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Control of Light-Activated Phosphorylation in Frog Photoreceptor Membranes†

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ABSTRACT: In this paper, we examine some factors which regulate the efficiency of light in activating rhodopsin phosphorylation. We have measured phosphate incorporation after illumination in suspensions of bullfrog rod outer segments incubated with [γ-^{32}P]ATP. We observed that delaying ATP addition after illumination causes maximum phosphate incorporation to decrease 80% within 2 h. This decay occurs in urea-treated, extracted rod outer segment membranes. The decay of the light effect is not influenced by regeneration of opsin to rhodopsin or the presence of long-lived photoproducts. However, regeneration of opsin increases the amount of phosphorylation initiated by a second exposure to light. Further phosphorylation can also occur after phosphate groups have been removed from the membranes by dephosphorylation. Finally, we have confirmed our earlier observation that small amounts of light (bleaching less than 5% of the rhodopsin present) are more effective, by tenfold, in initiating phosphorylation than are larger amounts.

In rod outer segments from vertebrate retinas, light triggers a sequence of reactions that convert the pigment rhodopsin to intermediate photoproducts and finally to opsin and all-*trans*-retinal (Hubbard et al., 1965). Alongside that classical photoproduct sequence, other light-dependent reactions have recently been discovered. Cyclic nucleotide levels in the rod outer segments seem to be regulated by light-dependent reactions (Keirns et al., 1975). In addition, illumination of rhodopsin triggers a Mg^{2+}-dependent transfer of the terminal phosphate group from ATP or GTP to serine and threonine residues on the protein moiety opsin. This light-activated phosphorylation of photoreceptor membranes has been observed in rod outer segments of frog (Bownds et al., 1972; Kühn, 1974) and cattle (Kühn and Dreyer, 1972; Frank et al., 1973; Shichi et al., 1974; Weller et al., 1975c; Chader et al., 1975). Inhibitors of the phosphorylation affect the light-induced permeability change of the outer segment plasma membrane, measured in vitro. In the presence of these inhibitors, small amounts of light produce greater changes in sodium permeability (Miller et al., 1975). Therefore, the phosphorylation reactions may be part of the mechanism linking rhodopsin photochemistry to the outer segment receptor potential and its control.

In this paper, we examine three factors which may control the effectiveness of light in activating phosphorylation: (1) dark reactions taking place in the receptor membrane after illumination, (2) the number of phosphate groups already bound to the rod outer segments, and (3) the proportion of the visual pigment that has been converted by light to photoproducts.

Materials and Methods

Rod outer segments were shaken into Ringer's solution from retinas of bullfrogs, which had been dark adapted for 12 h. The Ringer's solution contained 115 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, and 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5. The outer segments were sedimented by centrifugation at 1200*g* for 2 min at 22 °C and then resuspended in Ringer's solution. Portions of this suspension were illuminated with orange light calibrated to bleach known amounts of rhodopsin (Paulsen et al., 1975) and [γ-^{32}P]ATP of known specific activity was added to a final concentration of 4 to 5 mM. The suspensions were incubated at 22 °C in the dark, and 20-μL portions were withdrawn at intervals, added to 2 mL of 10% trichloroacetic acid–50 mM sodium phosphate, and put on ice. These samples were washed on Millipore filters and radioactivity was determined by scintillation counting. The regeneration of rhodopsin by 11-*cis*-retinal in rod outer segment preparations, the extraction

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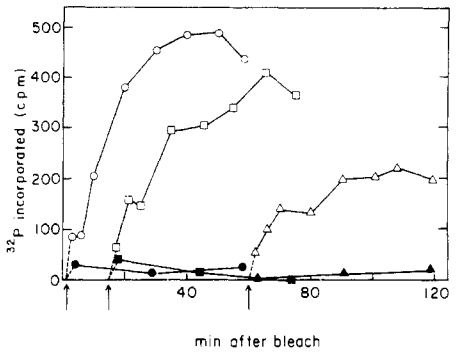

FIGURE 1: Effect of delayed ATP addition on maximum phosphate incorporation into illuminated rod outer segments. A suspension of rod outer segments was divided into six portions. Three portions were illuminated with an orange flash that bleached 60% of the rhodopsin present. ^{32}P incorporation into rod outer segments was measured after adding 5 mM ATP (0.1 Ci/mol of [γ-^{32}P]ATP) at 22 °C at 1 min (○), 15 min (□), and 60 min (△) after the flash (see arrows). Closed symbols refer to the corresponding dark control.

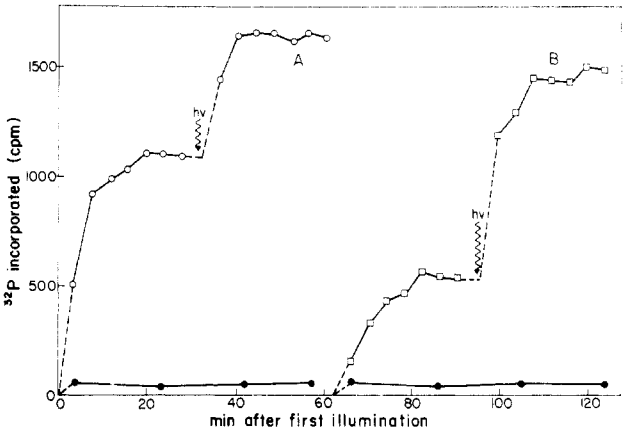

FIGURE 2: Effect of equal aging and of a second illumination on phosphate incorporation in outer segment membranes with delayed addition of ATP. Three portions of a rod outer segment suspension were prepared. One (portion B, □-□) was illuminated by an orange flash that bleached 39% of the rhodopsin present. Portions A and B then remained in the dark for 60 min. Portion A (○-○) was then illuminated, also bleaching 39% of the rhodopsin present. The third portion (●-●) remained in the dark throughout the experiment. Phosphate incorporation was initiated at 22 °C in the dark by adding 5 mM ATP (0.4 Ci/mol of [γ-^{32}P]ATP) to all three portions, 5 min after flash to portion A (○-○) and 63.5 min after flash to portion B (□-□). Thirty-three minutes after the addition of ATP both portions were exposed to a second flash (indicated by arrows), bleaching an additional 31% of the rhodopsin originally present.

of soluble components including kinase activity, and the identification of phosphorylated membrane components by sodium dodecyl sulfate gel electrophoresis have been described previously (Miller and Paulsen, 1975).

Results

Decay of the Light Effect. By incubating illuminated rod outer segments in the dark, we have investigated whether the maximum number of phosphate groups incorporated per photon absorbed decreases with time after illumination. Delaying addition of ATP after illumination did result in fewer phosphate groups being bound to the membrane (Figure 1). When ATP was added 1 h after illumination, the maximum phosphate incorporation was about 40% of the amount observed when ATP was added immediately after illumination. In addition the initial rate of phosphorylation declined as ATP addition was delayed. In samples with a 1-h lapse between light activation and ATP addition, the initial phosphorylation rate was 42% (SD 19%, n = 7) that of controls.

This decay of the light effect on rhodopsin phosphorylation cannot be explained as deterioration of the outer segment membranes. In Figure 2, phosphorylation was measured in portions of an outer segment suspension that had been treated identically, except that the portions were exposed to light at different times. One portion of the outer segment suspension (curve B) received illumination 60 min before the other (curve A). Maximum phosphorylation after 30 min was significantly less in the portion in which a 60-min time lapse occurred between illumination and ATP addition (Figure 2, curve B, first segment) than in the portion incubated with ATP immediately after illumination (curve A, first segment). The average maximum phosphate incorporation observed after a 1-h delay between illumination and ATP addition was 38% (SD 5%, n = 5) of that for conditions without delay. Addition of the antioxidant dithiothreitol (1 mM) to the outer segment suspension had no effect on this decrease in maximum phosphorylation.

By exposing rod outer segments to light for a second time (Figure 2, indicated by arrows), we have excluded the possibility that a specific deterioration, which occurs only after illumination, causes the decrease in maximum phosphorylation after delayed ATP addition. If light did initiate deterioration, less phosphate incorporation should be expected in portion B (illuminated 1 h earlier than A) than in portion A after a second exposure to light. In contrast, the second illumination resulted in a higher number of phosphate groups incorporated in portion B than in portion A. These differences in the quantum efficiency for rhodopsin phosphorylation will be discussed in more detail below.

The decrease in maximum phosphorylation observed after delayed addition of ATP does not result from phosphorylation of rhodopsin molecules by unlabeled nucleoside triphosphates endogenous to the outer segment. To demonstrate this, outer segments were sonicated and the membranes were extensively extracted with hypotonic Ringer's solution containing 5 mM EDTA[1] to remove buffer-soluble low-molecular-weight compounds and an essential soluble factor presumed to be the kinase (Kühn et al., 1973; Miller and Paulsen, 1975). The extracted membranes were divided into three portions. Then ATP, 10 mM $MgCl_2$, and a portion of the soluble component were added to one membrane sample immediately after illumination and to another sample 60 min after illumination. Maximum phosphate incorporation in the second portion was 42% of that in the first portion. Therefore, in the absence of phosphorylation by endogenous nucleoside triphosphates, a decrease in light activation of rhodopsin phosphorylation was still observed.

Thus far, the decay of the light effect has been observed only in the absence of added ATP. We examined whether this decay also occurs in the presence of 5 mM ATP by initiating phosphorylation (Figure 3a, ○-○), then causing dephosphorylation by removing external ATP from the outer segment suspension (Figure 3a, arrow A). We then asked whether the maximum phosphate incorporation stimulated by a second ATP addition (Figure 3a, arrow B) after dephosphorylation was different from a control (Figure 3a, △- - -△) in which ATP was added at arrow B for the first time. The maximum phosphate incorporation resulting from ATP addition 60 min after illumination

[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; SD, standard deviation.

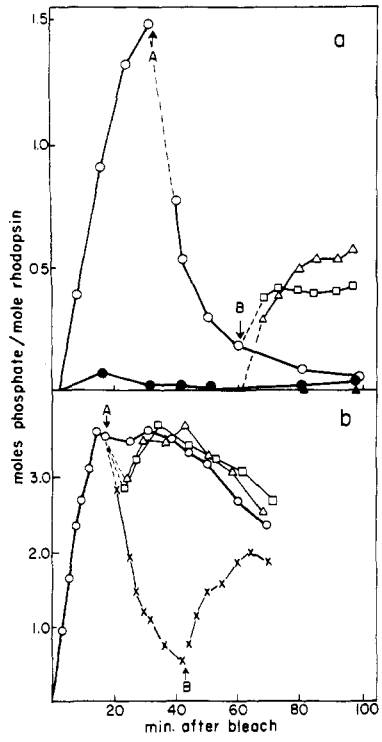

FIGURE 3: (a) Reversal of light activation during phosphorylation and dephosphorylation. A suspension of rod outer segments was divided into four portions. One portion (O–O) was illuminated to bleach 80% of the rhodopsin present and 5 mM [γ-^{32}P]ATP was added. This suspension was assayed for phosphate incorporation; after 30 min (arrow A), the suspension was centrifuged during the time indicated by the dashed line and the pellet was resuspended in Ringer's solution containing no ATP. The resulting dephosphorylation was monitored. At the time indicated by arrow B, the portion was divided and 5 mM [γ -^{32}P]ATP was added to half (□–□) of this suspension. The second portion of rod outer segments (●–●) was treated as the first, except that it was not illuminated. The third portion (▲–▲) was illuminated to bleach 80% of the rhodopsin present and incubated for 30 min in the dark in the absence of added ATP. At the time indicated by arrow A, this portion was also centrifuged and the pellet was resuspended in Ringer's solution. At arrow B, 5 mM [γ -^{32}P]ATP was added to this portion. The fourth portion (▲–▲) was treated as the third, but was not illuminated. (b) Phosphorylation of dephosphorylated outer segments. Phosphorylation was assayed in a rod outer segment suspension with 5 mM [γ-^{32}P]ATP after illumination bleaching 80% of the rhodopsin present. At the time indicated by arrow A, three portions of the suspension were centrifuged. The pellets were resuspended in either the supernatant (▲), Ringer's solution (x), or Ringer's solution having the same concentration and specific activity of [γ-^{32}P]ATP as the original incubation (□). A fourth portion, the control incubation, was not centrifuged (O). At the time indicated by arrow B, 5 mM [γ-^{32}P]ATP was added to the rod outer segments suspended in Ringer's solution (x). Maximum phosphate incorporation into a dark control has been subtracted from all other values.

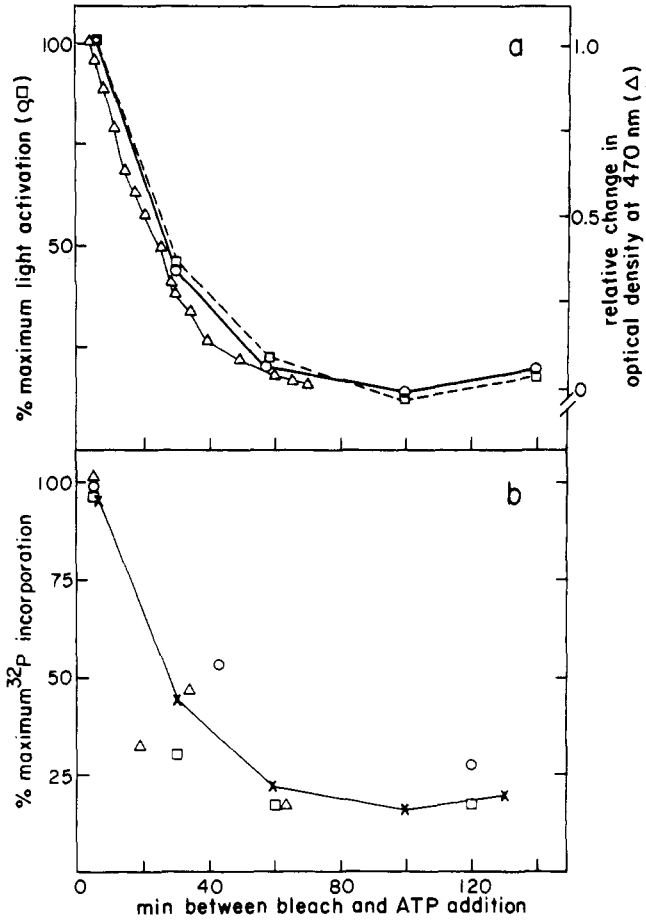

FIGURE 4: (a) Rhodopsin regeneration and the decay of light activation of phosphorylation. A rod outer segment suspension was divided into 12 portions. Two portions were illuminated with orange light, bleaching at 22 °C 79% of the rhodopsin present. Rhodopsin regeneration was initiated by adding 11-*cis*-retinal to one portion (□–□). The control remained without 11-*cis*-retinal (O–O); 4 mM [γ-^{32}P]ATP (1.3 Ci/mol) was added to both portions to initiate phosphorylation. This procedure was repeated with four other pairs of portions of the rod outer segment suspension, but varying delays were introduced between illumination and ATP addition. One pair of portions remained unilluminated. The phosphorylation measured in the unilluminated controls was subtracted from the other values. The maximum light-activated phosphate incorporation refers to the portion with the least delay between illumination and ATP addition. The decay of metarhodopsin III at 22 °C was measured spectrophotometrically, in outer segment suspensions of comparable composition, as change in optical density at 470 nm (▲–▲, from Paulsen et al., 1975). (b) Decay of light activation of phosphorylation in isolated retinas. Retinas were dissected from dark-adapted frogs and placed in Ringer's solution. Each retina was illuminated by a flash. After varying times of incubation in the dark, the rod outer segments were shaken from a retina, which was then discarded. 5 mM [γ-^{32}P]ATP (0.8 Ci/mol) was added to the rod outer segment suspension. The measured phosphate incorporation was adjusted for variation in the amount of rhodopsin present, as determined by difference spectroscopy. The maximum phosphate incorporation refers to the maximum level obtained after the shortest delay (5 min) between illumination and ATP addition. The other data are expressed as a fraction of the maximum incorporation. The different open symbols (□, O, ▲) represent three separate experiments. The solid line (x–x) shows reversal of light activation in a rod outer segment suspension.

was depressed in both, in comparison to phosphate incorporation observed immediately after illumination. Thus, a decay of the light effect on phosphorylation can take place when ATP is present, and during concurrent phosphorylation and dephosphorylation of rhodopsin molecules.

Decay of the Light Effect during Dark Reactions Involving the Rhodopsin Chromophore. The extent and time course of the decay of the light effect were not influenced by the regeneration of opsin to rhodopsin, nor by the presence of long-lived photoproducts. In the experiment of Figure 4a, regeneration of 86 to 100% of the rhodopsin, which occurred during a 30-min incubation with excess 11-*cis*-retinal, changed neither the time course of the decay nor the final level of phosphate incorporation (□–□, phosphorylation with regeneration; O–O, phosphorylation with no regeneration). At 22 °C, the time course of the decay of the light effect parallels the decay of metarhodopsin III (Figure 4a, ▲- - -▲) measured in bullfrog rod outer segments under comparable conditions (Paulsen et al., 1975). However, the decay of metarhodopsin III measured at 12 °C (Paulsen et al., 1975) was faster than the decay of light activation of rhodopsin phosphorylation (Figure 5). Thus, visual pigment molecules in the metarhodopsin III state are not a special substrate limiting the number of phosphate groups

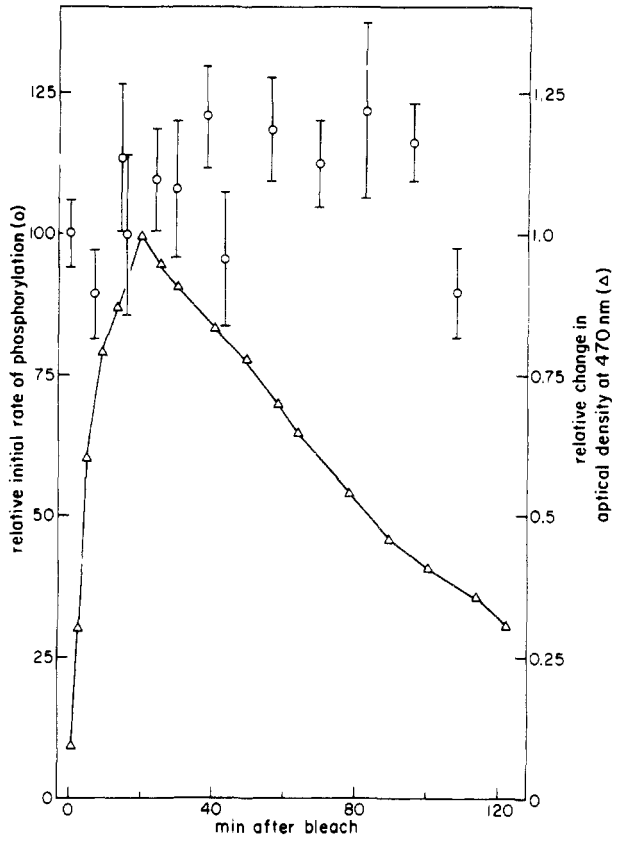

FIGURE 5: Time course of decay of the light effect on rhodopsin phosphorylation at 12 °C and metarhodopsin III decay. The decay of metarhodopsin III at 12 °C measured spectrophotometrically as change in optical density at 470 nm (Δ–Δ) in a comparable outer segment suspension was taken from Paulsen et al. (1975). A suspension of washed rod outer segments, maintained throughout at 12 °C, was illuminated with a flash that bleached 47% of the rhodopsin present. At the indicated intervals, aliquots were mixed with 5 mM [γ-^{32}P]ATP (1.4 Ci/mol). To obtain the initial phosphorylation rate, we sampled the incubations during a 5-min period. The initial rate of phosphorylation (O) was determined by fitting a line to the measured amounts of incorporation by linear-regression analysis. The error bars represents the standard error of the line.

TABLE I: Localization of Reversal of Light Effect.[a]

Condition	^{32}P incorp (cpm)	% of control phosphorylation
Dark		
membranes and extract kept at 22 °C	127	3.1
membranes and extract kept at 0 °C	140	3.4
Membranes illuminated		
ATP added immediately after illumination (control)	4126	100
ATP added 120 min after illumination:		
membranes and extract kept at 22 °C	349	8.5
membranes kept at 22 °C and extract at 0 °C	411	10.0
membranes kept at 0 °C and extract at 22 °C	3619	87.7
membranes and extract kept at 0 °C	4287	103.9

[a] Rod outer segments were lysed in 5 mM EDTA and the membranes were sedimented. The membrane pellet was sonicated in 2.7 M urea, washed, and finally resuspended in Ringer's solution. Both the membrane suspension and extract were divided into seven portions which were incubated as indicated. Membranes and supernatants were then recombined at 22 °C, and phosphorylation was assayed to determine the maximum phosphate incorporation, which is given in the table. Illumination was a flash bleaching 80% of the rhodopsin present, and 5 mM ATP (0.9 Ci/mol of [γ-^{32}P]ATP) with 10 mM $MgCl_2$ was used.

that can be incorporated into the outer segment membrane. (In Figure 5, initial rate of phosphorylation was assayed to monitor decay of light activation, because much longer times are required to reach maximum phosphate incorporation at 12 °C. We do not have sufficient data to compare initial rates at 12 and 22 °C.)

Maximum Decline of the Light Effect in Rod Outer Segments and Isolated Retinas. Maximum phosphate incorporation measured when ATP was added 120 min after illumination was 22% (SD 12%, n = 12) of the maximum phosphate incorporation observed when ATP was added immediately after illumination. To test whether this incomplete decay is due to missing intermediates from the inner segment of the photoreceptor cell, we examined the decay of the light effect in isolated intact retinas. Figure 4b shows that rods of the isolated retina also did not return completely to a dark-adapted state with respect to rhodopsin phosphorylation and that the time course of the decay of the light effect was not altered by isolating the outer segments.

While light-activated phosphorylation requires Mg^{2+} (Kühn and Dreyer, 1972; Bownds et al., 1972), light activation in isolated outer segments declined similarly at low and high levels of Mg^{2+}. When rod outer segments were incubated in Ringer's solution containing 5 mM EDTA without added $MgCl_2$, and at varying times after illumination 10 mM $MgCl_2$ was added with 5 mM ATP to start phosphorylation, the time course of the reversal was identical to that of a control incubated throughout with 5 mM EDTA and 10 mM $MgCl_2$. We have also observed no consistent difference in the time course of the decay of light activation after bleaching 1 to 80% of the rhodopsin present.

Localization of Processes Leading to the Decay of Light Activation. The decay of the light effect in urea-treated, extracted membranes mentioned above suggested a membrane locus for the process. To further investigate whether the kinase or other soluble factors control the decay of the light effect, we introduced temperature steps into experiments with extracted membranes. Lowering the temperature reduces the reversal of the light effect (compare Figures 4a and 5). The data in Table I demonstrate that keeping the rod outer segment extracts, containing kinase activity, at 0 °C during the interval between light exposure of extracted membranes and ATP addition has little effect on maximum phosphate incorporation into the membranes. On the other hand, the decay of the light effect was markedly reduced when the extracted membranes were held at 0 °C. Therefore, the observed decay of light activation of rhodopsin phosphorylation can be attributed to processes of extracted rod outer segment membranes.

Determination of Sites Available for Phosphorylation. We have examined phosphorylation in outer segments that had been illuminated to the same extent, but which were phosphorylated to different levels. As in the experiment described by Figure 2, a delay in ATP addition after the first bleach resulted in incorporation of less than half the number of phosphate groups than in the control. We then measured the maximum phosphate incorporation after a second bleach. Each illumination reduced the rhodopsin concentration by approximately the same amount. Under these conditions, the

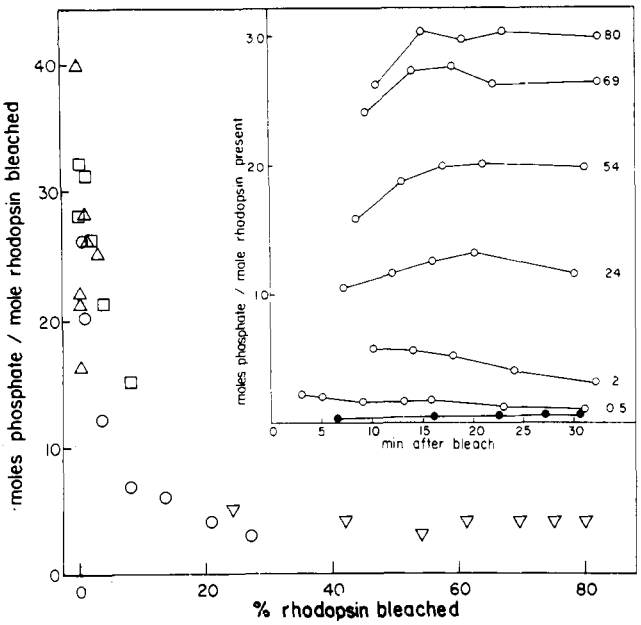

FIGURE 6: The efficiency of visual pigment phosphorylation as a function of illumination. The inset shows time courses of phosphorylation after rod outer segments were illuminated for less than 2 min to bleach with a continuous light source known fractions of the rhodopsin present by varying the light intensity. The number at the right of each curve is the percentage of the rhodopsin bleached. The main figure compiles data from 27 time courses in four experiments (represented by the four symbols). The efficiency of illumination (moles of phosphate incorporated per mole of rhodopsin bleached) on the ordinate was determined by dividing the maximum light-activated phosphorylation observed during a time course (maximum phosphorylation measured minus maximum phosphorylation in the dark control) by the fraction of rhodopsin bleached. The ATP concentration was 5 mM. (○, △, □, ▽) Illuminated membranes; (●) dark controls.

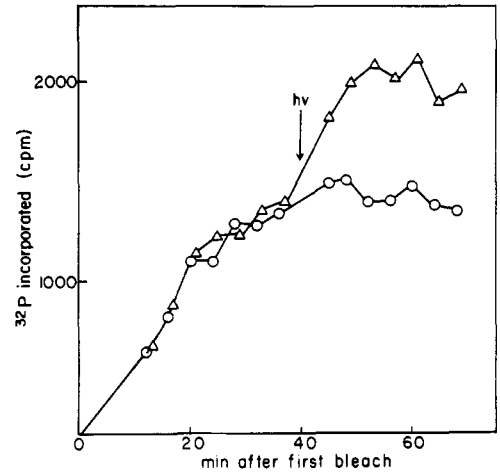

FIGURE 7: Effect of rhodopsin regeneration on quantum efficiency of phosphorylation. Phosphorylation was initiated by adding 5 mM [γ-^{32}P]ATP at 22 °C to rod outer segment suspensions after bleaching 82% of the rhodopsin present. 11-*cis*-Retinal was added to one portion (△) immediately after the bleaching but not to the control (○). In the portion supplemented with 11-*cis*-retinal, 96% of the bleached rhodopsin regenerated within 33 min. At the time indicated by the arrow, both portions were illuminated by an orange flash for a second time which reduced the rhodopsin concentration in the requested portion from 96 to 38% and in the control from 18 to 9%.

total phosphate incorporation after the two bleaches was similar (ratio of final phosphate incorporation in condition with delay to that of control = 1.02; SD = 0.21; n = 5), whether or not decay of light activation had decreased the response to the first bleach.

Figure 3a,b shows that binding sites for phosphate groups became available again when outer segment membranes are dephosphorylated. When dephosphorylation was induced after maximum phosphate incorporation by exchanging the Ringer's solution containing ATP for Ringer's solution with no ATP (Figure 3b, arrow A), a later addition of 5 mM ATP induced further phosphorylation (Figure 3b, arrow B). Control incubations in which dephosphorylation had not been induced showed no effect of a second addition of ATP. The total phosphorylation, after dephosphorylation and a second addition of ATP (Figure 3b after arrow B), did not reach the maximum level measured after the first addition of ATP. Instead, the maximum phosphate incorporation reached approximately the level of control samples where dephosphorylation of membranes in the presence of high ATP concentrations had been occurring (Figure 3b, ○-○, △-△, □-□). This result indicates that, in the presence of high ATP concentrations (5 mM) and concurrent phosphorylation and dephosphorylation of visual pigment molecules, the maximum number of phosphate groups bound decreases with the decay of the light effect.

Relation between Maximum Phosphate Incorporation and Extent of Rhodopsin Bleaching. When isolated rod outer segments were exposed to light that bleached between 10 and 80% of the rhodopsin molecules present, and then immediately incubated with 5 mM ATP, the total phosphate incubation in the dark was approximately proportional to the number of rhodopsin molecules bleached. However, bleaches which reduced the rhodopsin concentration by less than 8 to 10% were relatively more effective in activating phosphate incorporation than were larger bleaches. In Figure 6, portions of an outer segment suspension were exposed to a continuous light source to bleach a known amount of rhodopsin, 5 mM [γ-^{32}P]ATP was added in the dark and the time course of phosphorylation was measured (Figure 6, inset). The main Figure 6 summarizes data of 27 time courses from four separate experiments covering different, but overlapping, reductions in the rhodopsin concentration after light exposure. The number of phosphate groups incorporated per rhodopsin molecule bleached appeared to decrease exponentially from about 40 to 4 with bleaches of 0.2 to 10% of the rhodopsin present. For bleaches larger than 10%, the ratio of incorporated phosphate groups per rhodopsin bleached was 3.6 (SD 0.5). After both small and large reductions in the rhodopsin concentration, sodium dodecyl sulfate gel electrophoresis revealed a single radioactive band containing more than 90% of the radioactivity applied to the gel and appearing in the same position as opsin, the visual pigment protein.

Figure 2 (curve A) shows a difference in the quantum efficiency of the first and second illumination of a suspension of outer segments. When each flash bleached 30 to 40% of the rhodopsin originally present in the dark-adapted outer segments, the second bleach produced a maximum phosphate incorporation of only 43% (SD 27%; n = 5), the incorporation measured after the first bleach. The quantum efficiency of a second bleach for light-activated phosphorylation was increased, however, if bleached visual pigment molecules had been regenerated to rhodopsin. In Figure 7, the rhodopsin concentration was reduced by 82% after the first bleach. A second strong flash of light induced only a small additional amount of phosphate incorporation. However, if between the exposures of light opsin was regenerated to rhodopsin by addition of excess 11-*cis*-retinal, phosphate incorporation was stimulated markedly by the second flash.

Discussion

Beyond regeneration of the rhodopsin's spectral properties, dark adaptation of rod photoreceptor cells must include two membrane-associated reactions: dephosphorylation of rhodopsin and the decay of the light effect on rhodopsin phosphorylation. In this paper, we have described a decay of light activation of rhodopsin phosphorylation in rod outer segments and isolated retinas. Because this decay involves temperature-dependent changes in the receptor membrane (Table I) and is independent of dark reactions involving the chromophore of rhodopsin (Figures 4a and 5) or substances extractable from rod outer segment membranes, we suggest that the decay of the light effect represents a partial rearrangement of visual pigment molecules in the receptor membrane or conformational changes in the structure of opsin, which might be induced by the rapid rotational and translational movements of these molecules in the fluid membrane (Cone, 1972; Brown, 1972).

The maximum number of phosphate groups bound to the visual pigment protein in the rod outer segment membrane seems to be controlled by several processes. We hypothesize that illumination makes a specific number of sites available for phosphorylation, setting the maximum number of phosphate groups that can be incorporated. If this maximum is not reached during the period in which phosphorylation takes place, additional phosphate groups will be incorporated the next time phosphorylation is activated by light. If binding sites for phosphate groups become available after dephosphorylation, further phosphate groups can be incorporated up to the level determined by processes reversing light activation. Whether available sites actually become and remain phosphorylated is determined finally by the kinetics of the phosphorylation and dephosphorylation reactions which depend on the concentration of nucleoside triphosphates in the outer segment.

Even though phosphorylation is triggered by bleaching of rhodopsin, most of the present data suggest that the stoichiometry and time course of the subsequent phosphorylation is independent of the classical rhodopsin photoproduct and regeneration sequence. Regenerated rhodopsin can be phosphorylated as well as opsin (Miller and Paulsen, 1975); long-lived photoproduct decay and rhodopsin regeneration have no obvious effect on the decay of activation discussed in this paper (Figures 4a and 5), and dephosphorylation is not influenced by rhodopsin regeneration (Kühn et al., 1973; Miller and Paulsen, 1975; Weller et al., 1975c). These data suggest that the phosphate and chromophore attachment sites can be separately regulated, a distinction consistent with the demonstration that these sites are in fact on different parts of the opsin molecule (Virmaux et al., 1975).

Although phosphorylation, once set in motion, seems to proceed independently of subsequent reactions of the chromophore, we now find (Figure 7) that regeneration of rhodopsin can reset the "trigger" which activates the phosphorylation reaction. This represents our first observation of an effect of rhodopsin regeneration on the phosphorylation reaction.

Because neither dephosphorylation nor decay of light activation of phosphorylation seem to be controlled by later reactions of the rhodopsin chromophore, the photochemical reaction leading to activation must be considered as the crucial steps in controlling phosphate incorporation.

We have observed differences in the activation of phosphorylation with different amounts of light. As in our earlier reports (Bownds et al., 1972, 1974), small amounts of light were relatively most effective in promoting phosphate incorporation into frog photoreceptor membranes. The data do not establish whether the phosphate groups are spread evenly over many opsin molecules or confined to a few. However, it seems unlikely that 30 phosphate groups would bind to a single rhodopsin molecule; probably only a few of the approximately 40 serine and threonine residues on each opsin can be phosphorylated. Thus, it is likely that unbleached rhodopsin molecules are also phosphorylated.

Several laboratories (Kühn et al., 1973; Weller et al., 1975c; Frank and Buzney, 1975) have suggested a simple mechanism in which light activation is caused by exposure of sites on rhodopsin during bleaching. This model does not fit the observations that (1) regeneration does not reverse activation (Miller and Paulsen, 1975), (2) a second bleach in outer segments where the light effect has partly decayed can expose more bindings sites for phosphate groups than are expected from the amount of rhodopsin bleached, and (3) quantum efficiency for rhodopsin phosphorylation is higher at lower light levels.

We must eventually explain how a photoactivated rhodopsin molecule makes unbleached rhodopsin molecules susceptible to phosphorylation. It might, for example, interact with them in the plane of the membrane, or activate a soluble cofactor or enzyme which acts on unbleached rhodopsin molecules. All of the data obtained thus far emphasize the importance of the membrane fraction in light regulation of phosphorylation. The extractable "kinase" which restores phosphorylation when added back to extracted membranes is not light sensitive (Miller and Paulsen, 1975), and does not play a role in the activation–inactivation sequence described in this paper (Table I).

We think that the data are most simply interpreted as reflecting cooperative interactions between bleached and unbleached rhodopsin molecules in these photoreceptor membranes with the presence of bleached "excited" rhodopsin molecules making unbleached rhodopsin more accessible as substrate for the endogenous kinase.

Recent results indicate experiments required to extend our understanding of this phosphorylation reaction. Chader et al. (1976) have reported that GTP may be the preferred phosphate donor for phosphorylation in cattle photoreceptor membranes. The stoichiometry and light sensitivity of the reaction might be different if GTP is used as the phosphate donor. A further point is raised by the difference between high and low illumination in promoting phosphorylation. The mechanism of the reaction might differ at lower and higher light levels. Levels of illumination that bleach up to 1–5% of the visual pigment present, and are relatively most effective in promoting phosphorylation, are also in the range of illumination of normal physiological responses by frog rod outer segments. At higher intensities which bleach 10–100% of the rhodopsin present, the frog rod photoreceptor is "saturated" and does not respond to changes in illumination (Hood and Hock, 1975). In addition, Donner and Hemilä (1975) have reported that small bleaches cause different photoproduct kinetics in isolated retinas. It would thus be appropriate to repeat the various key experiments that have described the light-activated phosphorylation using illumination which bleaches 1% or less of the rhodopsin present.

The demonstration that inhibition of rhodopsin phosphorylation increases light sensitivity suggests that phosphorylation is involved in the relatively slower mechanisms of light and dark adaptation (Miller et al., 1975). Other laboratories

have also suggested that phosphorylation may play a role in adaptation processes (Kühn et al., 1973; Weller et al., 1975c). One possibility is that phosphorylation modifies an "enzymatic" activity of rhodopsin relevant to the control of the internal transmitter which is presumed to be the link between rhodopsin bleaching in the disk membrane and the permeability mechanism of the plasma membrane (Hagins, 1972). The examination of this or any other models (Weller et al., 1975a,b) will not proceed rapidly until the internal transmitter has been positively identified.

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