Light-Regulated Biochemical Events in Invertebrate Photoreceptors. 1. Light-Activated Guanosinetriphosphatase, Guanine Nucleotide Binding, and Cholera Toxin Catalyzed Labeling of Squid Photoreceptor Membranes†

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ABSTRACT: The occurrence of a guanine nucleotide binding protein activated by squid rhodopsin was established by examination of GTPase activity, guanine nucleotide binding, and cholera toxin catalyzed labeling of squid photoreceptor membranes. Purified squid (*Loligo opalescens*) photoreceptors exhibited GTPase activity that increased 3–4-fold by illumination. Half-maximal GTPase activity was observed when 2% of the rhodopsin was photoconverted to metarhodopsin. The K_m of the light-regulated activity was 1 μM GTP. Binding of the hydrolysis-resistant GTP analogue guanosine 5′-(β,γ-imidotriphosphate) [Gpp(NH)p] was enhanced >10 times by illumination. A protein, M_r 44 000, was identified as a component of the light-activated guanine nucleotide binding protein/GTPase through its specific labeling with [^{32}P]NAD catalyzed by cholera toxin: light increased the extent of ^{32}P incorporation 7-fold. The addition of ATP to the membrane suspension enhanced labeling, while guanine nucleotides inhibited labeling with the relative potency GTPγS $\gg$ GDP > GTP > Gpp(NH)p. The 44 000-dalton protein was membrane bound irrespective of variations in ionic strength and divalent ion concentration over a wide range. These results suggest that a G protein, which incorporates both GTP binding and hydrolysis functions, is intimately involved in the visual process of invertebrate photoreceptors.

The mechanism of visual transduction in invertebrate photoreceptors has remained elusive. The transduction process appears to be mediated through intracellular messengers, which transmit and amplify light absorption by rhodopsin into an increase in cell membrane permeability (Fuortes & Yeandle, 1964; Cone, 1973; Yeandle & Spiegler, 1973; Wu & Pak, 1975). Physiological studies established that invertebrate photoreceptors respond differently than vertebrate photoreceptors to the absorption of light: virtually all invertebrate photoreceptors depolarize due to a light-induced increase in permeability of the plasma membrane to sodium, while vertebrate rods and cones hyperpolarize as the result of a decreased sodium permeability [for a recent review, see Fain & Lisman (1981)]. The visual pigments, e.g., rhodopsin, also differ significantly in their optical spectroscopic characteristics, in the stability of photochemical intermediates, and in their molecular weight. Even morphologically the cells appear to be unrelated: the membrane of the outer segment of most invertebrate photoreceptors is arranged as numerous tightly packed microvillar projections, while vertebrate photoreceptors are modified cilia whose membrane forms disklike invaginations or pinched-off disks [for reviews, see Yamada (1982) and Eakin (1982)].

In spite of detailed electrophysiological characterization of the photoresponses, primarily in arthropods, biochemical studies of invertebrate photoreceptors are virtually nonexistent. Cephalopod retinas provide a unique opportunity to initiate studies of the biochemistry of invertebrate vision due to their relative abundance and the large size and lack of a chitinous exoskeleton of the eyes. Here and in the following paper (Vanderberg & Montal, 1984) we describe the occurrence of light-activated enzymes in squid photoreceptors and demonstrate that, unexpectedly, some of the light-activated biochemical events in invertebrate photoreceptors are similar to those operative in vertebrate photoreceptors.

Vertebrate photoreceptors possess a light-regulated protein, referred to as G protein or transducin, which exhibits GTPase activity and guanine nucleotide binding in response to absorption of light by the visual pigment rhodopsin (Wheeler et al., 1977; Godchaux & Zimmerman, 1979; Fung & Stryer, 1980; Fung et al., 1981). Through this protein, rhodopsin confers light activation to a cGMP photphodiesterase (Wheeler & Bitensky, 1977; Shinozawa et al., 1980). This enzyme cascade has been suggested as the first step in visual transduction for vertebrate photoreceptors.

A light-sensitive G protein also might be involved in invertebrate vision. This is consistent with the findings in the horseshoe crab *Limulus* that guanine nucleotide analogues, fluoride, and vanadate induce discrete waves in the dark resembling light-induced quantum bumps (Fein & Corson, 1979, 1981; Bolsover & Brown, 1980). Preliminary reports on octopus photoreceptors pointed to the existence of light-activated GTPase activity (Calhoon et al., 1980) and the ability of octopus photoreceptor membranes to confer light sensitivity to phosphodiesterase isolated from bovine photoreceptors (Ebrey et al., 1980).

Here we report a detailed characterization of light-activated hydrolysis of GTP, binding of guanine nucleotides, and labeling of a 44 000-dalton protein catalyzed by cholera toxin in the squid photoreceptor membranes. Preliminary accounts of this research have appeared elsewhere (Vandenberg & Montal, 1982, 1983).

Experimental Procedures

Materials. [^{3}H]Guanosine 5′-(β,γ-imidotriphosphate) [Gpp(NH)p][1] and [^{3}H]cAMP were obtained from New England Nuclear; [α-^{32}P]NAD was from New England Nu-

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); GTPγS, guanosine 5′-(γ-thiotriphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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clear or ICN; ATP, GTP, and Gpp(NH)p were from P-L Biochemicals; cholera enterotoxin was from Schwarz/Mann or Sigma; cAMP, creatine phosphokinase, creatine phosphate, NAD, GDP, ADP-ribose, bovine spleen NADase were from Sigma; guanosine 5′-(γ-thiotriphosphate) (GTPγS) was from Boehringer Mannheim; sodium dodecyl sulfate ($NaDodSO_4$) was from Gallard-Schlesinger; lauryl ester of sucrose L-1690 was from Ryoto Co., Ltd., Tokyo, Japan; 1-amino-2-napthol-4-sulfonic acid was from Eastman Chemicals; gel electrophoresis reagents were from Bio-Rad; poly(ethylenimine)–cellulose thin-layer chromatography sheets were from EM Reagents; bovine retinas were from Hormel.

Purification of Squid Photoreceptor Membranes. Squid (*Loligo opalescens*, Sea Life Supply, Monterey, CA) were dark adapted for 2 h, and then the eyecups were dissected under dim red light (Kodak Wratten 2) and stored at −80 °C. All subsequent procedures were carried out under infrared light with an infrared image converter for viewing (F. J. W. Industries, Mt. Prospect, IL.). Solutions were bubbled with argon prior to use. Fifty to seventy eyecups were thawed and gently shaken to dislodge the photoreceptors into 90 mL of 45% (w/v) sucrose, containing 300 mM KCl, 100 mM NaCl, 10 mM Tris-HCl, 5 mM $MgAc_2$, 1 mM EDTA, and 1 mM dithiothreitol, pH 8.0 (membrane buffer). The suspension was layered on a sucrose step gradient as follows: 15 mL of photoreceptor suspension, 13 mL of 40% (w/v) sucrose in membrane buffer, and 11 mL of membrane buffer. After centrifugation for 75 min at 22 000 rpm in a Beckman SW27 rotor, the band of photoreceptors at the 40% sucrose/buffer interface was collected, and aliquots were frozen in liquid nitrogen for storage at −80 °C.

For the assays of GTPase activity from freshly dissected retinas, suspensions of fresh squid (*Loligo pealei*) retinas were prepared as described below for the cholera toxin catalyzed labeling experiments in a solution containing 400 mM KCl, 50 mM Tris-HCl, 10 mM EGTA, 15 mM $MgSO_4$, 2 mM dithiothreitol, and 100 μM phenylmethanesulfonyl fluoride, pH 7.5 (0.5 mL/two retinas). Membranes were washed once (17 000*g*, 3 min) and resuspended in an equal volume of buffer prior to use.

Preparation of Bovine Phosphodiesterase and G Protein. Purified bovine photoreceptors were prepared under dim red illumination as described above for squid photoreceptors, except that the bovine membrane buffer consisted of 100 mM Tris-HCl, 2 mM $MgSO_4$, and 0.1 mM EGTA, pH 8.0. The membranes from the sucrose density gradient were washed twice with 30 mL of bovine membrane buffer (35000*g*, 20 min). The phosphodiesterase and G protein were obtained after freezing (liquid N_2) and thawing the purified photoreceptor membranes. The soluble enzymes were separated from the photoreceptor membranes by washing twice with 5 mM Tris-HCl–2 mM EDTA, pH 8.0 (10 mL), followed by centrifugation (40 000 rpm, 2 h) in a Beckman Ti65 rotor. The supernatant was removed carefully, and aliquots of it were stored at −80 °C. The second low ionic strength extract was used as the source of phosphodiesterase and G protein (Kuhn, 1980).

GTPase Assay. GTPase activity was measured by the method of Neufeld & Levy (1969), with [γ-^{32}P]GTP and by extracting hydrolyzed [^{32}P]P_i with molybdate and isobutyl alcohol. Suspensions of squid photoreceptors (10–15 μM rhodopsin) were incubated with various concentrations of [γ-^{32}P]GTP (0.1–0.5 μCi/sample) in 100 μL of a solution containing 5 mM creatine phosphate and 30 units/mL creatine phosphokinase. The addition of creatine phosphokinase and creatine phosphate preserved the [γ-^{32}P]GTP from nonspecific degradation and prevented transfer of ^{32}P to ADP to form [γ-^{32}P]ATP; in their absence [γ-^{32}P]GTP was degraded rapidly in the dark, as determined by thin-layer chromatography on poly(ethylenimine)–cellulose in 3.8 M sodium formate, pH 3.6 (Randerath & Randerath, 1964). After incubation of the membranes in the dark or following illumination, the hydrolysis of GTP was terminated by mixing it with 20 μL of 1 g/mL trichloroacetic acid–0.25 mM Na_2HPO_4. The samples were cooled on ice and then were centrifuged to remove precipitated protein (17000*g*, 2 min). An 80-μL aliquot of the supernatant was mixed rapidly with 10 μL of reducing solution (5.7 g of $Na_2S_2O_5$, 0.2 g of Na_2SO_3, 0.1 g of aminonaptholsulfonic acid in 100 mL) and then with 20 μL of acid ammonium molybdate (1.25 g of ammonium molybdate, 7 mL of concentrated H_2SO_4 in 100 mL). Inorganic phosphate was extracted into isobutyl alcohol by immediately vortexing for 15 s with 0.5 mL of isobutyl alcohol, followed by centrifuging briefly to separate the isobutyl alcohol and aqueous phases (17000*g*, 5 s). A 0.4-mL aliquot of the isobutyl alcohol extract was mixed with 10 mL of scintillation fluid for determination of radioactivity. Values from blank samples, in which trichloroacetic acid was added before [γ-^{32}P]GTP, were subtracted from all measurements.

Gpp(NH)p Binding Assay. Binding of the hydrolysis-resistant GTP analogue Gpp(NH)p to purified squid photoreceptor membranes was measured by incubating dark or illuminated membranes with 3 μM [^{3}H]Gpp(NH)p (0.1 μCi/sample), followed by filtration in the dark through Millipore HAWP filter disks (pore diameter 0.45 μm) to separate bound from free label. The filtered membranes were washed with 3 times 3 mL of membrane buffer. Filters were dissolved in scintillation fluid and counted.

Phosphodiesterase Assay. Phosphodiesterase activity was measured by the method of Thompson & Appleman (1971) as modified by Thompson et al. (1979). The phosphodiesterase assay was initiated by addition of $MgSO_4$ (7 mM), [^{3}H]cAMP (10 μM to 1 mM), and Gpp(NH)p (20 μM) to photoreceptor membranes in a volume of 80 μL. After incubation at room temperature in the dark or with illumination, the samples were boiled for 45 s to stop the reaction and then cooled on ice. Phosphodiesterase activity was quantitated following incubation with nucleosidase and chromatography on anion-exchange resin (Thompson et al., 1979).

Cholera Toxin Catalyzed Labeling. (*A*) *Photoreceptor Preparation.* All procedures except gel electrophoresis were carried out in the dark or with infrared illumination for viewing unless otherwise stated. Live squid (*L. opalescens*, Catalina Marine Science Center, Santa Catalina Island, CA) were dark adapted for at least 4 h before dissection of the retina. Retinas were rinsed in and then gently shaken into 50 mM Tris-HCl, 5 mM EGTA, 2 mM dithiothreitol, 100 μM ouabain, and 100 μM phenylmethanesulfonyl fluoride, pH 8.0 (referred to as membrane-labeling buffer), using 0.5 mL/two retinas. A suspension of photoreceptor outer segments was obtained by this procedure.

(*B*) *Radiolabeling.* Just before addition to the membranes, cholera toxin was activated by incubation with 20 mM dithiothreitol in 50 mM Tris-HCl buffer, pH 8, at 30 °C for 30 min. Photoreceptor membranes (5–10 μM rhodopsin) were incubated with activated cholera toxin (100 μg/mL) and [^{32}P]NAD (20 μM, 3 Ci/mmol) in 16 μL of membrane buffer, sometimes containing (as indicated in figure legends) 7.5 mM $MgAc_2$ and 1 mM ATP. After 30 min at 30 °C, the reaction was terminated by the addition of 2 volumes of 4.5% (w/v)

$NaDodSO_4$, 5% (v/v) glycerol, and 17 mM dithiothreitol (sample electrophoresis buffer). During the labeling period, samples either were kept in the dark or for most experiments were illuminated with a 100-W incandescent bulb positioned 0.5 m from the samples. In these cases, illumination was maintained during the labeling period. For experiments in which light intensity was varied (Figure 4), aliquots of membranes were illuminated prior to addition of [^{32}P]NAD and toxin. The samples at 0 °C were exposed to white light from an Ealing 12-V, 100-W tungsten halogen lamp at various intensities for 1 min with continuous stirring. An aliquot was immediately added to toxin and [^{32}P]NAD, and the membranes were incubated in the dark as described above. A membrane aliquot also was used for spectral determination of the extent of metarhodopsin formation.

(*C*) *Electrophoresis.* Samples were subjected to electrophoresis on 11.5% $NaDodSO_4$ polyacrylamide gels (Laemmli, 1970). Gels were run within 2 h of protein solubilization. Solubilized samples were not heated prior to electrophoresis. The gels were stained with Coomassie blue (Fairbanks et al., 1971), destained, and then dried on Whatman 3MM filter paper. The dried gels were exposed to Kodak XAR-5 X-ray film, sometimes with enhancement from Du Pont Lightning-Plus intensifying screens. For quantitation of the amount of ^{32}P incorporated into the protein, protein bands were cut from a parallel set of gels that had not been dried. Gel slices were dissolved in 0.4 mL of 30% hydrogen peroxide and incubated at 50 °C overnight (Tishler & Epstein, 1968). Samples were counted after addition of scintillation fluid.

(*D*) *Thin-Layer Chromatography.* Thin-layer chromatography for the identification of [^{32}P]NAD and its products was carried out on poly(ethylenimine)–cellulose sheets that were developed in 0.1 M LiCl–0.5 M formic acid (Cassel & Pfueffer, 1978). Sheets were exposed to Kodak X-AR5 film for determination of radioactive compounds or viewed with short-wave ultraviolet light for location of standards.

Quantitation of Rhodopsin and Protein. Rhodopsin concentration was quantitated spectroscopically on a Perkin-Elmer 550 UV–vis scanning spectrophotometer. Membrane aliquots were dissolved in 1.5% lauryl ester of sucrose L-1690 at 0–4 °C for 1–3 h (Nashima et al., 1978). To eliminate the absorbance due to retinochrome in samples containing unpurified retinal suspensions, 10 mM NH_2OH was added to the samples 1 h before recording the spectra (Hara & Hara, 1967). Insoluble material was removed by centrifugation (35000*g*, 30 min) and the pH of the solution adjusted to 10.5 by the addition of 0.1 volume of 0.5 M Na_2CO_3. Spectra were recorded at 8 °C. The concentration of rhodopsin was calculated from the difference spectrum between the sample in the dark and after illumination with a sharp cut-off yellow filter (Corning CS 3-69) that passes light ≥ 520 nm. At pH 10.5, the contribution of metarhodopsin to the absorbance at $\geq\lambda_{450nm}$ is negligible. A reliable measure of the rhodopsin concentration can be obtained at its λ_{max} of 490 nm with an extinction coefficient of 41 700 M^{-1} cm^{-1} (Hagins, 1973).

Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. All assays were performed at 19 ± 1 °C unless specified otherwise.

Results

Light-Activated GTPase. Squid photoreceptors have GTPase activity that is stimulated by light. The K_m of the light-dependent activity is about 1 μM, and the V_{max} is 480 pmol (mg of protein)$^{-1}$ min^{-1} (Figure 1). At higher substrate concentrations, low-affinity GTPase activity is apparent that has a K_m of about 50 μM and a V_{max} of 6500 pmol (mg of protein^{-1} min^{-1}. Because the V_{max} for the low-affinity GTPase activity is more than 10 times greater than the high-affinity light-sensitive GTPase, optimal measurement of light-stimulated GTPase is obtained with low concentrations of GTP. With 0.1 μM GTP, the light-stimulated GTPase activity was usually 1.6–3.0 times the dark activity with purified photoreceptors and was 3.9 times the dark activity with an unpurified retinal suspension from freshly dissected squid. The higher light-stimulated activity in freshly dissected retinas may be due to sensitivity of an essential sulfhydryl group to oxidation. Storage of purified photoreceptor membranes at 4 °C for several days resulted in loss of light-stimulated GTPase activity. This loss could be prevented or partially reversed by the addition of 5 mM dithiothreitol. Freshly dissected retinal suspensions also contained a soluble inhibitory factor, not apparent in purified photoreceptors, which reduced the GTPase activity elicited by light. Removal of the soluble fraction by washing the membranes was essential for obtaining optimal light-stimulated activity.

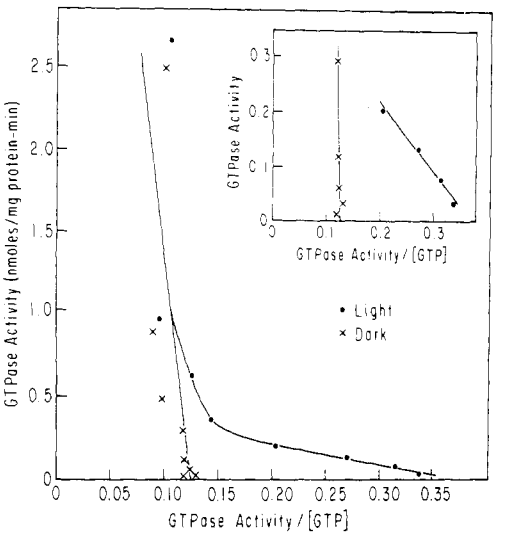

FIGURE 1: Eadie–Hofstee plot of rate of GTPase activity in the dark and after illumination. Purified squid photoreceptor membranes either were kept in the dark or were illuminated at 0 °C with light filtered through a 497-nm interference filter to produce about a 50:50 mixture of rhodopsin and metarhodopsin. With infrared illumination, 100-μL aliquots of the dark and illuminated membranes (1.1 mg/mL of protein) were incubated with [γ-^{32}P]GTP in concentrations from 0.1 to 100 μM for 1 min at 19 °C as described under Experimental Procedures. GTP hydrolysis was linear with time, indicating that initial rates of GTPase activity were measured. Results are the mean of duplicate samples, which usually differed by <3%. The inset shows an expanded *y* axis for data for samples that contained concentrations of GTP from 0.1 to 2.5 μM (also plotted in the main figure). (●) Illuminated; (×) dark samples. Light-activated GTPase had a K_m of approximately 1 μM and a V_{max} of 480 pmol (mg of protein)$^{-1}$ min^{-1}. These values were derived from the line plotted in the inset for low concentrations of GTP.

The low-affinity and high-affinity GTPase activities appear to arise from two distinct sites of GTPase activity (e.g., two enzymes or a single GTPase having two sites with different K_m and V_{max}) rather than from a light-induced change in affinity for GTP at a single site. The low- and high-affinity GTPase activities can be influenced independently by the addition of a phosphate-regenerating system. Creatine phosphokinase catalyzes the transfer of phosphate from creatine phosphate to ADP and GDP to produce ATP and GTP, respectively (Salomon & Rodbell, 1975). The addition of creatine phosphokinase and creatine phosphate to the photoreceptor membranes suppressed the dark GTPase activity without influencing the increment in light-stimulated activity.

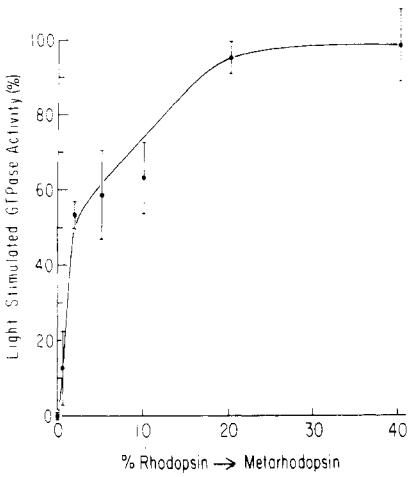

FIGURE 2: Relationship between metarhodopsin formation and GTPase activity in purified squid photoreceptor membranes. Purified squid photoreceptor membranes were incubated with 5 mM dithiothreitol prior to the assay. Membranes were illuminated during continuous stirring at 0 °C with light filtered through a 497-nm interference filter with an intensity of 3 $\mu W/cm^2$. At various times, samples were withdrawn and assayed for GTPase activity with 0.1 μM [γ-^{32}P]GTP as described under Experimental Procedures. Creatine phosphokinase and creatine phosphate were not added to the samples in this experiment. The light-stimulated GTPase activity is the percent activity between the dark and maximally illuminated samples. Maximal GTPase activity in the light was 1.2 mmol of GTP hydrolyzed (mol of rhodopsin)$^{-1}$ min^{-1}. This was twice the activity of dark samples. Each value represents the mean of duplicate measurements (or triplicate for dark samples) ± standard deviation.

Table I: Light Activation of Bovine Phosphodiesterase after Combination with Purified Squid Photoreceptor Membranes[a]

	phosphodiesterase activity (pmol/min)	
	dark	illuminated
squid photoreceptors	169 ± 26	170 ± 15
bovine phosphodiesterase and G protein extract	ND	742 ± 85
squid photoreceptors + bovine phosphodiesterase and G protein extract	921 ± 33	2632 ± 16

[a] Phosphodiesterase activity was measured as described under Experimental Procedures with 1 mM [^{3}H]cAMP. Purified squid photoreceptors (containing 0.3 pmol of squid rhodopsin) were assayed in the dark or after illumination, either alone or in combination with unpurified bovine phosphodiesterase and G protein extract (20 μg). The bovine extract contained approximately 20% phosphodiesterase, 75–80% G protein, and ≤5% other proteins on the basis of the intensities of Coomassie blue stained polyacrylamide gels. The bovine phosphodiesterase and G protein extract was not light sensitive and had been preexposed to light before addition to the squid membranes in the dark. Values represent mean ± standard deviation. ND = not determined.

However, concentrations of creatine phosphate higher than 5 mM also reduced light-stimulated activity. In addition, a phosphate-regenerating system prevented the formation of labeled ATP, as assayed by thin-layer chromatography. As with turkey erythrocyte membranes (Cassell & Selinger, 1976), one component of the low-affinity GTPase activity is probably a phosphotransferase that transfers the γ-phosphate of [γ-^{32}P]GTP to ADP to form [γ-^{32}P]ATP. Creatine phosphokinase prevents this transfer by reducing the availability of ADP. Other agents that might be expected to reduce nucleotide triphosphatase activity were not effective in reducing the dark GTPase activity. Little effect was observed after the addition of 10 μM dicyclohexylcarbodiimide or 10 μM ouabain, either alone or in combination with creatine phosphokinase and creatine phosphate. App(NH)p reduced both dark and light-stimulated GTPase activity.

GTPase activity was stimulated with low intensities of illumination. The amount of light required for GTPase activation was examined by illuminating membranes for various lengths of time and then assaying the GTPase activity of the illuminated samples (Figure 2). The GTPase activity of the purified squid photoreceptors was constant for at least 30 min after illumination. The light-stimulated GTPase activity was half-maximally stimulated when only 2% of the rhodopsin had been photoconverted to metarhodopsin. Because these experiments were performed with purified squid photoreceptors that were uncontaminated with the photopigment retinochrome, it is likely that GTPase activity is due to the interaction of illuminated rhodopsin with the GTPase. These purified membranes did not contain a band on $NaDodSO_4$–polyacrylamide gels corresponding to retinochrome, nor did they respond to the addition of 10 mM NH_2OH by a change in their visible absorption spectrum, indicating that retinochrome was not present.

Light-Activated Gpp(NH)p Binding. Binding of guanine nucleotides to purified squid photoreceptor membranes is regulated by light. By use of the hydrolysis resistant GTP analogue Gpp(NH)p as an indicator of GTP binding, it was found that the binding was 11 times greater (±6%, SD) in illuminated than dark membranes at a Gpp(NH)p concentration of 3.0 μM. Preliminary results showed that binding of [^{35}S]GTPγS also was enhanced by illumination.

Activation of Bovine Phosphodiesterase by Squid Photoreceptor Membranes. The combination of squid photoreceptor membranes with a soluble extract of bovine phosphodiesterase and G protein conferred light sensitivity to the bovine phosphodiesterase. Illumination of this mixture resulted in an increase in phosphodiesterase activity to 2.8–4.3 times the dark values, as illustrated in Table I. In the absence of the bovine extract, neither purified squid photoreceptors nor freshly dissected squid retinal suspensions exhibited light-regulated adenylate or guanylate phosphodiesterase activity nor adenylate cyclase activity under a variety of conditions (Vandenberg, 1982). The K_m of the squid retinal membrane phosphodiesterase activity for cAMP was 15 μM (Vandenberg, 1982), while the light-induced phosphodiesterase activity obtained after combination of squid membranes with bovine phosphodiesterase and G-protein extract was 3 mM, the same as that of purified bovine phosphodiesterase (Miki et al., 1975), suggesting that squid rhodopsin activated bovine phosphodiesterase. Light activation of bovine phosphodiesterase was measured both with freshly dissected squid retinal suspensions and with purified squid photoreceptor membranes. As noted above for the measurement of GTPase, freshly dissected squid membranes best demonstrated light-activated combination with phosphodiesterase after the membranes were washed to remove a soluble inhibitory factor.

Cholera Toxin Catalyzed Labeling. Cholera toxin catalyzed the transfer of label from [^{32}P]NAD to proteins in squid photoreceptor membranes (Figure 3). The label appeared predominantly on a protein with an apparent M_r of 44 000 on $NaDodSO_4$–polyacrylamide gels. In addition, faint bands of apparent M_r ≥95 000 were labeled. In the absence of cholera toxin, label only was incorporated into the low molecular weight (≤21 000) region of gels. The extent of labeling saturated at a toxin concentration of 100 μg/mL and at this concentration was maximal by 30 min at 30 °C. The labeled 44 000-dalton protein did not comigrate with either squid actin

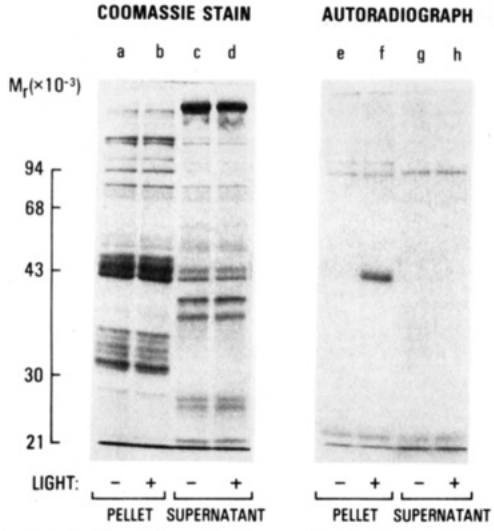

FIGURE 3: Light-dependent cholera toxin dependent labeling of photoreceptor membranes with [^{32}P]NAD. Squid retinal membranes were incubated with [^{32}P]NAD and cholera toxin in the dark or light as described under Experimental Procedures in the presence of 7.5 mM $MgAc_2$, 5 mM EGTA, and 1 mM ATP. After 30 min at 30 °C, the samples were chilled to 0 °C, and then membrane-associated and soluble material was separated by centrifugation (17000g, 2 min). In dim white light at 0 °C, the supernatant was removed and diluted with sample electrophoresis buffer to terminate the labeling reaction. The pellet was rinsed with membrane buffer before solubilization in sample electrophoresis buffer. Amounts of the pellet and supernatant on the gel are equivalent to their concentrations in the original membrane suspension. (Lanes a–d) Coomassie blue stained gel: (a) membrane-bound fraction from dark sample; (b) membrane-bound fraction from light sample; (c) soluble fraction, dark sample; (d) soluble fraction, light sample. (Lanes e–h) Corresponding autoradiograph.

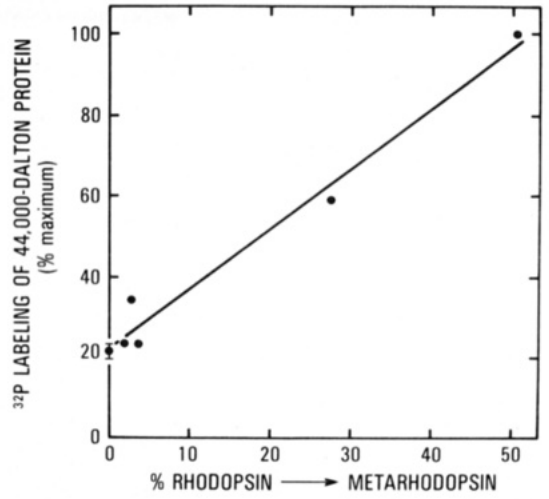

FIGURE 4: Relationship between metarhodopsin formation and cholera toxin catalyzed labeling of the 44 000-dalton protein. Samples of squid photoreceptor membranes were illuminated for 1 min at 0 °C with white light of various intensities. An aliquot of the sample was immediately combined with cholera toxin and [^{32}P]NAD, and incubation at 30 °C was initiated within 1 min of the illumination period. Solutions contained 13 mM $MgAc_2$, 3 mM EGTA, and 1 mM ATP. An aliquot of each sample was also used for spectral determination of the extent of metarhodopsin formation. Error bars for the dark value represent the standard deviation of three samples.

(M_r 43 000) or tubulin (M_r 52 000 and 54 000) and formed a narrow band overlapping approximately the second lowest one-fifth of the broad rhodopsin band centered at M_r 46 000 (compare Figures 1 and 5 with Figure 1; Vandenberg & Montal, 1984).

Light Dependence of Cholera Toxin Catalyzed Labeling. Radiolabeling of the 44 000-dalton protein required illumination of the membranes (Figure 3). Label was not observed in the 44 000-dalton protein if the membranes were not exposed to light (lane e). However, if the photoreceptor membranes were illuminated in the presence of cholera toxin and [^{32}P]-NAD, then ^{32}P was incorporated into the 44 000-dalton protein (Figure 3, lane f). The extent of labeling increased 7 times after illumination, to reach a final level of $1.9 \times 10^{-3} \pm 0.6 \times 10^{-3}$ (SD) mol of ^{32}P/mol of rhodopsin with a substrate concentration of 20 μM [^{32}P]NAD. Light-dependent labeling was observed with NAD concentrations from 10 μM to 6 mM. The maximum extent of labeling at 20 μM NAD was only $^1/_{12}$ as much observed with higher substrate concentrations, indicating that maximum labeling at high substrate concentrations is 1 phosphate/45 rhodopsin. This provides a lower limit for the number of 44 000-dalton proteins per rhodopsin (allowing for some inefficiency of labeling by cholera toxin) and is close to the estimate of the ratio of GTP binding protein to rhodopsin in vertebrate photoreceptors of 1:15 (Kuhn, 1980) or 1:8 (Kuhn, 1981).

An additional protein, M_r ~95 000, showed light-dependent labeling catalyzed by cholera toxin. Labeling was more extensive in dark than in illuminated membranes, but the difference between dark and light-exposed samples was less pronounced than for the 44 000-dalton protein. Because the 95 000-dalton polypeptide was a minor fraction of the total labeled protein, it was not studied further.

The light-intensity dependence of the toxin-catalyzed labeling was examined. Illumination was not required during the entire labeling period, but only for a brief time at the beginning. Membranes were exposed to various intensities of light and then immediately incubated with [^{32}P]NAD and toxin. Figure 4 illustrates the extent of labeling as a function of the fraction of rhodopsin that was photoconverted to metarhodopsin. Labeling was approximately linear with the formation of metarhodopsin. This relation provides an upper limit for the amount of light needed to cause labeling because the extent of labeling declined with a half-time of about 5 min when the addition of cholera toxin and [^{32}P]NAD was delayed after illumination. The limited time that the 44 000-dalton protein was available for labeling coupled with the very slow rate of ADP-ribosylation by cholera toxin (Gill, 1977) is probably responsible for the requirement for maximum metarhodopsin formation in order to achieve maximal labeling. The brief delay between illumination and addition of toxin and [^{32}P]NAD would also have the effect of shifting the intensity vs. labeling curve toward higher light intensities.

Specificity of [^{32}P]NAD for Cholera Toxin Catalyzed Labeling. To eliminate the possibility that the label was derived from a degradation product of [^{32}P]NAD, such as ADP-ribose, the fate of the labeled NAD was examined. Thin-layer chromatography of the $NaDodSO_4$-solubilized membrane solution after labeling (Figure 5B, lanes b and c) indicated that NADase activity was not present in the sample during the course of the experiment. Radiolabel remained associated exclusively with NAD. No differences were observed between dark and illuminated samples.

The possibility that an undetected trace of [^{32}P]ADP-ribose had contributed to the labeling was tested by addition of cold ADP-ribose to the membranes during labeling (Figure 5, lanes d and e). No changes were found in the extent of protein labeling (Figure 5A, lanes d and e) nor in the thin-layer chromatograms of the samples after labeling (Figure 5B, lanes d and e). Finally, to test the effect of [^{32}P]ADP-ribose as a substrate, the [^{32}P]NAD was enzymatically degraded with insoluble NADase prior to labeling and the NADase removed

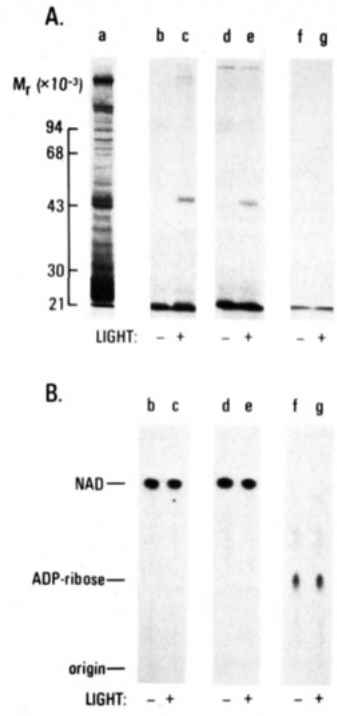

FIGURE 5: Requirements for cholera toxin catalyzed labeling. Comparison of [^{32}P]NAD and [^{32}P]ADP-ribose. Suspensions of squid retinal membranes were incubated in the dark or light as indicated with cholera toxin, 1 mM ATP, 13 mM $MgAc_2$, and 3 mM EGTA in the presence of (b and c) 20 μM [^{32}P]NAD, (d and e) 20 μM [^{32}P]NAD and 100 μM cold ADP-ribose, or (f and g) 20 μM [^{32}P]NAD pretreated with NADase as described under Experimental Procedures. (A) (a) Coomassie blue stained gel; (b–g) autoradiograph of gel. (B) (b–g) Autoradiograph of thin-layer chromatograph of $NaDodSO_4$-solubilized samples corresponding to lanes b–g in (A) above.

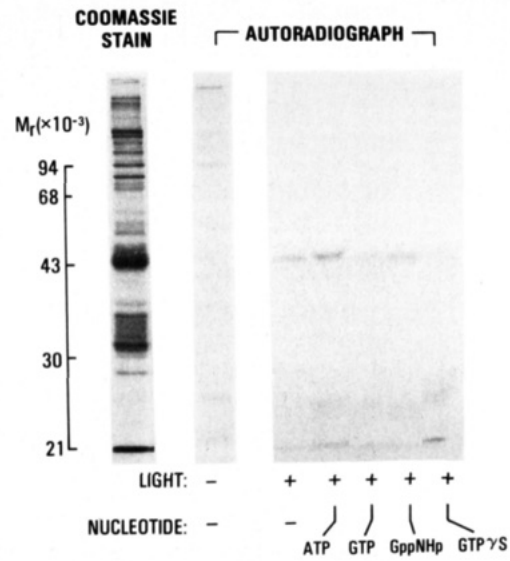

FIGURE 6: Effect of nucleotides on light-catalyzed and cholera toxin catalyzed labeling of squid photoreceptor membranes. Squid photoreceptors were incubated with [^{32}P]NAD and cholera toxin in the dark or light as indicated, in the presence of 7.5 mM EGTA and either no exogenously added nucleotide or 100 μM ATP, GTP, Gpp(NH)p, or GTPγS as indicated. Coomassie blue stained gel (left); autoradiograph (right). Dark samples (not shown) for all conditions were equivalent to the dark example shown.

by centrifugation (17000g, 5 min). The resulting [^{32}P]ADP-ribose was then incubated with membranes and cholera toxin. In this case, no label was incorporated into the 44 000-dalton protein (Figure 5A, lanes f and g), whereas the sample was shown by thin-layer chromatography to contain substantial amounts of [^{32}P]ADP-ribose but no [^{32}P]NAD (Figure 5B, lanes f and g).

The observed labeling was not due to phosphorylation from [^{32}P]ATP. Because the ^{32}P was on the AMP moiety of NAD, ATP that might have been produced would retain label in the α-phosphate, not in the γ-position that could be used for phosphorylation. In addition, radioactive ATP was not detected in the samples, and the labeling was enhanced, not reduced, by the addition of cold ATP. These results are consistent with the view that cholera toxin catalyzes the ADP-ribosylation of the 44 000-dalton protein with NAD as a substrate.

Effects of Guanine Nucleotides on Cholera Toxin Catalyzed Labeling. The addition of nucleotides to the membrane suspension influenced the labeling of the 44 000-dalton protein. However, unlike the adenylate cyclase GTP binding protein which requires GTP for labeling (Enomoto & Gill, 1980), the labeling of squid photoreceptor 44 000-dalton protein was decreased by most guanine nucleotides. The addition of 100 μM ATP enhanced the labeling, while GTP and GTPγS at the same concentration inhibited it. Gpp(NH)p had no apparent effect (Figure 6). The effect of the nucleotides was more apparent in solutions of low divalent ion concentrations that had been buffered with 10 mM EGTA. The addition of Mg^{2+} without nucleotide did not produce the same effect. Greater nucleotide specificity in the absence of Mg^{2+} could be due to inhibition of nucleotide diphosphate kinases that might otherwise enzymatically degrade the nucleotide under consideration to produce other nucleotide triphosphates (Enomoto & Gill, 1980). Inhibition of labeling by GTP and GTPγS could be due in part to the effects of GDP produced by hydrolysis or traces of GDPβS, respectively.

In the presence of ATP, guanine nucleotides also depressed the labeling. With 1 mM ATP present, an inhibition of labeling was observed with all guanine nucleotides tested, with a relative potency of GTPγS $\gg$ GDP > GTP > Gpp(NH)p. Addition of 100 μM GTPγS, the most potent inhibitory nucleotide tested, nearly eliminated the light-induced labeling. The extent of labeling in the presence of guanine nucleotides and 1 mM ATP, 13 mM $MgAc_2$, and 3 mM EGTA compared to illuminated samples with no added guanine nucleotide (100 ± 8%, SD) was as follows: (dark) no added guanine nucleotide 14 ± 1%; (light) 0.1 mM GDP 85%, 0.3 mM GDP 62%, 1.0 mM GDP 58%, 0.1 mM GTP 85%, 0.3 mM GTP 83%, 1.0 mM GTP 51%, 0.1 mM Gpp(NH)p 120%, 0.3 mM Gpp(NH)p 89%, 1.0 mM Gpp(NH)p 85%, 0.1 mM GTPγS 34%.

Additional Factors. Several other parameters regulate the labeling of the 44 000-dalton protein. A soluble factor was needed to achieve labeling. Membranes that had been centrifuged (17000g, 5 min) to remove soluble material and then resuspended in buffer were not labeled at the 44 000-dalton site, nor was label incorporated into the soluble fraction. Centrifuged membranes that were recombined with the soluble material retained their ability to incorporate label. An additional complexity is that freshly dissected retinas were found to be necessary for labeling. Membrane suspensions that had been frozen (–20 °C for 48 h) and then thawed did not express labeling of the 44 000-dalton protein. Addition of freshly isolated soluble fraction to previously frozen membranes did not reestablish the labeling capacity.

Properties of the 44 000-Dalton Protein. The labeled 44 000-dalton protein was membrane bound under all conditions examined. It appeared to be an integral membrane protein since it was not removed from the membrane by changes in ionic strength or divalent ion concentration. Ionic

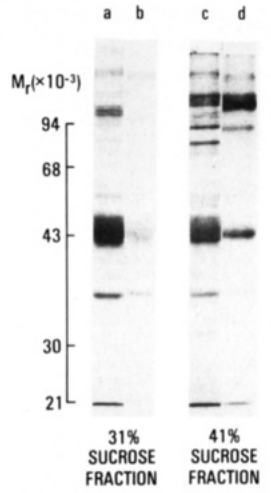

FIGURE 7: Purification of squid photoreceptor membranes after labeling. Squid photoreceptor membranes were labeled in the light with [^{32}]NAD and cholera toxin in the presence of 13 mM $MgAc_2$, 3 mM EGTA, and 1 mM ATP. After the labeling, membranes were centrifuged (17000g, 3 min) supernatants discarded, and the pellets rinsed in membrane buffer. In these samples, label was associated predominantly with the 44000-dalton protein (not shown). Membranes were stored at −80 °C. Thawed membranes were then resuspended in 5 mM Tris-HCl, 1 mM $MgAc_2$, and 0.1 mM EGTA, pH 8.0, and layered onto the top of a continuous sucrose gradient from 0 to 50% (w/v) sucrose containing the buffer above. The gradient was centrifuged at 4 °C for 3 h at 40000 rpm in a Beckman SW-50 rotor. Rhodopsin-containing membranes formed bands at 31 and 41% (w/v) sucrose. (a and b) 31% fraction of the sucrose gradient; (c and d) 41% fraction of the sucrose gradient; (a and c) Coomassie blue stained gel; (b and d) autoradiograph.

strength was varied from 5 mM Tris to 100 mM NaCl and divalent ions were varied from 10 mM Mg^{2+} to 10 mM EGTA without affecting the solubility of the 44000-dalton protein. It was however solubilized with Lubrol PX, a detergent.

The retinal membrane suspension was fractionated after labeling to ascertain which membrane was associated with the 44000-dalton protein. On a continuous sucrose density gradient, rhodopsin-containing membranes formed two distinct bands at 41 and 31% sucrose. Black-pigmented material was located as a pellet. Label was found predominantly in the denser rhodopsin-containing fraction (Figure 7). On Na$DodSO_4$–polyacrylamide gels, this fraction contained several protein bands not found in the less dense rhodopsin fraction. Label was associated predominantly with a protein of M_r 120000, in addition to the protein of M_r 44000. This appears to arise from freezing the membranes, since label only was observed in the 44000-dalton protein prior to freezing. Apparently, the 44000-dalton protein aggregated to produce the higher molecular weight form, perhaps by forming trimers with other 44000-dalton proteins.

Discussion

Squid photoreceptor membranes show light-activated binding of GTP analogues, hydrolysis of GTP, and labeling of a 44000-dalton protein that is catalyzed by cholera toxin. Similarities between the enzymatic activities of squid and vertebrate photoreceptors indicate that the squid photoreceptor also contains a G protein that incorporates both GTP binding and hydrolysis functions. The increase in GTPase and guanine nucleotide binding activities after illumination appears to arise from interaction of illuminated squid rhodopsin with the G protein. The somewhat lower light sensitivity of the squid GTPase compared to bovine GTPase (Wheeler et al., 1977) may arise from the fact that the squid G protein is membrane bound and, therefore, restricted in its interaction with as many rhodopsin molecules as the vertebrate protein. The K_m of the GTPase for GTP is the same as that of the bovine photoreceptor G protein (Wheeler & Bitensky, 1977) and similar to the G protein from turkey erythrocyte hormone-activated adenylate cyclase (Cassel & Selinger, 1976).

Further similarity between the squid and vertebrate photoreceptor enzymes is illustrated by the functional combination of bovine G protein and phosphodiesterase with squid photoreceptor membranes, demonstrating light activation of the bovine phosphodiesterase by squid rhodopsin. The functional combination could have arisen from interaction of either squid G protein with bovine phosphodiesterase or squid rhodopsin with bovine G protein. Both alternatives suggest homologies between the light-activated G proteins of squid and bovine photoreceptors. Exchange of components between enzyme systems also has indicated homology between the G proteins of octopus and bovine photoreceptors (Ebrey et al., 1980) and between those of bovine photoreceptor light-activated phosphodiesterase and hormone-mediated adenylate cyclase (Bitensky et al., 1982).

Light, a soluble factor, and [^{32}P]NAD are required for the cholera toxin catalyzed labeling of a 44000-dalton protein in squid photoreceptors. We consider it unlikely that the labeled protein is rhodopsin or actin due to differences in their locations on Na$DodSO_4$–polyacrylamide gels. The properties of the labeling make the 44000-dalton protein a good candidate for the light-activated GTP binding protein/GTPase. The known specificity of cholera toxin for the GTP binding protein from the hormone-activated cyclase is suggestive of this possibility. The cyclase GTP binding protein also exhibits both GTP binding activity and GTPase activity, which in that system is stimulated by hormone (Cassel & Selinger, 1976, 1978). The possibility that the 44000-dalton protein is the GTP binding protein/GTPase is further strengthened by the fact that light and guanine nucleotides influence its labeling in squid photoreceptors. It is not clear, however, whether the 44000-dalton protein represents the entire squid photoreceptor GTP binding protein or a subunit of an oligomer.

A number of similarities between the squid photoreceptor 44000-dalton protein, the hormone-activated cyclase guanine nucleotide binding protein, and the vertebrate photoreceptor guanine nucleotide binding protein are evident. The polypeptides labeled by cholera toxin are of similar molecular weight, being 44000, 43000, and 39000, respectively (Cassel & Pfueffer, 1978; Cooper et al., 1981; Abood et al., 1982). In some hormone-activated cyclase systems, a 52000-dalton protein is also labeled (Northup et al., 1980; Sternweis et al., 1981; Johnson et al., 1978). In these different systems cholera toxin dependent labeling is influenced by several variables: receptor-ligand or rhodopsin-light activation, guanine nucleotides, and a cytoplasmic factor.

The light regulation of squid photoreceptor labeling reported here clearly shows that cholera toxin catalyzed labeling requires activation of the receptor protein, in this case rhodopsin, for labeling. For the hormone-activated guanine nucleotide binding protein, Enomoto & Gill (1980) demonstrated that GTP, or its analogue was required for labeling and hormone-stimulated labeling only when guanine nucleotide binding was the rate-limiting step. The requirement for illumination of the squid photoreceptors in order to label the 44000-dalton protein is not related to the need to bind GTP because the addition of GTP was found to be inhibitory. Instead, absorption of light by a component of the photoreceptor, presumably rhodopsin, changes the conformation of

the 44000-dalton protein so that it can interact with cholera toxin. In addition to rhodopsin, retinochrome, a photosensitive protein located primarily in the photoreceptor inner segment, is another possible mediator for the light dependence of the labeling reaction. The experiments reported here do not distinguish between these alternatives. However, rhodopsin is the most plausible candidate for light activation because it interacts with the guanine nucleotide binding protein in response to light. Abood et al. (1982) found that illumination of rhodopsin in bovine photoreceptors, which lack retinochrome, also enhanced labeling with cholera toxin.

The inhibition of labeling by guanine nucleotides extends the range of effects produced by guanine nucleotides on cholera toxin catalyzed labeling of G proteins: GTP analogues have the opposite effect for the cyclase system (Enomoto & Gill, 1980), and guanine nucleotides exert an intermediate effect on the labeling of vertebrate photoreceptors—Gpp(NH)p enhanced labeling, while GTPγS, GTP, and GDP inhibited labeling (Abood et al., 1982). These differences may reflect conformeric variations of the GTP binding proteins that are induced by nucleotide binding. The influence of nucleotides on toxin-catalyzed protein labeling may be due in part to binding to sites other than the GTP binding site on the GTP binding protein. A second binding site on the GTP binding protein could explain the unexpected selectivity for ATP over guanine nucleotides. Alternatively, ATP could be interacting with other cofactors, not necesarily the GTP binding protein. Because rhodopsin is phosphorylated by ATP after illumination (Vandenberg & Montal, 1984), a secondary effect of ATP could be to turn off rhodopsin activity, thus accounting for the transient effect of light in promoting labeling.

A soluble factor is needed for labeling of the cyclase (Enomoto & Gill, 1980) and the squid photoreceptor guanine nucleotide binding proteins, but this requirement has not been examined for the vertebrate photoreceptor. Factor activity has been demonstrated from the cytoplasm of a variety of vertebrate erythrocytes (Enomoto & Gill, 1980). It will be of interest to determine if vertebrate and invertebrate factors are similar.

Variations in the guanine nucleotide binding proteins are apparent in their degree of association with the membrane. Both the cyclase (Northup et al., 1980; Sternweis et al., 1981; Hanski et al., 1981) and squid photoreceptor proteins are integral membrane proteins, while the vertebrate photoreceptor guanine nucleotide binding protein is membrane bound under some conditions of illumination, ionic strength, divalent ion concentration, and GTP concentration and is soluble for others (Kuhn, 1980, 1981).

The various guanine nucleotide binding proteins appear to possess structural and functional homology, with specific differences. It is of particular interest that guanine nucleotide binding proteins from different sources are activated by different receptor proteins—hormone receptor, vertebrate rhodopsin, and squid rhodopsin—and that they activate different effector proteins—adenylate cyclase, phosphodiesterase, and unknown proteins for the squid photoreceptors. These structural (Manning & Gilman, 1983) and functional (Bitensky et al., 1982) homologies indicate that the guanine nucleotide binding proteins, and perhaps also the receptor and effector components, may have been derived from common precursors during evolution.

The functional role of the G protein in squid photoreception remains unknown. Under conditions in which the squid membranes were capable of activating bovine phosphodiesterase through illumination of squid rhodopsin, neither endogenous cAMP phosphodiesterase nor adenylate cyclase activities were modulated by light in the squid photoreceptor (Vandenberg, 1982).

The high sensitivity of the squid GTPase to light and the large light-induced increase in both guanine nucleotide binding and GTPase activity suggest that the G protein is intimately involved in the visual process. It appears likely that squid photoreceptors represent a new class of enzyme cascade in which G proteins are involved as central regulatory intermediates.

Acknowledgments

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Registry No. GDP, 146-91-8; GTP, 86-01-1; Gpp(NH)p, 34273-04-6; NAD, 53-84-9; ATP, 56-65-5; GTPase, 9059-32-9; GTPγS, 37589-80-3; cyclic nucleotide phosphodiesterase, 9040-59-9.

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

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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).

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