not appreciably alter the labeling pattern.

The pattern of preferential labeling varies for the two proteins depending on whether ATP or ATP-γ-S participates in the reaction. As observed in Figure 1, phosphorylation leads to about twice as much ^{32}P incorporation into the 240-kDa protein than into the 220-kDa protein. This preference is reversed with thiophosphorylation. In that case, the rim protein is more intensely labeled by about a factor of 10 (see Figure 3).

Control experiments have established that light-induced labeling is not due to nucleotide binding. Rather, it involves the transfer of the terminal phosphate. For example, when the experiments were performed with [α^{32}P]ATP, [2,8,5′-^{3}H]ATP, or [^{35}S]ATP-α-S, with substrates in which the radioactive atoms are excluded from the terminal phosphate group, radioactive incorporation was drastically reduced for the two large proteins. Calculations indicate that at least 98% of the radioactivity in the bands of Figure 1 is due to the transfer of the terminal phosphate group. Therefore, the observed ^{32}P incorporations reflect kinase activity.

During the course of these experiments, light was also observed to alter the phosphorylated state of several proteins of low molecular mass. These appear to be the proteins described by Polans et al. (1979) and Biernbaum et al. (1983). They were routinely run off the gels to enhance the resolution of the larger proteins.

Subcellular Fractionation and Extraction. Because crude ROS preparations contain small amounts of retinal debris other than receptors, the cellular location of the labeled proteins was investigated. Subcellular fractionation was performed on a preparation that was already radioactively tagged. As a first step, the membranes of the ROS were purified by sequential centrifugations on sucrose gradients. The purified disks were then extracted with solutions of low ionic strength to remove all periheral membrane proteins. Extraction efficiency was based on the amount of phosphodiesterase observed on stained gels. In initial experiments, the sucrose gradients were prepared with normal salt solutions. When purified disks from such gradients were extracted with hypotonic media, only about 75% of their phosphodiesterase could be removed with three consecutive washes. To improve extraction, salts were also excluded for the sucrose centrifugations.

The ionic strength (μ) of the solubilized samples had a pronounced effect on subsequent gel electrophoresis. If μ was kept at 0.04 M, the protein content in the bands was greatly reduced for both rhodopsin and the 240-kDa polypeptide. The proteins reappeared if μ was increased to 0.14 M. Presumably, a moderate ionic strength is needed to adequately solubilize these molecules by $NaDodSO_4$. Such a salt effect was absent for the rim protein and the phosphodiesterase.

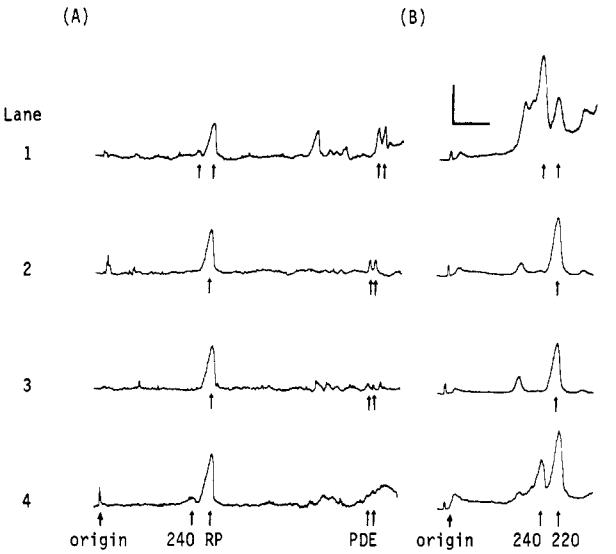

FIGURE 2: Subcellular fractionation of ^{32}P-labeled outer segments by sucrose centrifugation, with subsequent extraction of peripheral proteins with solutions of low ionic strength. Densitometry of Coomassie blue stained dried gel (A) and of the corresponding autoradiogram (B), with sensitivity of densitometer the same for all traces. (Lane 1) Sample from initial suspension; (lane 2) sample represented by lane 1 after sucrose density centrifugations; (lane 3) purified disks represented by lane 2 after extraction with Kuhn's medium of low ionic strength; (lane 4) disks represented by lane 3 after final extraction with 1 mM EDTA. Arrows mark the location of the following: major subunits of phosphodiesterase (PDE), rim protein (RP), protein at 240 kDa, and the phosphoproteins at 240 and 220 kDa. Rhodopsin peaks are not shown because they were off scale. During the course of these experiments, sample preparation for electrophoresis was found to be crucial for the detection of the 240-kDa protein. Solubilization for electrophoresis was performed by mixing 2 volumes of ROS suspension with 1 volume of 3 × Laemmli's sample buffer. If the ROS suspension had low ionic strength, the 240-kDa protein was absent on subsequent electrophoresis. Apparently, $NaDodSO_4$ is incapable of solubilizing this protein when the final ionic strength is 0.04 M. This is the reason for the absence of this protein in lanes 2 and 3 above. To show that the protein remained with the disks throughout the purification–extraction procedure, the pellet of membranes was resuspended in Ringer's following the final EDTA extraction. When this suspension was mixed with Laemmli's sample buffer, the final ionic strength was 0.15 M. As seen in lane 4, the 240-kDa protein reappears on both electrophoregram and autoradiogram, indicating that it was not extracted from the disks by the preceding washes. The inset gives the scale for all traces, with the ordinate being 0.2 OD units and abscissa 2 cm. The dried cellophane scatters light and is the source of background noise in (A).

Figure 2 illustrates the results of a typical fractionation experiment in which sucrose solutions of low ionic strength were employed. The effect of ionic strength can be easily seen on the densitometric traces of the Coomassie blue stained gel. Note the presence or absence of the 240-kDa protein and of the dimer of rhodopsin, which migrates just ahead of the phosphodiesterase. The latter enzyme is nearly absent on the last trace due to extraction with the preceding hypotonic washes.

Integral membrane proteins of the disks are expected to copurify with the visual pigment. Specifically, the concentrations of such proteins relative to rhodopsin should remain constant as disks are purified and extracted with hypotonic solutions. The experiment of Figure 2 was analyzed for this purpose. The rhodopsin content of each sample, electrophoresed per lane, was assayed by spectrophotometry. The protein content of the phosphodiesterase, rim protein, and 240-kDa protein was calculated by measuring their staining

Table I: Analysis of Experiment on Subcellular Fractionation Shown in Figure 2

lane[a]	μg of rho/lane[b]	μg of protein for PDE[c]	μg of protein for rim protein	μg of protein for 240-kDa protein	μg of protein/μg of rho for PDE	μg of protein/μg of rho for rim protein	μg of protein/μg of rho for 240-kDa protein
1	14.3	0.27	0.51	0.049	0.019	0.036	0.003
2	15.6	0.13	0.63		0.008	0.040	
3	20.9	0.05	0.72		0.002	0.035	
4	35.2	0.05	0.95	0.064	0.001	0.027	0.002

lane	cpm per band[d] 220-kDa protein	cpm per band[d] 240-kDa protein	cpm per μg of rho 220-kDa protein	cpm per μg of rho 240-kDa protein	cpm per μg of protein 220-kDa protein	cpm per μg of protein 240-kDa protein
1	46	105	3.2	7.4	90	2149
2	80		5.1		128	
3	77		3.7		107	
4	110	126	3.1	3.6	117	1973

[a] Refers to the lanes shown in Figure 2. [b] Rhodopsin content of each lane was based on a spectrophotometric assay. [c] Protein content of phosphodiesterase (PDE) was based on the α and β subunits. [d] Limiting error is due to uncertainty in base line when background radioactivity is subtracted on the densitometric traces. Error due to counting is less than 5%.

intensity relative to a known amount of myosin. Corrections, if any, were also made for differences in lane width and inhomogeneity in the bands. Finally, the mass ratio was derived for each protein, in units of microgram of protein per microgram of rhodopsin (see Table I). As judged by the constancy of its mass ratio, no loss of rim protein occurred during the entire subcellular fractionation. This also appears to be the case for the 240-kDa polypeptide, within the errors of the measurements. The two large proteins appear to be intrinsic membrane components, in contrast to the phosphodiesterase of which over 90% was released with the hypotonic washes.

The phosphodiesterase content of the initial ROS preparation was found to be about 0.019 μg/μg of rhodopsin, in reasonable agreement with previous reports. Miki et al. (1975), who were also working with frog, report a value that corresponds to about 0.007. For cattle, the estimate for this ratio varies from 0.004 (Kuhn, 1982) to a range of 0.03–0.11 (Baehr et al., 1979). The mass ratio for the rim protein was found to be about 0.035, in good agreement with a value of 0.04 calculated from the raw data of Molday & Molday (1979, their Figure 2).

Do the *phosphorylated* proteins also copurify with the disk? To determine whether their ^{32}P content remained constant with rhodopsin, the autoradiogram in Figure 2 was analyzed as described under Materials and Methods, with results shown in Table I. The errors in these calculations are greater than those for protein contents, especially for the initial sample where background corrections are more error prone. Nevertheless, the ^{32}P content of the two phosphoproteins (expressed per rhodopsin) remained relatively constant within the errors of the measurement throughout the entire purification and extraction procedure. These results suggest that the two large phosphoproteins are integral components of the outer segment membranes, residing either in the disk or plasma membranes.

Oxidation of Sulfhydryl Groups. Proteins were oxidized as a test for possible correspondence between rim protein and the 220-kDa phosphoprotein. To reduce interference from the labeled 240-kDa protein, the oxidation was performed with rods that have been radioactively labeled with thiophosphates. This was done because earlier observations established that of the two protein substrates light preferentially stimulates the thiophosphorylation of the 220-kDa molecule, incorporating

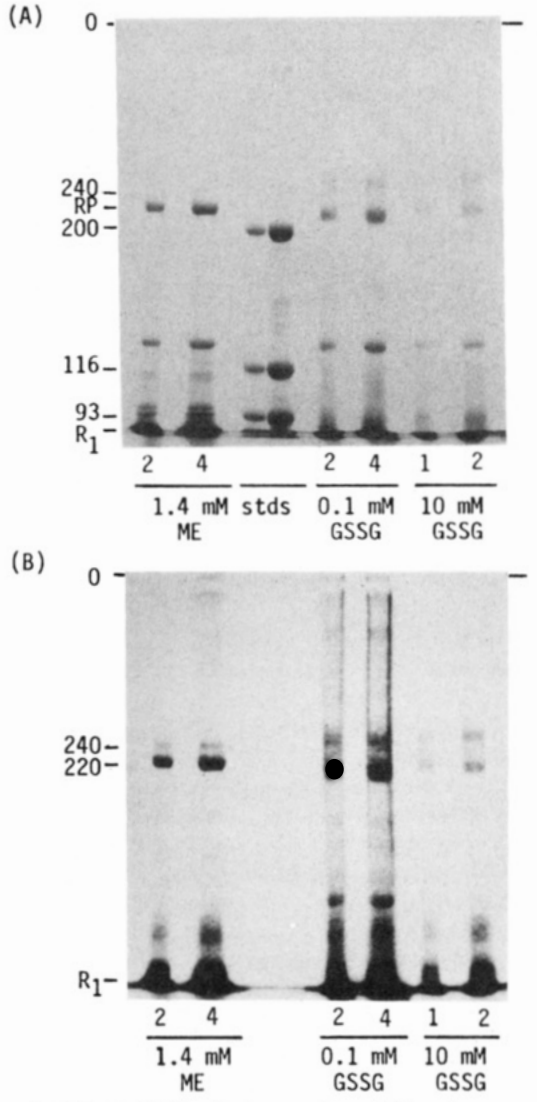

FIGURE 3: Effect of SH oxidation on the mobility of outer segment proteins that were previously thiophosphorylated. Electrophoregram (A) and corresponding fluorogram (B). Prior to electrophoresis, samples were reacted with indicated concentrations of either β-mercaptoethanol (ME) or oxidized glutathione (GSSG). The relative amount of sample, measured either as total protein or rhodopsin, is indicated below each lane. Two lanes in the center contain protein standards, which were also reduced with ME prior to electrophoresis. The gel was cast with GelBond (FMC Corp.) with an acrylamide concentration of 4% T/2.6% C. RP and 240 indicate the locations of the large membrane proteins. Size markers are in kilodaltons.

about 10 times more ^{35}S into it. In agreement with the observation of Papermaster et al. (1976), oxidized rim protein migrates in two bands on gel electrophoresis: one being slightly faster and the other slightly slower than that of the fully reduced sample (Figure 3). Oxidation also split the corresponding radioactive band into two bands, which superimposed with the displaced Coomassie blue stained proteins. Thus, both labeled and unlabeled proteins yield similar reaction products. This observation, together with the previous conclusion that both molecules are embedded in the ROS membranes, strongly suggests that the proteins are identical with each other. By extension, the results indicate that the rim protein is the phosphorylated substrate of the light-stimulated kinase.

Phosphorylation with Intact Cells. A general property of kinases is their nonselectivity toward their protein substrate (Weller, 1979). Although light-sensitive kinases are unlikely to occur outside photoreceptors, the enzymes responsible for the results of Figure 1 may have originated from cells other

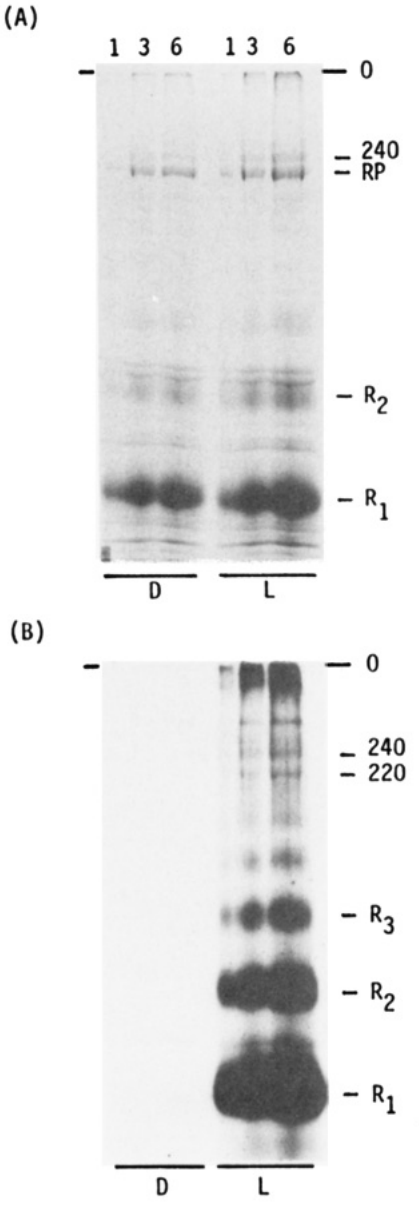

FIGURE 4: Light-induced phosphorylation of proteins in the receptor layer of excised retinas that were incubated under in vivo conditions with $^{32}PO_4$. Electrophoregram (A) and corresponding autoradiogram (B). Numbers above lanes refer to relative sample volumes electrophoresed for unilluminated control retina (D) and illuminated retina (L). The locations off the 240-kDa protein, rim protein, phosphodiesterase, and rhodopsin are respectively marked as 240, RP, PDE, and R_1.

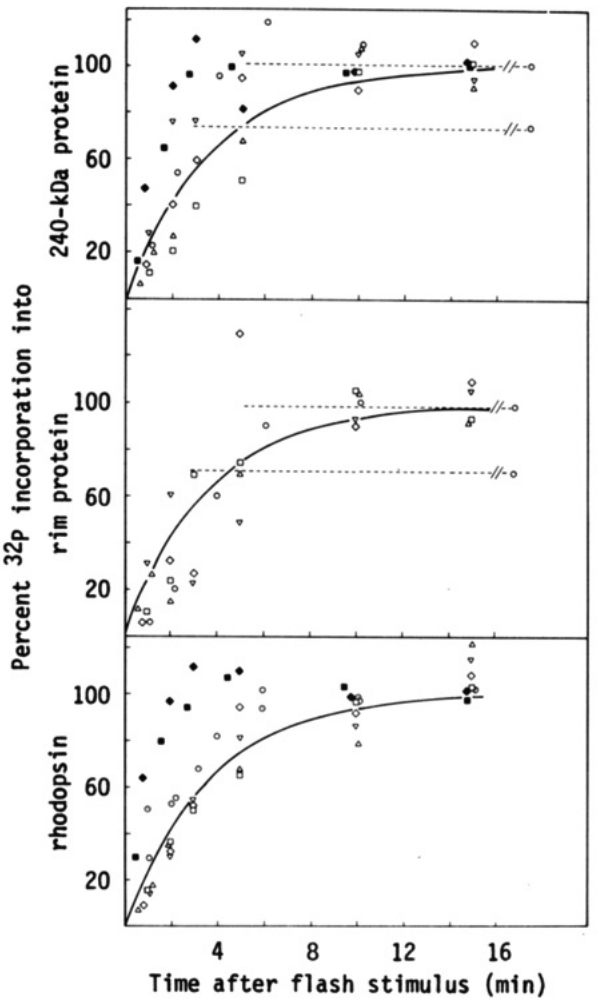

FIGURE 5: Time course of phosphorylation for 240-kDa protein, the rim protein, and rhodopsin, following a light flash that photoactivated 20% of rhodopsin in crude ROS preparations. The data derived by densitometry of autoradiogram from separate experiments are similar to those in Figure 1. A separate symbol is used for each experiment, with data from Figure 1 plotted as (◊). Filled symbols refer to experiments in which ATP was regenerated with the phosphocreatine system. The solid curve describes a first-order reaction with $t_{1/2}$ = 2.5 min. Horizontal dashed lines refer to tests of phosphoprotein turnover in one experiment [plotted as (○)]: aliquots were removed from the reaction mixture and mixed with 1 mM cold ATP at the time indicated by the start of the dashed lines; aliquots were then incubated for an additional 15 min before their reactions were quenched. Within each experiment, data points were normalized to the average of the data points taken at 10 and 15 min.

than photoreceptors. As an initial test of this possibility, phosphorylations were performed with excised retinas incubated with $^{32}PO_4$ under in vivo conditions. Figure 4 shows that ^{32}P incorporation into proteins of interest also occurred within the intact receptors of the retina and, moreover, was also light initiated. Thus, rods possess kinases that phosphorylate the 220- and 240-kDa proteins. Presumably, the same kinases are the active enzymes in the fragmented preparations of Figure 1. Proof of this, however, will require extensive characterization and comparison of the two reactions.

Light-induced phosphorylation of other minor proteins can also be seen in Figure 4. Their identity and reaction are currently under investigation.

Kinetics of Phosphorylation. Following a flash that photoactivates 20% of the rhodopsin, phosphorylation of the large proteins saturates within 10–15 min (Figure 5). Half-maximal incorporation occurs at about 2.5 min, when the initial concentration of ATP is 0.1 mM. Incubations longer than 15 min usually were marked by dephosphorylation in spite of the presence of 10 mM KF, a general inhibitor of phosphatases. The kinetics of rhodopsin phosphorylation was found to be similar in these very same experiments. All of the reactions deviate from first-order kinetics; there is an initial delay, with a large spread in data points. Variability due to experimental techniques is less than the point spread, as judged by the smaller deviations observed for thiophosphorylation (Figure 6). The latter experiments, which were performed several months before the experiments of Figure 5, resulted in less variability, possibly because thiophosphorylated proteins are hydrolysis resistant and because ATP-γ-S is not as labile with time as ATP. When ATP concentration was buffered at 0.1 mM with an ATP-regenerating system, time for half-maximal phosphorylation was reduced to about 1 min. Observed rates for thiophosphorylation were similar (Figure 6). The stoichiometry of ^{32}P incorporation has not yet been definitely

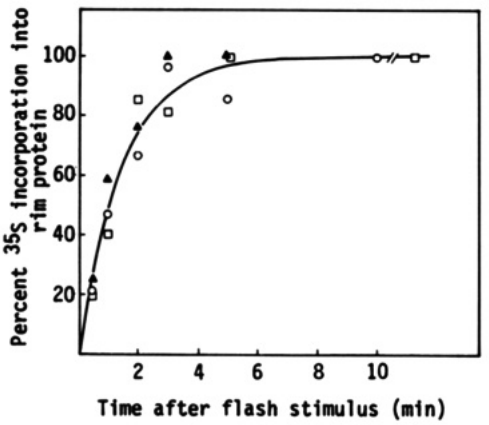

FIGURE 6: Time course of thiophosphorylation of rim protein, following a light flash that photoactivated 20% of rhodopsin in crude ROS preparations. Data are from three separate experiments, in which final concentrations for [^{35}S]ATP-γ-S and ATP-γ-S were 30–50 μCi/mL and 30–50 nM, respectively. The solid curve describes a first-order reaction with $t_{1/2}$ = 1 min. The last data point (□) was taken 30 min after the flash.

established. Preliminary experiments indicate that maximal labeling corresponds to about 1 and 0.1 phosphate per molecule of 240-kDa polypeptide and rim protein, respectively, when reaction is performed with an initial ATP concentration of 0.1 mM.

Is increased incorporation of ^{32}P due to an increase in the number of bound phosphates or to increased turnover of a fixed number of phosphates? This was tested with a "cold–chase" experiment [data plotted as (○) in Figure 5]. Following light stimulation, two aliquots were removed from the reaction mixture and were mixed with excess nonradioactive ATP. Mixings occurred 2.8 and 5 min after the onset of ^{32}P labeling (see dashed lines in Figure 5). These samples were incubated for an additional 15 min before their reactions were quenched. If light were to increase turnover, the cold–chase would release previously accumulated ^{32}P as "cold" phosphates would replace radioactive ones. On the other hand, if light were to increase bound phosphates, the "chase" should not have caused the loss of any ^{32}P. The amount of ^{32}P associated with the proteins remained high following the cold–chase, as if the labeling reactions were abruptly quenched at the moment of mixing with cold ATP. This result is inconsistent with increased turnover but is the predicted outcome for the alternative scheme. Thus, light's action is to increase the number of bound phosphates, as would be expected for a light-stimulated kinase reaction. Note that in cited experiment, light-induced incorporation of ^{32}P followed the same kinetics as in other experiments (see data points for samples not chased). Thus, the absence of turnover cannot be attributed to the lack of a light effect. Results of thiophosphorylation experiments were also consistent with a kinase scheme, requiring an increase in the number of bound phosphates.

Light Sensitivity of Phosphorylation. Analysis of experimental data, such as in Figure 7, indicates that the light sensitivity of the kinase is about the same for rim protein, 240-kDa protein, and rhodopsin (Figure 8), when expressed in relative terms. Half-maximal phosphorylation occurred with photoactivation of about 2% of the visual pigment. The threshold for measurable ^{32}P incorporation into the large proteins occurred at about 0.02% photoactivated pigment.

cGMP Dependence of Phosphorylation. Because cGMP plays a prominent role in phototransduction, its effect on protein phosphorylation was measured. In agreement with a previous paper (Shuster & Farber, 1984), cGMP inhibited

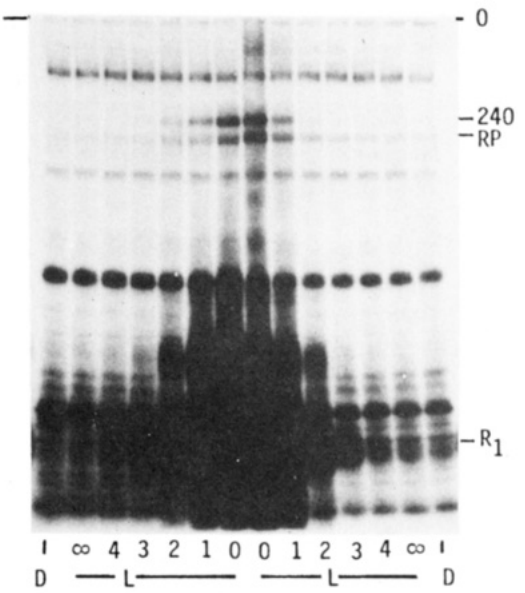

FIGURE 7: Light sensitivity of protein phosphorylation in crude outer segments. Autoradiogram shows ^{32}P incorporation following flashes of varied intensity for two separate experiments. Unexposed control samples are marked D. For the light-exposed samples (L), the stimulus flash was attenuated in log units as indicated below each lane. Unattenuated flash photoactivated 20% of rhodopsin. For an attenuation of ∞, a completely opaque "filter" was placed in the light path as a test for possible light leakage.

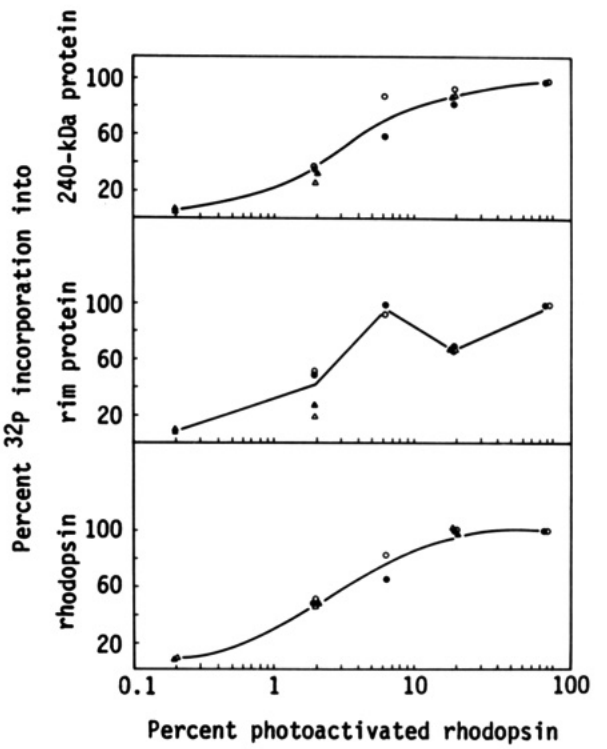

FIGURE 8: Light sensitivity for the phosphorylation of the 240-kDa protein, the rim protein, and rhodopsin. The ordinate represents the extent of phosphorylation 15 min following a single flash stimulus. Data from four experiments were normalized to levels obtained with maximum light intensity, with data in Figure 7 plotted as (△, ▲).

phosphorylation. The results tabulated in Table II are expressed relative to the endogenous cGMP concentrations of the starting preparation. This concentration was calculated to be less than 0.1 μM on the basis of dilution, assuming that all of the initial 50 μM cGMP (Woodruff & Bownds, 1979) leaked out of the fragmented rods and was evenly distributed throughout the incubation medium without any hydrolysis.

Table II: cGMP Dependence of Protein Phosphorylation[a]

[cGMP] (mM)	percent ^{32}P incorporation into		
	rhodopsin	240-kDA protein	rim protein
0.00[b]	100.0	100.0	100.0
0.05	45.0 ± 5.1 (3)	50.2 ± 12.0 (3)	43.7 ± 5.0 (3)
0.20	17.2 ± 6.3 (3)	23.8 ± 2.4 (2)	18.2 ± 3.7 (2)
1.00	11.1 ± 9.5 (3)	22.1 (1)	

[a] ^{32}P incorporation was measured 15 min after light stimulus that photoactivated 20% of rhodopsin. All experiments were performed in the presence of 0.1 mM ATP, phosphocreatine ATP-regenerating system, and 1 mM (isobutylmethyl)xanthine. The latter was included to prevent hydrolysis of cGMP by the light-activated phosphodiesterase and was also in the solutions during the isolation of the outer segments. Percent values expressed as mean ± SD (n), where n refers to number of experiments. [b] In the absence of any added cGMP, whose endogenous concentration was less than 0.1 μM.

Dilution factors were based on rhodopsin ratios, using a value of 3 mM for the concentration of the visual pigment within intact ROS (Harosi, 1975). Raising cGMP levels to 1 mM reduced ^{32}P incorporation into the large proteins so greatly that their radioactive levels were difficult to quantify. As listed in Table II, cGMP exerted the same relative inhibition for all three membrane proteins.

Discussion

The results of this paper demonstrate that light stimulates the phosphorylation of two large membrane proteins within vertebrate photoreceptors. Light's effect on these proteins may have been missed by others, partly because the incorporated radioactivity is low (about 3 orders of magnitude less than that for rhodopsin) and partly because resolution of the radioactive bands by gel electrophoresis requires adequate suppression of rhodopsin aggregation.

On the basis of four criteria (electrophoretic mobility, subcellular fractionation, binding of concanavalin A, and behavior following SH oxidation), the labeled protein at 220 kDa appears to be identical with the rim protein that Papermaster and his co-workers have previously characterized (Papermaster et al., 1976, 1978). As an additional test of this conclusion, immunological tests are planned for the future. The identity of the second protein, migrating at 240 kDa, remains unknown. It may reside either in the disk or plasma membranes. The latter possibility exists because subcellular fractionation cannot effectively separate disks from rod and cone plasma membranes. The 240-kDa protein appears to be distinct from the rim protein because of solubility differences with ionic strength (see Figure 2) and because of differential labeling when phosphorylation and thiophosphorylation reactions are compared (see Figures 1 and 3). Both of these molecules are minor proteins of the rod outer segment. In bullfrogs, about 100 rhodopsin molecules occur for each rim protein and about 1000 rhodopsins per 240-kDa protein.

Phosphorylation of the large proteins is possibly related to one of the two processes known to exist within outer segments: disk turnover (Young, 1976) and phototransduction (encompassing both receptor excitation and adaptation). The unique subcellular location of the rim protein would tend to favor the first possibility. Nevertheless, these alternatives were tested by investigating the kinase reactions for their kinetics, light sensitivity, and cGMP dependence. These form a good initial test for deciding whether the reactions are of relevance to phototransduction. The study was also extended to rhodopsin, so that phosphorylation of all three protein substrates could be observed within the same experiments. The need to include rhodopsin was 2-fold: to form a basis of comparison with previous studies and to compare the phosphorylation of the large proteins with a protein, whose phosphorylation is unquestionably linked to transduction.

Results of the current experiments on rhodopsin are in general agreement with those of previous papers. In the absence of ATP regeneration, the half-time for rhodopsin phosphorylation was found to be about 2.5 min. Using a slightly different protocol, Bownds et al. (1972) observed a similar half-time of about 5 min with frog ROS. Regarding light sensitivity, the experiments presented here show that 2% of the pigment needs to be photoactivated to achieve half-maximal phosphorylation. To reach this level, Miller et al. (1977) had to photoactivate about 35%, but their light stimulus was 5 min in duration as opposed to the 1 ms used in this paper. Increasing concentration of cGMP progressively reduced rhodopsin phosphorylation in agreement with Shuster & Farber (1984). Inhibition by cGMP was also reported by Hermolin et al. (1982), but they attributed the effect to the hydrolyzed product, GMP. The degree of inhibition that Swarup & Garbers (1983) reported was a function of light intensity, so that inhibition was replaced by stimulation as photoactivated rhodopsin increased from 1% to over 5%. Possible cause for all this discrepancy is that neither Hermolin et al. nor Swarup and Garbers used inhibitors to depress the light-activated activity of the cGMP-dependent phosphodiesterase.

One of the central observations of this study is the close resemblance between rhodopsin phosphorylation and phosphorylation of the large membrane proteins. This is the case whether kinetics, light sensitivity, or cGMP dependence is being compared. Thus, these kinase reactions are closely linked. Such linkage would occur if the same kinase were to react with all three of these proteins or, more likely, if phosphorylated rhodopsin were to closely regulate the activity of a set of kinases that are distinct from rhodopsin kinase. Due to the possible role of rhodopsin phosphorylation in excitation or adaptation, the large membrane proteins may also be linked functionally to transduction. If so, what is their specific role? One of the current hypotheses of phototransduction (Hagins, 1972) states that calcium is an intracellular messenger that is released from disks, diffuses to the plasma membrane, and is subsequently reaccumulated by the disks again. Because it is an integral membrane protein and because it is strategically located at the edges of disks, the rim protein is an ideal candidate to be the Ca pump envisioned by the original calcium hypothesis. Phosphorylation of the rim protein, however, does not resemble the properties of other pumps. Turnover rate of its phosphorylated form is very slow (in excess of 15 min), and because its radioactive level was invariant with acid and basic gel electrophoresis, its phosphate bond is not the acyl linkage usually encountered with "high-energy" intermediates (Avruch & Fairbanks, 1972). Similar considerations also rule out the 240-kDa protein as an ion translocator. Thus, the role these large proteins play in receptor physiology remains to be solved.

In conclusion, the work reported here shows that light stimulates the phosphorylation of two membrane proteins other than rhodopsin within vertebrate photoreceptors. Available evidence suggests that the physiological function of the large membrane proteins is more closely linked to transduction than to membrane turnover.

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Registry No. cGMP, 7665-99-8; protein kinase, 9026-43-1.

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