Light-Induced Dephosphorylation of a 33K Protein in Rod Outer Segments of Rat Retina†

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ABSTRACT: Phosphorylated proteins may play an important role in regulating the metabolism or function of rod photoreceptors. In mammalian retinas, a photoreceptor protein of 33 000 (33K) molecular weight is phosphorylated in a cyclic nucleotide dependent manner in vitro. Since light initiates the activation of a photoreceptor-specific phosphodiesterase and a rapid reduction in guanosine cyclic 3′,5′-phosphate concentration, phosphorylation of the 33K protein may be modulated by light in situ. In order to test this possibility, dark-adapted rat retinas were incubated for 30 min in the dark in phosphate-free Kreb's buffer containing [^{32}P]orthophosphate. Following incubation, rod outer segments were detached by shaking, and the ^{32}P-labeled rod outer segment proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, detected by autoradiography, and quantitated by densitometric scanning. The incorporation of radioactivity (^{32}P) into the 33K protein was higher than into any other rod outer segment protein, and the amount of ^{32}P-labeled 33K protein in the detached rod outer segments remained unchanged during 10 additional min of darkness. The addition of isobutylmethylxanthine to the incubation medium enhanced the incorporation of ^{32}P into 33K protein to about 400% of the original level. Exposure of freshly detached rod outer segments to room light for 90 s decreased the amount of labeled 33K protein to 45% of its original level. The dephosphorylation of labeled 33K protein continued, reaching 12% of the original dark value 10 min after the previously illuminated sample was returned to darkness. Light initiated the phosphorylation of rhodopsin, and rhodopsin phosphorylation continued during the postillumination period of darkness. These observations suggest that the 33K protein may be involved in light-initiated processes that are regulated in situ by changes in the intracellular concentration of guanosine cyclic 3′,5′-phosphate.

Protein phosphorylation is an important mechanism in the regulation of cellular metabolism or function (Cohen, 1982; Greengard, 1978). In the nervous system, phosphorylated proteins are postulated to be involved in the modulation of ion fluxes across the plasma membrane. In the rod photoreceptors of the vertebrate retina, there is evidence that phosphorylated proteins may participate in the regulation of visual function. Of particular relevance are proteins which exhibit light-dependent changes in the extent of their phosphorylation. The phosphorylation of rhodopsin by rhodopsin kinase is initiated by light and is suggested to modulate the activity of the photoreceptor cyclic nucleotide phosphodiesterase (Sitaramayya & Liebman, 1983; Aton & Litman, 1981; Liebman & Pugh, 1980). Others have suggested a role for the reaction in a slow adaptation process (Miller et al., 1977; Kuhn, 1974).

In the rod photoreceptors of frog retina, two small phosphoproteins (molecular weights of 12K and 13K) have been identified, and the levels of phosphorylation are modulated by light as well as by guanosine cyclic 3′,5′-phosphate (cGMP)[1] or phosphodiesterase inhibitors in situ. It is suggested that the 12K and 13K proteins may be involved in the light/dark adaptation or the phototransduction mechanism (Hermolin et al., 1982; Polans et al., 1979).

In the rod photoreceptors of mammalian retinas, a protein with 33 000 molecular weight (33K protein) on the sodium dodecyl sulfate ($NaDodSO_4$) gel (previously identified as the 30K protein) is phosphorylated by an endogenous cyclic nucleotide dependent protein kinase (Lee et al., 1982; Farber et al., 1979). The phosphorylation of the 33K protein is maximally stimulated by 10^{-4} M cGMP or by 10^{-5} M adenosine cyclic 3′,5′-phosphate (cAMP). The 33K protein is readily extractable from retinas or isolated rod outer segments (ROS) by hypotonic buffers (Lee et al., 1981a). It appears to be a soluble protein which is distributed within the cytoplasmic spaces of rod photoreceptors. In this paper, the phosphorylation and dephosphorylation of the 33K protein are investigated by using retinas incubated with [^{32}P]orthophosphate ([^{32}P]P_i). We found that the radioisotope is incorporated into the 33K protein during incubation in darkness and that the level of radioactivity (^{32}P) incorporation is reduced during subsequent illumination. The ability of light to modulate the extent of phosphorylation of the 33K protein is consistent with a role for this protein in the regulation of photoreceptor metabolism or function.

Experimental Procedures

Materials

The retinas used in this study were obtained from rats of either the Fischer CDJ strain (100–150 g), which are bred in our animal facility, or the Sprague-Dawley strain (100–150 g), which were purchased from Simonsen Laboratories, Inc., Gilroy, CA. All rats were reared in the vivarium under diurnal lighting conditions and dark adapted for 2–16 h before decapitation. The retinas were dissected immediately under dim red light after enucleation.

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[1] Abbreviations: ROS, rod outer segment(s); cAMP, adenosine cyclic 3′,5′-phosphate; cGMP, guanosine cyclic 3′,5′-phosphate; [^{32}P]P_i, [^{32}P]-orthophosphate; $NaDodSO_4$, sodium dodecyl sulfate; IBMX, isobutylmethylxanthine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Carrier-free [^{32}P]P_i and [γ-^{32}P]ATP (2.5–4 Ci/μmol) were purchased from Amersham and New England Nuclear, respectively.

Methods

Phosphorylation of Retinal Protein in Vitro. A freshly dissected retina from a dark-adapted rat was homogenized in the dark in 650 mL of ice-cold 50 mM Tris-HCl, pH 7.6. The retinal homogenate was used as the source both of the cyclic nucleotide dependent protein kinase and of its endogenous substrates. The phosphorylation reaction, in a final volume of 100 μL, contained 50 mM Tris-HCl, pH 7.6, 10^{-4} M isobutylmethylxanthine (IBMX), 5 mM $MgCl_2$, 60 μM [γ-^{32}P]ATP (sp act. ~2000 cpm/pmol), and 20 μL of retinal homogenate in the presence or absence of 10^{-5} M cAMP. The reaction was carried out at 30 °C for 10 min, either in the dark or under laboratory illumination as specified under Results. Phosphorylation was stopped by the addition of 19.0 μL of stopping solution (25% $NaDodSO_4$ and 25% mercaptoethanol). The samples were boiled at 100 °C for 3 min before being electrophoresed on $NaDodSO_4$–polyacrylamide gels.

Phosphorylation of ROS Protein in Vitro. Two freshly dissected retinas from a dark-adapted rat were transferred to a homogenizer containing 400 μL of ice-cold phosphate-free Kreb's solution (120 mM NaCl, 4.8 mM KCl, 1.3 mM $MgSO_4$, 10 mM glucose, 0.01 mM $CaCl_2$, 100 mM Tris-HCl, pH 7.4, and, unless otherwise indicated, 0.1 mM IBMX). The retinas were gently shaken to detach the ROS. After the retinas were removed, the ROS suspension was immediately homogenized, and two 150-μL aliquots each were transferred to phosphorylation reaction mixtures (200-μL final volume) containing either 10^{-5} M or no cAMP (see above). After the reaction mixtures stood in the dark at 30 °C for 10 min, 1.8 mL of ice-cold acetone was added, and each sample was kept in the ice bath for 2 h before being centrifuged in a clinical centrifuge. Each of the resultant precipitates was dissolved in 50 μL of lysis buffer and subjected to two-dimensional gel electrophoresis as described in a latter section.

In a separate series of experiments, phosphorylation of proteins in the ROS of frog retinas was studied. The ROS were prepared from dark-adapted frog retinas according to the procedures of Papermaster & Dreyer (1974) up to the stage that the crude ROS were pelleted by low-speed centrifugation. The ROS from six frog retinas were homogenized in 1 mL of 50 mM Tris-HCl, pH 7.5, and centrifuged at 100000*g*. Proteins in the resulting supernatant were phosphorylated in the above reaction mixture containing either 10^{-5} M cAMP or 10^{-4} M cGMP or no added cyclic nucleotide. The ^{32}P-labeled proteins were separated by $NaDodSO_4$ gel electrophoresis and detected by autoradiography, as described below.

Phosphorylation of ROS Protein in Situ. Freshly dissected retinas were incubated in the dark in a phosphate-free Kreb's solution containing [^{32}P]P_i to allow incorporation of ^{32}P into the endogenous phosphoproteins. In a typical experiment, six retinas were incubated at room temperature in 1.2 mL of Kreb's solution containing 2 mCi of [^{32}P]P_i for 30 min. At the end of the incubation, the retinas were transferred into 1 mL of Kreb's solution without the isotope and vortexed gently for 10 s to detach the ROS. The retinas were then removed with forceps, and the remaining ROS suspension was either immediately quenched with $NaDodSO_4$, allowed to stand in the dark, or exposed to laboratory illumination, as specified in each individual experiment. The ROS suspension was quenched by homogenizing multiple 100-μL aliquots in glass homogenizers, each containing 19.0 μL of stopping solution, before being processed further for $NaDodSO_4$–polyacrylamide gel electrophoresis. For samples to be analyzed by two-dimensional gel electrophoresis (Figure 2), the ROS suspension was quenched instead by 1 mL of ice-cold acetone.

In the experiments designed to evaluate the effect of light while ROS remained attached to the cell bodies, retinas for the light or dark samples were incubated in separate containers, exposed to light or darkness before the ROS were detached, and processed for $NaDodSO_4$–polyacrylamide gel electrophoresis. In another series of experiments designed to evaluate the effect of IBMX, the control and the sample retinas were incubated in separate media in the absence or presence of 0.1 or 1.0 mM IBMX.

In this study, illumination of the ROS or retina was carried out by exposing the sample(s) to laboratory light for 90 s. Using isolated bovine ROS, our laboratory illumination was determined to bleach 2.5% of the retinal rhodopsin per s. Thus, 90-s illumination essentially bleaches all the rhodopsin.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. ^{32}P-Labeled phosphoproteins of rat retinas or ROS were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis according to Laemmli (1970), using a Hoefer apparatus. Good resolution of rhodopsin and the 33K protein was achieved by using a 10% or 15% gel (14 × 9 cm) or a 6–20% gradient gel (14 × 20 cm). Routinely, a 15% gel was used, and the electrophoresis was carried out in a cold box (4 °C) by using a 20-mA current per gel until samples enter the reaction gel followed by 50 mA per gel until the end of the run. The electrophoresis was stopped 45 min after the bromophenol blue tracking dye left the bottom of the gel. Gels were then stained with Coomassie Brilliant Blue, destained, dried, and autoradiographed. The molecular weights of phosphoproteins were calibrated by using protein standards (Pharmacia) of phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soybean trypsin inhibitor (M_r 20000), and β-lactalbumin (M_r 14400).

Two-Dimensional Gel Electrophoresis. The procedures of O'Farrell (1975) were followed for two-dimensional gel electrophoresis. The ^{32}P-labeled ROS proteins were routinely precipitated with ice-cold acetone before being dissolved in the lysis buffer. In the first dimension, isoelectric focusing was carried out in tube gels (3 × 100 mm) prepared from Biolyte 4/6 (Bio-Rad). In the second dimension, electrophoresis was carried out by using 10% $NaDodSO_4$–polyacrylamide gels. The gels were then stained, dried, and autoradiographed.

Autoradiography and Quantitation of ^{32}P-Labeled Phosphoproteins. Autoradiography of the ^{32}P-labeled phosphoproteins was done by exposing the dried polyacrylamide gels to Cronex X-ray film (Du Pont) in X-ray cassettes lined with high-speed or lightening-plus intensifying screens (Du Pont). The cassettes were kept at −70 °C during exposure (Swanstrom & Shank, 1978). Gels containing ^{32}P-labeled proteins phosphorylated in vitro were exposed for 5–24 h. Gels containing phosphoproteins prepared from in situ experiments were exposed from 6 days to 2 weeks. The X-ray film was developed in a Kodak RP-X-omat processor and scanned with a densitometric scanner (Photovolt Corp., New York, NY) using either a 570- or a 595-μm filter. In each experiment, the sensitivity of the scanner was kept constant during the scanning of all samples. The level of ^{32}P-labeled 33K protein was routinely estimated by tracing the area of the corresponding scan peak (Figure 3a); the trace was cut and weighed and expressed in arbitrary units as mean ± SEM. This measurement was found to be proportional to the actual radioactivity in the gel band (determined by scintillation coun-

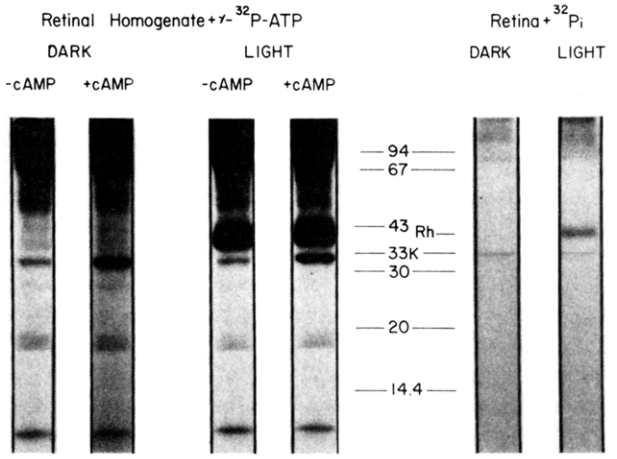

FIGURE 1: Phosphorylation of the 33K protein in vitro and in situ. Phosphorylation of the 33K protein in vitro was carried out by incubating a retinal homogenate with [γ-^{32}P]ATP, in the light or in darkness and in the presence or absence of 10^{-5} M cAMP (see Methods). At the end of the 10-min incubation, the phosphoproteins were quenched with $NaDodSO_4$ before electrophoresis. Phosphorylation of the 33K protein in situ was carried out by incubating the retinas in the dark in the presence of [^{32}P]P_i. At the end of the incubation, ROS was detached from retinas by shaking gently. The ROS suspension was either kept in darkness or exposed to light before the ROS proteins were quenched with $NaDodSO_4$. The phosphoproteins labeled in vitro and those labeled in situ were then electrophoresed in parallel on a 15% acrylamide gel, stained, dried, and autoradiographed as described under Methods. The polyacrylamide gel was calibrated with protein standards (Pharmacia) with molecular weights of 94 000, 67 000, 43 000, 30 000, 20 000, and 14 000, respectively. Rh designates rhodopsin.

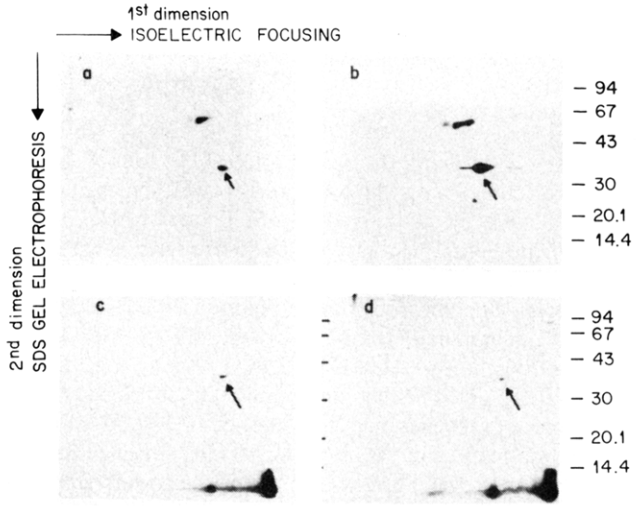

FIGURE 2: Identification of the ^{32}P-33K protein labeled in vitro and in situ by two-dimensional gel electrophoresis. Phosphorylation of the 33K proteins in vitro was carried out by incubating the homogenate of a freshly prepared ROS suspension in the dark with [γ-^{32}P]ATP. The reaction mixtures (see Methods) contain either (a) 0 or (b) 10^{-5} M cAMP, and the incubation was stopped by addition of ice-cold acetone. The 33K protein was labeled with ^{32}P in situ as described under Methods. At the end of incubation, ROS were gently detached. One aliquot of the ROS suspension was kept in the dark (c), and the other (d) was exposed to room light for 90 s before the samples were quenched with ice-cold acetone. The ^{32}P-33K protein was analyzed by two-dimensional gel electrophoresis according to O'Farrell (1975).

ting). The data in each series of experiments were compared by the Student's t test in order to determine the statistical significance of the differences in the level of ^{32}P-33K. It is considered as no difference when p is larger than 0.05.

Results

Phosphorylation of the 33K Protein in Vitro and in Situ. Figure 1 is an autoradiograph from a 15% acrylamide gel on which the retinal phosphoproteins labeled with ^{32}P either in vitro or in situ are compared. When the retinal homogenate of dark-adapted Fisher rats was incubated in the dark with [γ-^{32}P]ATP, several proteins became labeled with ^{32}P by endogenous protein kinases. The incorporation of ^{32}P into a protein with 33 000 molecular weight was stimulated at least 3-fold by 10^{-5} M cAMP. Cyclic GMP at 10^{-5} M also stimulated 33K protein phosphorylation, but to a lesser extent (results not shown). The extent of basal and cAMP-stimulated ^{32}P incorporation into the 33K protein was unchanged when the phosphorylation reaction was carried out in the light.

An additional band appeared on the autoradiograph of retinal proteins phosphorylated in the light. This band is apparently phosphorylated opsin because (1) it is observed only when ROS membranes are present during incubation but not when a soluble extract of the retina is phosphorylated (Lee et al., 1982), (2) it migrates with an apparent molecular weight of 35 000–40 000, (3) it is phosphorylated only in the light but not in the dark, and (4) it is phosphorylated in a cyclic nucleotide independent manner (Lee et al., 1981b). The incorporation of ^{32}P into other phosphoproteins is independent of added cyclic nucleotides or the light/dark condition of the phosphorylation reaction.

To study protein phosphorylation in situ, freshly dissected retinas of dark-adapted rats were incubated with [^{32}P]P_i in a phosphate-free Kreb's solution to allow incorporation of ^{32}P into ATP and then into phosphoproteins. It was found that incorporation of ^{32}P into phosphoproteins increased up to 2 h of incubation. In our experiments, incubation was carried out for 30 min in order to assure viability of the retinas and ROS. The ROS were then detached from the retinas, and the retinas were removed. The ROS suspension was exposed to either light or darkness before being solubilized in $NaDodSO_4$ and applied to a 15% acrylamide gel. Most labeled proteins in the ROS suspension have molecular weights higher than 80 000, and the extent of their phosphorylation was not affected by illumination. In the dark, the most extensively labeled phosphoprotein had an apparent molecular weight of 33 000, and the amount of ^{32}P-labeled 33K protein was reduced by illumination. The light-induced dephosphorylation of the 33K protein will be described in a later section. Exposure of the ROS suspension to light resulted in the appearance on the autoradiograph of a 37 000 molecular weight band, the phosphorylated opsin.

The phosphoproteins labeled in situ were also electrophoresed in parallel with those labeled in vitro by using 10% as well as 6–20% gradient gels. Under all electrophoretic conditions, it was found that the 33K protein labeled in situ comigrates with that labeled in vitro. The identity of the 33K protein phosphorylated in vitro and in situ was confirmed further by two-dimensional gel electrophoresis.

Panels a and b of Figure 2 depict the autoradiographs of the ^{32}P-labeled proteins which are phosphorylated by using the homogenate of a dark-adapted ROS suspension in the absence or presence of 10^{-5} M cAMP, respectively. The phosphorylation of a ^{32}P-labeled 33K protein, with an isoelectric point of pH 5.25, is extensively stimulated by the presence of 10^{-5} M cAMP, indicating the presence in the ROS suspension of the cyclic nucleotide dependent protein kinase and its endogenous substrates. Panels c and d of Figure 2 depict autoradiographs of phosphorylated ROS proteins which are labeled in situ and are exposed to either darkness or light, respectively. The phosphoprotein which shows a light-induced decrease in the content of ^{32}P labeling is identical with the 33K

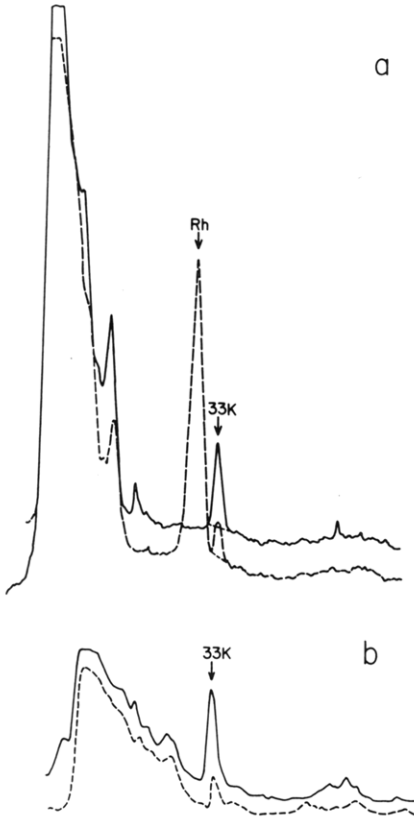

FIGURE 3: Densitometric scans of ^{32}P-labeled ROS proteins. (a) The phosphoproteins of ROS were labeled with ^{32}P in situ, exposed to light or darkness, and identified on the autoradiographs as described under Methods. The extent of ^{32}P incorporation was estimated by densitometric scanning. The upper scan (—) shows the ^{32}P-labeled ROS proteins which were labeled and kept in the dark, and the lower scan (---) shows those which are labeled in the dark and subsequently illuminated. The base lines of the two scans are displaced for clarity. (b) Freshly dissected retinas were incubated with [^{32}P]P_i in the dark in a phosphate-free Kreb's medium containing either 0 (—) or 1.0 mM (---) IBMX. At the end of the 30-min incubation, ROS were gently detached from the retina, and the ^{32}P-labeled ROS proteins were electrophoresed, autoradiographed, and scanned densitometrically as described under Methods. Rh is rhodopsin.

protein, labeled in vitro, as judged by its molecular weight or isoelectric point.

Levels of ^{32}P-Labeled 33K Protein at Various Times after ROS Detachment. When ROS are detached from the retina and kept in the dark, the level of ATP and GTP declines rapidly to reach a stable level (Biernbaum & Bounds, 1979), whereas cGMP declines gradually to reach 50% of the original dark level in about 30 min (Woodruff & Bownds, 1979). Since the 33K protein is a substrate of a cyclic nucleotide dependent protein kinase, reduction of the cGMP and ATP content may lead to a decline in the level of ^{32}P-labeled 33K protein, by reducing protein kinase activity as well as by reducing substrate concentration (ATP). To evaluate the levels of 33K as a function of time after the ROS detachment, the 33K protein was labeled in situ with ^{32}P as described under Methods. At the end of 30-min incubation, the ROS were detached, and the level of the labeled 33K protein was measured immediately in order to give a zero time level. The ROS suspension was kept in the dark, and the levels were measured again 5 and 10 min later. From the results of 4–15 independent experiments, the levels of labeled 33K were 10.22 ± 1.81, 9.30 ± 2.47, and 7.78 ± 1.82 (mean ● SEM) for the 0-, 5-, and 10-min samples, respectively. Though the mean decreases among the three samples, there is no statistical difference among the levels of labeled 33K protein. Therefore, in the

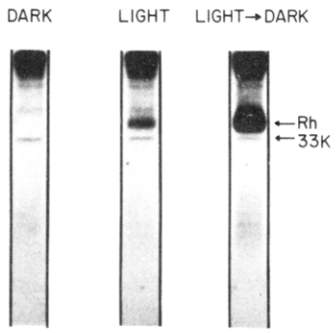

FIGURE 4: Effect of various illumination conditions upon the levels of ^{32}P-labeled ROS proteins. The phosphoproteins in ROS were labeled with ^{32}P in situ as described previously. After detachment, aliquots of the ROS suspension were either kept in the dark (DARK), illuminated for 90 s (LIGHT), or illuminated and subsequently returned to darkness (LIGHT → DARK) before the samples were quenched with $NaDodSO_4$. The ^{32}P-labeled ROS proteins were separated on an $NaDodSO_4$–polyacrylamide gel and located by autoradiography as described under Methods. Rh is rhodopsin.

dark, the phosphorylation and dephosphorylation of the 33K protein remained in equilibrium up to 10 min after the ROS are detached. The light/dark experiments described in the following section were done within 10 min after the ROS were detached from the retina.

Light-Induced Dephosphorylation of the ^{32}P-Labeled 33K Protein in Detached ROS. As shown in Figure 1, the ROS phosphoproteins become labeled when retinas are incubated in the dark with [^{32}P]P_i and the amount of the labeled 33K protein is reduced upon illumination. A typical densitometric scan of the labeled phosphoproteins of the dark-adapted and bleached ROS suspension is shown in Figure 3a. To quantitate the effect of light on the level of labeled 33K protein, the 33K protein was labeled in situ with ^{32}P as described under Methods. The ^{32}P-33K in the dark sample was measured by using aliquots taken immediately after the ROS were detached from the retina. The light sample was taken after the freshly detached ROS were exposed to 90 s of laboratory illumination. On the basis of the results from 15 independent experiments, the levels of the labeled 33K protein in the dark and light samples were 10.22 ± 1.81 and 4.59 ± 0.46 (mean ± SEM), respectively. If the level in the dark sample is taken arbitrarily as 100, the ^{32}P-33K in the light sample is 45% of the original level. An unpaired, one-tailed Student's t test showed that the difference has a p value of less than 0.005. The same extent of reduction in the level of ^{32}P-labeled 33K protein was observed also in experiments where the dark samples were quenched subsequent to the quenching of the light samples. This is consistent with results described in the above section and confirms that reduction in the level of labeled 33K protein in the light samples is due to light-induced events and not to ROS detachment-induced decay of the labeled 33K protein.

In another series of experiments, designed to determine whether the level of labeled 33K protein increases again after termination of illumination, the ROS suspension was first exposed to light for 90 s before being returned for 10 min of darkness (light → dark sample). We found that the amount of the labeled 33K protein decreased further in darkness to about 12.3% of the original dark level (mean ± SEM = 1.25 ± 0.15; $p < 0.005$). An autoradiograph obtained from such an experiment (Figure 4) shows that rhodopsin phosphorylation is initiated by light and it continues into the subsequent part of darkness. This indicates that, at the end of the 90-s illumination, [γ-^{32}P]ATP in the detached ROS was still available for rhodopsin phosphorylation and was probably available for 33K protein phosphorylation as well. Therefore,

if there is any rephosphorylation of the 33K protein which takes place after the illuminated ROS suspension is returned to darkness, apparently under our experimental conditions, the extent of rephosphorylation is less than that of dephosphorylation.

In another set of six independent experiments, retinas which were previously incubated in the dark with [^{32}P]P_i were either kept in the darkness or exposed to illumination immediately before the ROS were detached. The level of labeled 33K protein (4.16 ± 0.66; p <0.025) in the bleached ROS suspension was 58% of that in the dark adapted sample (7.12 ± 1.57). Since the ROS remained attached to the inner segment and cell body of the photoreceptors during illumination and, therefore, had access to the pool of [γ-^{32}P]ATP in the intact photoreceptor, we do not believe that the light-induced decrease in labeled 33K protein in the ROS suspension is due to a depletion of the ^{32}P-labeled trinucleotides. That the 33K dephosphorylation is a light-initiated event is further confirmed by the fact that the phosphorylation levels of the other rod phosphoproteins do not decrease under all experimental conditions we have tested.

Effect of IBMX Concentration on the Levels of Labeled 33K Protein. Freshly dissected retinas were incubated with [^{32}P]P_i in the dark for 30 min in the presence of 0, 0.1, or 1.0 mM IBMX. The levels of labeled 33K protein were measured immediately after the ROS were detached from the retina. From the results of 15 independent experiments, the amount of labeled 33K protein was 8.33 ± 1.60 (100%), and with the addition of either 0.1 or 1.0 mM IBMX to the Kreb's medium, the levels increased to 30.66 ± 4.43 (368.1%) and 32.60 ± 4.21 (391.4%), respectively. On the other hand, incorporation of ^{32}P into other phosphoproteins, as shown in Figure 3b, was not affected by IBMX in the incubation medium. Since incubation of the retina with IBMX results in an increased cGMP level (Woodruff & Bownds, 1979; Woodruff et al., 1977), this observation is consistent with the idea that the phosphorylation of the 33K protein is modulated by a cGMP-regulated mechanism.

In studying the effects of light on 33K phosphorylation, we chose to use the Kreb's solution containing 0.1 mM IBMX, because light-induced reduction in cGMP levels was observed in our earlier studies using rat retinas incubated in the same cocktail (Lolley et al., 1979). From the technical point of view, addition of IBMX stimulates enough incorporation of ^{32}P into the 33K protein to allow easy detection of light-induced changes within the sensitivity of autoradiography. In the rod photoreceptor cells, calcium is another cellular mediator which is shown to modulate the level of cGMP (Cohen et al., 1978) as well as that of protein phosphorylation (Polans et al., 1979). Its effect on the level of 33K phosphorylation is the topic of future study.

Discussion

Among the earliest responses to light which occur in rod photoreceptors is the activation of a cyclic nucleotide phosphodiesterase and the subsequent hydrolysis of cGMP (Woodruff & Bownds, 1979; Yee & Liebman, 1978; Miki et al., 1973).

Physiological investigations indicate that cGMP, applied intracellularly, both depolarizes rod photoreceptors in darkness and alters their response to light. These studies suggest that cGMP might act as an intracellular messenger between the photolysis of rhodopsin and the plasma membrane of ROS, controlling in some manner the movement of ions across the membrane. The concept implies that a series of biochemical reactions are triggered by the photolysis of rhodopsin, and these result in the eventual closure of Na^+ channels and hyperpolarization of the cell. The discovery and partial characterization of the role of bleached rhodopsin in the cascade of reactions which lead to the light activation of phosphodiesterase give credence to this concept. The question remains, however, whether light-initiated changes in cGMP concentration or cGMP turnover provide the appropriate signals for initiating changes in the plasma membrane. Opinions conflict on this issue, with investigators favoring a direct role for cGMP (Miller & Nicol, 1979), a role for H^+ (Pugh et al., 1982) that is produced during the hydrolysis of cGMP, or a role for specific phosphoproteins that are modulated by cGMP. The three possibilities are reasonable since each may contain information for the visual cell, but some of the information may relate to activities other than the control of membrane permeability.

In this investigation, we have explored the possibility that light can alter the level of phosphorylation of a 33K protein which is phosphorylated in a cyclic nucleotide dependent manner, in vitro. We have observed this phosphorylated protein in all mammalian ROS that we have examined, but, in each case, we have failed to identify the low molecular weight phosphoproteins identified in frog ROS by Polans et al. (1979). Our investigation of ROS from frog retinas (see Methods) confirms that small proteins with less than 14 000 molecular weight are phosphorylated in a cyclic nucleotide dependent manner, but phosphorylation of the 33K protein was not observed. It remains to be determined whether these findings are the result of species differences. We cannot rule out either the possibility that these phosphoproteins are lost during our preparation of frog ROS.

The 33K protein is phosphorylated also during incubation of rat retinas with [^{32}P]P_i, and its is dephosphorylated during illumination. Experiments with IBMX suggest that conditions which elevate cGMP levels in situ increase the level of 33K phosphorylation; these observations are consistent with the cyclic nucleotide dependent nature of 33K protein phosphorylation in vitro. In situ, several other ROS proteins incorporate lesser amounts of ^{32}P, and the phosphorylation of these proteins is unaffected by incubation with the phosphodiesterase inhibitor IBMX. Incorporation of ^{32}P into rhodopsin is not detected during the course of the dark incubation, indicating the absence of illumination throughout the operations of dissection, incubation, and ROS detachment. Detachment of ROS from the incubated retinas and subsequent incubation of isolated ROS did not significantly alter the content of ^{32}P-33K protein in dark-adapted ROS, suggesting that the turnover of the phosphate moiety of the 33K protein was balanced at near-steady-state conditions in darkness.

The current investigation does not give information regarding the rate at which the 33K protein is dephosphorylated, but it does demonstrate an ability of light to modulate the level to which the 33K protein is phosphorylated. The degree of phosphorylation of a protein is controlled by the relative rate of its phosphorylation and dephosphorylation. A decrease in the level of phosphorylated 33K protein could result from a decrease in the cyclic nucleotide dependent protein kinase activity, from an increase in the phosphoprotein phosphatase activity, or from a combination of both. A cyclic nucleotide dependent protein kinase from bovine ROS has been identified and partially characterized (Lee et al., 1981b). The phosphorylation of the 33K protein by the partially purified protein kinase is stimulated maximally by either 10^{-5} M cAMP or 10^{-4} M cGMP. The bovine ROS cyclic nucleotide dependent protein kinase had the characteristics of a cAMP-dependent

protein kinase, and our investigation of the bovine ROS did not reveal the presence of a specific cGMP protein kinase. Nevertheless, the level of cGMP in the dark-adapted retinas (10^{-5} M), being the highest among all tissues, is probably sufficient to regulate effectively the activity of the cyclic nucleotide dependent protein kinase.

Beyond the recognition that phosphoprotein phosphatase is present in ROS, little is known about the characteristics of the enzyme. Therefore, the mechanism by which the 33K protein is dephosphorylated upon exposure to light cannot be fully understood at this time. However, our partial knowledge suggests that the activity of cyclic nucleotide dependent protein kinase would be decreased in conjunction with a light-induced reduction in cGMP concentration. This plus the sustained activity of phosphoprotein phosphatase would be sufficient to cause a net dephosphorylation of the 33K protein. The function and regulation of the 33K protein as well as the phosphatase are topics for future study.

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Registry No. cAMP, 60-92-4; cGMP, 7665-99-8.

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