# A Monoclonal Antibody to Guanine Nucleotide Binding Protein Inhibits the Light-activated Cyclic GMP Pathway in Frog Rod Outer Segments

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ABSTRACT A monoclonal antibody that blocks the light-activated cyclic GMP (cGMP) pathway in frog photoreceptor outer segments (ROS) has been obtained. The antibody (4A) inhibits guanine nucleotide binding to G-protein, the intermediate that links rhodopsin excitation to cGMP phosphodiesterase (PDE), inhibiting light-induced PDE activity as a consequence. Antibody inhibition of the light-activated cGMP pathway is complete at a stoichiometry of approximately one antibody per G-protein in the mixture, which indicates high specificity of the inhibition. Inhibition is more pronounced than that caused by PDE inhibitors such as isobutylmethylxanthine (IBMX) or Ro 20-1724. Antibody 4A has the further effect of inhibiting the phosphorylation of two low molecular weight proteins, components I and II, whose phosphorylation normally can be stimulated by raising cGMP levels. The inhibition is not overridden by adding cGMP, which suggests that the G-protein influences these phosphorylations by a pathway distinct from its action on cGMP concentration. Antibody 4A may prove useful as a probe of the relevance of the cGMP pathway to visual transduction in living photoreceptors. Six other monoclonal antibodies to G-protein, as well as six monoclonal antibodies to rhodopsin and one to PDE, do not block light-activated guanine nucleotide binding, PDE activity, or ROS protein phosphorylations.

## INTRODUCTION

In recent years, biochemical and physiological evidence has suggested a role of cyclic GMP (cGMP) in visual transduction. Guanine nucleotide binding protein (G-protein) and phosphodiesterase (PDE) make up two-thirds of the total nonrhodopsin protein in the frog rod outer segment (ROS) (Hamm, H. E., and M. D. Bownds, manuscript submitted for publication). Light activates these proteins very rapidly (<100 ms) (Liebman and Pugh, 1979; Kuhn et al., 1981; Bennett,

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1982) with a high stoichiometry (100–500 G-protein molecules activated per rhodopsin bleached) (Fung and Stryer, 1980). The light-activated cGMP decrease measured in isolated ROS (Woodruff and Bownds, 1979) and in physiologically active ROS with inner segments attached (Biernbaum, M. S., C. S. Schobert, and M. D. Bownds, manuscript submitted for publication) has correspondingly fast kinetics and a high stoichiometry (Cote et al., 1984).

Cyclic GMP can influence the physiological light response. Bathing the retina with the PDE inhibitor isobutylmethylxanthine (IBMX) causes a depolarization of the plasma membrane that is suppressible by light (Lipton et al., 1977; Capovilla et al., 1982). Intracellular injection of cGMP increases the latency of the light response (Miller and Nichol, 1979; Miller, 1982). Although these findings suggest that cGMP may be involved in visual transduction (Miller, 1982), there is no simple relationship between cGMP levels and the light-induced electrical response (Meyertholen et al., 1980; Woodruff and Fain, 1982). Calcium is also thought to play a central role in transduction (Hagins, 1972), for it is rapidly extruded from outer segments upon illumination (Gold and Korenbrot, 1980; Yoshikami et al., 1980). Calcium and cGMP levels are interrelated in the outer segment cytoplasm (Cohen et al., 1978; Woodruff and Bownds, 1979; Polans et al., 1979, 1981; Kilbride, 1980; Cavaggioni and Sorbi, 1981; Woodruff and Fain, 1982; Hermolin et al., 1982), but the details of this interaction are not known.

To establish what pathways regulate transduction in vertebrate photoreceptors, it would be useful to obtain mutational blocks at a large number of different steps in the process. Use of such blocks has been crucial in establishing the sequence and branching pattern of complex metabolic pathways in microorganisms (Tatum, 1959; Yanofsky, 1964). An alternative has been to use drugs to block known reactions (for example, the use of IBMX to inhibit PDE), but the nonspecific effects of this and other pharmacological agents (Wells and Kramer, 1981) have made interpretation of data difficult.

Recent studies suggest that antibodies may prove to be generally useful as specific blocking agents. Conventional sera and, more recently, monoclonal antibodies have been found that bind specifically and tightly to the active sites of their target enzymes, thereby blocking their function (Frackelton and Rotman, 1980; Clark et al., 1981; Kaltoft et al., 1982; Kiehart et al., 1982). Some antibodies also restore function to mutant nonfunctional enzymes (Frackelton and Rotman, 1980). Inactivating monoclonal antibodies have been used to study enzyme mechanisms (Mountford et al., 1982), to identify the function of proteins (Jay et al., 1981; Clark et al., 1981), to investigate structure-function relationships on a protein's surface (Tzartos et al., 1981; Gabay and Schwartz, 1982), and to probe the intracellular role of particular enzyme activities (Kiehart et al., 1982).

This paper describes the actions of an antibody to the guanine nucleotide binding protein (G-protein) that blocks light-activated guanine nucleotide binding, cGMP phosphodiesterase activity, and the phosphorylation of components I and II. This antibody may prove useful in evaluating the importance of the cGMP pathway in transduction. The companion paper (Witt et al., 1984) provides

background material on generating and characterizing a first series of antibodies to the more prominent proteins. A subsequent paper will describe the effect of these antibodies on the physiological light response.

## MATERIALS AND METHODS

### *Reaction of Antibodies with ROS Proteins*

Details of frog (*Rana catesbeiana*) maintenance, retinal dissection, and ROS purification in Percoll step gradients are described in another paper (Hamm, H. E., and M. D. Bownds, manuscript submitted for publication). The characterization and purification of the monoclonal antibodies used in these studies are described in Witt et al. (1984). To allow antibody access to the cell interior, 20 μl of Percoll-purified intact ROS (~100 μM rhodopsin) are disrupted and permeabilized by passage through a 26-gauge needle in the presence of a purified antibody (~50 μM monoclonal IgG). Routinely a 10-fold excess of antibody over G-protein is used. This preparation is allowed to incubate for 15–30 min at room temperature to allow for antibody binding. The reaction mixture contains 50 kallikrein inhibitor units of Trasylol (aprotinin; FBA Pharmaceuticals, New York), a protease inhibitor. This serves to keep the dark level of PDE activity constant during the incubation step. Control treatments include Ringer's solution, commercially available IgG, and IgG purified from ascites fluid of mice carrying tumors induced by the NS-1 parent cell line (Witt et al., 1984).

### *PDE Activity Assay*

The proton evolution assay of PDE, first developed by Yee and Liebman (1978) and used in this laboratory by Kawamura and Bownds (1981), is used to screen for antibody effects. The assay takes advantage of the fact that for every cGMP cleaved by the PDE, a proton is produced. ROS are permeabilized as described above and incubated with antibody for 30 min, and then PDE activity is measured. The standard assay conditions are 4 mM cGMP substrate, 0.5 mM ATP, 0.5 mM GTP, 2.78 mM EGTA (free calcium, $10^{-9}$ M) in 200 μl frog Ringer's solution (115 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Hepes, pH 7.8) containing 5–7 μM rhodopsin; variations in conditions are specified in the figure legends. Proton evolution is monitored continuously, before and after flashes of light of increasing intensity. The maximal rate of proton evolution after a flash is defined as the flash response. Between pH 7.5 and 8.0, the pH change measured by a pH microelectrode is a linear function of PDE activity when the rhodopsin concentration is ~5–7 μM. The buffering of this pH change by any component of the assay mixture was determined as in Kawamura and Bownds (1981). Addition of antibodies does not change the buffering of the mixture. PDE activity is expressed as moles of cGMP hydrolyzed per mole rhodopsin per minute. The rhodopsin concentration was determined by difference spectroscopy (Bownds et al., 1971).

### *Guanine Nucleotide Binding*

Binding is measured by a method modified from Fung and Stryer (1980), which involves specific binding of labeled GTP to ROS membranes followed by washing away unbound nucleotide on Millipore filters and measuring bound nucleotide. To 50 μl of permeabilized ROS, prepared as described above, which have been incubated for 30 min with specific or control antibody, is added 200 μl of a reaction mixture containing 10 μM [8-$^3$H]GTP, 10 μM GDP, 1 mM cGMP, with various amounts of calcium (see figure legends). Aliquots are filtered through Millipore filters (type HA, 0.45 μM) in the dark before and at various

times after a flash of light; then filters are washed with Ringer's solution and bound tritium is measured by liquid scintillation counting.

*Protein Phosphorylation*

A mixture of 20 μl purified ROS disrupted as described above in the presence of specific or control antibody is incubated for 10 min with 30 μl of 10 μM [$\gamma^{32}$P]ATP (10 μCi), and various concentrations of GTP, cGMP, and calcium, depending on the experiment. After light treatment, samples are quenched with 200 μl 10% trichloroacetic acid, dissolved in solubilization solution (Polans et al., 1979), and subjected to electrophoresis and autoradiography according to the methods of Polans et al. (1979) and H. E. Hamm and M. D. Bownds (manuscript submitted for publication).

## RESULTS

*An Antibody to the Photoreceptor G-Protein Inhibits the Light-activated cGMP Pathway*

Monoclonal antibodies to several outer segment proteins (six to rhodopsin, seven to G-protein, and one to PDE) were examined for their effects on the cGMP pathway. These antibodies are characterized in the preceding paper (Witt et al., 1984). PDE activity was measured because it might be altered by antibody-induced perturbation at any of several earlier steps (rhodopsin–G-protein interaction, GTP binding, $G_\alpha$-$G_{\beta\gamma}$ subunit dissociation, or $G_\alpha$-PDE interaction; see Fung et al., 1981). ROS were disrupted in the presence of antibody to allow access to cytoplasmic space, and PDE activity was then measured.

Fig. 1 summarizes the effects on PDE activity of seven different G-protein antibodies, indicated by the different symbols used. (The antibodies bind to the $\alpha$ subunit of the G-protein [Witt et al., 1984], which contains the GTP binding site [Fung et al., 1981].) After a 30-min preincubation with antibody, PDE activity was measured in the dark (left set of data points). Then the maximal activity elicited by flash intensities causing threshold, intermediate, and near-saturating responses was determined. The dark- and light-stimulated PDE activities are two- to threefold higher than those reported by Kawamura and Bownds (1981) because lower rhodopsin concentrations were used (cf. Robinson et al., 1980) and the preparations aged during Percoll purification and preincubation with antibody (cf. Kawamura and Bownds, 1981).

All of the antibodies decrease the amplitude of light-induced PDE activity to some degree, and a complete inhibition is observed with the antibody designated 4A (bottom curve, filled circles). The PDE inhibition caused by 4A is complete at low and intermediate light levels, but at the highest light intensities, at which nearly all of the G-protein should be activated, a small light activation of PDE can be seen. This suggests that either a small pool of G-protein is inaccessible to the antibody, or that antibody inhibition is not complete. Control immunoglobulin not directed against ROS proteins (top curve, open triangles) has little effect on PDE activity, compared with Ringer's solution (data not shown). Fig. 1 shows slight effects of the antibodies on dark levels of PDE activity. In most experiments, no significant differences were found between dark levels of control and specific antibodies (see, for example, Table I).

G-protein activity is assayed more directly in the experiment of Fig. 2, in which guanine nucleotide binding to photoreceptor membranes is measured. This figure shows that antibody 4A can suppress light-activated guanine nucleotide binding. Permeabilized ROS were preincubated in the presence of control immunoglobulin (open symbols) or antibody 4A (closed symbols). Dark levels (left) of nucleotide binding are comparable in the two conditions, but antibody 4A suppresses >80% of light activation. This figure further shows that the antibody block is maintained through calcium concentration changes known to influence the PDE pathway (Kawamura and Bownds, 1981). The small differences in GTP binding caused by changing $Ca^{++}$ concentrations in the control condition are not significant. At lower light intensities, the effect of illumination

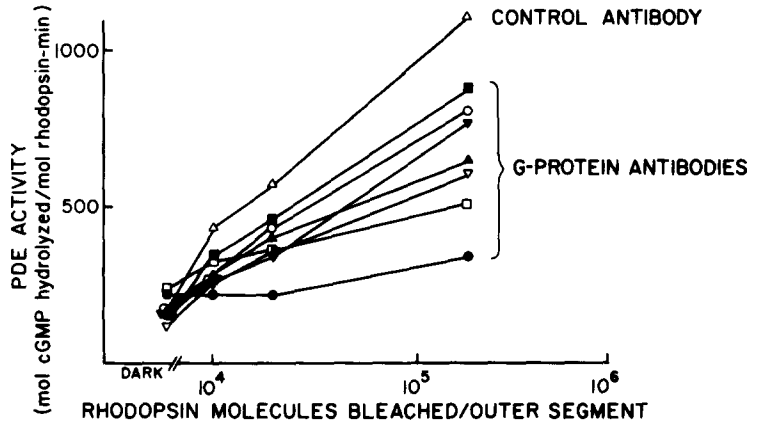


FIGURE 1. Several antibodies to G-protein influence light-activated PDE activity. Percoll-purified ROS were disrupted in the presence of antibody by passage through a 26-gauge needle and then incubated at a concentration of 50 $\mu$M rhodopsin, 25 $\mu$M antibody for 30 min at room temperature. Portions (20 $\mu$l) were diluted to 200 $\mu$l (final concentration, $10^{-9}$ M $Ca^{++}$, 0.5 mM GTP, 0.5 mM ATP, 5–7 $\mu$M rhodopsin in frog Ringer's, pH 7.8) and immediately assayed for PDE activity in the dark and after flashes of light of increasing intensities.

is more completely blocked by antibody 4A (data not shown). Thus, antibody 4A inhibits light-activated nucleotide binding to G-protein without changing binding in the dark.

Several other antibodies were screened for effects on PDE activity, including six antibodies to rhodopsin and one to PDE. Two of the rhodopsin antibodies decreased both dark- and light-activated PDE by ~30%, while the other had no effect. The PDE antibody had no effect on PDE activity.

### *Characterization of Antibody 4A Inhibition*

Fig. 3*A* shows that the antibody inhibition, measured by its effect on PDE activity, is evident by 5 min incubation with antibody 4A, and is essentially complete by 10 min. Thus, the antibody binding is rapid, a characteristic

necessary for experiments in which the antibody is introduced into living photoreceptors to examine its effects on the physiological light response. Fig. 3*B* demonstrates that the inhibition decreases with antibody dilution. The stoichiometry at which maximal inhibition occurs is approximately one antibody per G-protein molecule. (This is calculated by determining antibody concentration by absorbance at 280 nm [assuming $A^{1\%}_{1\,cm} = 13.5$; Kirschenbaum, 1973], rhodopsin concentration by difference spectroscopy [Bownds et al., 1971], and G-protein concentration relative to rhodopsin by the quantitation study in another paper

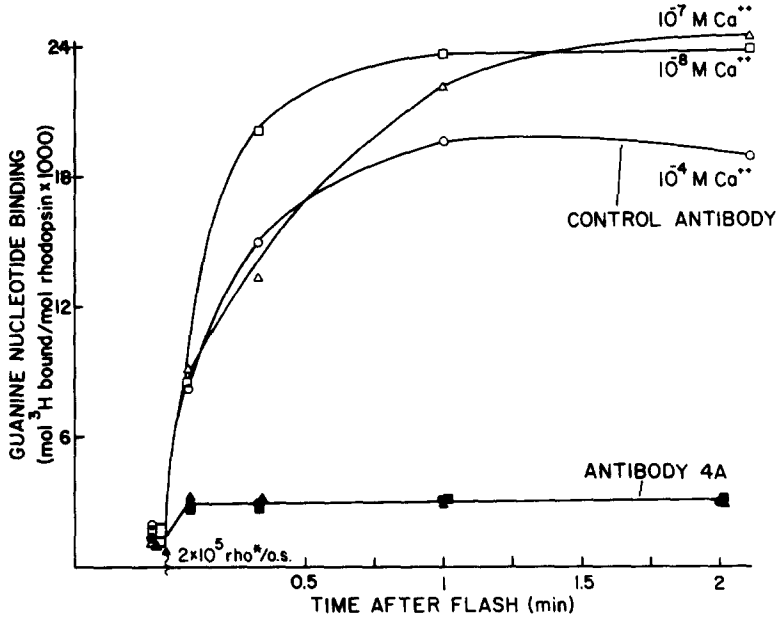


FIGURE 2. G-protein antibody 4A inhibits light-activated guanine nucleotide binding. Here, the effect of a near-saturating light on guanine nucleotide binding was measured after preincubation with commercially obtained nonspecific IgG (open symbols) or purified antibody 4A (closed symbols). Percoll-purified intact ROS were disrupted and incubated with antibody as in Fig. 1. Then a reaction mixture containing (final concentrations) 10 μM GTP (0.1 μM [8-³H]GTP), 10 μM GDP, 1 mM cGMP, and either $10^{-4}$ M (circles), $10^{-7}$ M (triangles), or $10^{-8}$ M (squares) calcium was added and guanine nucleotide binding was measured in the dark and after a flash of light bleaching $2 \times 10^5$ rhodopsin/outer segment by filtration through Millipore filters.

[Hamm, H. E., and M. D. Bownds, manuscript submitted for publication].) This result, combined with the lack of effect of control antibody, suggests that the inhibition is a specific antibody effect and is not caused by a contaminant in the purified antibody preparation. It further shows that the antibody binds strongly to G-protein.

The data shown in Table I contrast the inhibition of PDE caused by antibody 4A with that obtained by adding two competitive inhibitors of PDE used in

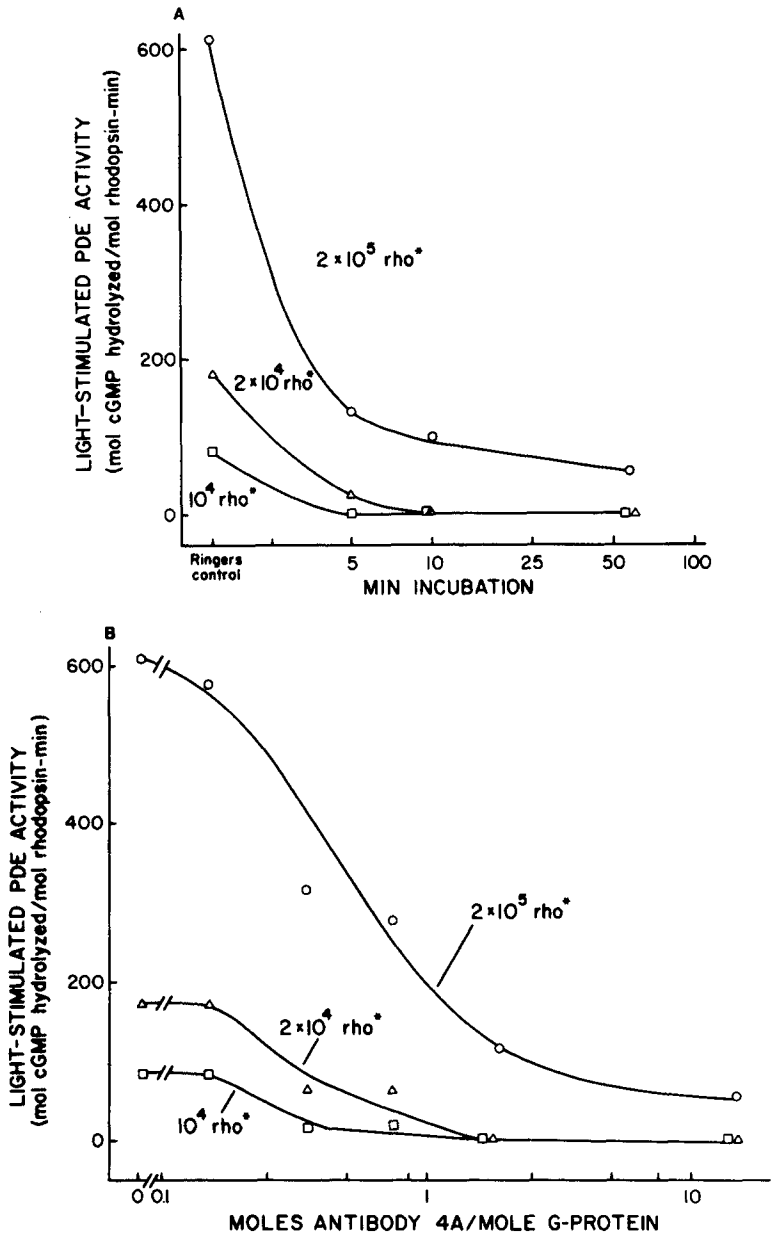


FIGURE 3. Time course and stoichiometry of antibody 4A inhibitory effect on PDE activity. (*A*) The antibody inhibition is apparent as soon after antibody addition as PDE activity can be measured, and is essentially complete after 10 min incubation. Permeabilized ROS were preincubated with antibody 4A for various amounts of time, and then PDE activity was measured under standard conditions. (*B*) Antibody inhibition is essentially complete when the G-protein antibody ratio is 1:1.5 (at a concentration of 3 $\mu$M G-protein, 4.5 $\mu$M antibody). Here, permeabilized ROS were preincubated for 30 min with the normal antibody concentration (15-fold molar excess) or with antibody diluted in Ringer's solution. Circles: flash bleaching $2 \times 10^5$ rhodopsins/outer segment; triangles: $2 \times 10^4$ rhodopsins bleached/outer segment; squares: $10^4$ rhodopsins bleached/outer segment.

recent electrophysiological studies (Lipton et al., 1977; Capovilla et al., 1982). The antibody inhibits only the light-activated PDE without perturbing dark activity. The drugs, even at high concentration, decrease dark and illuminated PDE activity to a similar degree and the relative activation by light over dark levels is unchanged (~300%). Thus, the effects of these drugs on the light-stimulated PDE are neither as potent nor as specific as the antibody effect.

*Antibody 4A Inhibits the Phosphorylation of Components I and II*

Cyclic nucleotide pathways frequently regulate multiple protein phosphorylations (cf. Cohen, 1982), and thus it is relevant to examine the effect of antibody 4A, as well as all of the other antibodies obtained, on the numerous protein phos-

TABLE I

*Effects of PDE Inhibitors on PDE Activity*

| Addition | | PDE activity* (moles cGMP hydrolyzed per mole rhodopsin per minute) Dark | Light | Percent light stimulation |
|---|---|---|---|---|
| | | | *2 × 10⁴ rhodopsins bleached* | |
| Ringer's solution | | 68.6±6.4 (5)‡ | 200±30 (5) | 292 |
| 4A | | 66±6 (3) | 66±6 (3) | 0 |
| IBMX | 4 mM | 26§ | 64 | 246 |
| | 2 mM | 26 | 56 | 215 |
| | 0.5 mM | 30 | 89 | 297 |
| | 0.25 mM | 40 | 116 | 290 |
| Ro 20-1724¶ | 4 mM | 38 | 95 | 232 |
| | 2 mM | 33 | 125 | 379 |
| | 0.5 mM | 40 | 149 | 373 |

* Assayed as described in Materials and Methods and the legend to Fig. 1.

‡ Numbers denote $x \pm$ SD ($n$).

§ Numbers denote an average of two determinations that were within 10% of each other.

¶ *d,l*-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone.

phorylations observed in the outer segment, particularly those that have been shown to be regulated by cGMP, light, or calcium (Hermolin et al., 1982). Fig. 4 shows one experiment in which disrupted ROS were incubated with Ringer's solution, control antibody, or G-protein antibodies and with [$\gamma^{32}$P]ATP in both the dark (left lane of each pair) and during illumination bleaching 1% of the rhodopsin present (right lane of each pair). The dark bands seen in the autoradiogram of the sodium dodecyl sulfate (SDS) gel indicate the typical protein phosphorylation patterns observed. In these disrupted ROS preparations, light causes phosphorylation of rhodopsin, but a dephosphorylation of two small proteins (components I and II) that occurs in vivo is lost (cf. Polans et al., 1979).

One might expect antibody 4A, because it inhibits only light-activated degradation of cGMP, to result in either no change or a slight increase in cGMP levels. Because cGMP stimulates phosphorylation of components I and II (Polans et al., 1979; Hermolin et al., 1982), corresponding effects in these phosphorylations should be observed. The opposite result is obtained; addition of 4A causes

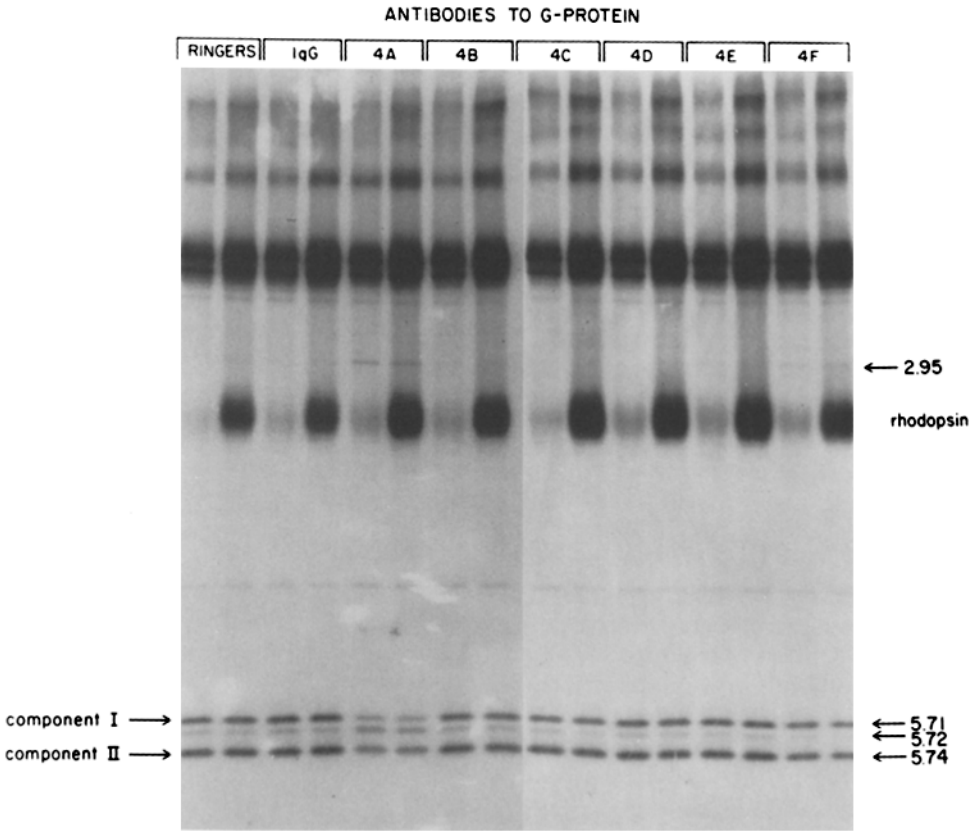


FIGURE 4. Antibody 4A has a specific inhibitory effect on component I phosphorylation and, to a lesser extent, component II phosphorylation. Six different G-protein antibodies, as well as nonspecific antibody and control Ringer's solution, were preincubated with disrupted outer segments as described in the Fig. 1 legend. Their effects on protein phosphorylation patterns were measured by incubation with [$\gamma^{32}$P]ATP in frog Ringer's solution containing $10^{-9}$ M calcium, followed by either 1 min continuous light bleaching $3 \times 10^7$ rhodopsins/outer segment (right lane of each pair) or dark (left lane of each pair).

inhibition of the phosphorylation of component I and, to a lesser extent, of component II in both the light and the dark (Fig. 4, lanes 5 and 6). In contrast, altering cGMP levels causes equal effects on the two phosphorylations (Polans et al., 1979; Hermolin et al., 1982). In addition to its inhibitory effect on the phosphorylation of components I and II, antibody 4A occasionally stimulated

the phosphorylation of proteins of 12,500 and 50,000 daltons (5.72P, four out of seven experiments, and 2.95P, one out of seven experiments, arrows, Fig. 4; see Hamm, H. E., and M. D. Bownds, manuscript submitted for publication). Other G-protein antibodies had no effect on phosphorylation levels of any protein (lanes 7–16).

The independence of the effects of cGMP and antibody 4A is further shown in Fig. 5, which demonstrates that 4A inhibition of phosphorylation of components I and II is not altered by cGMP addition. Thus, the antibody blocks the cGMP stimulation of the phosphorylation that can normally be observed in disrupted ROS. This same result is obtained in both high ($10^{-3}$ and $10^{-4}$ M) and low ($10^{-8}$ and $10^{-9}$ M) calcium (data not shown). Cyclic AMP also cannot override the antibody effect (data not shown).

Another interpretation of the antibody inhibition of the phosphorylation level of components I and II is that the antibody blocks dephosphorylation, and thus does not allow phosphate turnover and incorporation of radioactive phosphates. However, the data of Fig. 5, which show the stimulation by cGMP of the

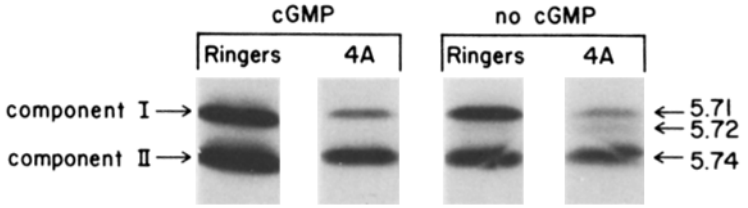


FIGURE 5. Antibody 4A inhibits component I and, to a lesser extent, component II phosphorylation independent of cGMP concentration. Here, the effects of antibody 4A or control Ringer's solution are measured in the dark either with or without 1 mM cGMP. Rods were treated as in Fig. 4.

phosphorylation of components I and II in the control lanes, suggest that more phosphorylatable sites are present.

Antibodies to rhodopsin and PDE, as well as to G-protein, were screened for effects on phosphorylation. None of the antibodies tested had any effect on rhodopsin phosphorylation or on any other protein phosphorylation, either in the light or dark.

## DISCUSSION

Antibody 4A, directed against G-protein, presents several useful features as an inhibitor of the light-sensitive cGMP pathway when compared with PDE inhibitors now in common use. Because it does not alter the dark activity of PDE, its introduction into a living cell might be expected not to perturb cGMP levels or aspects of dark current regulation that depend on these levels. Addition of PDE inhibitors to the system, on the other hand, causes a rise in dark cGMP levels and an increase in plasma membrane permeability (cf. Capovilla et al., 1982). A further characteristic of the antibody is that it causes almost complete inhibition

of the light-activated PDE activity, while the inhibition caused by even millimolar levels of PDE inhibitors does not exceed 50–70%.

If the light-induced cGMP decrease is an obligatory step in the reaction sequence connecting photon absorption to a plasma membrane conductance change, then one would predict that introduction of the antibody inside a living photoreceptor might lock it into a "dark state" in which normal cGMP levels are not perturbed, but their light-induced decrease and the consequent conductance change are blocked. If cGMP is not involved in the initial conductance decrease, but rather plays a role in the adaptation processes that follow, then its introduction into the cell might inhibit adaptation. Given the currently popular idea that cGMP regulates some aspect of calcium transport (cf. George and Hagins, 1983), it might prove interesting to examine the effect of the antibody on the activation of sodium/calcium exchange that occurs following illumination (Yau et al., 1981).

One practical issue has to be met before the G-protein antibody can be shown to be useful in physiological studies. G-protein accounts for 17% of the total protein of these structures with one copy being present for every 10 rhodopsin molecules (Hamm, H. E., and M. D. Bownds, manuscript submitted for publication). It may prove difficult to introduce enough antibody into the cell using conventional injection techniques to adequately block the cGMP pathway. Experiments currently in progress, in which the antibody is being introduced into tiger salamander photoreceptors by internal perfusion (Stern and Lisman, 1982), offer the prospect of overcoming the problem. It seems likely that this issue will be less important with antibodies targeted toward minor components, such as protein kinases, which are thought to be important in regulating transduction.

A detailed picture of the reaction sequence linking photoexcited rhodopsin to PDE activation is now available (Fung and Stryer, 1980; Fung et al., 1981; Fung, 1983; Fung and Nash, 1983). G-protein has three subunits, all of which must be present for the binding of G-protein to excited rhodopsin that is required for GTP attachment to the $\alpha$ subunit. As soon as GTP binds to the $\alpha$ subunit, it dissociates from the $\beta$ and $\gamma$ subunits, diffuses to PDE, and then activates it by removing a small inhibitory subunit. The protein blotting experiments described in the previous paper (Witt et al., 1984) demonstrate that antibody 4A and other G-protein antibodies bind to the $\alpha$ subunit of G-protein. Thus, the block in guanine nucleotide binding caused by antibody 4A introduces a lesion early in the activation sequence. The antibody might act near the guanine nucleotide binding site, at a more distant regulatory site, or at a site required for interaction of excited rhodopsin with G-protein. It should be possible to resolve these alternatives with the reconstitution systems now available (cf. Fung, 1983). Another possible mechanism of inhibition is an antibody cross-linking of G-protein molecules. To rule out an effect of cross-linking on G-protein inhibition, the experiments should be done with F(ab) fragments.

The finding that antibody 4A also causes inhibition of the phosphorylation of two small proteins, components I and II, illustrates a further potential benefit of studying antibody-induced lesions: new information on pathways may be obtained. The results can be taken to suggest a more complex system than is

explained by a linear pathway linking rhodopsin bleaching to a dephosphorylation of components I and II (i.e., rhodopsin + $h\nu$ → G-protein activation → PDE activation → cGMP decrease → dephosphorylation of components I and II). The observation that 4A inhibits the protein phosphorylations independent of light or cGMP levels suggests that a separate linkage between G-protein and the phosphorylation of components I and II must be considered. One possibility is that G-protein may have multiple functions; that is, it may also have the role, in addition to activating PDE, of regulating the (unknown) function of components I and II. (Guanine nucleotide binding proteins may have multiple roles in some other systems; for example, insulin appears to stimulate cAMP PDE and protein kinase via GTP and cholera toxin-dependent mechanisms [Heyworth et al., 1983*a*, *b*; Marchmont and Houslay, 1980]. There are also examples of guanine nucleotide binding proteins whose functions are independent of cyclic nucleotides [Gomperts, 1983; Kaziro, 1978; Margolis and Wilson, 1978].) Another possibility is that components I and II or their kinase is located very close to the G-protein on the disk membrane, which is involved in early steps leading to PDE activation, with the cGMP effect on their phosphorylation providing feedback control.

Several other possible explanations of the 4A inhibition, rather than suggesting new interactions, are compatible with existing information. If components I and II are localized very close to the G-protein–PDE complex on the disk membrane, binding of antibody to G-protein might sterically block access of protein kinase to components I and II. This would require that components I and II or their kinase be localized very close to the 4A antigenic site of G-protein, because several of the other antibodies that bind with affinity to G-protein have no effect on the phosphorylation of components I and II. Another possibility is that antibody 4A interacts directly with the cGMP-dependent protein kinase to inhibit it. Arguing against this, Witt et al. (1984) have observed that 4A binds only to G-protein in Western blots (detection limit: $5 \times 10^{-4}\%$ of total protein) and immunoprecipitates only G-protein.

It is interesting that of the 14 monoclonal antibodies directed against rhodopsin, G-protein, or PDE, one has been shown to function as a specific enzyme inhibitor, with potential usefulness in studies of the physiological role of the cGMP pathway. The other antibodies apparently bind to antigenic sites not involved in the functions we have measured. If this yield of inhibitory antibodies persists as we generate further antibodies to other outer segment proteins (rhodopsin kinase, other protein kinases, and plasma membrane components), then the approach of generating the monoclonal antibodies as pathway blockers may be justified. Antibodies that bind with high affinity but do not block activity are proving useful for immunocytochemical localization, affinity purification, or precipitation of active enzyme complexes.

G-protein, against which antibody 4A is directed, has been shown to have major structural and functional homologies with the guanine nucleotide binding proteins that mediate between hormone receptors and adenylate cyclase in hormone-sensitive cells (Bitensky et al., 1982; Abood et al., 1982; Manning and

Gilman, 1983). One might expect the antibody to produce inhibition in these other systems, and current experiments (Hamm, H. E., M. D. Bownds, and J. S. Takahashi, manuscript submitted for publication) have shown such inhibition in the β-adrenergic system of rat pineal membranes. Recent findings on these homologies suggest that G-protein might have multiple functions. For example, Manning and Gilman (1983) have found that the photoreceptor G-protein has structural similarities with both inhibitory and stimulatory guanine nucleotide binding proteins, and Abood et al. (1982) and VanDop et al. (1984) have found that bovine photoreceptor G-protein can be ADP-ribosylated at different sites by two toxins, cholera toxin and pertussis toxin, with different effects on its function. Several other antibodies that have been generated to G-protein may be useful in the future in establishing whether G-protein has roles other than PDE regulation in the photoreceptor.

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