Differential Phosphorylation by GTP and ATP in Isolated Rod Outer Segments of the Retina†

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ABSTRACT: Isolated bovine rod outer segment protein is phosphorylated with GTP-γ-^{32}P and ATP-γ-^{32}P and to a much lesser extent by CTP-γ-^{32}P and UTP-γ-^{32}P. Phosphorylation with both GTP (GTP-kinase activity) and ATP (ATP-kinase activity) is markedly stimulated by light; phosphorylation with GTP is lower in dark-adapted and higher in light-adapted rod outer segments than is phosphorylation with ATP. K_m values of 20 and 200 μM and V_{max} values of 2.1 and 5.9 nmol/(mg min^{-1}) were calculated using ATP and GTP, respectively, in light-adapted outer segments. When outer segments are incubated with GTP-γ-^{32}P under the usual conditions employed in these experiments, no formation of ATP-γ-^{32}P was detected by the techniques of high-pressure liquid chromatography and thin-layer chromatography. In intact, light-bleached outer segments, GTP appears to specifically phosphorylate rhodopsin. Histone and phosvitin are not phosphorylated to any appreciable extent by GTP. Histone appears to block rhodopsin phosphorylation by GTP while histone and, to some extent, phosvitin, both act as substrates for ATP-kinase activity. Cyclic AMP and other adenine derivates have a marked inhibitory effect on GTP-kinase activity. Phosphate also inhibits GTP-kinase activity but stimulates ATP-kinase activity. Such differences in phosphorylation with GTP and ATP indicate that these activities are either due to separate enzyme systems or, if only one enzyme is involved, the activities are under separate physiological control in the photoreceptor unit.

Protein kinase activity, utilizing ATP as phosphate donor, has been reported in rod outer segments, the photoreceptor units of the retina (Kuhn and Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973; Miller and Paulsen, 1975; Weller et al., 1975). Phosphorylation is low in dark-adapted outer segments but is increased by photobleaching of rhodopsin, the visual pigment which constitutes the bulk of outer segment protein.

Rod outer segments exhibit high guanylate cyclase (Pannbacker, 1973; Bensinger et al., 1974) and cyclic GMP phosphodiesterase (Chader, et al., 1974a-c) activities and recently we have found extraordinarily high concentrations of cGMP[1] in isolated outer segments (Krishna et al., 1975). This suggests a relatively unique function for guanine nucleotides in photoreceptor metabolism and, as demonstrated in a preliminary communication (Chader et al., 1975), the possibility that protein phosphorylation could occur with GTP as well as with ATP. In the present report, we compare GTP- and ATP-kinase activities in isolated bovine rod outer segments and present evidence for the presence of specific GTP-kinase activity in the photoreceptor unit.

Materials and Methods

Dark-adapted rod outer segments were prepared from bovine eyes by differential sucrose gradient centrifugation as previously described (Chader et al., 1974a). To reduce subcellular contamination, retinas were subjected to rapid swirling in 40% sucrose buffer rather than homogenization in the initial step of the isolation procedure. All manipulations were performed at 4 °C under dim red light. The final rod outer segment pellet was suspended at a concentration of approximately 1 mg of protein/ml in 40 mM Tris-HCl buffer, pH 7.6, containing 5 mM $MgCl_2$. Protein was determined by the method of Lowry et al. (1951). For bleaching, the rod outer segments were exposed to normal room light or, in timed experiments, were exposed under a tungsten bulb (100 ft-candles) after warming the outer segment suspension to 37 °C.

ATP-γ-^{32}P and GTP-γ-^{32}P were purchased from New England Nuclear Corp., Boston, Mass. CTP-γ-^{32}P, UTP-γ-^{32}P, and $^{32}P_i$ were obtained from ICN Pharmaceutical, Inc., Irvine, California. Nonradioactive nucleotides, histone (type II), phosvitin, and casein were obtained from Sigma Chemical Co., St. Louis, Mo.

Protein Kinase Assay. The assay tube contained 50-100 μg of outer segment protein in 40 mM Tris buffer, pH 7.6, 5 mM $MgCl_2$, and radioactive nucleotide at a specific activity of 60-200 cpm/pmol in a volume of 100 μl. Incubation was normally conducted under dim red light for 30 s at 37 °C. The assay was linear with time and protein concentration under these conditions. The reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid solution. Precipitated protein was collected on Metricel filters (0.45 μm; Gelman Inst. Co., Ann Arbor, Mich.) utilizing a Yeda filtration apparatus (Yeda Sci. Inst., Rehovot, Israel). Each sample was washed three times with 5 ml of 10% trichloracetic acid solution. When appropriate, the samples were also washed three times with 5 ml of chloroform-methanol (2:1, v/v) solution or chloroform-methanol (2:1, v/v) solution containing 1% HCl (1 N). Samples were then dried and assayed for radioactivity utilizing 1 ml of Cellosolve (Eastman Kodak, Rochester, N.Y.) and 10 ml of Aquasol (New England Nuclear Corp).

Chromatography. For determination of possible transfer of ^{32}P from GTP-γ-^{32}P to ADP, samples of rod outer seg-

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[1] Abbreviations used: cGMP, cyclic guanosine 3′,5′-monophosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; P_i, inorganic phosphate.

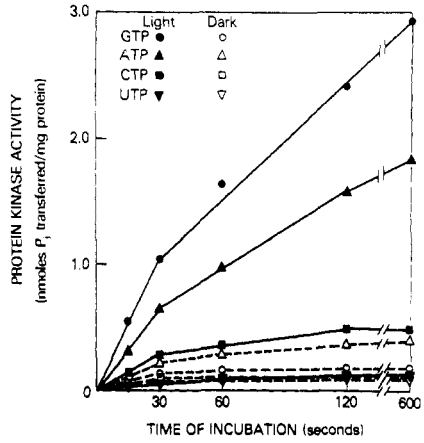

FIGURE 1: Time course of protein kinase activity in light- and dark-adapted rod outer segments. (●) GTP; (▲) ATP; (■) CTP; (▼) UTP; closed symbols are for bleached outer segments; open symbols are for dark-adapted outer segments. Assay contained radiolabeled nucleoside triphosphate at 0.1 mM with 50–100 μg of rod outer segment protein in 40 mM Tris buffer, 5 mM $MgCl_2$, pH 7.6. Incubation was at 37 °C. Values are averages of triplicate samples from three experiments for GTP and ATP and two experiments for CTP and UTP.

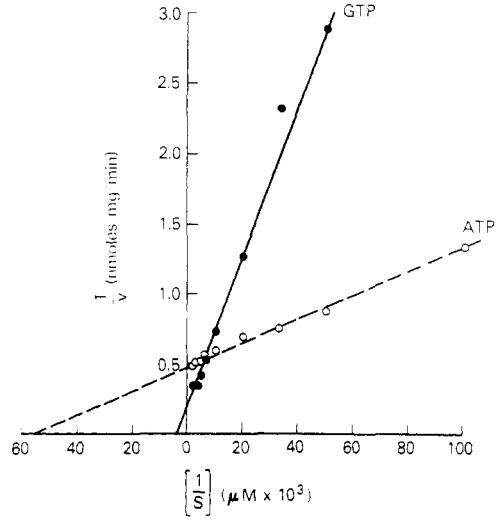

FIGURE 2: Effect of nucleotide concentration on protein kinase activity in light-adapted rod outer segments. (●) GTP; (○) ATP. The data correspond to the Michaelis–Menton inverse plot. Conditions were as in Figure 1.

ments were incubated as above; when appropriate, the incubation also included 0.1 mM ADP. After stopping the reaction with 0.2 ml of 10% trichloracetic acid solution, the samples were subjected to high-pressure liquid chromatography (Du Pont Model 841) on 6-ft Du Pont AAX anion-exchange columns eluted with 0.125 M or 0.250 M KH_2PO_4 buffer, pH 3.3. The flow rate was maintained at 1.3 ml/min by pressure adjustment (1000–1100 psi). Retention times were as follows: cGMP, 14.4 min; GTP, 18.5 min; ATP, 3.4 min; ADP 3.0 min; P_i, 2.2 min.

In other experiments, 20-μl portions of the reaction mixture (stopped with 0.1 ml of 1 N HCl) were subjected to thin-layer chromatography on Cellulose plates (Analtech, Inc., Wilmington, Del.). The solvent system consisted of isobutyric acid–2.1 N NH_4OH (66:34, v/v) as described by Kuo (1974). The R_f values observed were: ATP, 0.56; GTP, 0.28; inorganic phosphate, 0.44. Radioactive and nonradioactive standards of GTP, ATP, and P_i were used in these studies. After drying the plates, the cellulose from the individual tracks was scraped from the plates in 1-cm segments and radioactivity determined for each fraction. For studies involving charcoal adsorption, portions of the reaction mixture were treated with two 0.1-ml aliquots of activated charcoal (Sigma Chem. Co.) suspended in 0.1 N HCl at a concentration of 1 mg/ml. After centrifugation for 2 min in a Brinkmann Microfuge 3200, radioactivity in the supernatant was determined in a Packard liquid scintillation spectrometer utilizing 10 ml of Aquasol.

Gel Filtration. Phosphorylation of dark-adapted or bleached outer segments was conducted as described above. Regeneration was effected by addition of excess 9-*cis*-retinal (Sigma Chemical Co.) and incubation in the dark for 30 min at room temperature. Outer segments were then collected as a pellet by centrifugation (40 000*g*) and solubilized by treatment with 1.0% Emulphogene BC-720 (GAF Corp., New York, N.Y.) in 0.05 M Tris buffer, pH 7.8, for 15 min. The sample was then applied to a 1.5 cm × 1.7 m column of Bio-Gel A (100–200 mesh) and eluted with the Tris buffer containing 0.3% Ammonyx LO (Onyx Chem. Co., Jersey City, N.J.). The flow rate was 12 ml/h and 3.0-ml fractions were collected as described by O'Brien and Muellenberg (1975). Radioactivity in each fraction was determined and absorbance at 280 and 500 nm was measured. Phosphodiesterase activity was then measured in each sample. For this purpose, 0.05-ml portions of each fraction were incubated with 10 μM cGMP (approximately 120 000 cpm) and 100 μg of snake venom (ophiophagus hanna, Sigma Chem. Co.) in 30 mM Tris-HCl buffer, pH 7.6, containing 3.5 mM $MgCl_2$ for 3 min at 25 °C. The reaction was stopped by addition of 2.0 ml of a 50% slurry of Dowex AG1X8 (100–200 mesh, chloride form) ion-exchange resin (Bio-Rad Labs, Richmond Calif.). The radiolabeled guanosine in the supernatant fraction was then determined after addition of 15 ml of Aquasol.

Results

Nucleotide Specificity. Protein kinase activities in dark-adapted and light-bleached bovine rod outer segments using radiolabeled GTP, ATP, CTP, and UTP (1×10^{-4} M) are shown in Figure 1. For GTP, ATP, and CTP, activities in dark-adapted preparations are lower than in light-bleached outer segments. Increases of approximately 15-, 4-, and 2-fold were observed in the initial rates of the reaction for GTP, ATP, and CTP respectively. Phosphorylation in dark-adapted outer segments by UTP was low with no differences observed upon light exposure. In three experiments, the average initial rates of phosphorylation for ATP, GTP, CTP, and UTP in dark-adapted outer segments were 310 ± 20, 125 ± 15, 70 ± 13, and 11 ± 3 pmol/(mg min^{-1}) and in light-adapted outer segments were 1750 ± 70, 2300 ± 190, 180 ± 30, and 17 ± 4 pmol/(mg min^{-1}), respectively. In more detailed studies not shown here, initial rates of reaction remained linear for an incubation period of 40 s.

Nucleotide Concentration. At concentrations of nucleoside triphosphate below 20 μM, phosphorylation by ATP-γ-^{32}P is greater than that with GTP-γ-^{32}P, whereas above 80 μM phosphorylation by GTP is greater (Figure 2). Above 1–2 mM concentrations of nucleoside triphosphate, substrate inhibition occurs (unpublished observations). Kinetic calculations yield K_m values of 20 and 200 μM with ATP and GTP, respectively. The V_{max} value was 5.9 nmol/(mg min^{-1}) with GTP and 2.1 nmol/(mg min^{-1}) with ATP.

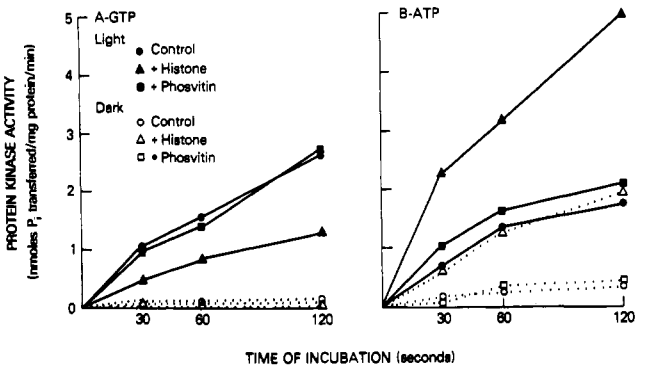

FIGURE 3: Effect of exogenous substrate on phosphorylation by protein kinase of light- or dark-adapted rod outer segments. (●) Rod outer segments alone; (▲) outer segments with 100 μg of histone added; (■) outer segments with 100 μg of phosvitin added. Solid lines correspond to light-adapted and dotted lines correspond to dark-adapted outer segments. Exogenous substrate and light- or dark-adapted outer segments were mixed immediately prior to incubation at 37 °C. Other conditions as in Figure 1.

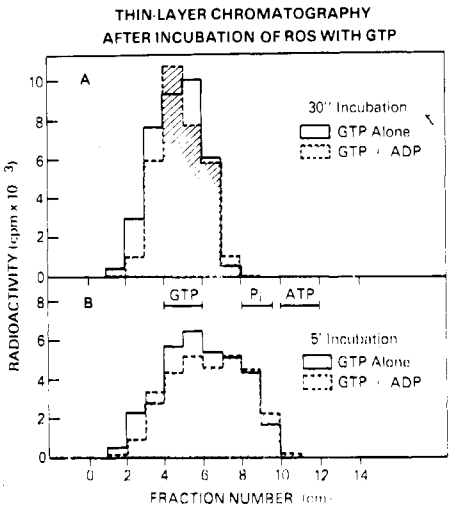

FIGURE 4: Thin-layer chromatographic patterns of incubations with GTP-γ-^{32}P and rod outer segments in the presence or absence of 0.1 mM ADP. Light-adapted outer segments were used in incubations of 30 s (Figure 5A) or 5 min (Figure 5B). Positions of standards are indicated by brackets. Other conditions as in Figure 1. The solvent system consisted of isobutyric acid–2.1 N NH_4OH (66:34, v/v).

Substrate Specificity. The effects of exogenous substrate on GTP- and ATP-kinase activities are shown in Figure 3. In dark-adapted rod outer segments, the initial rate of phosphorylation with GTP (140 pmol/(mg min^{-1})) was not greatly affected by the presence of histone or phosvitin. In bleached outer segments, GTP-kinase activity (2175 pmol/(mg min^{-1})) was inhibited by approximately 50% by histone (1050 pmol/(mg min^{-1})) and not affected by phosvitin. ATP-kinase activity in dark-adapted outer segments (300 pmol/(mg min^{-1})) was markedly increased by histone (1250 pmol/(mg min^{-1})) and essentially unaffected by phosvitin. In light-adapted outer segments, phosphorylation with ATP (1640 pmol/(mg min^{-1})) was stimulated by both phosvitin (2150 pmol/(mg min^{-1})) and histone (4460 pmol/(mg min^{-1})). Casein had little effect on ATP-kinase activity (103% of control) in light-adapted outer segments and inhibited GTP-kinase activity by 35%. Neither histone, phosvitin, nor casein had any marked effect on CTP- or UTP-kinase activities (data not shown).

In other experiments, the effects of exogenous substrate on the time course of light activation of outer segment protein kinase was examined. For this purpose, dark-adapted outer segments were exposed to light for specific time intervals, protein substrate and nucleoside triphosphate were added, and the mixture was incubated for 30 s in the dark as previously described (Chader et al., 1975). Initial rates of phosphorylation were thus measured for each time point of illumination. Activation of GTP-kinase activity by light was found to be consistently higher than that of ATP-kinase activity. Under these conditions, histone inhibited GTP-kinase activity by 90% but stimulated ATP-kinase activity by 250% above that seen in bleached native outer segments. Phosvitin had little effect on the activation of GTP-kinase activity and slightly stimulated that of ATP-kinase. With increasing time of illumination, the initial rate of phosphorylation (measured at 30 s of incubation) usually declined, although it did remain constant in some experiments over a 10-min period of illumination. The observed decrease in rate ranged from 30 to 50%. In a control experiment, part of an outer segment preparation was warmed to 37 °C and then bleached for a 10-min period while another portion was brought to 37 °C and remained in the dark for 9.5 min. The latter was then exposed to light for 30 s. Both samples were then incubated with 0.1 mM GTP-γ-^{32}P or ATP-γ-^{32}P at 37 °C for 30 s under dim red light. The rate of phosphorylation was 1150 pmol/(mg min^{-1}) in the sample exposed to light for 10 min and 1860 pmol/(mg min^{-1}) in the sample exposed to light for only 30 s of the 10-min period. Kinase inactivation thus does not appear to be merely due to denaturation or deterioration of the kinase enzyme(s) over the 10-min time period.

Specificity of P_i Transfer from GTP. Under the present conditions of rod outer segment incubation, i.e., 30 s of incubation at 37 °C with GTP-γ-^{32}P, no formation of ATP-γ-^{32}P could be detected by the technique of high-pressure anion-exchange chromatography. Virtually all of the radioactivity was recovered with the GTP peak at a retention time of 18.5 min.

As assessed by thin-layer chromatography, no formation of ATP-γ-^{32}P from GTP-γ-^{32}P was observed after an incubation period of 30 s when outer segments were incubated with 0.1 mM GTP-γ-^{32}P alone or in the presence of 0.1 mM ADP (Figure 4a). After 5 min of incubation (Figure 4b), radioactivity in the GTP-γ-^{32}P peak (R_f 0.28) was reduced and a new radioactive peak appeared with the same mobility as inorganic phosphate (R_f 0.44) but relatively close to the position of ATP (R_f 0.56). The newly formed peak was not increased (or reduced) by the presence of ADP during the incubation. To further verify that the newly formed peak was $^{32}P_i$ and not ATP-γ-^{32}P, outer segments incubated with GTP-γ-^{32}P were subjected to charcoal adsorption. Under these conditions, 96–97% of GTP and ATP present (measured as ATP-γ-^{32}P or GTP-γ-^{32}P) is adsorbed to the charcoal. This amounted to 38 900 ± 30 cpm bound of a total of 40 100 cpm of GTP-γ-^{32}P initially added. ATP (measured as ATP-γ-^{32}P) was similarly adsorbed. P_i remained in solution (98%). In the 30-s incubation, virtually all (98%) of the radioactivity was adsorbed to the charcoal, indicating it was still present as GTP-γ-^{32}P. In the 5-min incubation, about 40% (16 000 cpm) of the radioactivity remained in solution, a value similar to that in the newly formed peak observed in thin-layer chromatography (Figure 5b). Similar results were obtained in 5-min incubations with ATP-γ-^{32}P indicating that both GTP-γ-^{32}P and ATP-γ-^{32}P can be degraded to form $^{32}P_i$ under these

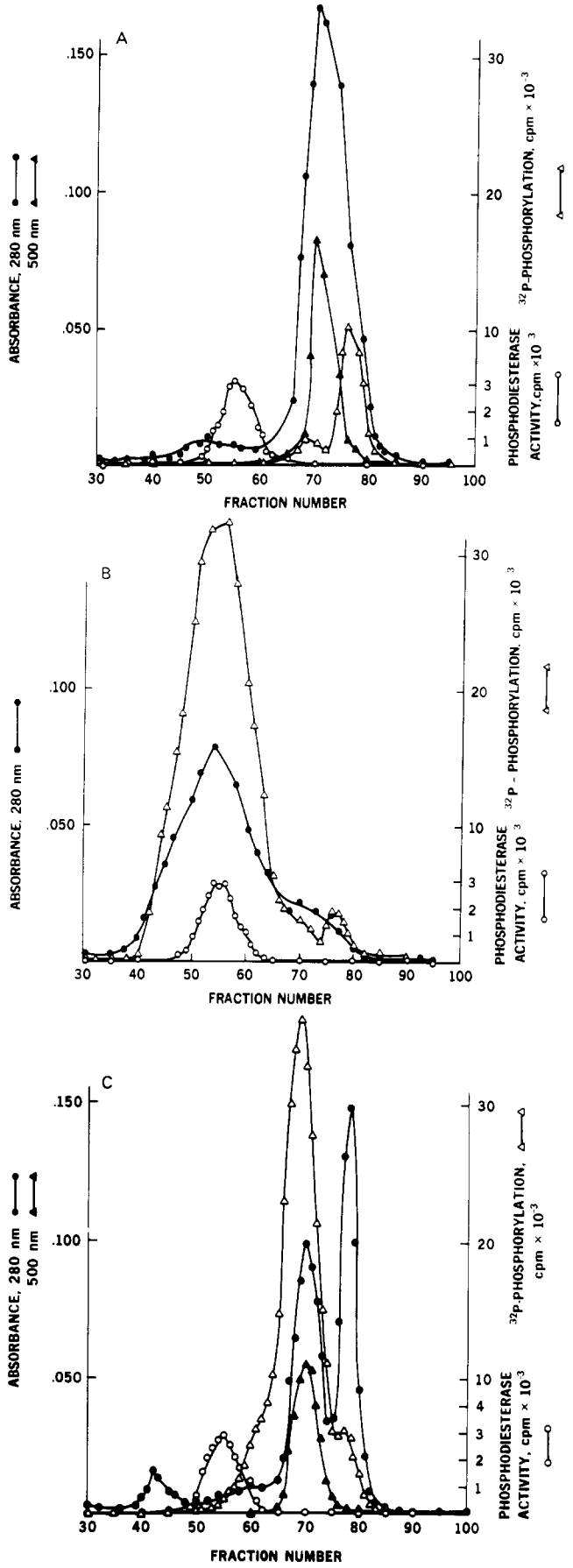

FIGURE 5: Gel filtration patterns of rod outer segments incubated with GTP-γ-^{32}P. (A) Dark-adapted, (B) light-adapted, (C) light-adapted and regenerated with 9-*cis*-retinal. After solubilization with 1.0% Emulphogene, samples were applied to 1.5 cm × 1.7 m columns of Agarose.

conditions but that $^{32}P_i$ transfer from GTP-γ-^{32}P to ADP forming ATP-γ-^{32}P is unlikely.

Specificity of Rhodopsin Phosphorylation. After phosphorylation of bleached rod outer segments, little of the radioactivity could be removed by extraction with solutions of chloroform–methanol or acidic chloroform–methanol. With chloroform–methanol, the percentages of remaining radioactivity were: ATP, 93 ± 3%; GTP, 94 ± 2%; CTP, 88 ± 6%; and UTP, 77 ± 12%. With acidic chloroform–methanol extraction, the percentages of remaining radioactivity were: ATP, 96 ± 2%; GTP, 91 ± 4%; CTP, 93 ± 8%; and UTP, 80 ± 10%. Although the percentage of radioactivity extracted was somewhat higher with CTP-γ-^{32}P and UTP-γ-^{32}P, the bulk of the material phosphorylated by all of the nucleoside triphosphates appears to be protein in nature.

Figure 5 demonstrates the relative specificity of rhodopsin phosphorylation by GTP-γ-^{32}P as assessed by Agarose gel filtration. As expected, phosphorylation of dark-adapted rod outer segments is low (Figure 5a) but two distinct phosphorylated species were observed. The smaller peak of radioactivity (fraction 70) was very close to the rhodopsin peak as adjudged by 500-nm adsorption. The second peak of radioactivity had a somewhat greater elution volume (fraction 78) and corresponded to the elution position of phospholipids under the present conditions. Cyclic GMP phosphodiesterase activity (fraction 55) did not coelute with the rhodopsin peak or either phosphorylated species.

Phosphorylation in the bleached preparation was considerably higher than when dark-adapted, and virtually all of the phosphorylation corresponded to the opsin peak (Figure 5b). The elution volume of bleached opsin (fraction 55) is markedly different from rhodopsin due to opsin aggregation (O'Brien and Muellenberg, 1975; Shichi et al., 1974) and corresponded in elution volume to phosphodiesterase activity (Figure 5b). Phosphodiesterase activity was no higher in the column fractions of the bleached preparation than those from the dark-adapted preparation. A radiolabeled peak was again observed at fraction 78.

When light-bleached, phosphorylated outer segments were incubated with 9-*cis*-retinal and subjected to gel filtration, the bulk of the protein was converted from opsin to isorhodopsin (Figure 5c). The 480–500-nm adsorption peak of rhodopsin was again seen at fraction 70 with a sharp 280-nm adsorption peak at fraction 81 due to excess 9-*cis*-retinal. The ^{32}P peak shifted from fraction 55 to fraction 70 in concordance with the rhodopsin peak with a small shoulder of radioactivity observed on the leading edge (fractions 58–63). No loss of radioactivity (dephosphorylation) was observed. As in the dark-adapted preparation, no radioactive peak was associated with the peak of phosphodiesterase activity.

Gel filtration of outer segment protein phosphorylated with ATP-γ-^{32}P was also performed with similar results (figures not shown). As with GTP-γ-^{32}P, two radiolabeled peaks were observed in dark-adapted outer segments with that corresponding to rhodopsin substantially increased in light-adapted outer segment preparations. Shichi et al. (1974) have reported the partial separation of ^{32}P-labeled protein from the main peak of rhodopsin when labeled with ATP-γ-^{32}P. We did not find this in our experiments, but rather found only a slight skew of the labeled peak with reference to the visual pigment as seen with GTP-γ-^{32}P in Figure 5a. No phosphorylation of protein in the area corresponding to phosphodiesterase activity was observed in dark-adapted or regenerated samples when ATP-γ-^{32}P was

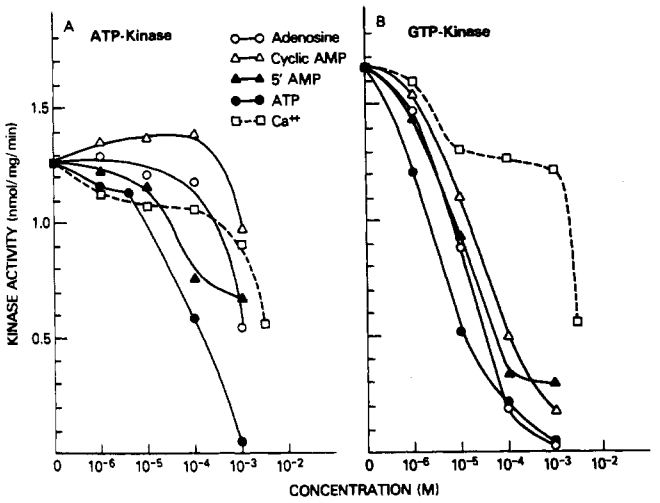

FIGURE 6: Effects of adenine derivatives and calcium on protein kinase activity in light-adapted rod outer segments. (A) ATP-kinase; (B) GTP-kinase; (■) control, no additions; (○) adenosine; (△) cAMP; (▲) 5′-AMP; (●) ATP; (□) Ca^{2+}. Incubation conditions as in Figure 1.

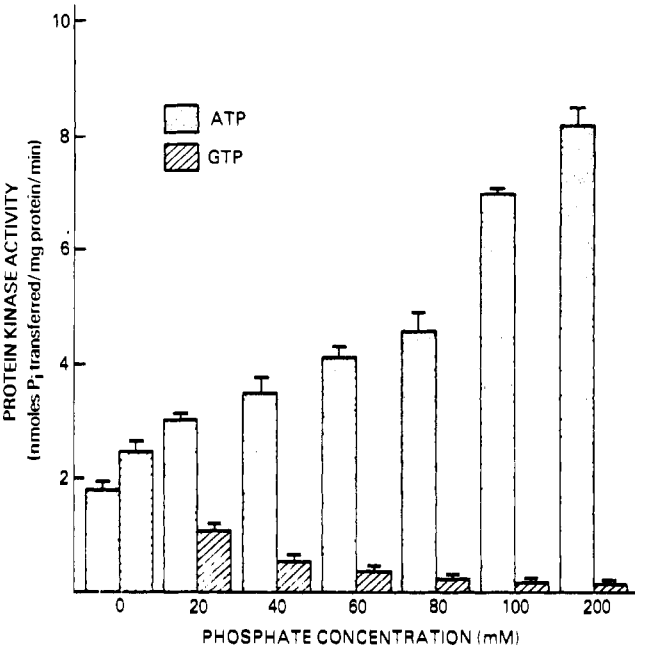

FIGURE 7: Effect of increasing phosphate concentration on protein kinase activity in light-adapted rod outer segments. Incubation conditions as in Figure 1 with and without increasing concentrations of potassium phosphate buffer, pH 7.8.

used as phosphate donor.

Activators and Inhibitors of Kinase Activity. Adenine nucleotides have a markedly inhibitory effect on GTP-kinase activity (Figure 6b). At 10^{-4} M concentrations (equimolar to GTP-γ-^{32}P used in these experiments), inhibition by unlabled ATP, adenosine, 5′-AMP, and cAMP was 88, 89, 81, and 71%, respectively. Under similar conditions, ATP-kinase activity is inhibited by 57, 8, and 60% by ATP, adenosine, and 5′-AMP, respectively (Figure 6a). A small stimulatory effect was seen with cGMP (results not shown). Calcium inhibited ATP-kinase activity by 30% at 1 mM concentration and 47% at 5 mM. Inhibition of GTP-kinase activity was also seen with calcium at 1 and 5 mM.

Guanine nucleotides were not as effective inhibitors as adenine nucleotides (figure not shown). Inhibition by guanosine, cGMP, and 5′-GMP was no greater than 50% even at 1 mM concentrations. cGMP at 100 μM inhibited ATP-kinase activity by 40% and GTP-kinase activity by 15%. As would be expected, due to substrate dilution, unlabeled GTP at 10^{-4} M inhibited apparent GTP-kinase activity by 48% and by about 90% at 10^{-3} M. Apparent ATP-kinase activity was inhibited 52 and 60% by concentrations of 10^{-4} and 10^{-3} M GTP, respectively.

Increasing concentrations of potassium phosphate had opposite effects on GTP- and ATP-kinase activities (Figure 7). Addition of 200 mM phosphate increased ATP-kinase activity by 4.5-fold. GTP-kinase activity was inhibited by over 90% at phosphate concentrations of 80 mM or above. Similar effects were observed when sodium was substituted for potassium when tested at 25, 50, and 100 mM concentrations (results not shown). Potassium chloride or sodium chloride had no effect on either kinase activity at concentrations of 25, 50, or 100 mM.

Discussion

Of the nucleotides studied in the present report, only ATP and GTP readily phosphorylated rod outer segment protein and demonstrated differential phosphorylation in light and dark. ATP-kinase activity has been well-documented in many tissues but little is known about GTP-kinase activity. Salomon and Rodbell (1973) have previously reported a "GTP-specific" protein kinase activity in fat cell preparations that phosphorylates added histone and is dependent on the addition of several other components such as ATP, cAMP, and a separate cAMP-dependent protein kinase. Kuo (1974) has shown more recently that this observed kinase activity may be due to transfer of ^{32}P from GTP-γ-^{32}P to AMP or ADP through various nucleotide kinases with subsequent protein phosphorylation by the newly formed ATP-γ-^{32}P. It is clear from the present study that phosphorylation in the rod outer segment system is markedly different from those studied by Salomon and Rodbell and by Kuo. Phosphorylation of rod outer segment protein proceeds efficiently with GTP-γ-^{32}P as well as with ATP-γ-^{32}P even in the absence of other nucleotide additives, exogenous substrates, etc. Although outer segments exhibit GTPase activity, we found no evidence of ATP-γ-^{32}P formation as assessed by high-pressure liquid chromatography and thin-layer chromatography. Rather, only increasing amounts of radiolabeled inorganic phosphate were observed. Further studies will be necessary, however, to rule out the possibility of transfer of very small amounts of ^{32}P from GTP-γ-^{32}P to adenine nucleotide and subsequent protein phosphorylation by ATP-γ-^{32}P or other as yet unknown intermediate(s).

Thus far, only light has been reported to stimulate protein kinase activity in rod outer segments. GTP-kinase activity appears to be more sensitive to light than is ATP-kinase in that phosphorylation from GTP is both lower in the dark and higher in the light. Both the rate of light activation and V_{max} of phosphorylation in fully bleached outer segments is greater with GTP than with ATP. Differences in ATP- and GTP-kinase activities are also apparent when exogenous substrate is added. With ATP-γ-^{32}P, phosphorylation of histone by outer segment kinase proceeds rapidly both in light and dark as previously shown by Frank and Bensinger (1974) utilizing the technique of disc gel electrophoresis. We find that addition of histone increases phosphorylation by ATP-kinase in dark-adapted outer segments to the level seen with a light-adapted preparation. A similar although smaller effect is seen with phosvitin in light-adapt-

ed outer segments. In contrast to these findings, Weller et al. (1975) have recently reported that ATP-kinase activity extracted from rod outer segments with Tris buffer is not capable of phosphorylating histone or phosvitin. These differences may be attributable to differences in control mechanisms, conformation, etc. of the kinase enzyme studied in its normal membrane milieu and when extracted in soluble form.

With GTP-γ-^{32}P, phosphorylation of outer segment protein appears to be markedly inhibited by the presence of histone. Little or no effect is seen with phosvitin, although we as yet cannot exclude the possibility that phosvitin rather than rhodopsin is phosphorylated in our experiments. We are presently separating the incubation products by disc gel electrophoresis to better examine the actual species phosphorylated in the presence of phosvitin and histone. ATP-kinase activity is thus able to utilize both histone and phosvitin as substrate besides its natural substrate, rhodopsin, while GTP-kinase activity does not appear to phosphorylate either histone or phosvitin and is markedly inhibited in phosphorylating its natural substrate by histone. Since ATP-kinase phosphorylates histone under light or dark conditions, our findings support the view that increased phosphorylation of rhodopsin in light-bleached outer segments is due to light-induced conformation changes in the rhodopsin substrate rather than activation of protein kinase activity by light. Phosphorylation with GTP, however, appears to be much more specific in that only rod outer segment protein (opsin) acts as substrate for the enzyme. It is presently not known if GTP-kinase activity is directly activated by light or if conformational changes in the substrate account for increased phosphorylation in light.

It appears that at least two different species are phosphorylated in light and in dark with both GTP and ATP with the larger component in light corresponding to opsin and the smaller most probably is a solvent extractable phospholipid. Regeneration of rhodopsin with retinal is not hindered by phosphorylation nor is dephosphorylation observed upon regeneration. Phosphodiesterase activity was easily separated from rhodopsin under dark-adapted conditions. Since no radioactive peak was associated with the enzyme activity in the dark-adapted or regenerated preparations, a mechanism for control of phosphodiesterase activity through phosphorylation does not appear likely. It is most interesting to note, however, that the level of activation of phosphodiesterase in light-bleached outer segments by GTP, ATP, CTP, or UTP (unpublished observations) can be correlated with the protein kinase activities shown in Figure 1. Phosphodiesterase activity was not greater in column fractions eluted from solubilized, light-adapted outer segments than those dark-adapted. As with protein kinase activity, control mechanisms may be disrupted or specific factors lost upon solubilization or extraction of outer segments.

This work and a previous study (Chader et al., 1975) indicate several possible routes for control of protein kinase in rod outer segments. Kinase activity does not always remain maximally expressed after illumination; an inactivation of kinase activity is often observed after 1–2 min of illumination as previously reported (Chader et al., 1975). This does not seem to be due to simple denaturation of the kinase enzyme, but rather to a decrease in the initial rate of phosphorylation and may afford a mechanism for the return of kinase activity to low, basal levels by photoproducts formed during bleaching. Cyclic nucleotides exhibit negative rather than positive control of kinase activity, in particular, cAMP at 10 μM inhibits GTP-kinase activity by over 40%. Other adenine compounds, adenosine, etc., are also inhibitory as has been reported in other systems studied. The effects of calcium and of phosphate offer interesting possibilities for control of kinase activity in vivo. The differential effects of phosphate on ATP- and GTP-kinase activities also further indicate the independent nature of the two activities in outer segments, although not yet demonstrating that two separate and distinct enzymes are involved.

Little is known about the nature of the reaction and chemical intermediate(s) involved in the conversion of the initial photic stimulus into a neural response in the photoreceptor cell of the retina. The high GTP-kinase activity in isolated rod outer segments and its specificity for light activation and utilization of opsin as substrate indicate that this activity as well as ATP-kinase activity may be intimately involved in the visual process.

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