Calcium and Cyclic GMP Regulation of Light-sensitive Protein Phosphorylation in Frog Photoreceptor Membranes

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ABSTRACT In frog photoreceptor membranes, light induces a dephosphorylation of two small proteins and a phosphorylation of rhodopsin. The level of phosphorylation of the two small proteins is influenced by cyclic GMP. Measurement of their phosphorylation as a function of cyclic GMP concentration shows fivefold stimulation as cyclic GMP is increased from 10^-5 to 10^-3 M. This includes the concentration range over which light activation of a cyclic GMP phosphodiesterase causes cyclic GMP levels to fall in vivo. Cyclic AMP does not affect the phosphorylations. Calcium ions inhibit the phosphorylation reactions. Calcium inhibits the cyclic GMP-stimulated phosphorylation of the small proteins as its concentration is increased from 10^-6 to 10^-3 M, with maximal inhibition of 70% being observed. Rhodopsin phosphorylation is not stimulated by cyclic nucleotides, but is inhibited by calcium, with 50% inhibition being observed as the Ca^{++} concentration is increased from 10^-9 to 10^-3 M. A nucleotide binding site appears to regulate rhodopsin phosphorylation. Several properties of the rhodopsin phosphorylation suggest that it does not play a role in a rapid ATP-dependent regulation of the cyclic GMP pathway. Calcium inhibition of protein phosphorylation is a distinctive feature of this system, and it is suggested that Ca^{++} regulation of protein phosphorylation plays a role in the visual adaptation process. Furthermore, the data provide support for the idea that calcium and cyclic GMP pathways interact in regulating the light-sensitive conductance.

INTRODUCTION

In the outer segment of the vertebrate rod photoreceptor cell, absorption of light by rhodopsin in the internal disk membrane system ultimately leads to a reduction in the permeability of the physically separate plasma membrane. There is compelling evidence that an internal transmitter mediates between photon capture and the permeability decrease (see Hubbell and Bownds, 1979). It has been suggested that Ca^{++} may play this role (Hagins, 1972;
Hagins and Yoshikami, 1974). Although evidence for the hypothesis that light induces movement of Ca" from disks to the plasma membrane has been elusive (Kaupp et al., 1979; Szuts, 1980), recent work has established that illuminated photoreceptor cells extrude Ca" into the external medium (Yoshikami et al., 1980; Gold and Korenbrot, 1980). Cyclic GMP has also been suggested as an internal transmitter based on evidence for its distribution in retinas (Orr et al., 1976), its rapid depletion upon illumination of isolated outer segments (Woodruff and Bownds, 1979), and the effects of intracellular injection of cyclic GMP (Miller and Nicol, 1979). Further work has shown that the cyclic GMP phosphodiesterase (PDE) responsible for the cyclic GMP drop is very rapidly activated by light (Yee and Liebman, 1978) and is sensitive to Ca" (Robinson et al., 1980; Kawamura and Bownds, 1981). Electrophysiological studies demonstrating that outer segments are depolarized by intracellular injections of either cyclic GMP or Ca" chelators (Miller and Nicol, 1979; Brown et al., 1977) have raised the possibility that Ca" and cyclic GMP controls may interact (see Lipton et al., 1977; Miller, 1981).

In many other systems cyclic nucleotides and Ca" appear to regulate protein kinases which in turn alter enzyme activities through phosphorylation (Greengard, 1978; Krebs and Beavo, 1979). In neural tissue from a variety of sources, phosphorylation of certain proteins has been correlated with neural activity. This includes mammalian brain (see Greengard, 1978), Torpedo synaptosomes (Michaelson and Avisar, 1979), crab nerve cells (Schoffeniels and Dandriofosse, 1980), and Aplysia nerve cells (Castellucciet al., 1980; Kaczmarek et al., 1980). In several of these instances the phosphorylation levels are influenced by cyclic nucleotides and/or Ca" (Greengard, 1978).

In rod outer segments several proteins are found to be phosphorylated (Farber et al., 1979; Polans et al., 1979). Two of these phosphorylations are light sensitive, and thus might play a role in transduction. Illumination of retinas induces a phosphorylation of rhodopsin (Bownds et al., 1972; Kühn et al., 1973; Frank et al., 1973) and a dephosphorylation of two low molecular weight proteins (Polans et al., 1979). The small proteins (designated components I and II and having molecular weights of 13,000 and 12,000, respectively) are phosphorylated in the dark and are dephosphorylated over the same range of illumination that causes decreases in the cyclic GMP content and permeability of isolated outer segments (Woodruff et al., 1977; Woodruff and Bownds, 1979). Their phosphorylation in the whole retina is stimulated by the addition of cyclic nucleotides or Ca"-chelating agents. One suggestion has been that dephosphorylation of these small proteins plays a role in the permeability change triggered by light. Rhodopsin phosphorylation induced by high light intensities has been partially characterized, but its function remains unknown (see Hermolin, 1981). The reaction seems too slow to play a role in the initial permeability change, but Liebman and Pugh (1980) have suggested that it might function in an ATP-requiring inactivation of the cyclic GMP phosphodiesterase. Others have suggested a role for the reaction in slower adaptation processes (Kühn, 1974; Miller et al., 1977).

The experiments reported in this study have examined phosphorylation
reactions in suspensions of permeabilized frog rod outer segments to further characterize Ca$^{++}$ and cyclic nucleotide controls. Minimal washing procedures are used, for Robinson et al. (1980) have shown that enzyme controls, such as the calcium control of PDE, can be easily eluted. The experiments address only the light-influenced phosphorylations, and a subsequent report will deal with Ca$^{++}$ and cyclic nucleotide regulation of those protein phosphorylation reactions that are not light sensitive. The goal of the work has been to see whether the Ca$^{++}$ and cyclic nucleotide effects observed have characteristics that might suggest a role for these controls in mediating the light-induced permeability change. The experiments have re-examined rhodopsin phosphorylation at the low light levels used in the cyclic nucleotide and permeability studies (Brodie and Bownds, 1976; Woodruff and Bownds, 1979; Robinson et al., 1980; Kawamura and Bownds, 1981) and have proven useful in evaluating the relationship between rhodopsin phosphorylation and control of the light-activated cyclic GMP phosphodiesterase.

**MATERIALS AND METHODS**

**Isolation of Rod Outer Segments**

Bullfrogs (*Rana catesbeiana* or *Rana grylio*) were maintained in tanks for 1–4 wk before use, fed ~5 g of Purina dog chow (Ralston Purina Co., St. Louis, Mo.) with vitamin supplements three times a week, and exposed to the photoperiod described by Woodruff and Bownds (1979). Animals were killed 2–3 h before the end of the dark period, and the retinas were removed and gently rinsed in Ringer's solution (115 mM NaCl, 2.5 mM KCl, 2 mM MgCl$_2$, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid], pH 7.5) as described in Woodruff and Bownds (1979). These operations, as well as subsequent isolation and incubation of rod outer segments, were performed at room temperature under infrared illumination using an image converter (FJW Industries, Mount Prospect, Ill.) for visualization.

Retinas were gently agitated to detach the rod outer segments in Ringer's solution (300 µl/retina). This Ringer's solution was buffered to 10$^{-5}$ M Ca$^{++}$ by making it 0.1 mM CaCl$_2$ and 2.78 mM EGTA (Caldwell, 1970). This was done both for convenience in buffering Ca$^{++}$ and also because earlier data describing cyclic GMP changes and phosphodiesterase activation used similar conditions (Woodruff and Bownds, 1979; Robinson et al., 1980). (This raised the question of whether the same effects would be observed in experiments in which outer segments were exposed to higher Ca$^{++}$ levels during detachment from the retina. Experiments in which detachment of outer segments was performed in either 10$^{-6}$ or 10$^{-3}$ M Ca$^{++}$, followed by readjustment to lower Ca$^{++}$ levels, yielded similar results.)

The outer segment suspension was then washed by centrifugation at 1,500 g for 15 s and then resuspended in Ringer's solution (500 µl/retina). The resuspension medium contained the various nucleoside triphosphate concentrations that are indicated in the text. Specific activities for nucleoside triphosphates are also given in the text.

Before centrifugation, >80% of the outer segments had intact plasma membranes, and thus were impermeable to the fluorescent stain dansyl cysteine used as described by Yoshikami et al. (1974). The shearing forces introduced in resuspension of the 1,500 g pellet were sufficient to permeabilize the outer segments, rendering virtually all of them permeable to the stain (i.e., introducing breaks in the plasma membrane) without altering their rodlike shape. The osmotic techniques of Korenbrot and Cone
(1972) could also be used to distinguish between “impermeable” and “permeabilized” outer segments. The resuspended preparation is referred to in the text as “permeabilized outer segments.” Intact rod outer segments that were not washed and resuspended in this manner failed to incorporate exogenous nucleoside triphosphates. Similar results have been noted by Paulson and Schurhoff (1979). Labeled nucleoside triphosphate was used to detect protein phosphorylation, because incorporation of phosphate into protein from added $^{32}$P was much less efficient in isolated rod outer segments. (The protein phosphorylations studied by Polans et al. [1979] were, in contrast, obtained by adding $^{32}$P to intact living retina. This label was incorporated into the nucleoside triphosphate pool, and the phosphorylated proteins in outer segments were analyzed after detachment of the outer segments from the retina.)

The outer segment suspension was then divided into 100-μl portions, which were adjusted to contain different Ca$^{2+}$ and cyclic GMP levels. Calcium was buffered using the EGTA and Ca$^{2+}$ levels given in Polans et al. (1981) and derived from Caldwell (1970). Different portions either were exposed to the various conditions of illumination indicated in the text or were left in the dark as controls. Various light intensities were obtained by passing a 1-ms flash (Sunpak Auto 411, Berkey Marketing Co., Woodside, N. Y.) through neutral density filters and the orange filter described by Brodie and Bownds (1976). The flash intensity was calibrated by bleaching rhodopsin. Rhodopsin concentration in each of the experiments was determined by difference spectroscopy (Bownds et al., 1971).

After a suitable period of incubation, incorporation of phosphate into protein was monitored by first quenching each of the outer segment portions with 200 μl of cold 10% trichloroacetic acid and then performing electrophoresis.

**Polyacrylamide Gel Electrophoresis (PAGE)**

Determination of the extent of phosphorylation was measured using PAGE with procedures similar to those outlined by Rudolph and Krueger (1979). The quenched and chilled precipitated samples were centrifuged at 5,000 g for 20 min, and the pellets were resuspended in 200 μl of Ringer’s solution and centrifuged again at 5,000 g for 20 min. The final pellet was dissolved in a sample solubilization solution (0.0625 M Tris-HCl, pH 6.8, 2.3% sodium dodecyl sulphate, 5% β-mercaptoethanol, 10% glycerol, and 0.2% bromphenol blue) to a final protein concentration of 0.5–1.0 mg/ml. The precipitation and washing procedure did not result in detectable loss of proteins.

Separation of proteins was carried out using procedures derived from O’Farrell (1975) based on those of Laemmli (1970), and fully described in Polans et al. (1979). Gels designed for separation and isolation of components I and II were run on a 28 × 14-cm slab gel apparatus (Hoefer Scientific Institute, San Francisco, Calif.), 26 cm long, using gradients of 6–20% acrylamide (pH 8.6) with a 4.5% acrylamide stacking gel (pH 6.8). Samples containing 15 μg rhodopsin were applied to the slots and gels were run at 10 mA per gel until the samples left the stacking gel; thereafter, the current was raised to 17.5 mA per gel until completion of the run, which was determined by the mobility of fluorescent dansylated cytochrome c added to one of the slots (prepared as described in Inouye, 1971).

Gels were then stained with Coomassie Brilliant Blue according to the methods of Fairbanks et al. (1971), dried with a Hoefer slab gel dryer and autoradiograms prepared by exposing X-ray film (Kodak V-Omat R, Eastman Kodak Co., Rochester, N. Y.) to the gel. After exposure for 5 d films were developed in Kodak liquid X-ray developer (6 min), stopped, and fixed in Kodak Rapid Fixer. Previous exposures of autoradiograms for various times indicated that 5-d exposures resulted in band
densities within the linear range of film sensitivity. Band densities were quantitated using a Joyce-Loebel Co. microdensitometer (Princesway, England) to scan the gels and a Hewlett-Packard 9820 calculator (Hewlett-Packard Co., Palo Alto, Calif.) interfaced with a Numonics 264 digitizer (Numonics Corp., Lansdale, Pa.) to integrate the areas under the peaks. In some experiments, components I and II were excised from the dried gel, dissolved in 0.5 ml H2O2, and 32P incorporation was determined by scintillation counting in 5.0 ml Aquasol (New England Nuclear, Boston, Mass.) using a Searle Mark II liquid scintillation counter (Searle Radiographics Inc., Des Plaines, Ill.).

Gels used for many of the studies of rhodopsin phosphorylation used the same procedures except that the running gel was 10% acrylamide and 8 cm in length. Samples containing 15 µg rhodopsin were applied to the gels, which were run at 10 mA per gel until stacking was complete and at 25 mA per gel thereafter. The length of the run was determined by the rate of migration of the bromphenol blue marker. After staining, the rhodopsin band was excised and 32P incorporation was determined by scintillation counting as above. Autoradiograms of the remaining portions of the gels revealed that significant polymerization of rhodopsin had not taken place.

Chemicals

Acrylamide, N,N’-methylene bisacrylamide and Coomassie Brilliant Blue R-250 were purchased from Bio-Rad Laboratories, Richmond, Calif., sodium dodecyl sulfate was obtained from British Drug Houses (Poole, England), and carrier free 32Pi, γ-[32P]-ATP, and γ-[32P]-GTP were purchased from New England Nuclear. All other nucleotide compounds were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

The initial figures of this paper (Figs. 1–5) illustrate effects of Ca++ and cyclic nucleotides on the phosphorylation of the two small proteins (components I and II), a reaction that occurs in the dark. Effects of Ca++ and nucleotides on light-activated rhodopsin phosphorylation are described in Figs. 6–9. Both ATP and GTP were added during incubation of outer segments in all experiments, except where noted, because these nucleotides are normally found in millimolar levels in the rod outer segment cytoplasm (Biernbaum and Bownds, 1979). The GTP concentration, which was found to be important in regulating rhodopsin phosphorylation (see below), but not the phosphorylation of components I and II, was usually maintained at 5 × 10⁻⁴ M. The level of radiolabeled γ-[32P]-ATP phosphate donor was generally maintained at 10⁻⁵ M. This relatively low level proved optimal for detecting and quantitating the incorporation of radioactivity into both rhodopsin and components I and II. Qualitatively similar results were obtained with millimolar ATP levels. ATP was used as radioactive phosphate donor because all outer segment protein phosphorylations, with the exception of rhodopsin phosphorylation (see below), used ATP with at least sevenfold greater efficiency than GTP (Hermolin, 1981).

Phosphorylation of Components I and II

Fig. 1 shows a typical autoradiogram demonstrating the effects of Ca++ and cyclic nucleotides on the phosphorylation levels of components I and II (see arrows). These effects will be discussed in more detail in the following figures.
Cyclic GMP enhances the phosphorylation level of both components I and II (compare slot 1, with no added cyclic GMP, with slot 4, containing $5 \times 10^{-4}$ M cGMP). Cyclic AMP does not have such an effect (slot 3). The cyclic GMP-stimulated phosphorylation is inhibited by Ca$^{++}$ (slots 4-7 have $10^{-9}$, $5 \times 10^{-9}$, and $5 \times 10^{-8}$ M Ca$^{++}$, respectively).
10^{-6}, 5 \times 10^{-5}, and 5 \times 10^{-4} \text{M Ca}^{++}, respectively, in the presence of 5 \times 10^{-4} \text{M cGMP}; however, calcium alone, in the absence of cyclic GMP, has no effect (slots 1 and 2 contain 5 \times 10^{-4} and 10^{-9} \text{M Ca}^{++}, respectively, with no added cyclic GMP). Addition of 5'-GMP did not mimic the cyclic GMP effect, and the results were not altered by varying the amount of GTP added from 0 to 10^{-3} \text{M}. Examination of bands other than components I and II in several experiments reveals that some are affected by either Ca^{++} or cyclic GMP addition and these are mentioned briefly below. Current studies are showing that some of these phosphorylations are endogenous to rod outer segments and some are not. A future paper will deal with this point in more detail.

A densitometric scan of an autoradiogram in the region of components I and II from a separate experiment is shown in Fig. 2. This illustrates the sort of data used in the quantitation used in Figs. 3–5, and provides another example of the Ca^{++} (5 \times 10^{-4} \text{M}) and cyclic nucleotide (5 \times 10^{-4} \text{M}) effects noted in Fig. 1. The control scan in Fig. 2, containing 10^{-9} \text{M Ca}^{++} and no
added cyclic GMP, can be compared with slot 2 in Fig. 1. Similar data were obtained in 30 separate experiments using different incubation conditions (with various nucleoside triphosphate levels). In Figs. 1 and 2, component II is labeled slightly more heavily than component I, whereas on the average, the opposite is the case (see Figs. 3–5). The differences, however, are not large. Note also that in some of the incubation conditions shown in Fig. 1 a radioactive band is apparent between components I and II, a band that is not seen in Fig. 2. This band is not observed in all experiments and may derive from contaminants of the outer segment suspension (Polans et al., 1979). Because it is sometimes difficult to separate this variable band from components I and II when excising gel bands for direct scintillation counting, the quantitation in Figs. 3–5 uses the area of the peaks of components I and II on the autoradiogram as a relative measure of $^{32}$P$_i$ incorporation.
The time course of the phosphorylation of components I and II both before and after the addition of Ca++ and/or cyclic GMP is shown in Fig. 3. This figure demonstrates that cyclic GMP and Ca++ influence the absolute amount of phosphorylation, not just its kinetics. Incorporation of phosphate clearly reaches a plateau value in the absence of added Ca++ or cyclic GMP. The plateau values probably are not a result of saturation of the phosphorylation sites or depletion of ATP since the addition of $5 \times 10^{-4}$ M cyclic GMP (arrow in Fig. 3) causes approximately fourfold stimulation of phosphorylation. The presence of $5 \times 10^{-4}$ M Ca++ diminishes this stimulation by decreasing the amount of phosphate incorporated. Calcium, to exert its effect, must be present during the phosphorylation reaction. It acts to arrest the phosphorylation but does not cause dephosphorylation. This was shown in separate experiments in which Ca++ was added at 0, 30, and 180 s after cyclic GMP addition. In the last case Ca++ had no effect, and it caused approximately half-maximal inhibition when added after 30 s. Finally, the figure demonstrates that Ca++, added alone instead of in the presence of cyclic GMP, has little effect on phosphorylation.

Having defined the time to reach plateau values, one can examine the concentration dependence of the cyclic GMP and Ca++ effects (Figs. 4 and 5). Cyclic GMP stimulation becomes significant at $5 \times 10^{-8}$ M and reaches a maximum between $5 \times 10^{-4}$ and $5 \times 10^{-3}$ M (Fig. 4). It was determined that...
the kinetics shown in Fig. 3 were similar for cyclic GMP stimulation of phosphorylation up to 10^{-2} M cyclic GMP and that the cyclic GMP added to give the concentrations indicated in Fig. 4 was not significantly depleted by phosphodiesterase activity present in the preparation, as measured by the procedures of Kawamura and Bownds (1981).

The Ca^{++} inhibition of cyclic GMP-stimulated phosphorylation is shown graphically in Fig. 5. It is approximately half-maximal at 2 \times 10^{-5} M Ca^{++}. In these experiments outer segments were shaken from the retina in low Ca^{++} media (10^{-9} M) and then transferred to solutions with differing Ca^{++} concentrations.

In the more than 30 experiments performed under a variety of incubation conditions, components I and II were phosphorylated with similar kinetics and were similarly affected by addition of cyclic nucleotides and/or Ca^{++}, with these effects being manifested over similar concentration ranges. The similarity in response suggests that these proteins are related in both structure and function. This is further indicated by preliminary results using two-dimensional PAGE (isoelectric focusing followed by sodium dodecyl sulphate [SDS] electrophoretic separation), which shows that these two protein bands have the same isoelectric point. It seems unlikely that these proteins are
derived from a larger precursor or that the smaller is derived from the larger precursor because of proteolysis during outer segment isolation and treatment. Isolation and electrophoresis of outer segments with all solutions containing the protease inhibitor phenylmethylsulfonylfluoride (PMSF, 0.1 mM final concentration) yielded no different results.

**Phosphorylation of Rhodopsin**

Previous studies of rhodopsin phosphorylation have used relatively high light intensities. It was felt necessary initially to examine this reaction at the low light intensities used to study cyclic GMP changes (Woodruff and Bownds, 1979), the dephosphorylation of components I and II (Polans et al., 1979), and cyclic GMP phosphodiesterase activation (Robinson et al., 1980) under similar incubation conditions. One striking difference between the phosphorylation of components I and II and rhodopsin phosphorylation was found and is illustrated by Fig. 6. The presence of either GTP, ATP, or β-γ-methylene-ATP, a nonhydrolyzable analogue of ATP (all 5 × 10⁻⁴ M), in addition to γ-[³²P]-ATP caused a marked stimulation of the incorporation of ³²P into rhodopsin after a flash-bleaching of 3 × 10⁴ rhodopsins per outer segment. In the case of ATP this occurred even though there was a 50-fold dilution of the isotope, corrected by the normalization described in the legend of Fig. 6. The stimulating effect of GTP was maximal at a concentration of 5 × 10⁻⁴ M and observed at light intensities of 3 × 10⁶ rhodopsin molecules per outer segments bleached. This suggests that a regulatory binding of nucleotide apart from binding of the ATP substrate for phosphorylation stimulates rhodopsin phosphorylation. All subsequent experiments with rhodopsin phosphorylation were done in the presence of 5 × 10⁻⁴ M GTP, both to keep the stimulation constant and optimal, and also because the outer segment cytoplasm contains millimolar levels of the nucleotide.

Fig. 7 demonstrates that the rhodopsin phosphorylation initiated by a dim 1-ms flash reaches a plateau level within 2 min. Although the time course appears uninfluenced by the intensity of the flash over a range that bleaches from 3 × 10⁴ to 3 × 10⁶ rhodopsin molecules per outer segment, the final extent increases with increasing light intensities. This was also seen in experiments where 3 × 10⁷ rhodopsin molecules per outer segment were bleached (data not shown). The data of this figure were taken as the basis for examining the effects of Ca²⁺ and cyclic nucleotides by measuring phosphate incorporation into rhodopsin 90 s after bleaches of various intensities. This was done, however, only after determining that changes in light intensity and cyclic GMP or Ca²⁺ concentration did not influence the time to reach plateau rhodopsin phosphorylation, but rather its extent.

Fig. 8 shows the plateau levels of phosphorylation caused by flashes of increasing intensity for three different Ca²⁺ concentrations. At the highest intensity tested increasing Ca²⁺ from 10⁻⁹ to 10⁻³ M caused >50% reduction in phosphorylation, and half of this effect was observed by raising Ca²⁺ to 10⁻⁶ M. This agrees with Chader et al. (1980). The phosphorylation at all three Ca²⁺ concentrations did not become easily measurable until at least 3
X 10⁴ rhodopsin molecules per outer segment were bleached (of the total of 3 X 10⁹ rhodopsins present in each outer segment). Thus, this reaction is not detectable at light intensities where many other light-induced reactions have been recently characterized: a GTP decrease (Biernbaum and Bownds, 1979), a cyclic GMP decrease (Woodruff and Bownds, 1979), a dephosphorylation of components I and II (Polans et al., 1979), and a cyclic GMP phosphodiesterase activation (Robinson et al., 1980). Furthermore, the phosphodiesterase activation, unlike rhodopsin phosphorylation, is sensitized as Ca²⁺ levels increase. Similar data were obtained with γ-³²P-GTP as phosphate donor (instead of ATP), except that the reaction was even less light sensitive (see below).

**Figure 6.** Nucleotide addition stimulates light-induced rhodopsin phosphorylation. Outer segment suspensions were exposed to a flash bleaching 3 X 10⁴ rhodopsin molecules per outer segment (0.001% bleach) in the presence of 10⁻⁵ M ATP (20 mCi/jumol) alone (○), or with the further addition of 5 X 10⁻⁴ M GTP (△), 5 X 10⁻⁴ M β,γ-methylene ATP (□), or 5 X 10⁻⁴ M ATP (▽). Calcium concentrations were buffered to 10⁻⁹ M Ca²⁺ in all cases. Closed symbols indicate dark controls. To correct for dilution of specific activity in the 5 X 10⁻⁴ M ATP curve, data for phosphorylation have been normalized, with dark levels at this ATP concentration being defined as 1. Data are from one of three similar experiments. In this and subsequent rhodopsin phosphorylation experiments, radioactive phosphate incorporation was monitored by excising the rhodopsin band from stained and destained polyacrylamide gels after electrophoresis, followed by scintillation counting (see Materials and Methods). Dark levels at time = 0, for each condition were arbitrarily defined as 1.0.
The data in Fig. 8 also reinforce a point made previously (Bownds et al., 1972; Miller et al., 1977): more phosphate is incorporated per molecule of rhodopsin bleached at low light intensities. Thus, one can calculate from the data in Fig. 8 that 10 phosphates are incorporated for each rhodopsin bleached when $3 \times 10^4$ rhodopsin molecules/outer segment are bleached in $10^{-9}$ M Ca$^{++}$. This number falls to 1 with light that bleaches $3 \times 10^5$ rhodopsin molecules/outer segment. This stoichiometry is obtained in spite of the fact that ATP is present at a concentration ($10^{-5}$ M) close to the $K_m$ for this enzyme (unpublished data of this laboratory and Chader et al., 1976; Shichi and Somers, 1978).
The effects of cyclic nucleotides on the rhodopsin phosphorylation were examined. Cyclic AMP had no effect, which agrees with previous reports in the literature (Shichi and Somers, 1978; Farber et al., 1979). Cyclic GMP addition, however, caused the inhibition shown in Fig. 9, plotted as a function of nucleotide concentration. The cyclic GMP effect, however, may well be due to its hydrolysis product 5'-GMP, for GMP also causes inhibition (Fig. 9). GDP was without effect, as were guanosine, cyclic AMP, ADP, AMP, and adenine at these concentrations. As was the case with the Ca" effects noted previously, plateau levels of phosphorylation were influenced, but not time to reach plateau levels of phosphorylation. These effects are due to inhibition of phosphorylation, not stimulation of dephosphorylation, for neither cyclic GMP nor GMP decrease the level of phosphorylation if they are added after the phosphorylation has reached its plateau level. Given that the concentrations at which these nucleotide effects are being observed are close to the supposed in vivo levels (Woodruff and Bownds, 1979), one might conclude that they are of physiological significance. However, the inhibitions are abolished if ATP concentration is raised from 10^-5 to 10^-3 M, and this latter ATP level is closer to that occurring in the normal rod outer segment.

Unlike most protein phosphorylations, rhodopsin phosphorylation in response to intense flashes used either radiolabeled ATP or GTP equally well (Hermolin, 1981). These observations are in basic agreement with Chader et
al. (1980). Light-induced rhodopsin phosphorylation with GTP as the phosphate donor showed several similarities and one major difference compared with ATP as the phosphate donor. In both cases, the rate of phosphorylation was enhanced in the presence of other nucleoside triphosphates. The time taken to reach plateau levels of phosphorylation after a flash bleaching $3 \times 10^6$ rhodopsins per outer segment was 90 s and light-induced phosphorylation was inhibited by cyclic GMP or 5'-GMP ($5 \times 10^{-5}$ M) or $10^{-3}$ M Ca++. However, the light intensity threshold was significantly different when GTP was provided as the phosphate donor. A bleach of $3 \times 10^6$ rhodopsins per outer segment was required to detect significant rhodopsin phosphorylation with GTP as phosphate donor, whereas bleaches of $3 \times 10^4$ rhodopsins were sufficient when ATP was the donor.
Other Protein Phosphorylations in Outer Segments

This paper has concentrated on the two light-sensitive phosphorylations of outer segment proteins where the effects of Ca" and cyclic GMP were highly consistent. A number of other protein phosphorylations were observed to be influenced by Ca" and/or cyclic nucleotides but not by light and some can be identified in Fig. 1. These are listed here for they may prove to be of interest, but a detailed account will be presented in subsequent work. Two proteins, with apparent molecular weights of 90,000 and 120,000, were observed always to be present and phosphorylated, with Ca" (5 × 10^{-4} M) inhibition of their phosphorylation noted in 7 out of 11 experiments. The variability in the control of their phosphorylation may be due to differential elution of control elements during washing of the outer segments (cf. Robinson et al., 1980). Phosphorylation of a 50,000-mol wt protein showed Ca" inhibition (5 × 10^{-4} M) in all of the experiments. Finally, a 25,000-mol wt protein that was present in 60% of the experiments had phosphorylation stimulated by cyclic GMP but not cyclic AMP (5 × 10^{-4} M) and inhibited by Ca" (5 × 10^{-4} M). This protein may be either differentially eluted during preparation of the outer segments, or derived from variable contaminating material. Its apparent molecular weight is similar to a protein in cattle outer segments whose phosphorylation is stimulated by both cyclic AMP and cyclic GMP (Farber et al., 1979).

DISCUSSION

The phosphorylation of rhodopsin and the cyclic GMP-dependent phosphorylation of components I and II are inhibited by Ca". Previously observed effects of Ca" on enzyme activities in the photoreceptor include a desensitization of light-induced phosphodiesterase (Robinson et al., 1980; Kawamura and Bownds, 1981) and inhibition of a particulate-associated guanylate cyclase (Fleischman and Denisevich, 1979; Troyer et al., 1978). In other systems Ca" often stimulates kinases, thereby enhancing phosphorylation levels (see Wolff and Brostrom, 1979). In these experiments it seems most likely that Ca" acts by inhibiting kinase activity. Addition of Ca" after phosphorylation has reached steady levels does not cause dephosphorylation and thus it seems unlikely that a Ca"-sensitive phosphatase is involved.

The locus of the Ca" effect or its mechanism of action is not specified in this work: it may act on either the kinase(s) or the protein substrate(s). The Ca" inhibition does not appear to be mediated by the decrease in cyclic nucleotide levels that have been noted in intact cells or outer segments as Ca" levels are increased (Cohen et al., 1978; Polans et al., 1981). In the permeabilized outer segment preparations used in these experiments, endogenous cyclic nucleotide levels are reduced, and we have determined that the cyclic GMP added in the experiments described in Figs. 1–5 was not differentially degraded in the different Ca" concentrations.

Calcium often exerts its effect on protein phosphorylation via calmodulin, although recent studies suggest the existence of Ca"-dependent kinases requiring phospholipids as co-factors (Takai et al., 1979; Kuo et al., 1980;
Wrenn et al., 1980. The inhibition of Ca\(^{++}\) studied here appears not to be mediated by calmodulin, for the addition of the calmodulin blocker trifluoroperazone to a final concentration of 10^{-5} M (Levin and Weiss, 1978) does not block the effects of Ca\(^{++}\). Other work in this laboratory (P. R. Robinson, M. J. Radeke, and M. D. Bownds, manuscript in preparation) shows no evidence for calmodulin involvement in the Ca\(^{++}\)-influenced phosphodiesterase.

The Ca\(^{++}\) concentrations at which half-maximal inhibition of components I and II phosphorylation occurs is \(\sim 10^{-4}\) M. A central question is whether this is too high to be of physiological relevance for intracellular control processes, which normally are thought to be modulated by Ca\(^{++}\) concentration changes in the range of 10^{-8}-10^{-6} M (see Wolff and Brostrom, 1979). Maximal effects of Ca\(^{++}\) at concentrations of 5 \times 10^{-5} have been observed for calmodulin-mediated protein phosphorylations (O'Callaghan et al., 1980) and as high as 10^{-3} M for phospholipid-mediated phosphorylations (Kuo et al., 1980). Our present knowledge of Ca\(^{++}\) homeostasis in outer segments is too preliminary to outline a normal range of Ca\(^{++}\) levels inside the rod outer segment. There is growing evidence that Ca\(^{++}\) is differently buffered in different regions of cells (Tillotson and Gorman, 1980; Harary and Brown, 1981), and that its diffusion in outer segments is restricted (McLaughlin and Brown, 1981). Thus, it is possible that high local concentrations of Ca\(^{++}\) might occur in the outer segment at the sites of the phosphorylations.

Similarly little is known about localization of cyclic GMP within the outer segment and how specific pools may be differently influenced by light. The concentration range over which cyclic GMP is observed to stimulate the phosphorylation of components I and II (10^{-5}-10^{-3} M) approximately matches the high cyclic GMP levels known to exist in rod outer segments, where cytoplasmic dark levels are \(\sim 10^{-4}\) M and are decreased by illumination to the 10^{-5} M range (Woodruff and Bownds, 1979).

A possible physiological role for Ca\(^{++}\) and cyclic GMP regulation of protein phosphorylation might involve their interaction in the desensitizing adaptation processes that follow a bright flash of light. One speculation, based on suggestions by Bownds (1980), would be as follows: the initial flash of light hitting a dark-adapted photoreceptor causes a relatively large cyclic GMP phosphodiesterase activation and cyclic GMP decrease. This cyclic GMP decrease causes the dephosphorylation of components I and II. The dephosphorylation is presumed to cause, directly or indirectly, a decrease in plasma membrane permeability (see Bownds, 1980). A further effect of illumination is to cause extrusion of Ca\(^{++}\) from the outer segments (Gold and Korenbrot, 1980; Yoshikami et al., 1980). We suggest that this extrusion follows the cyclic GMP decrease and lowers cytoplasmic Ca\(^{++}\) levels. This releases Ca\(^{++}\) inhibition of the phosphorylation of components I and II. Thus, components I and II are rephosphorylated shortly after their dephosphorylation by light, even though cyclic GMP levels may not have returned yet to their dark value. A second flash of light now must be brighter if it is to cause a dephosphorylation (or permeability change) as large as the original one. This is because the
lowered Ca\(^{++}\) has both reduced the light sensitivity of the phosphodiesterase (Robinson et al., 1980; Kawamura and Bownds, 1981) and enhanced phosphorylation of components I and II. One might further expect after a bright flash of light a return of permeability in the dark (i.e., an increase in the phosphorylation of components I and II caused by lowering Ca\(^{++}\)) even while the sensitivity of phosphodiesterase to a subsequent flash of light remains inhibited by low Ca\(^{++}\). Sensitivity of the phosphodiesterase would return more slowly as calcium returns to its normal levels. (Several electrophysiological studies have shown that dark permeability returns more rapidly than light sensitivity during dark adaptation [Fain, 1976; Albiani and Yoshikami, 1980; Kleinschmidt and Dowling, 1975].) Finally, in the dark-adapted photoreceptor, Ca\(^{++}\) has returned to its relatively high level (~10\(^{-4}\) M), but its inhibition of phosphorylation is counteracted by high cyclic GMP levels.

It should be emphasized that this is only one of many possible models and, indeed, it is based on a view of Ca\(^{++}\) changes that is contrary to that held by most investigators in the field (see Hubbell and Bownds, 1979). It is offered as an illustration of the sort of correlations one is seeking between the biochemistry and electrophysiology of the system. Recent electrophysiological studies demonstrating dramatic effects of both calcium and cyclic GMP on the light-sensitive conductance (Yau et al., 1981; Miller, 1980; Miller and Nichol, 1979), as well as data of the sort presented in this paper, suggest that both calcium and cyclic GMP pathways are important in regulating the light response.

**Rhodopsin Phosphorylation**

The work presented here examines rhodopsin phosphorylation at lower and more physiologically relevant light intensities than had previously been used and reveals an influence of Ca\(^{++}\) and cyclic GMP not seen before. The lack of a cyclic AMP effect on this reaction has been shown previously (Kühn and Dreyer, 1972; Bownds et al., 1972; Frank and Buzney, 1975; Lolley et al., 1977; Shichi and Somers, 1978).

The Ca\(^{++}\) regulation of rhodopsin phosphorylation might have physiological relevance in the model just indicated for components I and II. The central idea is that this phosphorylation decreases the sensitivity of the transduction process in some way (Kühn, 1974; Miller et al., 1977; and see below). Thus, a decrease in Ca\(^{++}\) levels after a flash might enhance rhodopsin phosphorylation and thus depress sensitivity. Again, as is the case with phosphodiesterase and the phosphorylation of components I and II, this role for rhodopsin phosphorylation in adaptation assumes that light causes a decrease in cytoplasmic Ca\(^{++}\) levels (see Bownds, 1980).

The data in this paper prove to be relevant to one proposed role for rhodopsin phosphorylation in phosphodiesterase regulation. Sitaramayya et al. (1977) suggest that phosphorylated rhodopsin is less effective in triggering phosphodiesterase activation. Liebman and Pugh (1980) have noted that phosphodiesterase, after its light activation, can be rapidly inactivated by an ATP-dependent process (see also Kawamura and Bownds, 1981). They suggest
that this ATP-dependent process is rhodopsin phosphorylation, which quenches the ability of bleached rhodopsin to activate phosphodiesterase. Our experiments do not support these ideas for the following reasons: (a) rhodopsin phosphorylation only reaches detectable levels at much higher light intensities than those at which significant PDE activation (and ATP-dependent inactivation) are observed (Fig. 8; cf. Kawamura and Bownds, 1981); (b) rhodopsin phosphorylation occurs at a slower rate than PDE activation and deactivation (Fig. 7); (c) rhodopsin phosphorylation can be almost completely suppressed under conditions (cyclic GMP or GMP and low ATP added) in which the PDE activation and ATP-dependent deactivation remain normal (Fig. 9; cf. Kawamura and Bownds, 1981). None of these arguments eliminates the possibility that a very small amount of total phosphorylated rhodopsin might act as a scavenger that quenches the ability of photoexcited rhodopsin to activate phosphodiesterase. The techniques used would not detect reactions of less than one phosphorylation per 10^6 rhodopsins. However, numerous other outer segment protein phosphorylations, particularly those mentioned above that are Ca^{2+} sensitive, as well as other ATP-dependent processes, must also be considered candidates for the ATP-dependent PDE controls.

Several further interesting features of the rhodopsin phosphorylation have emerged during these studies. Transfer of phosphate from a given triphosphate donor to rhodopsin is enhanced if another nucleoside triphosphate, or non-hydrolyzable analogue, is present. Thus, addition of GTP to an ATP-supported phosphorylation enhances the transfer of phosphate from ATP and vice versa. This suggests the presence, on either the kinase or rhodopsin substrate, of nucleotide binding site(s) that regulate the extent of phosphorylation. This regulatory effect does not appear to require covalent binding of the terminal phosphate group because the nonhydrolyzable analogue β,γ-methylene ATP is as effective a stimulus as other nucleoside triphosphates. GTP is also found to act as a phosphate donor, which confirms the work of Chader et al. (1980). However, this occurs only at higher light levels (i.e., bleaching 0.1% or more of the rhodopsin present). The same stimulation of phosphorylation by added nucleoside triphosphate and inhibition by added cyclic GMP or GMP or Ca^{2+} is observed. At the lower of the light levels shown in Fig. 8 the light-induced rhodopsin phosphorylation by GTP is not detectable, whereas use of ATP yields the data shown.

To better assess the importance of the phosphorylation of rhodopsin and other proteins, as well as the effects of cyclic GMP and Ca^{2+}, it would be desirable to study this chemistry in a preparation that maintains normal physiological function. Neither the permeabilized outer segments used in this study nor the intact outer segments used to measure nucleotide changes (Woodruff et al., 1977; Biernbaum and Bownds, 1979) maintain the ability to perform normal conductance and adaptation changes. We have recently developed procedures that permit isolation of pure suspensions of outer segments still attached to the mitochondria-rich inner segment. These inner segment-outer segment structures maintain their normal electrophysiology and can also be used to follow the phosphorylation reactions discussed in this
paper. Use of these preparations should permit simultaneous assay of changes in conductance, sensitivity, nucleotide levels, and protein phosphorylation.

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