Regulation by Light of Cyclic Nucleotide-dependent Protein Kinases and Their Substrates in Frog Rod Outer Segments

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ABSTRACT Cyclic nucleotides (both cAMP and cGMP) stimulate the phosphorylation of several proteins of 65–70, 50–52, 21, 13, and 12 kD in rod outer segments (ROS) of the frog retina. Subcellular fractionation showed that phosphopeptides of 67, 21, 13, and 12 kD were soluble and phosphopeptides of 69, 67, 50–52, and 12 kD were membrane associated at physiological ionic strength. Components I and II, 13 and 12 kD, respectively, are the major cyclic nucleotide-dependent phosphoproteins of ROS and have been reported to be phosphorylated in the dark and dephosphorylated in the light. Under unstimulated conditions, phosphorylated Components I and II were found in the soluble fraction. Cyclic nucleotide stimulation of phosphorylation resulted in increased phospho-Components I and II in the soluble fraction, and phospho-Component II on the membrane. Light had no effect on the phosphorylation level of soluble Components I and II, but it caused a depletion within 1 s of the membrane-bound phospho-Component II. A half-maximal decrease in membrane-bound Component II was seen at 5×10^5 rhodopsins bleached per outer segment.

The cyclic nucleotide-dependent protein kinase(s) were found primarily in the peripheral membrane fraction of ROS proteins. 8-bromo cyclic AMP was two orders of magnitude more effective than 8-bromo cyclic GMP at stimulating Component I and II phosphorylation. An active peptide of the Walsh inhibitor of cAMP-dependent protein kinase [PKI(5-22)amide] blocked the phosphorylation with an IC_{50} of 10 nM. Photoaffinity labeling studies with 8-N_3-cAMP and 8-N_3-cGMP revealed the presence of a 52-kD band specifically labeled with 8-N_3-cAMP, but no specific 8-N_3-cGMP labeling. These data suggest that cyclic nucleotide-dependent protein phosphorylation in ROS occurs via the activation of a cAMP-dependent protein kinase.

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INTRODUCTION

The phototransduction cascade in vertebrate rod outer segments (ROS) is an example of a physiological process in which cyclic nucleotides act directly, without the intermediacy of protein kinases and protein phosphorylation. In the dark-adapted photoreceptor, cGMP levels are 100-fold higher than in most tissues (Ferrendelli and De Vries, 1983). Cyclic GMP-gated cation channels in the plasma membrane (Fesenko et al., 1985; Yau and Nakatani, 1985) allow the flow of the dark current. Light activates an enzyme cascade, linking rhodopsin excitation to a rapid decrease in cGMP levels in the cytoplasm, leading to the closure of cGMP-gated channels. The proteins responsible for this cascade make up >95% of the protein mass of the outer segment (Hamm and Bownds, 1986). Absorption of a photon of light by rhodopsin leads to a conformational change allowing binding and activation of a GTP-binding protein (G protein) called transducin (Godchaux and Zimmerman, 1979; Fung and Stryer, 1980; Kühn, 1980; Fung et al., 1981). GTP-GDP exchange by the α subunit causes release from rhodopsin and from its $\beta\gamma$ subunit. The activated G protein α subunit then interacts with an inactive cGMP phosphodiesterase and activates it by causing the dissociation of an inhibitor (Hurley and Stryer, 1982; Deterre et al., 1986), resulting in catalytic breakdown of cGMP (Woodruff and Bownds, 1979; Cote et al., 1984; Blazynski and Cohen, 1986).

Although the primary action of cGMP in visual receptors is directly upon channels, rods also contain cyclic nucleotide-dependent protein kinases and prominent cyclic nucleotide-stimulated phosphoproteins. Since cyclic nucleotide levels change significantly with light stimulation, cyclic nucleotide-dependent phosphorylation may play a role in ROS physiology (Farber et al., 1978; Farber, 1982). Such reactions could potentially play feedback and feed-forward regulatory roles in the desensitization and dark adaptation phenomena of vision. For example, they might mediate ATP- and cGMP-dependent changes in channel conductance (Ertel et al., 1989; Filatov et al., 1989).

Most studies on cyclic nucleotide-dependent kinases and their substrates have concentrated on a 33-kD phosphoprotein in mammalian ROS (Lee et al., 1981, 1984, 1987) and two phosphopeptides of 12 and 13 kD in amphibian ROS (Components I and II, Polans et al., 1979; Hermolin et al., 1982; Brewer and Bownds, 1986; Hamm and Bownds, 1986; Shinozawa and Yoshizawa, 1986; Hayashi et al., 1987). Several considerations suggest that they may be functional homologues. They are the major cyclic nucleotide-stimulated phosphopeptides in each species. Their phosphorylation level is high in the dark, and light causes dephosphorylation in both cases (Polans et al., 1979; Lee et al., 1984). They are both associated with the G protein (Hamm and Bownds, 1984; Lee et al., 1987; Suh and Hamm, 1988). Since visual adaptation may involve regulation of the gain of the excitation pathway, it is of interest to examine whether these phosphoproteins are regulating some aspect of G protein behavior.

Evidence that Components I and II are associated with G protein was first obtained using a monoclonal antibody to the G protein, 4A. This antibody blocked G protein activation by light, and also blocked phosphorylation of Components I and II (Hamm and Bownds, 1984). Lee et al. (1987) showed that the 33-kD protein copurified with the $\beta\gamma$ subunit of the G protein, and Suh and Hamm (1988) have

shown that Components I and II comigrate in sucrose gradients with $G_{\beta\gamma}$. The role of these phosphoproteins in G protein regulation or in ROS physiology has not yet been elucidated.

In this paper, the regulation of phosphorylation levels by light and cyclic nucleotides is examined and the kinases involved in phosphorylation of Components I and II are defined.

MATERIALS AND METHODS

Materials

Guanosine triphosphate and 8-bromo cGMP were from Pharmacia P/L Biochemicals, Milwaukee, WI. Aprotinin, GDPβS and GTPγS were from Boehringer-Mannheim Biochemicals, Indianapolis, IN. Cyclic GMP, 2′-deoxy-cGMP and protein kinase inhibitor were from Sigma Chemical Co., St. Louis, MO. 8-2-[aminoethyl]thio cGMP was synthesized using the method of Lincoln et al. (1977). γ-[^{32}P]ATP was made from $^{32}P_i$ by the Gamma-Prep A kit from ProMegaBiotec, Madison, WI. 8-(5-thioacetamidofluorescein)-cGMP (8-TAAF-cGMP) was a generous gift of Prof. Andrea Cavaggioni, Parma, Italy. 8-azido-cGMP was a gift of Prof. B. Haley, Lexington, KY and 8-parachlorophenylthio-cGMP (8-PCPT-cGMP) was a gift of Prof. J. Corbin, Nashville, TN.

Preparation and Purification of ROS

The methods used for dissection of frog (*Rana catesbeiana*) retinas and purification of ROS are described in Hamm and Bownds (1986). Briefly, frogs that had been housed in cyclic light conditions (12 h light, 12 h dark) were dark adapted for at least 2 h. Retinas were dissected under infrared illumination, rinsed in frog Ringer's solution (105 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, and 10 mM HEPES, pH 7.5) containing 6% Percoll, then gently shaken to release ROS from the retina. The suspension containing ROS was layered onto a discontinuous isoosmotic Percoll gradient consisting of 6, 45, and 70% Percoll and centrifuged for 4 min at 2,500 rpm in an International Equipment Co. rotor #816 (Needham Heights, MA). Purified intact ROS were harvested from the 45–70% Percoll interface, then diluted with Ringer's solution and centrifuged at 500 *g* for 15 s to remove Percoll.

Fractionation of ROS Proteins

To release soluble proteins, purified outer segments were disrupted by passage through a 27-gauge needle, and centrifuged for 30 s at 10,000 *g*. Membranes were washed twice with Ringers solution containing 10^2 KIU (Kallikrein Inhibitor Units) Trasylol/ml, and 2 μM leupeptin. Unless stated otherwise in the figure legend, 0.39 mM EGTA was added to the Ringer's solution containing 0.1 mM $CaCl_2$ to give a free Ca^{2+} concentration of $\sim 10^{-8}$ M. To remove peripherally bound proteins, the resulting pellet was resuspended in water and washed twice. Centrifugations were performed in a Beckman Airfuge (Beckman Instruments Inc., Palo Alto, CA) at 100,000 *g* for 10 min.

Phosphorylation of ROS Proteins

Samples of 50 μl purified, disrupted rods (~200 μg rhodopsin per 50 μl) were incubated for 8 min in frog Ringer's solution containing 30 μM γ-[^{32}P]ATP (30 μCi) and 10^{-8} M Ca^{2+} (solution A); other additions were made as indicated in the figure legends. After incubating some samples in the dark, they were exposed to light, bleaching a known amount of rhodopsin. Light intensities of the flash and continuous light sources were calibrated as described by

Brodie and Bownds (1976). Samples were then quenched by the addition of 200 μl 20% trichloroacetic acid (TCA). Alternatively, for subcellular fractionation of phosphoproteins, the phosphorylation reaction was stopped by the addition of 5 mM KH_2PO_4 and 5 mM EDTA in Ringer's solution (stop solution). Samples were centrifuged, and the supernatant was removed and quenched with TCA. The pellet was washed once in the same buffer and centrifuged. The supernatant was then removed and the pellet was quenched by the addition of solubilization solution (2% sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 10% glycerol, 2% bromphenol blue). Control experiments showed that the stop solution prevented further phosphorylation or dephosphorylation during the washing steps. When ROS were incubated first in reaction mixture for 8 min, then for various amounts of time after the addition of stop solution, the phosphorylation pattern remained stable for up to 2 h. Fractions quenched with TCA were allowed to precipitate for 2 h at 0°C.

Polyacrylamide Gel Electrophoresis and Analysis

Samples were suspended in solubilization solution and proteins were separated on long 8–20% gradient Laemmli polyacrylamide gels that had been optimized for the separation of low molecular weight polypeptides (Polans et al., 1979). Gels were dried and subjected to autoradiography on Kodak XAR film in the presence of Cronex intensifier screens (Dupont Co., Wilmington, DE). Phosphorylation levels were quantitated by densitometry (densitometer: E-C Apparatus Corp., St. Petersburg, FL; integrator: Hewlett-Packard Co., Palo Alto, CA) of autoradiograms that were within the linear range of the film. Quantitation is expressed as arbitrary units of optical density. In cases where phosphorylated bands were incompletely separated, the recorded scans were photocopied, and the peaks were cut out and weighed.

RESULTS

Subcellular Distribution of Cyclic Nucleotide-dependent Protein Kinase Substrates

The proteins from Percoll-purified ROS, phosphorylated by ROS kinases in the dark, are shown in Fig. 1. In the absence of any exogenous activators, the proteins shown in lane *1* were phosphorylated. Incubation with 8-bromo-cGMP or 8-bromo-cAMP (500 μM) increased the phosphorylation of peptides of molecular mass ~65–70, 50–52, 21, 13, and 12 kD (Fig. 1, lanes *2* and *3*). The phosphorylation of the 12.5 kD protein, 5.72, was inhibited by both cyclic nucleotides (see also Fig. 3, below).

The two major phosphorylated bands at 13 and 12 kD, also called Components I and II (Polans et al., 1979; Hermolin et al., 1982; Brewer and Bownds, 1986; Hayashi et al., 1987) or Bands I and II (Shinozawa and Yoshizawa, 1986), are the most prominent proteins in ROS whose phosphorylation is enhanced by cyclic nucleotides. They were reported by Hermolin et al. (1982) to be sensitive to Ca^{2+}, but in this more highly purified preparation, their phosphorylation is not enhanced by Ca^{2+} either in the absence or the presence of cyclic nucleotides (data not shown).

When a crude fractionation was performed by centrifugation of membranes after phosphorylation of ROS proteins (Fig. 1, lanes *4* and *5*, control; lanes *6* and *7*, with 8-bromo-cGMP), the phosphopeptides were fractionated into soluble and membrane-bound components. One band of ~67 kD is soluble, and two other bands of ~67 and 70 kD are membrane associated. One of these may be rhodopsin kinase, which is autophosphorylated but is also a substrate for cyclic AMP-dependent protein kinase (Hargrave, P. A., personal communication). The phosphopeptides of

~50 kD were membrane-bound and the 20-kD phosphopeptide partitioned to the soluble fraction.

In the unstimulated condition, most of the phosphorylated Components I and II were found in the supernatant fraction, while the phosphopeptide 5.72 was mostly membrane-bound (Fig. 1, lanes *4* and *5*). After incubation with 8-bromo cGMP (Fig. 1, lanes *6* and *7*), Components I and II phosphorylation was enhanced and the phosphorylation of 5.72 was inhibited. Phospho-Components I and II were then found in the supernatant, but a portion of phospho-Component II was found on the membrane.

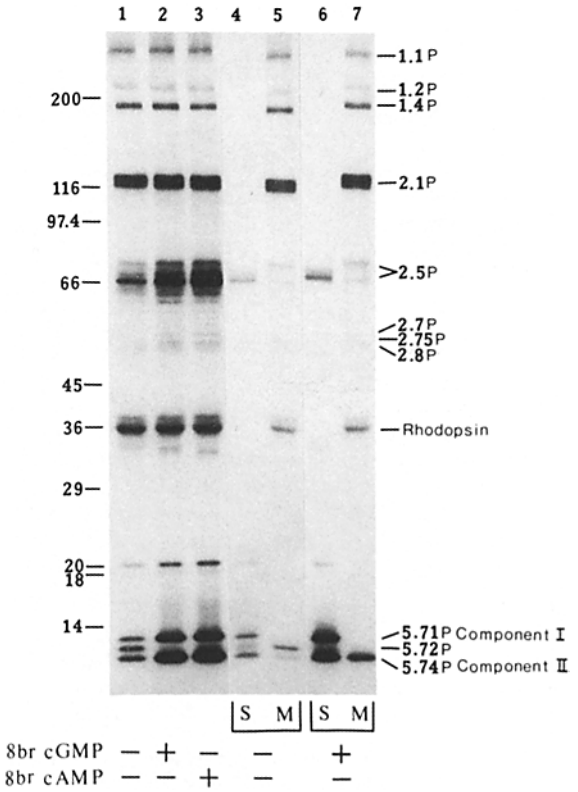

FIGURE 1. Protein phosphorylation pattern in frog ROS. The names of identified phosphorylated bands are shown at the right of the figure. Numbers correspond to the numbering scheme introduced in Hamm and Bownds (1986). Percoll-purified ROS were incubated in the dark for 8 min with 500 μM GTP and additions as described in Materials and Methods. Final concentrations of 8-bromo cyclic nucleotides were 500 μM. (Lanes *1–3*) Samples were quenched with 10% TCA and subjected to electrophoresis and autoradiography. (Lanes *4–7*) The reaction was stopped with 5 mM EDTA and phosphate buffer, and samples were centrifuged at 100,000*g* for 5 min to separate soluble (*S*) and membrane (*M*) fractions.

It was of interest to know whether the phospho-Component II redistribution was of all the protein mass or of only a subfraction that was phosphorylated. In experiments where sufficient protein was loaded onto one-dimensional gels so that Components I and II could be detected by Coomassie blue staining, proteins of molecular weights 12,000 and 13,000 were detected in the membrane fraction only, and no protein stain was found in the soluble fraction (data not shown). This suggests that the phosphorylated Components I and II detected in these experiments are only a minor fraction of the total Components I and II protein.

To estimate the amount of Components I and II present in ROS, the soluble and peripheral membrane protein fractions were pooled and two-dimensional gels were

loaded with 200 μg of protein (data not shown). The Coomassie blue–stained Components I and II protein spots have a pI of 6.5, and do not comigrate with the single spots of phosphorylated Components I and II detected on autoradiograms that have a pI of 6.2. The mass of Components I and II was estimated by comparing the density of Coomassie blue in these spots with the γ subunit of G protein. Equal amounts of Component I and Component II were measured, and each spot was 0.17 of Gγ. Since it is known that the G protein heterotrimer is present at 1 copy per 10 rhodopsins, the approximate amount of Components I and II is 0.17/10 rhodopsins. Since frog ROS contain 3×10^9 rhodopsins/outer segment, there are $\sim 6 \times 10^6$ copies of Components I and II per outer segment.

TABLE I

Specificity of the cGMP Effect on Component II Redistribution

	Phosphorylated Component II	
	Soluble	Membrane-bound
Control	3.94*	1.00
+ cGMP (500 μM)‡	15.9	4.42
+ 8-bromo-cGMP	24.8	12.5
+ 8-N_3-cGMP	32.3	10.7
+ 8-PCPT-cGMP	30.66	28.9
+ 8-TAAF-cGMP	23.54	25.1
+ 2-deoxy-cGMP	3.15	1.40
+ 5′GMP	0.18	0.47
+ 8-bromo-5′GMP	0.63	1.36
+ GDP	0.50	0.98
+ cAMP	6.76	14.5
+ 8-bromo-cAMP	9.44	22.9
+ 5′AMP	2.82	4.14
+ 8-bromo-5′AMP	0.81	2.26
+ ADP	0.45	0.71

*Arbitrary units of optical density.

‡ROS were incubated in the dark with 500 μM additions, then membranes and the supernatant were separated and analyzed. 500 μM GTP was present in all samples, since it was found to enhance the effectiveness of agents to stimulate the membrane phosphorylation of Component II. *8-TAFF-cGMP*, 8-(5-thioacetamidofluorescein)-cGMP; *8-PCPT-cGMP*, 8-parachlorophenylthio-cGMP.

Chemical Specificity of the Cyclic Nucleotide Effect on Subcellular Distribution of Phosphorylated Component II

Earlier in vivo and in vitro studies of Components I and II phosphorylation had shown parallel behavior of the two phosphopeptides under all conditions (Polans et al., 1979; Hermolin et al., 1982; Brewer and Bownds, 1986; Shinozawa and Yoshizawa, 1986). A difference in their behavior was unmasked by examining their subcellular distribution (Fig. 1). To further evaluate the nature of the membrane association of phosphorylated Component II, the specificity of cyclic nucleotides to effect the phosphorylation of Component II in the two fractions was examined in the presence of 500 μM GTP (Table I).

The most potent stimulators of the endogenous kinase activity were cyclic nucleotide analogues substituted at the 8′ position of the guanine ring, which is similar to other cyclic nucleotide kinases. The 2′-substituted cGMP analogue was less effective than cGMP. Metabolically active cyclic nucleotides cGMP and cAMP were not as effective as stimulating phosphorylation as the 8′-substituted analogues. Other metabolically active nucleotides, 5′-GMP, GDP, and ADP, decreased the level of phosphorylation. This may be in part because they can be used by transphosphorylating enzymes for the synthesis of ATP, decreasing the specific activity of the γ-[^{32}P]ATP.

Although all cyclic nucleotides could stimulate phosphorylation of Component II, cyclic AMP and its analogues were more effective than cGMP and its analogues at stimulating phosphorylation of its membrane-associated form. The fact that the cyclic AMP and cyclic GMP analogues had different effects on the subcellular distribution of phosphorylated Component II suggested that two different kinases might be present. Phosphorylated Component I was not found on the membrane in any of the above conditions.

Effect of Light on Phosphorylation of Components I and II

Polans et al. (1979) reported a light-induced dephosphorylation of Components I and II if the retinas were illuminated. However, physiological levels of light had no effect on phosphorylation levels in isolated ROS (Polans et al., 1979; Hermolin et al., 1982). It was of interest to reexamine the effect of light on the soluble and membrane-bound fractions of Components I and II. Light bleaching 1% rhodopsin caused a 60% depletion of phosphorylated Component II from the membrane, while it had no effect on soluble phosphorylated Components I and II.

To examine the kinetics of the light-induced depletion of Component II from the membrane, the reaction was quenched at various times after a light stimulus, bleaching a total of 1% of the rhodopsin during 10 s. Fig. 2 shows that light caused a decrease in the phosphorylated Component II on the membrane, which is complete by 1 s, the earliest time measured.

The light intensity needed to evoke the response of Component II to light is shown in Fig. 3. There was relatively little effect of light on the phosphorylation of Components I and II in the soluble fraction, while light caused a decrease in the amount of membrane-bound phosphorylated Component II. A flash of light bleaching 5×10^5 rhodopsin molecules caused a decrease in membrane-bound phosphorylated Component II, and a maximal 60% decrease was observed after a flash that bleached 5×10^6 rhodopsin molecules. The effect of these flashes on rhodopsin phosphorylation is shown on the lower part of the figure for comparison. The dephosphorylation of membrane-bound Component II occurs over a range of light intensities corresponding to 100 or more rhodopsins bleached per disk.

It is not known whether the light-dependent decrease in membrane-bound phosphorylated Component II is due to a release of protein from the membrane or dephosphorylation by a phosphatase. To test whether dephosphorylation of Component II occurs on the membrane, stop solution (consisting of 5 mM EDTA to chelate Mg, and 5 mM phosphate to serve as a phosphatase inhibitor) was added to the reaction mixture before the light stimulus, and the extent of light-evoked decrease

of membrane-bound Component II phosophorylation was measured. Approximately 50% of the light-dependent decrease in phosphorylated Component II was blocked by this treatment (data not shown). The fact that some decrease in phosphorylation levels persisted after blockade of the phosphatase suggests that light-evoked release also occurs. This is supported by two other observations. First, the light-induced decrease in Component II phosphorylation occurred both in the presence of cGMP and the slowly hydrolyzable analogue 8-bromo-cGMP, which should not have been broken down within 1 min. Second, no light-induced decrease in phosphorylated membrane-bound Component II occurred in the absence of guanine nucleotides (data not shown). It is known that guanine nucleotides are required for the light-induced release of G protein from the disk membrane into the cytoplasm. The interaction of Components I and II with G protein will be discussed further below.

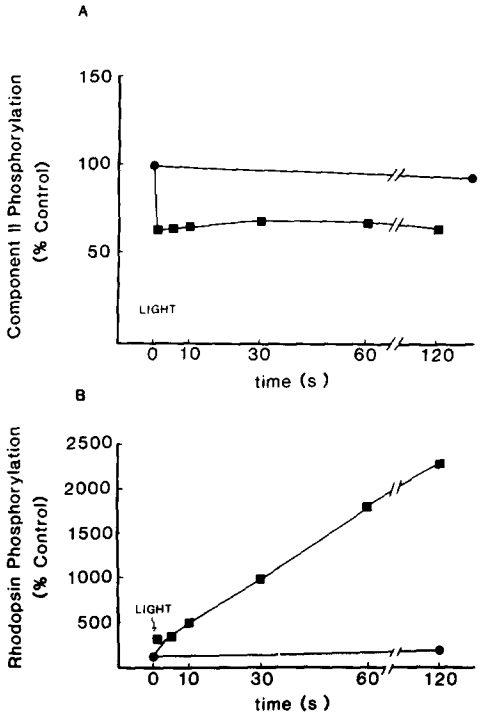

FIGURE 2. Light causes a rapid depletion of membrane-bound Component II which is complete within 2 s. ROS were incubated in the dark in the presence of 500 μM cGMP and 500 μM GTP for 7 min, then illuminated for 10 s with continuous light bleaching a total of 1% rhodopsin. Samples were quenched at the times indicated, and separated by centrifugation into soluble and membrane fractions as described in Materials and Methods. Phosphorylation levels in the membrane fraction were quantitated by densitometry of autoradiograms. (*A*) Phosphorylation levels of Component II in the dark (*filled circles*) or after illumination (*filled squares*). (*B*) The time course of rhodopsin phosphorylation in the same experiment in the dark (*filled circles*) or after illumination (*filled squares*).

Subcellular Distribution of Cyclic Nucleotide-dependent Protein Kinases

Since light selectively affected the membrane-bound pool of phospho-Component II, it was of interest to examine the regulation of this phosphorylation by kinases and phosphatases in more detail.

The subcellular distribution of the ROS kinases was inferred by first fractionating ROS proteins into soluble, peripherally membrane-bound, and tightly membrane-bound protein fractions, and then incubating the fractions with γ-[^{32}P]ATP (Fig. 4). Fractions that contain both the kinase and the substrate should contain phosphoryl-

ated bands stimulated by cyclic nucleotides, whereas fractions that lack either kinase or substrate should not. Addition of exogenous kinase should determine which fractions contain phosphorylatable substrates.

In the soluble fraction, Components I and II were phosphorylated, but not in a cyclic nucleotide–dependent manner (Fig. 4, lanes *1* and *2*). The kinase responsible for this phosphorylation is not known. This result is in contrast to the data obtained

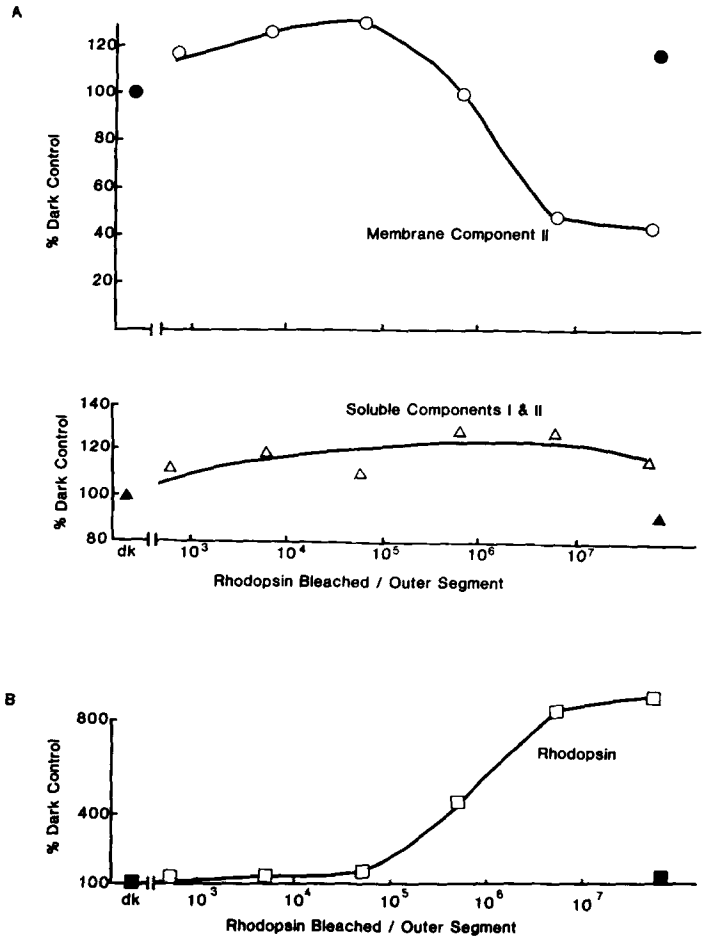

FIGURE 3. Intensity response curve for the effect of light on membrane-bound Component II phosphorylation level. Calibrated flashes of varying intensities were delivered to outer segments that had been incubated for 7 min in the presence of 500 μM 8-bromo-cGMP and 500 μM GTP. Samples were quenched after 1 min and separated by centrifugation into soluble and membrane fractions. Phosphorylation levels of soluble and membrane-bound Component II (*A*), and for comparison, rhodopsin monomer (*B*), were quantitated by densitometry of autoradiograms, and data were expressed as the percent of change compared with the dark control level. Filled symbols are dark control points taken at the beginning and end of the series; open symbols denote samples that received flashes of light.

when the phosphorylation reaction was carried out in whole ROS followed by ROS fractionation (Fig. 1, lanes *4–7*). One possible explanation of this observation is that no active cyclic nucleotide-dependent kinase is present in the soluble fraction. However, addition of exogenous cGMP-dependent protein kinase did not change this result (Fig. 4, lane *3*).

In the peripheral membrane fraction, there was no detectable basal phosphorylation level of Components I and II, suggesting that no other kinases act on the peripheral fraction of Components I and II. Addition of cGMP caused a significant level of phosphorylation (Fig. 4, lanes *4* and *5*). This demonstrates that the peripheral membrane fraction contains cyclic nucleotide–dependent kinase. Exogenous cGMP-dependent protein kinase phosphorylated Components I and II with a different pattern than the endogenous kinase (Fig. 4, lane *6*), suggesting that the endogenous kinase may be different from cGMP-dependent protein kinase.

The pattern of phosphorylation of peripherally bound Components I and II was different in the presence and absence of membranes. In the presence of membranes, phosphorylated Component I was found solely in the soluble fraction and

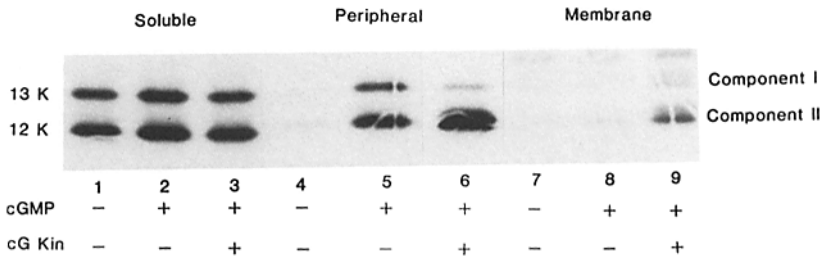

FIGURE 4. Subcellular distribution of endogenous kinase. ROS proteins were fractionated as described in Methods and then incubated with cGMP (500 μM) or cGMP-dependent protein kinase (10 μg/ml) as indicated in the figure. After 8 min of incubation, the reaction was quenched with 20% TCA, and samples were subjected to electrophoresis and autoradiography.

not on membranes (Fig. 1, lane 7). If, however, peripherally bound membrane proteins were stripped from the membrane, Component I was then phosphorylated by the endogenous kinase (Fig. 4, lane *5*).

No phosphorylation of Components I and II, either basal- or cGMP-stimulated, was observed in the integral membrane fraction (Fig. 4, lanes *7* and *8*), suggesting that the kinase had been extracted by hypotonic washes. The washed membranes still contained some Component II, as judged by the ability of the exogenous kinase to phosphorylate it (lane *9*).

Nature of the Endogenous Kinase(s)

To determine the specificity of the cyclic nucleotide–stimulated kinase, the potency of 8-bromo analogues of cAMP and cGMP to stimulate phosphorylation was assessed. Fig. 5 shows that 8-bromo cAMP was 100-fold more potent than 8-bromo-cGMP at phosphorylating Component II in both soluble (*A*) and membrane (*B*) fractions. The higher potency of cAMP with respect to cGMP has previously been

reported by Krapivinskii et al. (1987). The concentrations of various cyclic nucleotides that result in half-maximal stimulation of soluble and membrane-bound Component II is presented in Table II. Similar data were obtained for all the other cyclic nucleotide–stimulated phosphoproteins, with cAMP or its analogues being 100–200-fold more potent than cGMP or its analogues. These data suggest that the endogenous protein kinase is a cAMP-stimulated enzyme.

At similar concentrations of cyclic nucleotides that stimulated phosphorylation of

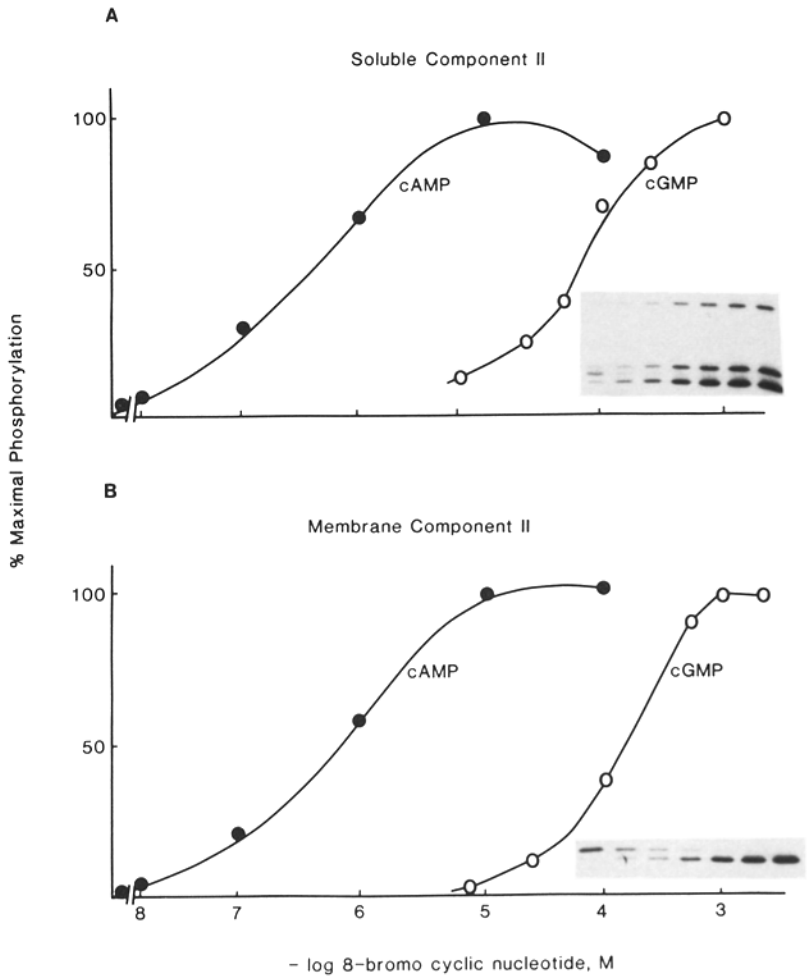

FIGURE 5. Cyclic nucleotide concentration dependence of Component II phosphorylation in soluble and membrane fractions. ROS were incubated with 500 μM GTP and 8-bromo-cyclic AMP or 8-bromo-cGMP (0–1 mM) for 8 min. The reaction was terminated with stop buffer. Then samples were centrifuged for 30 s at 10,000 *g*, quenched with 20% TCA, and subjected to electrophoresis and autoradiography. Phosphorylation levels in each lane were quantitated by densitometry of autoradiograms. (*Insets*) Phosphorylation of Components I and II stimulated by 8-bromo-cGMP.

TABLE II

Potency of Cyclic Nucleotides to Stimulate Component II Phosphorylation

	Apparent K_m Component II phosphorylation	
	Soluble	Membrane-bound
	μM	μM
8-bromo-cAMP	0.37	0.67
8-bromo-cGMP	65	140
cGMP	170	600

ROS were inclubated for 8 min with increasing amounts of cyclic nucleotide and γ-[^{32}P]ATP, and then soluble and membrane fractions were separated and analyzed as in Fig. 5.

Components I and II and the other cyclic nucleotide–stimulated substrates, the inhibition of phosphorylation by cyclic nucleotides of the phosphopeptide 5.72 was released (see inset). Thus there is a reciprocal regulation of this phosphoprotein.

The heat-stable inhibitor (Walsh inhibitor) specifically inhibits the cAMP-dependent but not the cGMP-dependent kinase. The effect of the active synthetic peptide from the Walsh inhibitor, PKI(5-22)amide (Cheng et al., 1986), was tested to further verify the nature of the endogenous kinase (Fig. 6). ROS were incubated with 500 μM 8-bromo-cAMP and various amounts of the inhibitor peptide. The peptide completely blocked the phosphorylation of Component II at a potency similar to that found for blockade of cAMP-dependent protein kinase in other tissues (IC_{50}, 10 nM). A similar potency was found for the other substrates of 21, 55, and 85–90 kD. The inhibitor peptide also reversed the inhibition of 12.5 kD peptide phosphorylation (data not shown). The Walsh inhibitor isolated from skeletal muscle blocked the phosphorylation with a potency similar to its active fragment (data not shown).

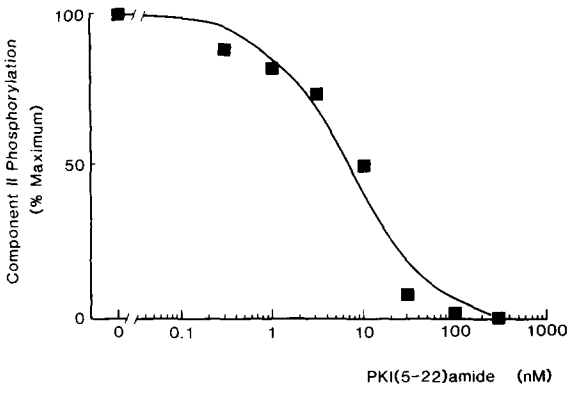

FIGURE 6. The active peptide fragment of the Walsh inhibitor, PKI(5-22), blocks the phosphorylation of Components I and II. ROS were incubated for 8 min with 500 μM 8-bromo-cAMP and the inhibitor peptide (0–200 nM). Samples were prepared as in the legend of Fig. 5. Phosphorylation levels were quantitated by densitometry of autoradiograms. The curve was drawn with a computer-generated fit of the data to a Michaelis-Menten–type equation for a competitive inhibitor, which predicted an IC_{50} of 7 nM.

Identification of ROS Kinases

The above studies demonstrate that the kinase that phosphorylates Components I and II and several other substrates in vitro is a cAMP-dependent enzyme. It is of interest to ascertain whether ROS contain any cGMP-dependent protein kinase, since cGMP levels in ROS are significantly higher than cAMP levels (Ferrendelli and DeVries, 1983), and because light causes rapid changes in cGMP concentration (Cote et al., 1984; Blazynski and Cohen, 1986).

To identify the cyclic nucleotide–dependent kinases present in ROS, photoaffinity labeling studies were carried out. ROS fractions were incubated with [^{32}P]-8-azido-cAMP and kinases were identified by their ability to specifically bind to these probes. Nonspecific binding was detected in the presence of excess cold cyclic nucleotides, either cyclic AMP or cyclic GMP. Covalently attached label was identified by autoradiography after electrophoresis. The soluble fraction (Fig. 7, lanes *1–3*) has label in two bands, at 39 and 50 kD. The label was not competed off with

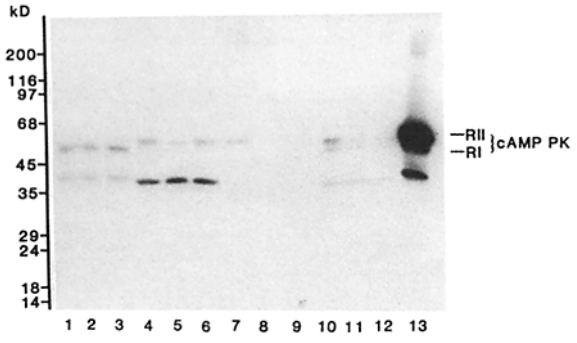

FIGURE 7. Photoaffinity labeling of ROS kinases with [^{32}P]-8-N_3-cAMP. Fractionated ROS proteins or ROS were incubated in the dark for 5 min with [^{32}P]-8-N_3-cAMP in the absence of cold cyclic nucleotides (lanes *1, 4, 7, 10,* and *13*), or in the presence of 10 μM cAMP (lanes *2, 5,* and *8*), or 10 μM cGMP (lanes *3, 6,* and *9*) as described in Materials and Methods. Then they were illuminated with UV light for 1 min. Samples were quenched, precipitated by 20% TCA, and subjected to electrophoresis and autoradiography. Lanes *1–3,* soluble proteins; lanes *4–6,* peripheral membrane proteins; lanes *7–9,* washed membranes; lanes *10–12,* whole ROS; lane *13,* 1 μg cAMP-dependent protein kinase.

cold cyclic nucleotides (Fig. 7, lanes *2* and *3*). The peripheral membrane fraction (Fig. 7, lanes *4–6*) contains a band of 54 kD, which is eliminated by incubation with excess cold cAMP but not with cGMP. A band at 39 kD, which comigrates with G_α, is labeled even in the presence of cold cyclic nucleotides. The integral membrane fraction (Fig. 7, lanes *7–9*) contains a minor band at 54 kD, which is eliminated by preincubation with cold cAMP. Whole ROS (Fig. 7, lanes *10–12*) contain all three bands; only the 54-kD band is specific. The purified cAMP-dependent protein kinase is heavily labeled with [^{32}P]-8-azido-cAMP in lane *13*. Radioactive bands at 54 and 47 kD corresponding to type II and type I regulatory subunits of cAMP-dependent protein kinase are visible in less exposed autoradiograms. The two nonspecific bands comigrate with G_α (39 kD) and arrestin (50 kD); both proteins contain nucleotide-binding pockets and thus could have some affinity for the label. It is clearly seen in less exposed autoradiograms that the 54-kD band present mostly in the peripheral membrane fraction and to a small extent in the integral membrane frac-

tion comigrates with the type II (54 kD) regulatory subunit of cAMP-dependent protein kinase. Thus it appears that ROS contain a cAMP-binding protein with properties similar to type II isozyme of cAMP-dependent protein kinase.

No bands were seen in any fraction in the region of 74 kD that could correspond to cGMP-dependent protein kinase, nor was any band seen that could be more efficiently competed with cGMP than cAMP.

To investigate with an independent method whether frog ROS contain cGMP-dependent protein kinase, cross-reactivity of antisera against the soluble bovine lung cGMP-dependent protein kinase was determined using Western blotting. This antisera cross-reacted with authentic cGMP-dependent protein kinase. Immunostaining of ROS proteins showed that a 68-kD band in the integral membrane fraction comigrated with authentic cGMP-dependent protein kinase, but this band was also stained with preimmune serum, suggesting that the ROS protein was recognized by some other component of rabbit sera (data not shown).

The ability of certain kinases to undergo autophosphorylation can be used to determine their presence in protein mixtures. ROS proteins or purified cGMP-dependent protein kinase were phosphorylated in the presence of [^{32}P]ATP. Autoradiograms of these proteins revealed no phosphorylated ROS band comigrating with autophosphorylated cGMP-dependent protein kinase (data not shown).

DISCUSSION

Physiological Regulation of Phosphorylation Levels

The cyclic GMP concentration in dark-adapted ROS is ~50 μM, and bright light causes a breakdown of 40–50% of this cGMP within a second. (Woodruff and Bownds, 1979; Cote et al., 1984; Blazynski and Cohen, 1986). Decreased cyclic nucleotide levels could deactivate cyclic nucleotide–dependent kinases, and, in the presence of phosphoprotein phosphatases, lead to decreased phosphorylation of substrate proteins. Such light-dependent dephosphorylation of proteins have been observed in mammalian (Lee et al., 1984) and amphibian ROS (Polans et al., 1979; Hermolin et al., 1982). Light-dependent decreases of phosphorylation levels of Components I and II were reported in intact frog retinas by Polans et al. (1979), but it was hard to duplicate the observation in isolated ROS (Hermolin et al., 1982), or at physiological light levels in outer segments with inner segments attached (Brewer and Bownds, 1986). Polans et al. (1979) reported that phosphorylated Components I and II are peripherally bound to membranes. In this report, both a cytoplasmic and membrane-bound pool was found. The subcellular fractionation of ROS kinases and substrates allowed the observation of a light-induced decrease in the phosphorylation of membrane-bound Component II. Release of phosphorylated Components I and II into the cytosol may be exaggerated in the in vitro experiments, where dilution of cytosol after ROS breakage alters the normal equilibrium between membrane binding sites and cytoplasm. Released phosphorylated Components I and II are not light sensitive. This may explain why no effect of light has been reported in vitro, since a large excess of soluble phosphorylated Components I and II would mask a light effect on the membrane-bound form.

A relevant question is which cyclic nucleotide activates this kinase in situ. Dark-adapted ROS contain ~5 μM cAMP (Ferrendelli and Cohen, 1976; DeVries et al., 1978; Cohen, 1982; Blazynski and Cohen, 1984) and ~50 μM cGMP (Orr et al., 1976; Woodruff et al., 1977), and bright light causes ~50% decrease in both cAMP content (DeVries et al., 1978; Blazynski and Cohen, 1984; Cohen, 1982) and cGMP content (Woodruff and Bownds, 1979; Cote et al., 1984; Blazynski and Cohen, 1986). The activation constant for the type II cAMP-dependent protein kinase is 10 nM for cAMP and 2 μM for cGMP (Walter and Greengard, 1981). It seems possible that either cyclic nucleotide could activate the kinase. The light-dependent changes of cyclic GMP and its role in the ROS transduction process are well documented (Liebman et al., 1987). Although the free concentration of cGMP in photoreceptors is not known, estimates based upon electrophysiological measurements of ~5 μM in the dark and <1 μM in the light (Nakatani and Yau, 1988) would allow cGMP activation of the cAMP-dependent protein kinase in the dark and inactivation in the light.

Cyclic AMP appears to play a role in light-dependent adaptational movements in both rods and cones in lower vertebrates (Burnside et al., 1982; Dearry and Burnside, 1985), and light-dependent and circadian ROS turnover (Besharse et al., 1982; Pierce and Besharse, 1985). Adenylate cyclase activity is present in photoreceptors in both rod- and cone-dominant retinas, and can be stimulated by the hydrolysis-resistant GTP analogue Gpp(NH)p and several neurotransmitters (DeVries et al., 1982; Cohen and Blazynski, 1987; Blazynski et al., 1986). Several neurotransmitter receptors have been reported to be present on ROS plasma membranes including D_2-dopamine receptors (Brann and Jelsema, 1985; Brann and Young, 1986; Cohen, 1989), insulin-like growth factor (Zick et al., 1987), acidic fibroblast growth factor (Plouet et al., 1988), and an indoleamine receptor (Cohen and Blazynski, 1987) similar to the D_2-dopamine receptor (Cohen, 1989). Adenylate cyclase-coupled receptors, or other unknown physiological stimuli, could thus regulate cAMP concentrations in ROS, resulting in activation of the ROS cAMP-dependent protein kinase to these changes. Components I and II, as well as the other substrate phosphoproteins of M_r 21, 50–52, and 65–70 are candidates for a role in the regulation of any of these processes in amphibians.

Subcellular Distribution of Kinases and Kinase Substrates in ROS

An interesting paradox was noted in the data shown in Figs. 1 and 4. In the soluble fraction, Components I and II are not substrates for cyclic nucleotide–dependent kinases and the cyclic nucleotide–stimulated kinase is peripherally bound to membrane (Fig. 4). However, after incubation of ROS with γ-[^{32}P]ATP and 8-bromo-cGMP, most phosphorylated Components I and II are in the soluble fraction (Fig. 1, lanes *6* and *7*). It seems plausible that the locus of phosphorylation is the membrane, and that phosphorylated Component II can be released from the membrane into the soluble fraction. In fact, release of phosphorylated Components I and II from membranes was experimentally demonstrated. After washing away cytoplasmic components, reincubation of ROS membranes with γ-[^{32}P]ATP and cGMP stimulated phosphorylation of Components I and II, which partitioned into the superna-

tant fraction after centrifugation. This cycle of washing and reincubation was repeated several times, and each time a similar amount of phosphorylated Components I and II were released.

In other experiments in which sufficient protein was loaded onto gels to detect Components I and II by Coomassie blue stain, the bulk of stained Components I and II were found on the membrane, whereas most radiolabeled phosphorylated Components I and II were in the soluble fraction.

All of these data support the notion that the unphosphorylated pool of Components I and II is found on the membrane, where a membrane-bound kinase phosphorylates a small subfraction of the Components which are then released from the membrane. However, the studies reported here cannot shed light on such mechanisms, since only phosphorylation levels were measured. To further understand the regulation of membrane association of Components I and II, and implications of possible redistribution between membrane and soluble pools for its function, more sensitive methods of quantitation of protein levels of Components I and II must be found. In this regard, recently several monoclonal antibodies against Components I and II have been produced and are currently being characterized (Salov, L., K. Suh, and H. Hamm, unpublished).

ROS Kinases

Several facts suggest that the ROS cyclic nucleotide–dependent kinase may be a cAMP-dependent protein kinase. (*a*) Cyclic AMP is more potent than cGMP at stimulating Components I and II phosphorylation. (*b*) The active peptide of the protein kinase inhibitor (Walsh inhibitor), which specifically inhibits the cAMP-dependent protein kinase (Cheng et al., 1986), inhibits the phosphorylation potently. (*c*) Cyclic GMP–dependent protein kinase (purified from bovine lung) has a different substrate specificity than the endogenous kinase, selectively phosphorylating Component II over Component I (Fig. 4, lanes *5* and *6*), which suggests that the endogenous kinase is not identical to cGMP-dependent protein kinase. (*d*) Cyclic AMP–dependent protein kinase is known to be present in bovine (Lee et al., 1981), rat (Lee et al., 1984), and frog ROS (Yamazaki et al., 1980). The fact that a band of 54 kD was specifically radiolabeled by the photoaffinity ligand [^{32}P]8-N_3-cAMP is evidence that frog ROS contain the RII isoform of cAMP-dependent protein kinase.

In this study, no endogenous cGMP-dependent protein kinase was detected in ROS by a variety of techniques, including photoaffinity labeling, immunoblotting with antisera against the soluble bovine lung enzyme, or phosphorylation at the molecular weight where kinase autophosphorylation would occur. The most conclusive evidence that a ROS-specific cyclic GMP–dependent protein kinase is not active in these experiments is complete inhibition of endogenous phosphorylation by the Walsh inhibitor peptide, which specifically inhibits cAMP-dependent protein kinases.

In experiments in which ROS were first fractionated and then phosphorylated, Components I and II in the soluble fraction were phosphorylated in a cyclic nucleotide–independent manner. The level of phosphorylation in this fraction was never stimulated by cyclic nucleotides and was variable between experiments. Two possibilities may be suggested to explain the phosphorylation of soluble Components I

and II. (*a*) Another kinase may phosphorylate soluble Components I and II. Hayashi et al. (1987) have reported a phosphatidylinositol kinase that phosphorylates soluble Components I and II. Binder et al. (1989) showed that Components I and II are substrates of protein kinase C. (*b*) Alternatively, the kinase in the soluble fraction is not a different kinase, but one that is rendered independent of cAMP regulation by some mechanism such as proteolysis or autophosphorylation. This type of kinase regulation is known to occur in several other systems (Keeley et al., 1975; Hirota et al., 1985; Saitoh and Schwartz, 1983). Purification and characterization of the kinases from the two fractions will be required to resolve these possibilities.

Studies on the chemical specificity of the ROS cyclic nucleotide–dependent kinase suggest that this kinase is similar to other cyclic nucleotide–dependent protein kinases. The chemistry of the active cyclic nucleotide analogues is similar to analogues activating other cyclic nucleotide–dependent protein kinases (Takio et al., 1984; Corbin et al., 1986). The 8′-substituted analogues are more effective, and bulky side groups are more effective than small ones, probably because they favor the *cis*-conformation of molecule. The 8′-substituted cGMP analogues are also more potent at activating the ROS light-sensitive channel (Yau and Nakatani, 1985; Zimmerman et al., 1985; Stern et al., 1986) and the cGMP-induced conductance on disc membranes (Caretta et al., 1985; Koch and Kaupp, 1985). 2-deoxy-cGMP is ineffective in all tested systems (Caretta et al., 1985; Koch and Kaupp, 1985; Corbin et al., 1986). That the cyclic nucleotide binding sites on the kinase and the channel have similar stereochemistry suggests that these molecules might be homologous (Takio et al., 1984).

Physical Characterization of Phosphorylated Components I and II

The relationship between Components I and II and the reason for preferential binding of phosphorylated Component II to the membrane are not known. Shinozawa and Yoshizawa (1986) suggested that the Components may exist as a dimer, since the native molecular weight in sucrose gradients is double the size determined in sodium dodecyl sulfate gels. The differential membrane localization of phosphorylated Component II compared with Component I suggests that phosphorylated Components I and II do not form a complex on the membrane.

Analysis of phosphorylated Components I and II on two-dimensional gels shows that the pI of the phosphorylated Components is 6.2, while for the unphosphorylated Components it is 6.5. The presence of only one phosphorylated spot suggests that Components I and II are phosphorylated on a single residue by the cAMP-dependent protein kinase. Shinozawa and Yoshizawa (1986) reported that this phosphorylation occurs on serine residues.

Relationship of Components I and II with G Protein

Robinson et al. (1986) showed that cGMP increases the affinity of G protein for GTP relative to GDP, an effect that has been suggested to play a role in the desensitization of the cGMP cascade to light. In those experiments, cGMP had no effect on the affinity of the G protein for GTP or GDP alone; both GTP and GDP were required. The half-maximal cGMP concentrations needed for this effect are very

similar to those needed for stimulation of phosphorylation of Components I and II, suggesting a possible role for these phosphorylations.

A relationship between Components I and II and G protein was previously suggested by Hamm and Bownds (1984). They found a monoclonal antibody (MAb), 4A, cross-reactive with the α subunit of the G protein, which blocks G protein activation by light. The antibody also blocked the phosphorylation of Components I and II. Further evidence for a relationship between Component II and G protein comes from the finding that the addition of excess purified G protein to the membrane increases the amount of phosphorylated Component II bound to the membrane (Hamm, 1987).

Suh and Hamm (1988) reported further evidence for a physical interaction between G protein and Components I and II. They showed that MAb 4A elutes G protein and Component II from the membrane with a similar time course. They also separated frog ROS proteins in sucrose density gradients and found that G protein and Components I and II comigrate under all conditions. Under conditions in which G protein subunits dissociate, Components I and II comigrate with the $\beta\gamma$ subunits.

In bovine ROS, a 33-kD protein whose phosphorylation is stimulated by cGMP also forms a complex with $G\beta\gamma$ (Lee et al., 1987). This protein is also phosphorylated by cAMP-dependent protein kinase (Lee et al., 1981), and light causes its dephosphorylation in rat retina or ROS (Lee et al., 1984). When Lee et al. (1987) purified the 33-kD protein, they found it associated in a 1:1 stoichiometric complex with $G\beta\gamma$. This protein is localized to ROS and rod inner segments (Lee et al., 1988). We have attempted to find a 33-kD protein in frog ROS whose phosphorylation can be stimulated by cyclic nucleotides, but we have never seen evidence for this protein. Conversely, we have looked for evidence of Components I and II in mammalian retinas and ROS, and have not found them. Thus it is possible that Components I and II and the 33-kD phosphoprotein are functional homologues in amphibians and mammals.

The effect of the binding of the Components to G protein is not known. One candidate reaction that might be modulated by Components I and II is the ATP-dependent turnoff of cGMP phosphodiesterase (Liebman and Pugh, 1980). This ATP-dependent turnoff is commonly thought to be mediated by rhodopsin phosphorylation. However, cGMP is always included in this assay for the measurement of phosphodiesterase, and thus the cyclic nucleotide–dependent kinase is also activated. The phosphodiesterase turnoff has been reconstituted using purified rhodopsin, G protein and phosphodiesterase, and a partially purified rhodopsin kinase (Sitaramayya and Liebman, 1983; Sitaramayya, 1986). It is clear from these studies that rhodopsin phosphorylation provides an important mechanism for phosphodiesterase turnoff. Whether cyclic nucleotide–dependent kinase activation also plays a role in phosphodiesterase turnoff has not been examined, since the partially purified rhodopsin kinase preparation may also contain cyclic nucleotide–dependent kinase. We are currently examining this point further using purified components. These studies should clarify whether cyclic nucleotide–dependent protein phosphorylation plays a role in the turnoff of the light-activated cGMP cascade of visual transduction.

Other functions of Components I and II interaction with G protein are also pos-

sible. (*a*) Phosphorylation-dependent interaction of Components I and II with G protein could play a role in the down-regulation of the excitatory cascade during light adaptation. (*b*) Interaction of Components I and II with $\beta\gamma$ could represent a branch point in the excitatory pathway, with the $\beta\gamma$-phosphoprotein complex modulating some other ROS process. This possibility has been suggested for the 33-kD protein of mammals (Lee et al., 1987). The relationship between G protein and Components I and II is being investigated further using purified components to test these possibilities.

A Potential Physiological Role of Phosphorylation in ROS

Under the in vitro conditions of these experiments, light caused a 60% decrease in membrane-bound phospho-Component II. The kinetics of the light-induced changes in Component II phosphorylation were fast enough that Component II could be involved in the modulation of rapid physiological processes in ROS. Polans et al. (1979) demonstrated that in vivo, agents that increase cGMP concentrations increase the phosphorylation of Components I and II, suggesting that cGMP could indeed be an endogenous activator of the kinase. As stated earlier, the levels of both cAMP and cGMP do change in response to light and other stimuli within a range of concentrations that would modulate this kinase activity. Thus these phosphorylations could play a role in cAMP- or cGMP-mediated processes. One particularly intriguing possibility is that the phosphorylation of Components I and II serves an integrating function in the modulation of both cAMP and cGMP-mediated processes in ROS.

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