Phosphorylation in Sealed Rod Outer Segments: Effects of Cyclic Nucleotides†

Terrence A. Shuster and Debora B. Farber*

ABSTRACT: Rod outer segments (ROS) from rat were purified on Percoll gradients. These ROS had intact plasma membranes since they were impermeable to small molecules. Protein phosphorylation in the purified ROS was studied after the plasma membrane was disrupted by freeze/thawing. [γ-^{32}P]ATP was used as phosphate donor. ATP concentration, time, temperature, and light or dark adaptation were varied in the assays. The ^{32}P-labeled proteins were separated by polyacrylamide gel electrophoresis and autoradiographed. Rhodopsin was the dominant phosphorylated protein, and the addition of adenosine cyclic 3′,5′-phosphate (cAMP) or guanosine cyclic 3′,5′-phosphate (cGMP) (10^{-4} M) did not qualitatively alter the ROS phosphorylation pattern. The only cyclic nucleotide effect we could establish in these experiments was the inhibition of rhodopsin phosphorylation by cGMP. This inhibition did not appear to be competitive with ATP since cAMP was much less inhibitory than cGMP and the phosphorylation in the presence of cGMP reached a plateau at a much lower level than in control conditions. Hypotheses implying an involvement of protein phosphoryltion/dephosphorylation in dark adaptation have been formulated [Miller, J. A., & Paulsen, R. (1975) *J. Biol. Chem. 250*, 4427–4432; Kuhn, H., McDowell, J. H., Leser, K. H., & Bader, S. (1977) *Biophys. Struct. Mech. 3*, 175–180]; we suggest that cGMP may control this process through the modulation of the extent of inhibition of phosphorylation of the visual pigment.

Rod outer segments (ROS)[1] of retinal photoreceptors are characterized by very dynamic metabolism of cGMP. In all species studied, the ROS of dark-adapted retinas possess high levels of cGMP and the enzymes required for cGMP synthesis and degradation (Fletcher & Chader, 1976; Orr et al., 1976; Virmaux et al., 1976; Miki et al., 1975; Farber, 1982). Changes in cGMP levels upon exposure to light for milliseconds (Woodruff et al., 1977) as well as the effect of intracellularly injected cGMP on the plasma membrane polarization of rod visual cells (Miller & Nicol, 1979) suggest that cGMP may play a specific role in photoreceptor cell function. Cyclic GMP mediated biochemical reactions could link the bleaching of rhodopsin to the electrophysiological response of the photoreceptor plasma membrane. Models have been proposed (Farber et al., 1978; Hubbell & Bownds, 1979) in which the Na^+ permeability of the ROS membrane would be controlled by a protein (or proteins) that becomes phosphorylated through the catalytic action of a cyclic nucleotide dependent protein kinase. These models represent a major hypothesis in photoreceptor biochemistry. However, definitive evidence is still lacking for a physiological involvement of cGMP in the visual process.

To establish a ROS-specific event, particularly an enzymatic event, it is necessary to have reasonable quantities of fresh ROS which remain stable during the study. The ROS should be as homogeneous as possible, and it is essential that they maintain an intact plasma membrane during purification to prevent loss of endogenous proteins and also to minimize the uptake of exogenous contaminants. The criteria for sealed ROS have been discussed by Schnetkamp et al. (1979) and Schnetkamp & Daeman (1982) in their work describing the isolation of sealed bovine ROS on sucrose/Ficoll gradients. We have chosen to prepare rat ROS by utilizing self-generating Percoll gradients. Percoll is thought to be innocuous, it does not interfere with our enzyme assays, and the injured particles generally float at densities lower than those for healthy particles (Pharmacia Fine Chemicals AB, 1980; Pertoft et al., 1977). In this paper, we report the successful, rapid isolation of homogeneous preparations of sealed ROS from rat. Our criteria for a sealed plasma membrane involve demonstrating a permeability barrier to small molecules. We have utilized these rat ROS to study phosphorylation patterns and the sensitivity of those patterns to cyclic nucleotides. During these studies, we have varied substrate concentrations, temperature, light adaptation, and time. Our results indicate that, in sealed ROS from rat, there are no cAMP- or cGMP-dependent increases of protein phosphorylation and that cGMP, and to a much lesser extent cAMP, inhibits the phosphorylation of rhodopsin. This latter observation is in partial agreement with the results of Hermolin et al. (1982) obtained with frog preparations, and also in partial agreement with Swarup & Garbers (1983), who used porcine ROS. Thus, we find a ROS-specific role for cGMP which apparently is restricted to the control of the phosphorylation of rhodopsin.

Experimental Procedures

Materials

RPMI 1640 is a product of Grand Island Biological Co. (GIBCO). ATP, cAMP, and cGMP were purchased from Sigma. [γ-^{32}P]ATP was purchased from New England Nuclear. Molecular weight standards, Percoll, and density marker beads were purchased from Pharmacia Fine Chemicals. Materials for PAGE were Bio-Rad products. All other

† From the Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, California 90024. *Received April 20, 1983; revised manuscript received September 14, 1983.* This work was supported in part by the National Retinitis Pigmentosa Foundation, Baltimore, MD (T.A.S. Fellowship), and by NIH Grants EY 02651, EY 00331, and RCDA EY 00144 (to D.B.F.).

[1] Abbreviations: ROS, rod outer segment(s); cAMP, adenosine cyclic 3′,5′-phosphate; cGMP, guanosine cyclic 3′,5′-phosphate; SIP, stock isoosmotic Percoll; PAGE, polyacrylamide gel electrophoresis; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; $NaDodSO_4$, sodium dodecyl sulfate; G6DP, glucose-6-phosphate dehydrogenase; NADP, nicotinamide adenine dinucleotide phosphate; Cl_3AcOH, trichloroacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/84/0423-0515$01.50/0

chemicals were reagent grade. Cronex X-ray film was from Du Pont.

Methods

ROS Isolation. All isolation steps were carried out in plastic tubes at 0–4 °C. Retinas from Sprague-Dawley or Outbred Simonson albino rats, usually 10–14 per isolation, were dissected in RPMI 1640 culture medium (GIBCO) or in buffer A (130 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, and 5 mM HEPES, pH 6.9) and then transferred to test tubes containing 2 mL of RPMI 1640 (pH 7.5) or buffer A. The retinas were vortexed full speed for 30 s, and large particles were allowed to settle for 5 min. The upper 1.5 mL of crude ROS was carefully transferred to a polycarbonate centrifuge tube. New buffer was then added to the retinas; they were briefly vortexed and again allowed to settle for 5 min. The crude ROS supernatant was added to the polycarbonate tube, and these steps were repeated until 3.75 mL of outer segment supernatant had been collected; 4.25 mL of SIP (SIP consists of 9 mL of Percoll plus 1 mL of 10× buffer A at pH 6.0) was then added to the crude ROS and mixed gently. This preparation was centrifuged at 20000 rpm for 30 min in a type 40 fixed-angle rotor with no brake applied. The ROS band near the center of the gradient was collected with a plastic pipet. This preparation could be used as collected in the presence of Percoll; however, a further step was generally employed to reduce the amount of Percoll. The ROS obtained from the gradient were centifuged again in a type 40 rotor at 35000 rpm for 1 h. This resulted in a hard pellet of Percoll, with the ROS in a very loose pellet above the Percoll. The reduction of the Percoll facilitates analysis by gel electrophoresis and reduces the possibility of a Percoll effect in our assays. These techniques were used for both light-adapted and dark-adapted (dim red light) preparations. An alternate preparatory technique involved simply layering 1.5 mL of crude ROS supernatant over 6 mL of Percoll diluted with RPMI 1640 (4 mL of SIP + 2 mL of RPMI 1640). This Percoll overlayed with crude ROS was then centrifuged at 20000 rpm for 30 min, and the ROS were collected from a band about 40% down the gradient. ROS from either procedure gave the same experimental results. When ATPase activities were determined, the ROS were prepared in HEPES-buffered saline (pH 7.2) to minimize phosphate contamination.

Phosphorylation of Endogenous Substates. All phosphorylation reactions contained 0.5–1.0 mM dithiothreitol. When light-adapted ROS were phosphorylated, the ROS were exposed to bright room light for 20–30 min at 0–4 °C prior to starting the experiment. ROS membranes were disrupted by freeze/thawing 3 times. For electrophoretic analysis of phosphorylated proteins in light-adapted ROS, an appropriate amount of ROS suspension was added to a test tube at 0–4 °C; $MgCl_2$ concentration was usually adjusted to 10 mM, cyclic nucleotides were added to a final concentration of 10^{-4} M (when present), and the reaction was started by addition of [γ-^{32}P]ATP. Reactions generally were run at (0.5–1.5) × 10^{-6} M ATP with a specific activity of 1000 dpm/pmol. Higher ATP concentrations also were used, and then the specific activity was reduced (e.g., 100 dpm/pmol at 7 × 10^{-5} M ATP). Reactions were run at either 0–4 or 37 °C. In some time studies, samples of the reaction mixture at 4 °C were removed and then incubated at 37 °C for additional time to determine if the phosphorylated protein pattern was temperature sensitive. Reaction aliquots were taken at various times; the phosphorylation was stopped by addition of the reaction aliquots to test tubes containing 20% $NaDodSO_4$ (final concentration of $NaDodSO_4$ was 4%) and vortexing immediately. For electrophoretic analysis of dark-adapted ROS preparations, all steps were run under dim red light. When the phosphorylation was initiated by light, the ATP was added to the reaction mixture in the dark, and the tubes were wrapped in foil and kept at 0–4 °C. The reactions were started with the exposure of the tubes to light by removing the foil in bright room lights. Reaction aliquots for PAGE were taken at the appropriate time and stopped with $NaDodSO_4$ as described for light-adapted ROS.

When the phosphorylation of rhodopsin was used to determine if the ROS preparation had an intact plasma membrane, high specific activity ATP was added to a final concentration of 1 × 10^{-6} M to light-adapted ROS. These reactions were conducted by using fresh ROS which had not been frozen. As a control, ROS from the same preparation which had been broken by freeze/thawing were tested. These reactions were carried out for various times at 0–4 °C and were stopped by the addition of 20% $NaDodSO_4$ (as above) for electrophoretic analysis.

The phosphorylation of rhodopsin also was studied and quantified by liquid scintillation counting. The assays were carried out at 37 °C essentially according to Rubin et al. (1974). A final volume of 0.1–0.2 mL contained 10 mM $MgCl_2$, 1 mM DTT, 10^{-4} M cyclic nucleotide (if indicated), and an appropriate amount of broken ROS. Reactions were carried out in the light and in the dark; they were initiated by adding ATP and were stopped with 0.5 mL of ice-cold 20% trichloroacetic acid (Cl_3AcOH). Protein was pelleted by centrifugation at 2000*g* for 30 min, and the supernatant was discarded. The pellet was redissolved briefly in 100 μL of ice-cold 1 N NaOH and reprecipitated immediately with 1 mL of cold 20% Cl_3AcOH. The precipitate was collected on Millipore HAWP filters and washed with 2 mL of cold 20% Cl_3AcOH, and the filters were counted in PCS scintillation cocktail (Amersham)/xylene (2:1).

Polyacrylamide Gel Electrophoresis (PAGE). Linear gradient gels of 8–13% acrylamide containing $NaDodSO_4$ were used to analyze phosphorylation patterns. The lower separating gel contained 0.35 M Tris-HCl and 0.1% $NaDodSO_4$ at pH 8.8. The acrylamide:*N*,*N*′-methylenebis(acrylamide) ratio was 30:0.8. Gradients were stabilized by the addition of 10% glycerol to the high-percentage acrylamide during formation. Stacking gels were 4.5% acrylamide in 0.125 M Tris-HCl and 0.1% $NaDodSO_4$ at pH 6.6. Gel cross-linking was initiated by the addition of 60 μL of 10% ammonium persulfate and 15 μL of TEMED for 20 mL of gel. Gels were 1.5 mm thick and were run at 15 mA per slab until the tracking dye entered the separating gel; at this point, the current was increased to 30 mA. Gradient gels with stacking gels of at least 1 cm were necessary to focus the proteins when Percoll was present in the samples. All test samples contained 5% glycerol and tracking dye. Gels were stained with Coomassie blue R250 (0.4 g/L) in isopropyl alcohol/acetic acid/H_2O (25:1:65) for 16 h and destained slowly in 10% acetic acid or rapidly with methanol/H_2O/acetic acid (5:5:1).

Autoradiography. Analysis of ^{32}P-labeled proteins was accomplished by autoradiography. After the gel was stained and destained, it was shaken in 5% glycerol for at least 30 min and vacuum dried on filter paper. Cronex X-ray film was exposed to the gel at room temperature using intensifying screens.

Protein Determination. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as standard.

Glucose-6-phosphate Dehydrogenase. This assay was conducted according to Schnetkamp & Daeman (1982). To 500 μL of buffer A or RPMI 1640 medium were added 6 μL of 20 mM glucose 6-phosphate and 6 μL of 20 mM NADP. The reaction was started by the addition of 50–100 μL of ROS preparation. The conversion of NADP to NADPH was measured spectrophotometrically. Results from intact ROS were compared to results from freeze/thawed samples of the same ROS preparation. In other experiments, 5 μL of a 10% Triton X-100 solution was added to the intact ROS assay, the spectrophotometer was reset to zero, and the absorbance at 340 nm was noted with time.

Cytochrome c Oxidase and ATPase Assays. These assays were run essentially as described by Nagy et al. (1983).

Results

ROS Preparation. We have successfully prepared sealed ROS from rats ranging in age from 4 weeks to 6 months by means of Percoll gradients. These gradients resulted in a band of sealed ROS at a density of 1.06 g/mL as determined by density gradient marker beads (Pharmacia). Routinely, it is important to be very careful when removing the ROS band from the gradient to avoid contamination by retinal fragments. Phase-contrast microscopy showed these preparations to be virtually homogeneous, without inner segments of portions of the cell body attached to the ROS. Confirmation of the purity of these ROS was obtained by measuring the (Na,K)-ATPase activity, which is present in inner segments but not in ROS (Zimmerman et al., 1976; Bok & Filerman, 1979), and the activity of cytochrome *c* oxidase, a mitochondrial marker. (Na,K)-ATPase activities of retina, crude ROS supernatant, and purified ROS (after high-speed centrifugation) were 169, 109, and 5 μmol of P_i min^{-1} (mg of protein)$^{-1}$, respectively. Thus, contamination by (Na,K)-ATPase was minimal. Cytochrome *c* oxidase activity of purified ROS was zero while retina and crude supernatant gave values of 31 and 5 μmol min^{-1} (mg of protein)$^{-1}$. We did not determine the A_{500nm}/A_{280nm} ratio used to characterize ROS preparations since Percoll absorbs in the 280-nm range. As will be discussed below, even after centrifugation at 35 000 rpm for 1 h, a very small amount of Percoll remains with the ROS.

We have used different buffer systems to prepare the ROS such as RPMI 1640 and buffer A. Although the Percoll was adjusted osmotically with 10× buffer A (SIP) in most cases, the results differed. When the retinas were isolated in buffer A, the ROS preparation from the gradient was present in two distinct bands, each representing about 50% of the total ROS. The higher density band represented sealed ROS, and the lower density band (near the top of the gradient) consisted of ROS which were leaky, as discussed later under ATP permeability. The shape of the ROS prepared in pure buffer A changed rapidly, and they became round within 2 h. When RPMI 1640 was used to isolate the ROS, they maintained their shape for at least 4 h, and 80% or more of the ROS banded at the sealed density. A typical preparation in RPMI 1640 resulted in approximately 0.5–1.5 mL of ROS at a concentration of 0.3–1.0 mg/mL of protein. $NaDodSO_4$–PAGE showed that these preparations contained several proteins in addition to rhodopsin, which is the protein present in highest concentration in ROS (Figure 1). The integrity of the plasma membrane of sealed ROS was established by measuring the permeability to ATP, required for rhodopsin phosphorylation in light-adapted samples, and also by measuring glucose-6-phosphate dehydrogenase activity.

ATP Permeability. That the plasma membrane was still intact and represented a barrier to small molecules was determined by phosphorylation of rhodopsin with [γ-^{32}P]ATP. As can be seen in Figure 2, the autoradiograph of proteins (higher density band) from light-adapted ROS, rhodopsin was not phosphorylated in sealed ROS (lane A), but phosphorylation proceeded in the same preparation when it was freeze/thawed 3 times (lane B). Freeze/thawing did not activate an enzyme or substrate and thereby promote phosphorylation. This was demonstrated by comparing the phosphorylation of upper, presumed unsealed, ROS prior to freeze/thawing with that after freeze/thawing. In both conditions, the incorporation of ^{32}P was the same. Freze/thawing caused no change in the apparent enzyme activity. These results were obtained by using ROS separated from the gradient before the Percoll was pelleted. Identical observations were made with ROS prepared in buffer A or in RPMI 1640 and also when most of the Percoll was removed from the ROS preparation by centrifugation at 35 000 rpm for 1 h as described under Methods. The sealed nature of the ROS is indicated in Figure 2 where lanes C and D show rhodopsin phosphorylation after gentle resuspension of the pellet. Lane C indicates that the ROS were still sealed after high-speed centrifugation, whereas lane D shows that freeze/thawing

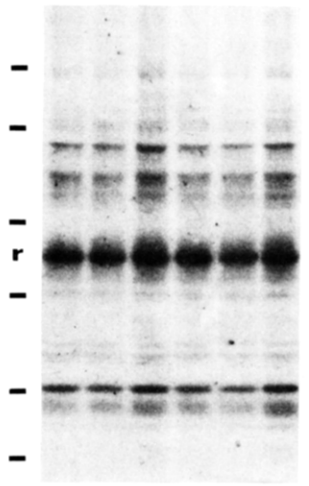

FIGURE 1: Protein profile of isolated ROS after high-speed centrifugation to remove Percoll. The gel is an 8–13% polyacrylamide gradient which contains $NaDodSO_4$. Molecular weight markers are 94 000, 68 000, 43 000, 30 000, 20 000, and 14 400. This figure shows the portion of the gel used to generate sections B and C of the autoradiogram in Figure 4. Each third lane contains 30% more protein. The letter r indicates the band corresponding to rhodopsin.

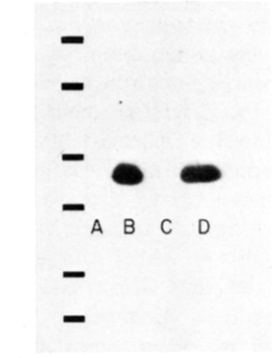

FIGURE 2: Autoradiograph demonstrating [γ-^{32}P]ATP permeability of ROS prepared in RPMI 1640 prior to and after high-speed centrifugation to remove Percoll. Lanes A and B contain ROS as isolated from the gradient (in the presence of Percoll). Lane A is prior to freeze/thawing, and lane B is after freeze/thawing. This ROS preparation was then centrifuged at 35 000 rpm (type 40 rotor) for 1 h, and [γ-^{32}P]ATP permeability was tested before (lane C) and after (lane D) freeze/thawing. Phosphorylation reactions were run at 0–4 °C. Molecular weight markers are the same as in Figure 1.

Table I: Glucose-6-phosphate Dehydrogenase Activity[a]

sample	A_{340}/10 min
buffer A prepared high-density ROS	
intact initial	0.001
freeze/thawed	0.081
intact aged 1 h	0.025
buffer A prepared low-density ROS	
initial	0.072
freeze/thawed	0.074
RPMI 1640 prepared ROS	
intact initial	0.006
Triton X-100 added	0.030
freeze/thawed	0.021

[a] Assays were conducted by using various individual ROS preparations as they were taken from the Percoll gradient. No attempt was made to normalize for the protein concentrations of the different groups; however, the protein concentrations were equal within each group. Assays were conducted as described under Methods.

again disrupted the plasma membrane. The ROS pellet from the 35 000 rpm spin was very loose and was easily resuspended by gently swirling the centrifuge tube. Since a small amount of Percoll is evident on gels run on the 35 000-rpm pellet, the ROS may be floating on a small amount of high-density, unpelleted Percoll after the centrifugation. This could preserve the integrity of the plasma membrane of the ROS and avoid their disruption by pelleting and resuspending. Light-adapted ROS isolated from the Percoll gradient and prepared with RPMI 1640 maintained their permeability barrier to ATP for at least 2 h, long enough for all of our manipulations.

When dark-adapted ROS were prepared under dim red light, the phosphorylation of rhodopsin was not observed, as expected. These ROS were sealed and also impermeable to ATP since, when light adapted, no phosphorylation of rhodopsin could be obtained unless the ROS were disrupted.

Glucose-6-phosphate Dehydrogenase (G6PD) Assay. As another indication of the sealed nature of our ROS preparation, we adopted the G6PD assay suggested by Schnetkamp & Daeman (1982) in their characterization of bovine ROS. When ROS were prepared in buffer A exclusively and tested for G6PD activity (permeability to glucose 6-phosphate and NADP), the activity was substantially enhanced by freeze/thawing. These results are shown in Table I. By contrast, the low-density band of ROS from buffer A preparations had activity levels which were generally intermediate between those for the sealed and the freeze/thawed high-density band of ROS, and this activity was unaffected by the freeze/thawing process. The fragility of buffer A prepared ROS also was indicated by the increase in G6PD activity with time. Table I shows that whereas some preparations had an absolute barrier to small molecules, there was a substantial increase in G6PD activity after 1 h in the presence of Percoll. It was noted earlier that these ROS preparations lost their characteristic shape rapidly. ROS prepared in RPMI 1640 also showed an increase in G6PD activity either when they were freeze/thawed or when Triton X-100 was added to the sample (Table I). These G6PD results indicate that the RPMI 1640 prepared ROS also had an intact plasma membrane which maintained a barrier to small molecules.

Protein Phosphorylation Patterns. Our assay technique, which involves the use of low ATP concentrations and low temperature, was adopted in an attempt to limit the rate and extent of phosphorylation. We had hoped to determine whether ROS proteins were sequentially phosphorylated and the effect of cyclic nucleotides in these reactions. Assays could then be shifted to 37 °C to observe dephosphorylation. Experiments conducted on crude preparations had indicated some success. However, as we continued to purify the ROS, the protein phosphorylation pattern became simpler until all of the apparent cAMP effect was lost in the ROS fractions obtained from the Percoll gradient. Examples of ROS protein

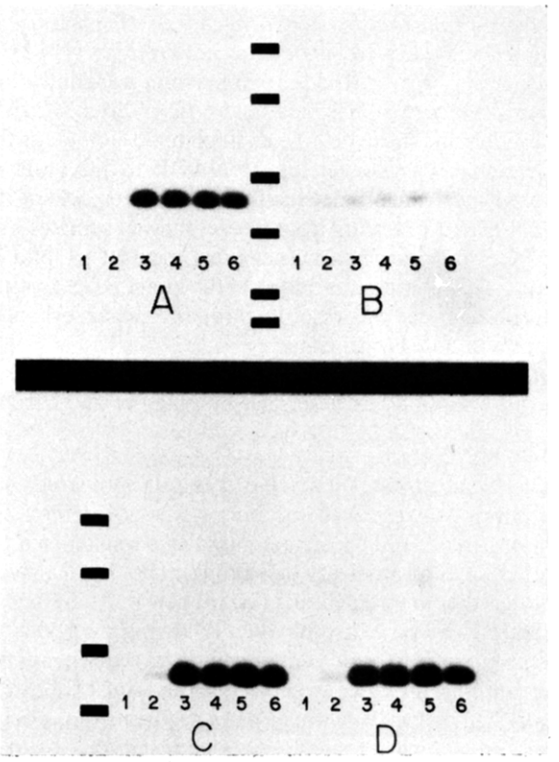

FIGURE 3: Autoradiograph demonstrating the effects of cyclic nucleotides and theophylline on ROS phosphorylation patterns. Each series contains equal amounts of protein. Reactions were initiated with [γ-^{32}P]ATP on light-prepared ROS constantly maintained at 0–4 °C which had been preincubated for 10 min with cAMP (series A), cGMP (series B), and 1 mM theophylline (series D) or with no additions (series C). Lanes 1, 2, 3, and 4 correspond to reactions carried out at 0–4 °C for 10 s, 1 min, 15 min, and 30 min, respectively. Lanes 5 and 6 consist of aliquots of reaction carried out at 0–4 °C for 14 min and then transferred to 37 °C for 1 and 15 min, respectively, to allow dephosphorylation to begin.

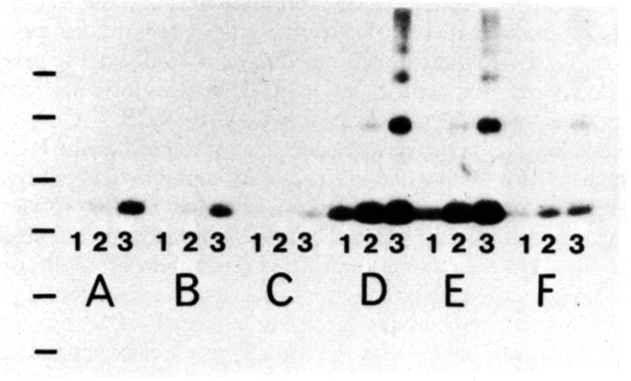

FIGURE 4: Autoradiograph demonstrating cGMP inhibition of ROS phosphorylation at 4 and 37 °C in samples where phosphorylation was initiated with light. ROS samples were prepared under red light and maintained in the dark through all purification techniques. Cyclic nucleotides and [γ-^{32}P]ATP were added, and tubes were wrapped in foil and brought to the light, all at 0–4 °C. Reaction was initiated by removing the foil. Series A (no additions), series B (10^{-4} M cAMP), and series C (10^{-4} M cGMP) were run at 0–4 °C. Series D (no additions), series E (10^{-4} M cAMP), and series F (10^{-4} M cGMP) were run at 37 °C. Lanes 1, 2, and 3 correspond to reaction times of 1, 5, and 20 min, respectively. Each lane 3 contains 30% more protein than lanes 1 and 2.

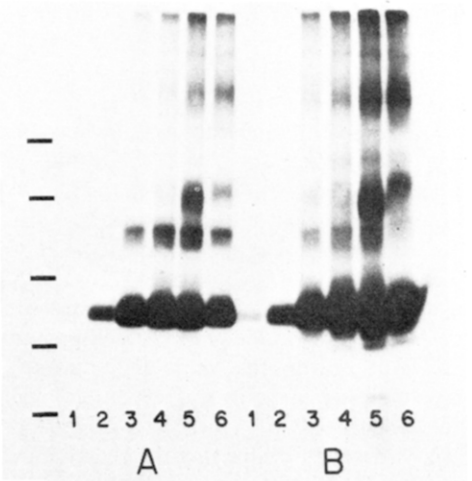

FIGURE 5: Autoradiograph demonstrating the phosphorylation patterns of light-adapted crude ROS supernatants prior to purification on Percoll gradients. Series A has no additions, and series B contains 10^{-4} M cAMP. Lanes 1–6 are reaction times as described in Figure 3. Molecular weight markers are 94 000, 68 000, 43 000, 30 000, and 20 000. There is cAMP-enhanced phosphorylation of proteins in the 30 000–35 000 molecular weight range as well as of other proteins.

phosphorylation in the absence of cyclic nucleotides are shown in Figure 3, series C, and Figure 4, series A and D. Electrophoretic analysis indicated no qualitative change in phosphorylation patterns when cAMP was added to the reaction (Figure 3, series A, and Figure 4, series B and E) or in response to cGMP (Figure 3, series B, and Figure 4, series C and F). The possibility existed that the cAMP-enhanced phosphorylation of a particular protein (30 000–35 000 molecular weight) could have been masked by the large amount of phosphorylated rhodopsin. This protein has been described as a soluble component of ROS (Farber et al., 1979) and has been suggested to be involved in the control of the permeability to sodium of the ROS plasma membrane (Farber et al., 1978). However, dark-adapted preparations of sealed ROS which were freeze/thawed and phosphorylated under dim light showed no signs of any cAMP- or cGMP-enhanced protein phosphorylation after separation by $NaDodSO_4$ gel electrophoresis. Phosphorylation studies using crude ROS supernatant isolated directly from the vortexed retinas prior to purification on a gradient detected small amounts of cAMP-enhanced phosphorylation in the 30 000–35 000 molecular weight range. Figure 5 shows that even though cAMP-enhanced phosphorylation is present in the crude ROS supernatant, the predominant phosphorylated protein is still rhodopsin. Figure 5 also shows that the ROS that had not been purified on the Percoll gradient contain several other proteins which accept ^{32}P from ATP. These proteins may be components of cellular fractions other than the ROS such as inner segments, photoreceptor soma, subretinal matrix, etc. Furthermore, Figure 5 shows a protein band which has the characteristics of an autophosphorylated type II cAMP-dependent protein kinase regulatory subunit. These characteristics include an appropriate molecular weight of approximately 55K (Shuster et al., 1983) and phosphorylation under conditions (low ATP concentration, low temperature, no cAMP) designed to enhance the regulatory subunit autophosphorylation (Rubin et al., 1979; Rangel-Aldao & Rosen, 1976). This band does not copurify with rhodopsin and is clearly lacking in Figures 2–4 although comparable amounts of rhodopsin phosphorylation occur.

Experiments were carried out in which type II cAMP-dependent protein kinase was added to the ROS preparation to determine if the phosphorylation pattern changed. Our results (not shown) were similar to reports of other investigators

Table II: Effect of Cyclic Nucleotides on Light-Stimulated Phosphorylation[a]

sample	protein phosphorylation (cpm)
ROS, no additions	3164 ± 55
ROS plus 10^{-4} cAMP	2845 ± 5
ROS plus 10^{-4} cGMP	1706 ± 30

[a] Assays were conducted with ROS preparations as they were taken from the Percoll gradient. The reaction was carried out for 3 min as described under Methods. ROS used for light assays were kept under laboratory illumination for 30 min at 0–4 °C, and cyclic nucleotides were added 10 min prior to starting the reaction.

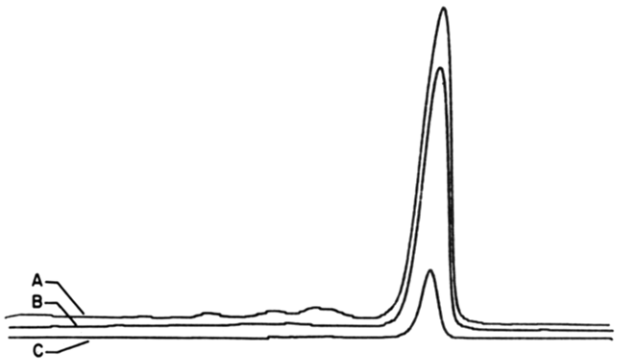

FIGURE 6: Densitometric scans of the plateau level of rhodopsin phosphorylation from autoradiographs obtained as in Figure 3. Similar quantitative ratios of rhodopsin phosphorylation in the presence and absence of cyclic nucleotides were obtained from scans of experiments carried out as in Figure 4. (A) Rhodopsin phosphorylation with no added cyclic nucleotides; (B) with 10^{-4} M cyclic AMP; (C) with 10^{-4} M cyclic GMP. Integration of the peaks gives relative areas of 7:5:1, respectively.

(Frank & Buzney, 1975) who found that the additional bands phosphorylated upon addition of exogenous protein kinase were characteristic of the added enzyme. The phosphorylation pattern in our ROS preparation was consistent at low and high ATP concentrations and high or low temperatures, or if the reaction was initiated by ATP or light (discussed later). In any condition, the ROS were free of cAMP-stimulated phosphorylation, although the ATP and Mg concentrations, temperature, and pH were chosen to ensure the activity of type II cAMP protein kinase (Shuster et al., 1983; Rubin et al., 1979). These results also were confirmed by test tube assays. As seen in Table II, no detectable phosphorylation increase took place with the addition of cAMP. We also determined that Percoll did not interfere with cAMP-dependent type II protein kinase phosphorylation of histone by running assays in the presence and absence of Percoll (results not shown).

While cAMP did not increase the number of ^{32}P-labeled proteins in our preparations, it did seem to inhibit the overall phosphorylation slightly. More importantly, quite substantial inhibition of phosphorylation, particularly rhodopsin phosphorylation, occurred in the presence of cGMP at 10^{-4} M concentration (Table II and Figures 3, 4, and 6). Comparison of series C (base line) and series B of Figure 3 indicates a clear inhibition of protein phosphorylation by cGMP, which is quantitated in Figure 6. It had been suggested (Weller et al., 1975) that phosphodiesterase inhibitors also might inhibit rhodopsin phosphorylation. Series C and D of Figure 3 compare phosphorylation pattern in the absence and presence of theophylline, and no difference can be observed. Since theophylline did not cause any change in the ROS phosphorylation patterns when it was added to the reaction mixture, generally it was not used.

It can be seen in Figure 3, series B, that the phosphorylation of rhodopsin seems to plateau at a level lower than that reached in series A, C, and D. This result was always observed in our experiments. In addition, it is evident that cAMP inhibits phosphorylation less than cGMP. The cGMP inhibition of protein phosphorylation was also detected in test tube assays. Results in Table II show that when assays were run with dark-adapted ROS, and this level of phosphorylation was subtracted from phosphorylation values obtained with the same dark preparations after light exposure, there was substantial reduction of light-stimulated phosphorylation by cGMP. While the autoradiograms demonstrate the inhibition of plateau levels of rhodopsin phosphorylation by cGMP, the test assays demonstrate the cGMP inhibition of phosphorylation in a time frame (3 min) consistent with linear phosphorylation activity in fully bleached preparations.

The lack of cAMP stimulation and the cGMP inhibition were observed in ROS preparations in the presence of Percoll and also after Percoll was pelleted in the 35 000 rpm centrifugation. The inhibition of phosphorylation by cGMP was consistent at low and high temperatures, and when the reaction was initiated with light in dark-adapted ROS. Figure 4 shows light-initiated reactions run at 0–4 °C (series A–C) and 37 °C (series D–F). Reactions run at 37 °C not only increased the incorporation of ^{32}P into rhodopsin at comparable times of incubation but also often resulted in the phosphorylation of additional high molecular weight proteins which appear to be multimers of rhodopsin; the incorporation of ^{32}P into these proteins was not stimulated by cAMP (Figure 4, series D and E). Cyclic GMP also inhibited protein phosphorylation at high ATP concentrations (70 μM), and the cGMP metabolite, 5′-GMP, was more inhibitory than cAMP (results not shown).

We have used the same procedures to prepare frog and bovine ROS. Cyclic GMP inhibition of protein phosphorylation was also present in these samples.

Discussion

Intact, purified ROS are indispensable for the study of the involvement of cyclic nucleotides in physiological mechanisms such as phototransduction and light/dark adaptation. Otherwise, artifactual observations resulting from the loss of soluble components of the ROS, or from exogenous contamination, could be obtained. The studies described in this paper were carried out by using sealed ROS from rat purified on Percoll gradients. The rat was the animal of choice because it can be light or dark adapted easily, and the initial ROS can be prepared rapidly after sacrifice with reasonable yield. We found that with bovine eyes, the procedure was less predictable since the eyes had a variable history (temperature, time of sacrifice of the cow, light conditions, time elapsed until dissection of the retina, ROS preparation, etc.). In addition, eyes obtained after more than 2 h postmortem often yielded no sealed ROS on the Percoll gradients.

Percoll is a colloidal silica material coated with polyvinylpyrrolidone, which has high density but low osmolarity and viscosity. This material forms self-generating gradients readily, is thought to be innocuous, and generally allows the banding of viable particles at a higher density than that of injured particles (Pharmacia Fine Chemicals AB, 1980; Pertoft et al., 1977). Pertoft et al. (1977) suggest that the buoyant density at which particles band represents the native density of the particles; in the case of sealed ROS of rat, it is 1.06.

As expected, we could obtain two clearly separated bands of rat ROS from the Percoll gradients, one with leaky and the other with intact ROS plasma membranes. This was confirmed by testing for permeability to ATP, which was used for rhodopsin phosphorylation, and by measuring glucose-6-phosphate dehydrogenase activity.

We have used low ATP concentrations in most of the experiments. There were several reasons for this choice. (1) Our purpose was to adjust substrate concentrations and temperature to slow down the phosphorylation/dephosphorylation reactions. We had hoped to detect the initial protein phosphorylated at 4 °C and then, by raising the temperature to 37 °C, to determine the initial protein dephosphorylated. However, by changing the temperature from 4 to 37 °C or the ATP concentration within the range of 0.5–70 μM, we did not alter the patterns of phosphorylation of the Percoll-prepared ROS. (2) Low ATP concentrations would minimize the risk of cGMP phosphodiesterase activation which, in bovine ROS, occurs with a K_a for ATP of 36 μM (Yee & Liebman, 1978). Since we were interested in studying the cGMP effect on the phosphorylation of ROS proteins, we did not want to stimulate cGMP degradation. (3) Low ATP concentrations and low temperature have been used to demonstrate autophosphorylation of cAMP-dependent type II protein kinase (Rubin et al., 1979), a physiologically important event which is best detected at nonphysiological conditions. Essentially our results indicate that the purified, intact ROS are free of cAMP-dependent protein kinase activity. This result was consistent during all manipulations. We had previously observed cAMP-dependent stimulation of phosphorylation in ROS obtained by sucrose density gradient centrifugation; these preparations probably contained many truncated ROS/rod inner segments (Farber et al., 1979). In the present study, cyclic AMP dependent protein kinase activity was apparent only when crude ROS supernatants were used. The sealed nature of our ROS preparation is crucial to this conclusion since we had to demonstrate that the cAMP-dependent protein kinase proposed as a modulator of the ROS response to light and the phosphorylated 30 000–35 000 molecular weight substrate protein were not lost during purification. It is clear from comparing Figure 5 with Figures 2–4 that proteins phosphorylated in a cAMP-stimulated manner do not copurify with rhodopsin. This is also true for the approximately M_r 55 000 protein (Figure 5), which we suggest may be the cAMP-dependent regulatory subunit of type II protein kinase.

The only cyclic nucleotide effect we could find in our experiments was the inhibition of phosphorylation. This is in partial agreement with the findings of Hermolin et al. (1982) and with the recent report of Swarup & Garbers (1983). Hermolin et al. (1982) demonstrated the cGMP inhibitory effect in the initial period of phosphorylation. However, they concluded that it was not physiologically relevant, although the cGMP-stimulated phosphorylation they suggested is relevant occurs at a higher K_a than the K_i for cGMP inhibition of rhodopsin phosphorylation. We do not find cGMP-stimulated protein kinase activity under any assay condition. Swarup and Garbers also demonstrated the inhibition of phosphorylation by cGMP; however, unlike Hermolin et al. (1982) and this report, they concluded that GMP is an activator of rhodopsin phosphorylation. Furthermore, Swarup and Garbers stated that at higher levels of bleaching and after longer periods of time, cGMP stimulates rhodopsin phosphorylation, presumably after conversion to GTP. We do not see this in our preparations. Cyclic AMP was less effective as an inhibitor than cGMP. This inhibition occurred in all preparations, regardless of whether the samples were light or dark prepared, and at all the ATP concentrations and temperatures studied. Densitometric quantitation of the incorporation of ^{32}P into rhodopsin indicated that the reaction

carried out in the presence of cGMP reached a much lower plateau than when it was run without cyclic nucleotide added (Figures 3B, 4F, and 6). These results suggest that cGMP does not compete with ATP for the ATP binding sites. If cGMP were simply a competitive inhibitor of ATP, the phosphorylation could continue taking place with time at a slower rate, and eventually, the same final amount of phosphate incorporation would be reached in the inhibited as well as in the uninhibited reaction. Another possible interpretation for our observations would be the presence in ROS of either phosphoprotein phosphatase or ATPase stimulated by cGMP. It is becoming clear that cGMP plays a role in the control of rhodopsin phosphorylation separate from that ascribed to it by recent models which suggest cGMP stimulation of a protein kinase.

The levels of cGMP in ROS vary according to the amount of light that has reached them. Woodruff et al. (1977) found that the decrease of the high cGMP concentration of dark-prepared ROS becomes larger as illumination increases and varies linearly with the logarithm of light intensity at levels that bleach between 5×10^1 and 5×10^4 rhodopsin molecules (outer segment)$^{-1}$ s^{-1}. It also has been shown that rhodopsin kinase is not activated by light (Shichi & Somers, 1978) but that the light activation of rhodopsin phosphorylation is due to exposure of phosphorylation sites on the rhodopsin molecule during bleaching (Frank et al., 1973; Kuhn et al., 1973; Weller et at., 1975). These observations, in addition to the inhibitory action of cGMP described in this paper, may explain why in vivo, in the dark, rhodopsin is not phosphorylated. At saturating levels of light, the concentration of cGMP is reduced to a minimum, and the phosphorylation of bleached rhodopsin proceeds freely.

Hypotheses have been formulated about the phosphorylation/dephosphorylation of rhodopsin being implicated in the process of dark adaptation. Miller & Paulsen (1975) studied the regeneration of rhodopsin from opsin with 11-*cis*-retinal before, during, and after phosphorylation of the opsin moiety in frog ROS. They concluded that dephosphorylation and the inactivation of rhodopsin were two reactions that had to be part of dark adaptation in vivo. Kuhn et al. (1977) showed that the time required for dephosphorylation of rhodopsin in isolated frog retinas is similar to the time required for dark adaptation after strong bleaching illumination. Since cGMP levels recover slowly during dark adaptation (Woodruff & Bownds, 1979; Kilbride & Ebrey, 1979), it is probable that they will gradually inhibit the incorporation of phosphate into the visual pigment. Thus, we suggest that a physiological relevant role for cGMP in ROS may be the control of the process of dark adaptation through the modulation of rhodopsin phosphorylation.

Acknowledgments

We thank Dr. Agnes Nagy for many helpful suggestions and for her technical help. We also thank Andrea G. Williams for her assistance in preparation of the manuscript.

Registry No. Cyclic GMP, 7665-99-8.

References

Bok, D., & Filerman, B. (1979) *Invest. Ophthalmol. Visual Sci. 18* (*Suppl.*), 224.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Farber, D. B. (1982) *Hand. Exp. Pharmacol. 58*, 465–524.

Farber, D. B., Brown, B. M., & Lolley, R. N. (1978) *Vision Res. 18*, 497–499.

Farber, D. B., Brown, B. M., & Lolley, R. N. (1979) *Biochemistry 18*, 370–378.

Fletcher, R. T., & Chader, G. J. (1976) *Biochem. Biophys. Res. Commun. 70*, 1297–1302.

Frank, R. N., & Buzney, S. M. (1975) *Biochemistry 14*, 5110–5117.

Frank, R. N., Cavanagh, H. D., & Kenyon, K. R. (1973) *J. Biol. Chem. 248*, 596–609.

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

Hubbell, W. L., & Bownds, M. D. (1979) *Annu. Rev. Neurosci. 2*, 17–34.

Kilbride, P., & Ebrey, T. G. (1979) *J. Gen. Physiol. 74*, 415–426.

Kuhn, H., Cook, J. H., & Dreyer, W. J. (1973) *Biochemistry 12*, 2495–2502.

Kuhn, H., McDowell, J. H., Leser, K. H., & Bader, S. (1977) *Biophys. Struct. Mech. 3*, 175–180.

Miki, N., Baraban, J. M., Keirns, J. J., Boyce, J. J., & Bitensky, M. W. (1975) *J. Biol. Chem. 250*, 6320–6327.

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

Miller, W. H., & Nicol, G. D. (1979) *Nature* (*London*) *280*, 64–66.

Nagy, A., Shuster, T. A., & Rosenberg, M. D. (1983) *J. Neurochem. 40*, 226–233.

Orr, H. T., Lowry, O. H., Cohen, A. I., & Ferrendelli, J. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4442–4445.

Pertoft, H., Rubin, K., Kjellen, L., Laurent, T. C., & Klingeborn, B. (1977) *Exp. Cell Res. 110*, 449–457.

Pharmacia Fine Chemicals AB (1980) *Percoll Methodology & Applications*, Piscataway, NJ.

Rangel-Aldao, R., & Rosen, O. M. (1976) *J. Biol. Chem. 251*, 7526–7529.

Rubin, C. S., Erlichman, J., & Rosen, O. M. (1974) *Methods Enzymol. 38*, 308–315.

Rubin, C. S., Rangel-Aldao, R., Sarkar, D., Erlichman, J., & Fleischer, N. (1979) *J. Biol. Chem. 254*, 3797–3805.

Schnetkamp, P. P. M., & Daemen, F. J. M. (1982) *Methods Enzymol. 81*, 110–116.

Schnetkamp, P. P. M., Klompmakers, A. A., & Daemen, F. J. M. (1979) *Biochim. Biophys. Acta 552*, 379–389.

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

Shuster, T. A., Nagy, A., & Rosenberg, M. D. (1983) *Enzyme 30*, 1–11.

Swarup, G., & Garbers, D. L. (1983) *Biochemistry 22*, 1102–1106.

Virmaux, N., Nullans, G., & Goridis, C. (1976) *J. Neurochem. 26*, 233–235.

Weller, M., Virmaux, N., & Mandel, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 381–385.

Woodruff, M. L., & Bownds, M. D. (1979) *J. Gen. Physiol. 73*, 629–653.

Woodruff, M. L., Bownds, M. D., Green, S. H., Morrisey, J. L., & Shedlovsky, A. (1977) *J. Gen. Physiol. 69*, 667–679.

Yee, R., & Liebman, P. A. (1978) *J. Biol. Chem. 253*, 8902–8909.

Zimmerman, W. F., Daemen, F. J. M., & Bonting, S. L. (1976) *J. Biol. Chem. 251*, 4700–4705.