Hara, T., & Hara, R. (1967) *Nature (London) 214*, 572–575.
Johnson, G. L., Kaslow, H. R., & Bourne, H. R. (1978) *J. Biol. Chem. 253*, 7120–7123.
Kuhn, H. (1980) *Nature (London) 283*, 587–589.
Kuhn, H. (1981) *Curr. Top. Membr. Transp. 15*, 171–201.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, A. J. (1951) *J. Biol. Chem. 193*, 263–275.
Manning, D. R., & Gilman, A. C. (1983) *J. Biol. Chem. 258*, 7059–7063.
Miki, N., Baraban, J. M., Keirns, J. J., Boyce, J. J., & Bitensky, M. W. (1975) *J. Biol. Chem. 250*, 6320–6327.
Nashima, K., Mitsudo, M., & Kito, Y. (1978) *Biochim. Biophys. Acta 536*, 78–87.
Neufeld, A. H., & Levy, H. M. (1969) *J. Biol. Chem. 244*, 6493–6497.
Northup, J. K., Sternweis, P. C., Smigel, M. D., Schleifer, L. S., Ross, E. M., & Gilman, A. G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6516–6520.
Randerath, K., & Randerath, E. (1964) *J. Chromatogr. 16*, 111–125.
Salomon, Y., & Rodbell, M. (1975) *J. Biol. Chem. 250*, 7245–7250.
Shinozawa, T., Uchida, S., Martin, E., Cafiso, D., Hubbell, W., & Bitensky, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1408–1411.
Sternweis, P. C., Northup, J. K., Smigel, M. D., & Gilman, A. G. (1981) *J. Biol. Chem. 256*, 11517–11526.
Thompson, W. J., & Appleman, M. M. (1971) *Biochemistry 10*, 311–316.
Thompson, W. J., Terasaki, W. L., Epstein, P. M., & Strada, S. J. (1979) *Adv. Cyclic Nucleotide Res. 10*, 69–92.
Tishler, P., & Epstein, C. J. (1968) *Anal. Biochem. 22*, 89–98.
Vandenberg, C. A. (1982) Ph.D. Dissertation, University of California, San Diego.
Vandenberg, C. A., & Montal, M. (1982) *Biophys. J. 37*, 195a.
Vandenberg, C. A., & Montal, M. (1983) *Biophys. J. 41*, 25a.
Vandenberg, C. A., & Montal, M. (1984) *Biochemistry* (following paper in this issue).
Wheeler, G. L., & Bitensky, M. W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4238–4242.
Wheeler, G. L., Matuo, Y., & Bitensky, M. W. (1977) *Nature (London) 269*, 822–824.
Wu, C.-F., & Pak, W. L. (1975) *J. Gen. Physiol. 66*, 149–168.
Yamada, E. (1982) *Methods Enzymol. 81*, 3–17.
Yeandle, S., & Spiegler, J. B. (1973) *J. Gen. Physiol. 61*, 552–571.

Light-Regulated Biochemical Events in Invertebrate Photoreceptors. 2. Light-Regulated Phosphorylation of Rhodopsin and Phosphoinositides in Squid Photoreceptor Membranes†

Carol A. Vandenberg‡ and Mauricio Montal*

ABSTRACT: Phosphorylation of squid photoreceptor membrane components by Mg-[γ-^{32}P]ATP is regulated by light. Illumination of squid photoreceptors (*Loligo opalescens* or *Loligo pealei*) resulted in phosphorylation of rhodopsin and a 55 000-dalton protein. Rhodopsin phosphorylation was increased 15–20-fold by light, to an average of 0.9–1.8 phosphates/metarhodopsin. The linear dependence of rhodopsin phosphorylation on photoconversion of rhodopsin to metarhodopsin suggests that metarhodopsin is a light-activated substrate for phosphorylation. Phospholipids also were phosphorylated by [γ-^{32}P]ATP. In the dark, ^{32}P was incorporated into phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate, and phosphatidic acid. Illuminated membranes showed increased ^{32}P incorporation into phosphatidic acid and decreased incorporation into the phosphorylated phosphoinositides. These results suggest, for the first time, the participation of a light-activated phospholipase C in squid photoreceptors.

Phosphorylation of cellular constituents is a mechanism commonly involved in cellular regulation of enzymatic activities [for reviews, see Glass & Krebs (1980) and Cohen (1982)]. There is ample evidence suggesting that the cellular response to several neurotransmitters and hormones is mediated through modulation of the phosphorylation state of specific proteins (Greengard, 1981; Nestler & Greengard, 1983). In the vertebrate retinal rod cells, the visual pigment rhodopsin can be phosphorylated up to an extent of 9 mol of phosphate/mol of rhodopsin (Wilden & Kuhn, 1982). Phosphorylation of vertebrate rhodopsin is a light-dependent reaction catalyzed by a specific rhodopsin kinase that recognizes illuminated rhodopsin as a substrate for phosphorylation (Bownds et al., 1972; Kuhn & Dreyer, 1972; Kuhn et al., 1973; Frank et al., 1973; Weller et al., 1975b; Frank & Buzney, 1975; McDowell & Kuhn, 1977; Schichi & Somers, 1978). While it is unlikely that this reaction is linked to excitation, it has been suggested that rhodopsin phosphorylation could be associated with light adaptation (Weller et al., 1975a; Kuhn et al., 1977) or could cause rapid deactivation of cGMP phosphodiesterase activity following illumination in the presence of ATP (Liebman & Pugh, 1980). Preliminary reports pointing to the existence of light-dependent phosphorylation in invertebrate photoreceptors have appeared (Paulsen & Hoppe, 1978; Yoshioka et al., 1981).

† From the Departments of Neurosciences, Biology, and Physics, University of California, San Diego, La Jolla, California 92093. *Received September 6, 1983.* This investigation was supported by a research grant from the National Institutes of Health (EY-02084). C.A.V. was the recipient of a Grass Foundation Fellowship.
‡ Present address: Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90024.

0006-2960/84/0423-2347$01.50/0

Here, we characterize light-dependent phosphorylation of rhodopsin and phospholipids from the squids *Loligo pealei* and *Loligo opalescens*. We provide evidence that metarhodopsin is a light-activated substrate for phosphorylation and that light regulates the incorporation of phosphate into phosphoinositides. A preliminary account of this research has appeared elsewhere (Vandenberg & Montal, 1982).

Experimental Procedures

Materials. [γ-^{32}P]ATP was purchased from Amersham; [γ-^{32}P]GTP was from New England Nuclear; ATP, phosphatidylinositol (PI),[1] phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2) were from Sigma; silica gel 60 thin-layer chromatography sheets and poly(ethylenimine)–cellulose thin-layer chromatography sheets were from EM Reagents; lauryl ester of sucrose L-1690 was from Ryoto Co., Ltd., Tokyo; sodium dodecyl sulfate was from Gallard-Schlesinger; gel electrophoresis reagents were from Bio-Rad; chloroform, methanol, and ammonium hydroxide were from Mallinckrodt.

Photoreceptor Preparation. All procedures except gel electrophoresis and thin-layer chromatography were carried out in the dark or with infrared illumination for viewing (F. J. W. Industries, Mt. Prospect, IL) unless otherwise stated. Squid were obtained from the Marine Biological Laboratories, Woods Hole, MA (*L. pealei*), and the Catalina Marine Science Center, Catalina Island, CA (*L. opalescens*). Live squid were dark adapted for at least 2 h before dissection of the retina. A suspension of photoreceptor outer segments was obtained by rinsing and then gently shaking the dissected retina in a solution containing 50 mM Tris-HCl, 10 mM EGTA, 2 mM dithiothreitol, 0.1 mM ouabain, and 0.1 mM phenylmethanesulfonyl fluoride, pH 8.0, using 0.5 mL for two retinas.

Radiolabeling. Membranes were illuminated by exposure to light from an Ealing 12-V, 100-W tungsten halogen lamp, which was sometimes filtered with a narrow-band interference filter (λ_{max} of 497 nm). After illumination, the lamp was turned off; then with infrared illumination for viewing, the dark and illuminated samples were combined immediately with $MgAc_2$ and [γ-^{32}P]ATP in 16-μL aliquots. The composition of the labeling medium was 50 mM Tris-HCl, 18 mM $MgAc_2$, 10 mM EGTA, 4 mM [γ-^{32}P]ATP (15–30 Ci/mol), 2 mM dithiothreitol, 0.1 mM ouabain, and 0.1 mM phenylmethanesulfonyl fluoride, pH 8.0. Each sample contained retinal membranes with 5–15 μM rhodopsin, unless indicated otherwise. The membrane suspension was incubated at room temperature (18.5 °C) for 30 min except where indicated otherwise. Labeling was terminated by solubilization in sodium dodecyl sulfate (for gel electrophoresis) or by addition of methanol or trichloroacetic acid (for lipid extraction). The concentration of rhodopsin in the samples and the amount of metarhodopsin formed by illumination were determined spectroscopically as described previously (Vandenberg & Montal, 1984b).

Gel Electrophoresis. Labeled samples were denatured by the addition of 2 volumes of 4.5% (w/v) $NaDodSO_4$, 5% (v/v) glycerol, and 17 mM dithiothreitol ($NaDodSO_4$ gel electrophoresis solution). Samples were subjected to electrophoresis on 10% $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970). Gels were run within 2 h of protein solubilization. Samples were not heated prior to electrophoresis. The gels were stained with Coomassie blue (Fairbanks et al., 1971) and then dried on Whatman 3MM filter paper. The dried gels were exposed to Kodak XAR-5 X-ray film with Du Pont Lightning-Plus intensifying screens. The amount of ^{32}P that was incorporated into the gel was quantitated by cutting bands from a parallel gel that had not been dried. Gel slices were dissolved in 0.4 mL of 30% hydrogen peroxide by incubation at 50 °C overnight (Tishler & Epstein, 1968). Samples were counted after addition of scintillation fluid. The extent of rhodopsin phosphorylation is expressed as mol of phosphate/mol of rhodopsin. In this context, "rhodopsin" is used in a general sense to refer to the total amount of rhodopsin present in the sample regardless of its conformational state (i.e., rhodopsin plus metarhodopsin).

Thin-Layer Chromatography of Nucleotides. Thin-layer chromatography of the $NaDodSO_4$-solubilized samples was carried out to ensure that the [γ-^{32}P]ATP was not degraded during the labeling procedure. Samples were applied to poly(ethylenimine)–cellulose sheets, which were then developed in 1.2 M LiCl (Randerath & Randerath, 1964). Chromatography sheets were exposed to Kodak XAR-5 X-ray film for location of radioactive compounds or viewed with short-wave ultraviolet light for location of standards.

Lipid Extraction and Thin-Layer Chromatography. Membranes were labeled and the reaction was stopped either by precipitation with 5% trichloroacetic acid for 10 min at 0 °C or by direct extraction with methanol and chloroform. Trichloroacetic acid was removed after centrifugation (3 min, clinical centrifuge) and the precipitate washed with water (3 min, clinical centrifuge). Omission of the trichloroacetic acid precipitation and wash did not change the results of the lipid extraction. For each 16-μL sample, lipids were extracted by vortexing briefly after the addition of methanol (0.3 mL), then chloroform (0.6 mL), and finally 1 M KCl (50 μL). Brief sonication (10–30 s, Bransonic 220 bath sonicator) was sometimes used to disperse material in the chloroform/methanol/KCl suspension. The suspension was centrifuged (3 min, clinical centrifuge) and the lower phase removed and dried with a gentle stream of nitrogen. The dried lipids were dissolved in chloroform/methanol (2:1) and spotted on silica gel thin-layer chromatography sheets. Chromatographs of samples together with lipid standards were developed in one dimension in chloroform/methanol/15 M ammonium hydroxide/H_2O (90:90:7:22) (Schacht, 1978). Radioactive lipids were located by radioautography on Kodak XAR-5 X-ray film. Nonradioactive lipids were located by exposure to iodine vapor or by spraying the chromatogram with phosphate reagent (Dittmer & Lester, 1964). PI, PA, PIP, and PIP_2 were identified by the mobility of standards. Other phospholipids were identified by their reaction with ninhydrin or Dragendorff reagents (Skipski & Barclay, 1969).

Results

Light-Regulated Phosphorylation of Distinct Membrane Components of Squid Photoreceptors. Light regulated the phosphorylation of several components of squid photoreceptor membranes, as indicated by the appearance of label in distinct bands on $NaDodSO_4$–polyacrylamide gels (Figure 1). In the dark, phosphate was incorporated primarily into a low molecular weight region of the gel, which migrated with tracking dye (pyronin Y), and was not associated with Coomassie blue staining material. This radiolabeled material was shown to be phospholipids (see below). After illumination, the extent of lipid phosphorylation decreased. Illuminated membranes showed phosphorylation of several protein bands. A broad

[1] Abbreviations: CAPS, 3-(cyclohexylamino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; Tris, tris(hydroxymethyl)aminomethane.

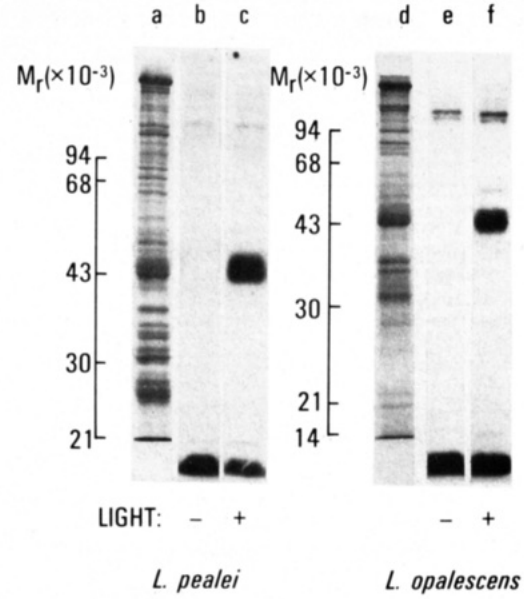

FIGURE 1: $NaDodSO_4$–polyacrylamide gel of phosphorylated squid photoreceptor membranes. Photoreceptor membranes were phosphorylated as described under Experimental Procedures in a solution containing 15 mM $MgAc_2$ and 1 mM [γ-^{32}P]ATP. (a–c) *L. pealei* photoreceptors (10% polyacrylamide gel); (d–f) *L. opalescens* photoreceptors (10.5% polyacrylamide gel); (a and d) Coomassie blue stained gel; (b, c, e, and f) autoradiograph of gel from dark and illuminated samples.

band centered at M_r 46 000, identified as rhodopsin, was labeled prominently. Light-dependent phosphate incorporation was observed also, but was less conspicuous, in two bands of apparent M_r 55 000 and ≤14 000, which were not identified. Light-independent labeling was minimal. All phosphorylated material was membrane bound after centrifugation. Qualitatively, the phosphorylation of *L. pealei* and *L. opalescens* photoreceptors was similar. However, the extent of rhodopsin phosphorylation was greater with the *L. pealei* photoreceptors because optimal conditions were more thoroughly examined.

Several criteria were used to identify the phosphorylated 46 000-dalton protein as rhodopsin. Squid rhodopsin can be made fluorescent by reducing the labile Schiff base that links retinal to opsin with sodium borohydride to form the stable amine-bonded *N*-retinylopsin (Hagins, 1973). After this treatment, a fluorescent band was observed under long-wave ultraviolet light that corresponded in location and extent to the phosphorylated protein located by autoradiography. Rhodopsin purified by DEAE-cellulose chromatography also gave a broad band at this molecular weight. In this case, the protein was determined to be rhodopsin on the basis of its visible absorption spectrum (Vandenberg & Montal, 1984a).

Squid photoreceptors purified by sucrose density centrifugation also exhibited light-activated phosphorylation of the M_r 46 000 protein (not shown). In this preparation, rhodopsin comprised the major protein, and the radiolabel was clearly associated with rhodopsin. Because the background level of dark phosphorylation of other proteins was higher with the sucrose-density purified preparation, an unpurified freshly dissected retinal suspension was used routinely for the phosphorylation experiments.

Nucleotide Specificity of Rhodopsin Phosphorylation. ATP was the phosphate donor for rhodopsin phoshorylation. With the addition of 100 μM ouabain to the photoreceptor suspension, little breakdown of the [γ-^{32}P]ATP was found during the 30-min phosphorylation period. However, if ouabain was omitted, 50% of the [γ-^{32}P]ATP was hydrolyzed within 5 min.

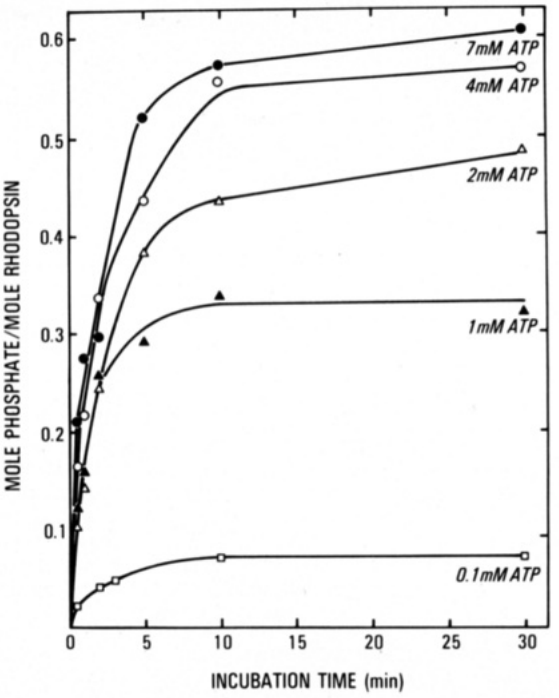

FIGURE 2: Effect of ATP concentration on the rate and final extent of rhodopsin phosphorylation. Membranes (*L. pealei*) were illuminated with white light for 1 min before addition of [γ-^{32}P]ATP and $MgAc_2$ and then were incubated in the dark for various periods of time. (□) 0.1 mM ATP, 14 mM $MgAc_2$; (▲) 1.0 mM ATP, 15 mM $MgAc_2$; (△) 2.0 mM ATP, 16 mM $MgAc_2$; (○) 4.0 mM ATP, 18 mM $MgAc_2$; (●) 7 mM ATP, 21 mM $MgAc_2$. All samples contained, in addition, 50 mM Tris-HCl, 10 mM EGTA, 2 mM dithiothreitol, 0.1 mM ouabain, and 0.1 mM phenylmethanesulfonyl fluoride, pH 8.0. Dark values were 15–20% of the light values shown here.

Transfer of ^{32}P from ATP to GTP was not detected. Comparison of [γ-^{32}P]ATP and [γ-^{32}P]GTP showed that ATP was the preferred substrate for rhodopsin phosphorylation. With 1 mM [γ-^{32}P]GTP, phosphorylation of rhodopsin was not observed in the dark or light. By a decrease in the concentration of GTP to 1 μM, phosphorylation of rhodopsin could be demonstrated due to the 1000-fold increase in specific activity of the label. However, at this concentration of GTP, the rapid light-activated hydrolysis of GTP consumed the [γ-^{32}P]GTP before it could be used in phosphorylation of the illuminated samples [see Vandenberg & Montal (1984b)], and less radiolabel was observed on illuminated membranes than on dark samples.

Kinetics of Rhodopsin Phosphorylation. With [γ-^{32}P]ATP as a substrate, the half-time of rhodopsin labeling was between 1 and 5 min. Figure 2 shows the rate of rhodopsin phosphorylation over an ATP concentration range of 0.1–7.0 mM. The half-time of labeling was 1–2 min throughout the concentration range tested. ATP concentrations of ≥4 mM were required to achieve high final extents of phosphate incorporation. With 0.1 mM ATP, a final rhodopsin phosphorylation of only 0.07 phosphate/rhodopsin was observed, even though this sample contained an 8-fold molar excess of ATP over rhodopsin. In contrast, with 4 mM ATP, 0.56 phosphate/rhodopsin was incorporated. Low phosphorylation extents also were reported for the phosphorylation of bovine rhodopsin at low ATP concentrations [e.g., Wilden & Kuhn (1982) and Schichi & Somers (1978)]. A decline in rhodopsin phosphorylation that would reflect dephosphorylation was not observed during the 30-min labeling period.

An Eadie–Hofstee plot of the initial rate of phosphorylation at various substrate concentrations revealed a K_m for ATP of approximately 1 mM. For most experiments, 4 mM ATP and

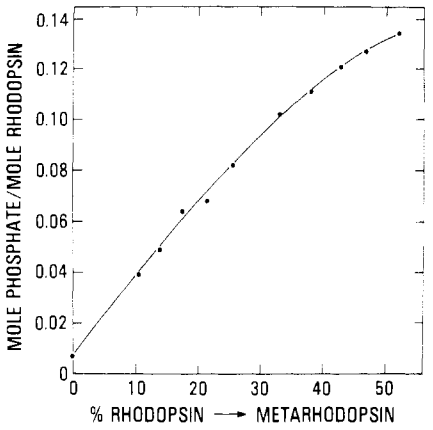

FIGURE 3: Effect of metarhodopsin formation on phosphorylation. A suspension of *L. opalescens* photoreceptors was illuminated with light filtered through a narrow-band interference filter (λ_{max} of 497 nm). At intervals from 0.5 to 15 min, samples were removed and added to 15 mM $MgAc_2$ and 1 mM ATP. The highest extent of phosphorylation in this experiment, 0.135 phosphate/total rhodopsin, is less than usually observed because 1 mM [γ-^{32}P]ATP was used rather than 4 mM [γ-^{32}P]ATP as in other experiments.

a 30-min incubation time were used to obtain maximal phosphorylation.

Light Dependence of Rhodopsin Phosphorylation. The effect of light on rhodopsin phosphorylation indicates that phosphorylation is a dark reaction following illumination. After membranes were exposed to continuous moderate-intensity light, metarhodopsin was produced until a photostationary equilibrium of rhodopsin and metarhodopsin was established with prolonged illumination containing approximately 50% each rhodopsin and metarhodopsin. Subsequent addition of [γ-^{32}P]ATP caused phosphorylation of the visual pigment to the extent of 0.4–0.6 phosphate/total rhodopsin. The number of moles of phosphate per mole of metarhodopsin, obtained by quantitation of the amount of metarhodopsin formed, was 0.9–1.8. Twelve-fold more phosphate was incorporated into illuminated than into dark samples. The effect of varying the amount of metarhodopsin indicates that metarhodopsin (not rhodopsin) was the substrate for the kinase. The amount of metarhodopsin formed by illumination was controlled by varying the duration of illumination with a low-intensity light prior to incubation with [γ-^{32}P]ATP. Phosphate incorporation into the protein was approximately linear with the formation of metarhodopsin (Figure 3). If rhodopsin kinase were activated by light and caused unilluminated rhodopsin to become phosphorylated, then phosphorylation would be expected to saturate when only a fraction of rhodopsin had absorbed light. Alternatively, if rhodopsin formed by photoregeneration from metarhodopsin could be phosphorylated in addition to metarhodopsin, then prolonged illumination after formation of a 50:50 mixture of rhodopsin and metarhodopsin would show an increased level of phosphorylation. Neither of these alternatives was observed, although very high light intensities that promote rapid recycling of rhodopsin were not tested. This indicates that metarhodopsin is a light-activated substrate for the kinase. The slight saturation of rhodopsin phosphorylation with high concentrations of metarhodopsin may have arisen from a slow decline of phosphorylation capacity caused by the delay between illumination and addition of [γ-^{32}P]ATP. When a dark interval was imposed between illumination and ATP addition, the extent of rhodopsin phosphorylation was reduced about 60%, with a half-time of 20 min.

Effects of pH, Buffer Composition, and Divalent and Monovalent Ions on Rhodopsin Phosphorylation. Inorganic

Table I: Effect of Ions on Rhodopsin Phosphorylation[a]

	mol of phosphate/mol of rhodopsin			
	dark	light	light − dark	% max
30 mM Tris-HCl, 6 mM EGTA	0.018	0.108	0.090	20
+ 18 mM $CaCl_2$	0.019	0.022	0.001	0
+ 18 mM $MgAc_2$	0.033	0.449	0.452	100
+ 18 mM $MgAc_2$, 100 mM NaCl	0.047	0.440	0.393	87
+ 18 mM $MgAc_2$, 400 mM NaCl	0.033	0.237	0.201	45
+ 18 mM $MgAc_2$, 100 mM KCl	0.025	0.421	0.399	88

[a] Photoreceptor membranes (*L. pealei*) were incubated with [γ-^{32}P]ATP in the presence of the ions indicated after being kept dark or illuminated for 2 min through a narrow-band interference filter with a λ_{max} of 497 nm. In addition to the added ions, all samples contained 30 mM Tris-HCl, 6 mM EGTA, 4 mM [γ-^{32}P]ATP, 1.2 mM dithiothreitol, 60 μM ouabain, and 60 μM phenylmethanesulfonyl fluoride, pH 8.0.

Table II: Calcium Inhibition of Rhodopsin Phosphorylation[a]

	phosphate/rhodopsin (% max)	
ions added	dark	illuminated
6 mM $MgAc_2$	23	100
5 mM $MgAc_2$, 0.1 mM $CaCl_2$	14	58
5 mM $MgAc_2$, 1 mM $CaCl_2$	17	42
5 mM $MgAc_2$, 5 mM $CaCl_2$	14	34
5 mM $CaCl_2$	14	14

[a] A photoreceptor suspension (*L. pealei*) in 50 mM Tris-HCl, 2 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, and 0.1 mM ouabain, pH 8.0, was centrifuged (35000*g*, 20 min, 4 °C) to remove endogenous divalent ions and was resuspended in the same buffer. A portion was illuminated with white light for 5 min, then $MgAc_2$, $CaCl_2$, or EGTA was added to aliquots of the dark or illuminated membrane suspension, followed by 4 mM [γ-^{32}P]ATP. After 30 min, the reaction was stopped by the addition of $NaDodSO_4$. Phosphorylation of rhodopsin was quantitated by scintillation counting of the rhodopsin band of the polyacrylamide gel.

ions and buffers affect the extent of rhodopsin phosphorylation. Membranes buffered with 100 mM Tris-HCl showed more than twice as much rhodopsin phosphorylation as those buffered with either 100 mM sodium phosphate or 100 mM CAPS/NaOH. The optimum pH for rhodopsin phosphorylation was 8.5.

Magnesium was needed for membrane phosphorylation (Table I). In the absence of magnesium, when divalent ions were chelated with EGTA, little phosphorylation was observed (Tables I and II). Calcium did not substitute for magnesium in promoting phosphorylation. On the contrary, the addition of calcium in the presence of magnesium inhibited rhodopsin phosphorylation (Table II). This effect of calcium cannot be attributed to activation of calcium-sensitive proteases. Calcium-induced degradation of rhodopsin was not observed with *L. pealei* (the species of squid used for this experiment) although many species of squid including *L. opalescens* (Vandenberg & Montal, 1984a), *Todarodes*, and *Watasenia* (Nashima et al., 1979) do show calcium-promoted proteolysis. In the presence of calcium, no change was found in the Coomassie blue stained polyacrylamide gel band pattern or staining intensity, nor was there an increase in low molecular weight phosphorylated bands that might be produced if rhodopsin were proteolyzed.

The addition of NaCl or KCl to the phosphorylation medium also reduced the extent of rhodopsin phosphorylation in the presence of magnesium (Table I). High ionic strength may promote resealing of the photoreceptor fragments, interfere

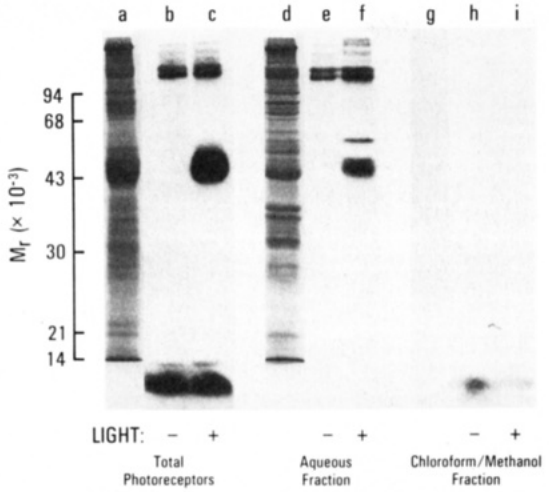

FIGURE 4: $NaDodSO_4$–polyacrylamide gel of phosphorylated chloroform/methanol-extracted photoreceptors. Labeled membranes (*L. opalescens*) were extracted with chloroform/methanol as described under Experimental Procedures. The chloroform/methanol extract was dried under nitrogen and then gently rinsed with H_2O to 0 °C to remove KCl before solubilization in $NaDodSO_4$ gel electrophoresis solution. The chloroform/methanol-insoluble material was centrifuged and the pellet solubilized in $NaDodSO_4$ gel electrophoresis solution. (a–c) Unextracted membranes; (d–f) chloroform/methanol-insoluble material; (g–i) chloroform/methanol extract.

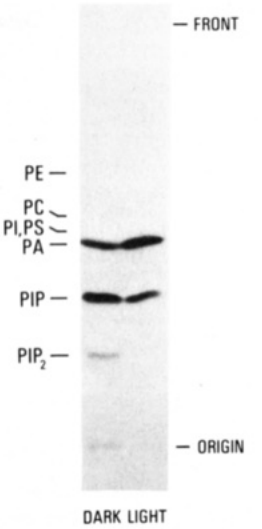

FIGURE 5: Silica gel thin-layer chromatograph of phosphorylated lipids. Squid photoreceptor membranes (*L. opalescens*) were labeled in the dark after being kept in the dark or illuminated for 2 min through a narrow-band interference filter with a λ_{max} of 497 nm. Material extracted with chloroform/methanol was chromatographed as described under Experimental Procedures. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

with metarhodopsin–kinase interactions, or promote activation of a phosphatase. In addition, it is possible that rhodopsin kinase was solubilized under these conditions. A reduction in kinase activity due to the high salt concentration might not affect phosphorylation in vivo because the kinase concentration would be about 300 times greater than under the assay conditions.

Lipid Phosphorylation. The low molecular weight phosphorylated material that runs fastest on $NaDodSO_4$–polyacrylamide gels is lipid. Extraction of labeled membranes with chloroform/methanol removed this material from the raiolabeled protein fraction (Figure 4). Thin-layer chromatography of the chloroform/methanol extract on silica gel resolved several lipids that were identifed as PA, PIP, and PIP_2 (Figure 5). Following illumination, phosphate incorporation into PA was increased, and phosphate incorporation of PIP and PIP_2 was decreased with respect to dark samples.

Phosphorylation of the total lipid fraction (determined from polyacrylamide gels) decreased to a variable extent following illumination. With *L. opalescens* photoreceptor preparations, little difference in the incorporation of phosphate into the total lipid fraction was found between dark and light samples, although the label on individual lipid classes was influenced by light. The decrease in PIP and PIP_2 labeling was equivalent to the increase in PA phosphate incorporation. *L. pealei* photoreceptors more consistently demonstrated a light-induced decrease in total lipid phosphorylation, but the distribution of label among lipid classes was not determined. Of 15 experiments with *L. pealei* with different membrane preparations, 12 showed a light-induced decrease in total lipid phosphorylation, with their illuminated samples averaging 60 ± 15% (SD) of the label found in dark samples. Lipid phosphorylation in the dark was similar in amount to the maximum light-induced rhodopsin phosphorylation. The light effect on lipid labeling was rapid, and could be detected readily within 3 min after the addition of [γ-^{32}P]ATP (the earliest time examined).

The effect of light on lipid phosphorylation also involves a dark reaction following illumination. The decrease in lipid labeling is more sensitive to illumination than rhodopsin phosphorylation: the light-induced decrease in lipid labeling was two-thirds complete when only ~2% of the rhodopsin had been photoconverted to metarhodopsin.

The effect of ions on total lipid phosphorylation was similar to their effects on rhodopsin phosphorylation (see Table I). Magnesium was required for lipid labeling. Calcium did not substitute for magnesium and was slightly inhibitory in the presence of magnesium. NaCl and KCl also decreased lipid phosphorylation both in the light and in the dark. In the presence of these ions, the light-induced change in lipid labeling still was observed.

Discussion

The results presented here show that squid rhodopsin is phosphorylated following illumination. The simplest interpretation of the linear dependence of rhodopsin phosphorylation on metarhodopsin formation is that metarhodopsin is the light-activated substrate for the kinase. This interpretation is consistent with the observations of Paulsen & Hoppe (1978) with octopus photoreceptors that phosphorylation was induced by light that converted rhodopsin to metarhodopsin but that light that was only absorbed by metarhodopsin did not initiate phosphorylation.

The average phosphorylation extent of 0.9–1.8 phosphates/metarhodopsin establishes that a fraction of the metarhodopsin was phosphorylated in at least two sites. If the phosphate is not equally distributed, then some metarhodopsin might contain a considerably higher complement of phosphate. Although Paulsen & Hoppe (1978) also reported phosphorylation extents of 0.3–2.0 phosphates/rhodopsin, their results are not directly comparable because they did not measure rhodopsin phosphorylation but rather considered phosphorylation of the entire photoreceptor.

Phosphorylation of squid rhodopsin is remarkably similar to phosphorylation of vertebrate rhodopsin. Until recently, the highest average phosphorylation extents reported were 4 phosphates/rhodopsin in frog rod outer segments (Miller et al., 1977) and 2.4 phosphates/rhodopsin in bovine rod outer

segment (Kuhn & McDowell, 1977). Using very bright continuous illumination during labeling and freshly prepared [γ-^{32}P]ATP, Wilden & Kuhn (1982) recently showed average phosphorylation extents of 7 phosphates/rhodopsin with both frog and bovine photoreceptors. It will be of interest to test if high phosphorylation extents are also produced in squid under the conditions reported for the vertebrates.

The function of light-induced phosphorylation of squid rhodopsin has not been determined. Because it appears to be a relatively slow process, it is probably not involved in excitation. Rhodopsin phosphorylation is not required, for example, for activation of the GTP binding protein because it can be activated by light when ATP is not present (Vandenberg & Montal, 1984b). Perhaps phosphorylation provides a way for the cell to distinguish between newly formed metarhodopsin that initiates visual excitation and metarhodopsin that had been formed previously. This is a particular problem for invertebrates that normally have a large proportion of metarhodopsin in their photoreceptors and rely on photoregeneration rather than chemical regeneration of rhodopsin. The role of metarhodopsin phosphorylation might be to inactivate the metarhodopsin shortly after its formation so that its physiological effect is only transient, as is the electrical response of the plasma membrane.

The labeling of lipids with phosphate in squid reveals striking parallels with lipid phosphorylation in other systems [for reviews, see Michell (1975, 1979, 1982) and Putney (1981)]. For the visual system, this represents a new light-regulated event, since light-dependent lipid phosphorylation has not been reported in vertebrate photoreceptors. The effect of light on the rise in PA labeling and decrease in PIP and PIP_2 labeling is consistent with the activation of just one enzyme, a phospholipase C, as proposed by Michell (1982) to explain transmitter-stimulated phosphatidylinositol metabolism. The requirement for only low amounts of illumination to achieve a decrease in total lipid phosphorylation suggests that illuminated rhodopsin efficiently activates the phosphatidylinositol phospholipase C.

It is interesting to note that Anderson et al. (1978) did not detect phosphatidylinositol when they analyzed the phospholipid content of *Loligo plei* and *L. pealei* photoreceptors, perhaps the result of light-induced degradation of phosphoinositides. Yoshioka et al. (1981) also reported phosphorylation of lipids in squid (*Watasenia scintillans*) photoreceptors, although using their frozen and thawed preparations they only detected labeling of PIP, with minimal labeling of PA and no labeling of PIP_2. Differences in the treatment of the membrane preparation and labeling conditions may account for our ability to measure phosphate incorporation into PA and PIP_2.

PI metabolism often has been associated with calcium fluxes across membranes (Michell, 1982). With the squid photoreceptor membrane suspension, calcium concentrations would not be expected to change in regions accessible to [γ-^{32}P]ATP. However, in intact invertebrate photoreceptors, light induces an influx of calcium, which may contribute to light adaptation and to the shape of the light-induced membrane current with continuous illumination (Brown, 1977). It is conceivable that light-stimulated PI turnover is associated with these phenomena. The clear effect of light on lipid phosphorylation indicates that PI turnover is an inherent property of the visual process.

Registry No. ATP, 56-65-5; Tris, 77-86-1; Mg, 7439-95-4; Ca, 7440-70-2; Na, 7440-23-5; K, 7440-09-7; rhodopsin kinase, 54004-64-7; phospholipase C, 9001-86-9.

References

Anderson, R. E., Benolken, R. M., Kelleher, P. A., Maude, M. B., & Weigand, R. D. (1978) *Biochim. Biophys. Acta 316*, 316–326.
Bownds, D., Dawes, J., Miller, J., & Stahlman, M. (1972) *Nature (London), New Biol. 237*, 125–127.
Brown, J. E. (1977) *Biophys. Struct. Mech. 3*, 141–143.
Cohen, P. (1982) *Nature (London) 296*, 613–620.
Dittmer, J. C., & Lester, R. L. (1964) *J. Lipid Res. 5*, 126–127.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.
Frank, R. N., & Buzney, S. M. (1975) *Biochemistry 14*, 5110–5117.
Frank, R. N., Cavannagh, H. D., & Kenyon, K. R. (1973) *J. Biol. Chem. 248*, 596–609.
Glass, D. B., & Krebs, E. G. (1980) *Annu. Rev. Pharmacol. Toxicol. 20*, 363–388.
Greengard, P. (1981) *Harvey Lect. 75*, 277–331.
Hagins, F. M. (1973) *J. Biol. Chem. 248*, 3298–3304.
Kuhn, H., & Dreyer, W. J. (1972) *FEBS Lett. 20*, 1–6.
Kuhn, H., & McDowell, J. H. (1977) *Biophys. Struct. Mech. 3*, 199–203.
Kuhn, H., Cook, J. H., & Dreyer, W. J. (1973) *Biochemistry 12*, 7495–7502.
Kuhn, H., McDowell, J. H., Leser, K. H., & Bader S. (1977) *Biophys. Struct. Mech. 3*, 175–180.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Liebman, P. A., & Pugh, E. N., Jr. (1980) *Nature (London) 287*, 734–736.
McDowell, J. H., & Kuhn, H. (1977) *Biochemistry 16*, 4054–4060.
Michell, R. H. (1975) *Biochim. Biophys. Acta 415*, 81–147.
Michell, R. H. (1979) *Trends Biochem. Sci. (Pers. Ed.) 4*, 128–131.
Michell, R. H. (1982) *Nature (London) 296*, 492–493.
Miller, J. A., Paulsen, R., & Bownds, M. D. (1977) *Biochemistry 16*, 2633–2639.
Nashima, K., Mitsudo, M., & Kito, Y. (1979) *Biochim. Biophys. Acta 579*, 155–168.
Nestler, E. J., & Greengard, P. (1983) *Nature (London) 305*, 583–588.
Paulsen, R., & Hoppe, I. (1978) *FEBS Lett. 96*, 55–58.
Putney, J. W. (1981) *Life Sci. 29*, 1183–1194.
Randerath, K., & Randerath, E. (1964) *J. Chromatogr. 16*, 111–125.
Schacht, J. (1978) *J. Lipid Res. 19*, 1063–1067.
Schichi, H., & Somers, R. L. (1978) *J. Biol. Chem. 19*, 7040–7046.
Skipski, V. P., & Barclay, M. (1969) *Methods Enzymol. 14*, 530–598.
Tishler, P., & Epstein, C. J. (1968) *Anal. Biochem. 22*, 89–98.
Vandenberg, C. A., & Montal, M. (1982) *Biophys. J. 37*, 195a.
Vandenberg, C. A., & Montal, M. (1984a) *Biochim. Biophys. Acta* (in press).
Vandenberg, C. A., & Montal, M. (1984b) *Biochemistry* (preceding paper in this issue).
Weller, M., Goridis, C., Virmaux, N., & Mandel, P. (1975a) *Exp. Eye Res. 21*, 405–408.
Weller, M., Virmaux, N., & Mandel, P. (1975b) *Proc. Natl. Acad. Sci. U.S.A. 72*, 381–385.
Wilden, U., & Kuhn, H. (1982) *Biochemistry 21*, 3014–3022.
Yoshioka, T., Inoue, H., Inomata, K., Hayashi, F., Takagi, M., & Tanekaka, T. (1981) *Proc. Jpn Acad., Ser. B 57*, 309–313.