Inhibition of Monoclonal Antibody Binding and Proteolysis by Light-Induced Phosphorylation of Rhodopsin†

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ABSTRACT: Light-induced phosphorylation of rhodopsin in bovine rod outer segment disk membranes inhibits the binding of three carboxyl-terminal-specific anti-rhodopsin antibodies and the cleavage of the carboxyl-terminal region of rhodopsin by trypsin and *Staphylococcus aureus* V-8 protease. Two monoclonal antibodies, rho 3A6 and rho 1C5, which previously have been shown to preferentially bind to the 8′–12′ and the 9′–18′ carboxyl-terminal segments of rhodopsin, respectively, are both highly sensitive to phosphorylation. When an average of one phosphate is incorporated per rhodopsin, the binding reactivity of rhodopsin for these antibodies decreases to 30% that of nonphosphorylated rhodopsin as measured in radioimmune competition assays. Reactivity of the rho 1D4 antibody whose primary binding site is localized in the 1′–8′ C-terminal segment of rhodopsin is unaffected at this level of phosphorylation but decreases to 30% when three phosphates on average are incorporated per rhodopsin. Direct binding studies using ^{125}I-labeled antibodies indicate that phosphorylation of rhodopsin decreases the maximum extent of rho 3A6 and rho 1C5 binding to rhodopsin. For rho 1D4, the maximum extent of binding is unaffected by phosphorylation, but the dissociation constant is increased by 10-fold. Phosphorylation of rhodopsin also inhibits cleavage of the 1′–9′ and 1′–7′ carboxyl-terminal peptides by trypsin and *S. aureus* V-8 protease, respectively. When an average of one phosphate per rhodopsin is incorporated, cleavage decreases to 40% that of nonphosphorylated rhodopsin as measured by high-performance liquid chromatography. Phosphorylation of rhodopsin had no effect on *S. aureus* cleavage of rhodopsin into the F_1 (M_r 25 000) and F_2 (M_r 12 000) fragments. These results suggest that the 9′-Thr or possibly the 11′-Ser which is in the primary binding domain of rho 3A6 and rho 1C5 antibodies and is in close proximity to the cleavage sites for trypsin and *S. aureus* protease is preferentially phosphorylated at low levels of phosphate incorporation. In related experiments, the binding of these carboxyl-terminal-specific antibodies to rhodopsin in rod outer segment membranes is shown to inhibit light-induced phosphorylation of rhodopsin. These studies indicate that these carboxyl-terminal antibodies and proteolysis are useful probes for distinguishing phosphorylated from nonphosphorylated rhodopsin for in vitro and in vivo analysis.

The carboxyl-terminal segment of the photoreceptor protein rhodopsin is exposed on the cytoplasmic surface of rod outer segment (ROS)[1] disk membranes where it is phosphorylated by ATP in a light-dependent, kinase-catalyzed reaction (Kuhn & Dreyer, 1972; Bownds et al., 1972). The role of light-induced phosphorylation in the visual process has not been firmly established, but recent studies indicate that phosphorylation of rhodopsin inhibits the light-dependent activation of cGMP-specific phosphodiesterase (Sitaramayya et al., 1977; Sitaramayya & Liebman, 1983). This has led to the suggestion that this reaction serves as an important control mechanism in the visual process (Liebman & Pugh, 1980).

Analysis of the sequence of rhodopsin indicates that as many as 7 serine and threonine residues along the 15 amino acid C-terminal segment can serve as potential sites of phosphorylation (Hargrave et al., 1980). Multiple species of phosphorylated rhodopsin have been detected (Wilden & Kühn, 1982; Aton et al., 1984), but the importance of these species and the preferential sites of phosphorylation have not been identified.

A number of monoclonal antibodies which specifically bind to the carboxyl-terminal segment of rhodopsin have been produced (Molday & MacKenzie, 1983). Recently, synthetic peptides and specific proteases have been used to localize the peptide binding determinants for several antibodies along the carboxyl-terminal 1′–18′ segment of bovine rhodopsin (Arendt et al., 1983; MacKenzie et al., 1984). Since the seven serine and threonine residues which can serve as sites of light-induced phosphorylation of rhodopsin are localized within the binding domains of these antibodies and are in close proximity to protease cleavage sites, it was of interest to determine if phosphorylation of rhodopsin affected the binding of these specific monoclonal antibodies and cleavage by specific proteolytic enzymes.

In this paper, we report that phosphorylation of rhodopsin differentially inhibits the binding of three C-terminal-specific monoclonal antibodies, designated rho 1D4, rho 3A6, and rho 1C5, and inhibits cleavage of the C-terminal segment of rhodopsin by trypsin and *Staphylococcus aureus* V-8 protease. The properties of these specific probes in relation to light-induced phosphorylation are investigated.

Experimental Procedures

Preparation of Bovine ROS. Purified ROS membranes were prepared from frozen bovine retinas (Hormel) by cen-

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[1] Abbreviations: ROS, rod outer segment(s); DTT, dithiothreitol; Cl_3CCOOH, trichloroacetic acid; PBS, phosphate-buffered saline; rho, rhodopsin; Con A, concanavalin A; RIA, radioimmunoassay; Ig, immunoglobulin; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; HPLC, high-performance liquid chromatography; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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trifugation on a discontinuous sucrose gradient as described by McDowell & Kühn (1977). Unbleached ROS membranes solubilized in 50 mM CTAB had an A_{400nm}/A_{500nm} ratio of 0.24. The rhodopsin concentration was determined from the change in absorbance at 500 nm upon bleaching of CTAB-solubilized ROS using a molar extinction coefficient of 40 500.

Radioiodination of Monoclonal Antibodies. Rho 1D4, rho 3A6, and rho 1C5 monoclonal antibodies (Molday & MacKenzie, 1983; MacKenzie et al., 1984) were purified from mouse ascites fluid by ammonium sulfate fractionation and DEAE ion-exchange chromatography (Garvey et al., 1977). One milligram of the rho 3A6, rho 1C5, or goat anti-mouse Ig antibodies in 0.5 mL of PBS was iodinated by using the chloramine T method (Hunter & Greenwood, 1962); the rho 1D4 antibody, which was inactivated by chloramine T, was iodinated by using Enzymobeads (Bio-Rad Laboratories) containing immobilized glucose oxidase and lactoperoxidase. The iodinated proteins were separated from free [^{125}I]iodide and other reactants by diluting the reaction mixture with 0.5 mL of PBS and centrifuging this solution through 0.5 g of AG1X10-Cl ion-exchange resin (Bio-Rad) placed in the reservoir of a 0.2-μm microfiltration device (BioAnalytical Systems). The binding activities of the iodinated monoclonal antibodies were determined by quantifying the amount of antibody which bound to excess rhodopsin–Sepharose 2B beads prepared by the method of Cuatrecasas (1970). Briefly, 2 μg of ^{125}I-labeled antibody was incubated with 0.25 mL of rhodopsin–Sepharose beads for 30 min. The beads were diluted with 2.5 mL of buffer, and the amount of radioactivity in the supernatant was determined. Retention of binding activity after iodination was 57% for rho 1D4, 79% for rho 3A6, and 70% for rho 1C5. The specific activities were 7.8×10^4 dpm/μg for rho 1D4 and $(1–3) \times 10^6$ dpm/μg for rho 3A6, 1C5, and goat anti-mouse Ig antibodies.

The monoclonal antibodies were subtyped by using a mouse immunoglobulin identification kit from Mannheim-Boehringer.

Light-Induced Phosphorylation of Rhodopsin. The light-stimulated activity of rhodopsin kinase in ROS membrane preparations was determined essentially as described by Kühn & Wilden (1982). ROS membranes were suspended in 1 mL of buffer A (0.1 M potassium phosphate, pH 7.0, 2 mM $MgCl_2$, and 1 mM DTT) at a rhodopsin concentration of 15 μM and gently homogenized in the dark. Radiolabeled [γ-^{32}P]ATP (2000–3000 cpm/nmol) was added to a concentration of 3 mM. The membranes were bleached and incubated at 37 °C in a water bath. Phosphorylation was quenched at various times by transferring 50-μL aliquots to 0.5 mL of ice-cold 25% Cl_3CCOOH containing 10 mM ATP. Precipitates were pelleted in an Eppendorf microcentrifuge (13 000 rpm for 5 min). The pellets were washed 2 more times with cold 10% Cl_3CCOOH and 5 mM H_3PO_4, solubilized with 1.5 mL of ACS scintillation fluid (Amersham) containing 10% by volume of 0.6 M NCS tissue solubilizer (Amersham), and counted in a Phillips PW4700 liquid scintillation counter.

The inhibition of light-stimulated phosphorylation of rhodopsin by different monoclonal antibodies was measured by preincubating ROS membranes with varying concentrations of the specific antibody in buffer A in the dark at room temperature. After 60–90 min, [γ-^{32}P]ATP was added, the membranes were bleached in room light, and the level of ^{32}P incorporation was determined as above.

Solid-Phase Radioimmune Competition Assays. The effect of rhodopsin phosphorylation on the binding of monoclonal anti-rhodopsin antibodies was studied by using the RIA competitive inhibition assay previously described (Molday & MacKenzie, 1983). Fifty-microliter aliquots of ROS were removed at different times after the onset of phosphorylation, and the extent of ^{32}P incorporation was determined as described above. Additional 50-μL aliquots were removed and quenched in RIA buffer (PBS, pH 7.4, 1% fetal calf serum, 1% BSA, and 0.1% NaN_3) containing 5 mM EDTA to complex free Mg^{2+}. From this suspension, 25-μL aliquots were serially diluted into 50-μL volumes of hybridoma culture fluid containing the monoclonal antibody. After a 60-min incubation at 23 °C, 25-μL aliquots were removed, and the level of unbound antibody was determined by using the solid-phase RIA employing Triton X-100 solubilized ROS as the immobilized antigen and ^{125}I-labeled goat anti-mouse Ig as a tracer second antibody (Molday & MacKenzie, 1983). The level of ^{125}I was measured in a Beckman 8000 γ counter.

Direct Binding Studies. The binding of radioiodinated monoclonal antibodies to phosphorylated rhodopsin was measured by using detergent-solubilized rhodopsin immobilized on concanavalin A coated flex vinyl microtiter plate wells. Coated wells were prepared by treatment with 25 μL of a 0.2 mg/mL solution of Con A in 5 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, and 0.5 mM $MnCl_2$ for 2 h at 23 °C. The wells were then rinsed with buffer, and nonspecific protein binding sites were quenched by incubating the wells with Con A binding buffer (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1 mM $CaCl_2$, 0.5 mM $MnCl_2$, 1% BSA, and 0.1% NaN_3) for 1 h. ROS membranes were solubilized with 1% Triton X-100 and diluted to a rhodopsin concentration of 5 μM in Con A binding buffer. Aliquots of 25 μL were incubated in Con A coated wells for 60 min at 23 °C. The assay wells were then rinsed in PBS and treated for a further 60 min with 25 μL of ^{125}I-labeled monoclonal antibody at various concentrations in Con A binding buffer. After a final rinsing in PBS, individual wells were cut out and counted in a Beckman 8000 γ counter. The binding of ^{125}I-labeled antibody to Con A treated wells was used as a control for a measure of background binding. Typically, the binding of the rho 1D4 and rho 1C5 antibodies was less than 10% that observed when rhodopsin had been first immobilized onto the Con A coated wells. The ^{125}I-labeled rho 3A6 antibody gave a 3-fold higher level of background binding, indicating the possible presence of Con A specific oligosaccharides on this antibody.

Proteolysis of Phosphorylated Rhodopsin. The effect of phosphorylation of rhodopsin in ROS membranes on trypsin and *S. aureus* protease cleavage of the C-terminal 1′–9′ and 1′–7′ peptides was studied as follows: ROS membranes (15 μM rhodopsin) were bleached and incubated with 3 mM [γ-^{32}P]ATP (2000–3000 cpm/pmol) at 37 °C, and at various times, aliquots were removed and diluted 10-fold into protease-containing solution. The extent of ^{32}P incorporation at each time was determined as previously described. Proteolytic digestion was performed for 2 h at 25 °C with TPCK–trypsin (Worthington Biochemical) or *S. aureus* protease (strain V-8, Pierce Chemical Co.) at a ROS protein to enzyme ratio of 10:1 by weight in 5 mM Tris-HCl buffer, pH 7.4, containing 1 mM $CaCl_2$. Digested membranes were pelleted by centrifugation (27000*g* for 30 min), the supernatant fractions were concentrated, and the quantity of 1′–9′ or 1′–7′ peptide was determined by HPLC on a Waters μBondapak C18 (39 × 300 mm) column eluted with a linear gradient of 0–30% acetonitrile in 0.1% trifluoroacetic acid using a Varian Vista 54 liquid chromatography system. Eluted peptides were detected by the absorbance at 215 nm, and the relative changes in peptides were measured by integrating areas under the appropriate peaks.

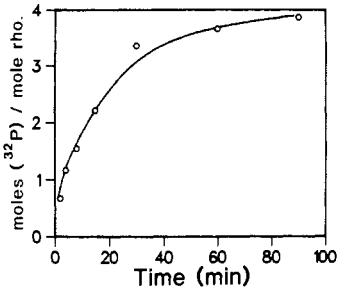

FIGURE 1: Time course for the light-induced phosphorylation of rhodopsin. Bovine ROS (15 μM rhodopsin) in 0.1 M potassium phosphate, pH 7.0, 2 mM $MgCl_2$, 1 mM DTT, and 3 mM [γ-^{32}P]ATP were bleached and incubated at 37 °C under continuous white light illumination. At various times, aliquots were removed, and the level of Cl_3CCOOH-precipitable ^{32}P was determined.

$NaDodSO_4$ Gel Electrophoresis. Bleached ROS membranes which had been incubated in the presence or absence of 3 mM ATP for 90 min at 37 °C and subjected to proteolytic digestion using TPCK–trypsin (Worthington Biochemical) or *Staphylococcus aureus* protease (strain V-8, Pierce Chemical) were analyzed by $NaDodSO_4$ gel electrophoresis. Proteolytic digestion was carried out for 3 h at 23 °C with a ROS protein to enzyme ratio by weight of 10:1 in a volume of 0.5 mL. Digestion was terminated by the addition of 50 μL of soybean trypsin inhibitor (4 mg/mL) for trypsin or 0.5 mL of 0.4 mM phenylmethanesulfonyl fluoride for *S. aureus* protease. Membranes were washed 3 times by centrifugation (27000*g* for 30 min) in 5 mM Tris-HCl, pH 7.4, and resuspended to 0.1 mL in the same buffer. Samples of digested ROS were subjected to $NaDodSO_4$ gel electrophoresis and gel transfer as previously described (Molday & MacKenzie, 1983). Transfer blots were incubated with ^{125}I-labeled rho 1D4 (5 μg/mL, 7.8 × 10^4 dpm/μg) in RIA buffer for 2 h at room temperature. After extensive rinsing in PBS plus 0.2% Sarkosyl, the immunoblots were subjected to autoradiography for 36 h.

RESULTS

Kinase Activity of ROS Membranes. Bovine ROS which had been prepared from frozen retinas in the presence of moderate ionic strength buffers maintained much of their kinase activity and were able to undergo light-stimulated incorporation of ^{32}P to a level similar to that reported by Kühn & Wilden (1982). The time course for rhodopsin phosphorylation is shown in Figure 1. After a 90-min incubation at 37 °C, approximately 4–6 mol of phosphate on average could be incorporated per mol of rhodopsin. The half-time for rhodopsin phosphorylation under these conditions was observed to be about 12 min. Unbleached ROS membranes incubated with [γ-^{32}P]ATP for 90 min in the dark generally showed only 5% of the maximum level of phosphate incorporation seen with bleached membranes.

Inhibition of Antibody Binding by Rhodopsin Phosphorylation. Phosphorylation of rhodopsin was found to inhibit the binding of three C-terminal-specific anti-rhodopsin monoclonal antibodies, rho 1D4, rho 3A6, and rho 1C5. This was initially measured by solid-phase RIA competition assays in which aliquots of ROS membranes removed at various times during the kinase-catalyzed phosphorylation reaction were tested for their capacity to inhibit the binding of the monoclonal antibodies to immobilized rhodopsin. As shown in Figure 2, ROS membranes which had been phosphorylated for long time periods were less effective inhibitors. The protein concentration necessary to produce half-maximal inhibition (I_{50}) of antibody

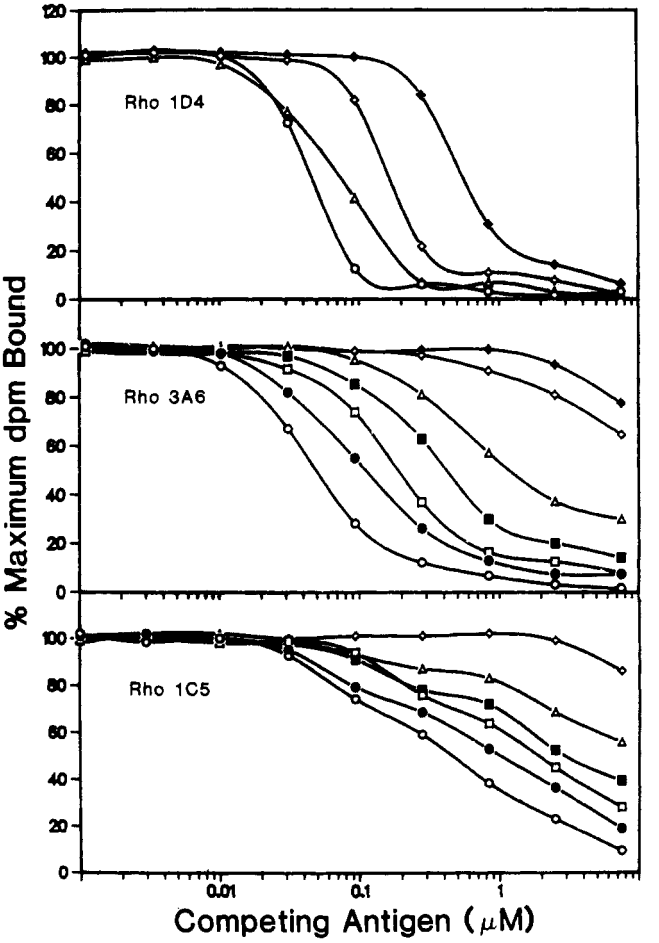

FIGURE 2: Effect of phosphorylation of rhodopsin on antibody reactivity to rhodopsin. ROS membranes were removed at 0 (○), 2 (●), 4 (□), 8 (■), 15 (△), 30 (◇), and 90 min (◆) after light-induced, [γ-^{32}P]ATP-dependent phosphorylation of rhodopsin and used as competitive inhibitors of rho 1D4, rho 3A6, and rho 1C5 binding to Triton X-100 solubilized ROS membrane proteins immobilized on flex vinyl microtiter wells.

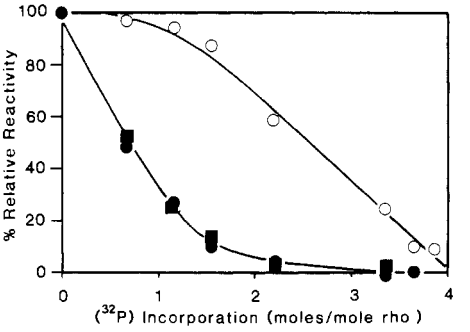

FIGURE 3: Inhibition of rho 3A6 (●), rho 1C5 (■), and rho 1D4 (○) antibody binding as a function of the extent of phosphorylation. Percent relative reactivity is defined as [I_{50}(nonphosphorylated rhodopsin)/I_{50}(phosphorylated rhodopsin)] × 100 where I_{50} is the concentration of rhodopsin required to obtain half-maximum inhibition of antibody binding to immobilized rhodopsin as determined from the competitive inhibition profiles in Figure 2.

binding to immobilized rhodopsin was determined from these inhibition curves. The immunoreactivity of phosphorylated rhodopsin relative to nonphosphorylated rhodopsin as a function of ^{32}P incorporation is shown in Figure 3. Both rho 3A6 and rho 1C5 antibodies were found to be equally sensitive to phosphorylation. When an average of one phosphate per rhodopsin was incorporated, the reactivity decreased to 30% that of nonphosphorylated rhodopsin. In contrast, little loss

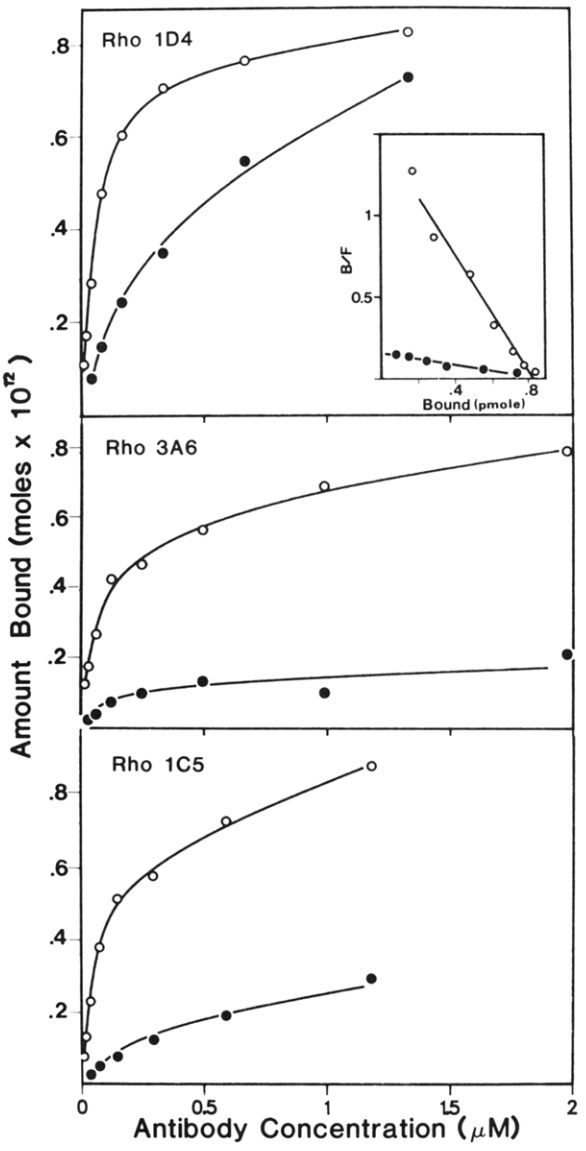

FIGURE 4: Direct binding of the three carboxyl-terminal-specific monoclonal antibodies to detergent-solubilized nonphosphorylated ROS (○) and phosphorylated rhodopsin (●) immobilized on Con A coated wells. A Scatchard analysis of ^{125}I-rho 1D4 binding is also shown in the insert for rho 1D4.

in reactivity toward the rho 1D4 antibody was observed at this level of phosphorylation. An average of over three phosphates per rhodopsin was required to decrease this antibody reactivity to 30%.

Direct Binding of ^{125}I-Labeled Monoclonal Antibodies to Rhodopsin. Direct binding of the monoclonal antibodies to nonphosphorylated and phosphorylated rhodopsin having an average of four phosphates per rhodopsin was carried out in order to determine if phosphorylation of rhodopsin affected the maximum extent of binding or the apparent affinity. As shown in Figure 4, the three monoclonal antibodies bound with high affinity to nonphosphorylated rhodopsin. Scatchard analysis of the binding curves indicated a single class of binding sites with apparent dissociation constants on the order of 10^{-8} M (Table I). The maximum extent of binding of ^{125}I-labeled rho 1D4 antibody to phosphorylated rhodopsin was similar to that observed for nonphosphorylated rhodopsin (Figure 4). The apparent dissociation constant (K_d), however, was 10 times greater for phosphorylated rhodopsin. In contrast, the maximum extent of binding of both rho 3A6 and rho 1C5 to phosphorylated rhodopsin was less than 20% and 30%, respectively, that of nonphosphorylated rhodopsin.

Table I: Direct Binding of ^{125}I-Labeled Monoclonal Antibodies to Bovine Rhodopsin

antibody	subtype	app K_d (M)
rho 1D4	IgG_1, κ	3.8×10^{-8}
		$(3.2 \times 10^{-7})^a$
rho 3A6	IgG_1, κ	8.4×10^{-8}
rho 1C5	IgG_1, κ	5.7×10^{-8}

[a] Dissociation constant for binding of ^{125}I-labeled rho 1D4 to phosphorylated rhodopsin containing an average of 4 mol of phosphate per mol of rhodopsin.

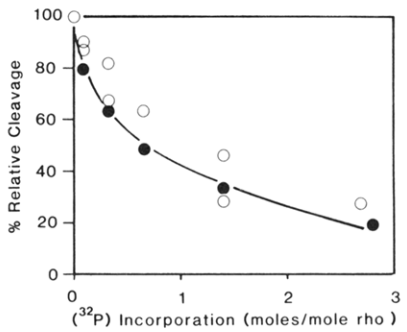

FIGURE 5: Inhibition of trypsin and *S. aureus* protease cleavage at the carboxyl-terminal region of rhodopsin following light-stimulated phosphorylation. Bovine ROS membranes which had been phosphorylated to different levels with [^{32}P]ATP were subjected to proteolytic digestion with either TPCK–trypsin (○) or *S. aureus* (●) for 2 h at a protein to enzyme ratio of 10:1. The extent of proteolysis of phosphorylated ROS relative to nonphosphorylated ROS was determined by measuring the change in concentration of either the 1′–9′ or the 1′–7′ C-terminal peptide present in the supernatant fraction from digested ROS membranes. Released peptides were quantitated by reversed-phase HPLC as described in the text.

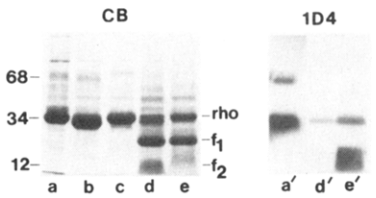

FIGURE 6: $NaDodSO_4$ gel electrophoresis of nonphosphorylated and phosphorylated ROS membranes treated with proteolytic enzymes. (Left panel) Coomassie Blue (CB) stained gels: (a) undigested, nonphosphorylated ROS; (b) trypsin-treated nonphosphorylated ROS; (c) trypsin-treated phosphorylated ROS; (d) *S. aureus* treated nonphosphorylated ROS; (e) *S. aureus* treated phosphorylated ROS. (Right panel) Immunoblots labeled with ^{125}I-rho 1D4 antibody: (a′) undigested nonphosphorylated ROS; (d′) *S. aureus* treated nonphosphorylated ROS; (e′) *S. aureus* treated, phosphorylated ROS.

Effect of Phosphorylation on Proteolysis of Rhodopsin. The effect of phosphorylation on cleavage of the 1′–9′ and 1′–7′ C-terminal peptides from rhodopsin by trypsin and *S. aureus* protease was analyzed by HPLC. A shown in Figure 5, the quantity of peptide released from phosphorylated rhodopsin relative to nonphosphorylated rhodopsin decreased with increasing phosphate incorporation. When an average of one phosphate was incorporated per rhodopsin, cleavage was reduced to 40% that of nonphosphorylated rhodopsin. The HPLC profile of peptides obtained from cleaved samples of phosphorylated rhodopsin showed no additional peaks characteristic of phosphorylated peptides.

Inhibition of trypsin and *S. aureus* protease cleavage at the C-terminal region of rhodopsin was also observed by $NaDodSO_4$ gel electrophoresis (Figure 6). Whereas digestion of nonphosphorylated rhodopsin (gel a) with trypsin results in a small decrease in the apparent molecular weight of rho-

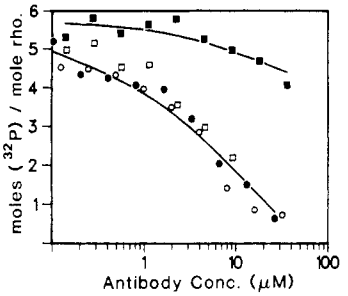

FIGURE 7: Inhibition of phosphorylation of rhodopsin by C-terminal-specific monoclonal antibodies. Unbleached ROS were incubated for 1 h in the dark with varying concentrations of the rho 1D4 (●), rho 3A6 (○), or rho 1C5 (□) antibodies. After the addition of [γ-^{32}P]ATP, the membranes were bleached and incubated for 90 min at 37 °C, and the incorporation of ^{32}P was determined as described under Experimental Procedures. In the control experiments, membranes were treated in the dark with the monoclonal antibody rho 4A2 specific for the N-terminal region of rhodopsin (■).

dopsin (gel b) as previously reported (Molday & Molday, 1979), treatment of phosphorylated rhodopsin with trypsin had no effect (gel c).

Digestion of nonphosphorylated rhodopsin with *S. aureus* protease (Findley et al., 1980) initially releases a seven amino acid segment from the C-terminus of rhodopsin (doublet at M_r 34 000 in gel d) and subsequently cleaves rhodopsin into a M_r 25 000 F_1 fragment containing the N-terminus and a F_2 fragment derived from the C-terminus end of rhodopsin (gel d). Phosphorylation of rhodopsin inhibits *S. aureus* cleavage of the C-terminal fragment of rhodopsin as shown by a single band of undigested rhodopsin (gel e). Cleavage of rhodopsin into the F_1 and F_2 fragments (gel e), however, is not affected. The F_2 fragment of phosphorylated rhodopsin migrates with a higher apparent molecular weight than the corresponding F_2 fragment of nonphosphorylated rhodopsin as seen in Coomassie Blue stained gels. Immunoblotting studies indicate that the rho 1D4 antibody binds to undigested rhodopsin (gel a′) and the F_2 fragment of phosphorylated rhodopsin (gel e′), but not the F_2 fragment of nonphosphorylated rhodopsin (gel d′). This confirms that the C-terminal segment required for rho 1D4 antibody binding (Molday & MacKenzie, 1983) is removed from the F_2 fragment of nonphosphorylated rhodopsin but is present on the F_2 fragment of phosphorylated rhodopsin.

Inhibition of Rhodopsin Phosphorylation by Monoclonal Antibodies. The effect of C-terminal anti-rhodopsin antibodies on light-stimulated phosphorylation of rhodopsin was also studied. As illustrated in Figure 7, the three monoclonal antibodies, rho 1D4, rho 3A6, and rho 1C5, were equally effective in inhibiting phosphorylation. Half-maximum inhibition is reached at an antibody concentration of 5.3 μM when the rhodopsin concentration is 13.5 μM. The antibody-mediated inhibition of rhodopsin phosphorylation was specific since substitution of these C-terminal antibodies with the N-terminal-specific anti-rhodopsin monoclonal antibody rho 4A2 (Molday & MacKenzie, 1983) showed a low degree of inhibition only at high antibody concentration. Extrapolation of the inhibition curve for the rho 4A2 in Figure 7 indicates that half-maximum inhibition requires an antibody concentration over an order of magnitude greater. Nonspecific mouse Ig exhibited the same inhibition profile as rho 4A2 antibody (not shown).

DISCUSSION

Previous studies employing limited proteolysis and synthetic peptides have indicated that the three monoclonal antibodies used in this study bind to different sites along the carboxyl-

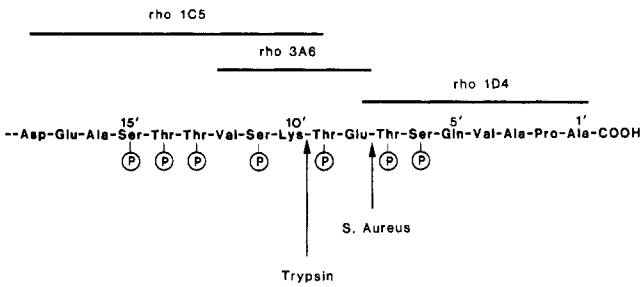

FIGURE 8: Sequence of the carboxyl-terminal 1′–18′ segment of bovine rhodopsin (Hargrave et al., 1980) showing sites of phosphorylation, cleavage by trypsin and *S. aureus* protease, and binding of rho 1D4, rho 3A6, and rho 1C5 monoclonal antibodies (MacKenzie et al., 1984).

terminal region of bovine rhodopsin (MacKenzie et al., 1984). As shown in Figure 8, the primary peptide binding determinants have been identified as the 1′–8′ C-terminal peptide segment for the rho 1D4 antibody, the 8′–12′ peptide segment for the rho 3A6 antibody, and the 9′–18′ peptide segment for the rho 1C5 antibody. Seven Ser and Thr residues which can serve as sites for light-induced phosphorylation (Hargrave & Fong, 1977; Hargrave et al., 1980) lie within these antibody binding determinants.

The location of preferential site(s) of phosphorylation can be deduced from the effect of phosphorylation on monoclonal antibody binding and proteolysis at the C-terminal segment of rhodopsin. The marked decrease in binding of rho 3A6 and rho 1C5 observed at low levels (one P_i per rhodopsin) of phosphorylation (Figure 3) indicates that the initial sites of phosphorylation must reside within binding sites of these antibodies. The peptide segment on rhodopsin which is part of the binding sites for both these antibodies is the 9′–12′ segment (MacKenzie et al., 1984). This segment contains a Thr in the 9′-position and a Ser in the 11′ which if phosphorylated would be likely to inhibit the binding of these antibodies as observed in both competitive inhibition and direct binding studies. We have previously shown (MacKenzie et al., 1984) that replacement of a single amino acid in the binding domain of the rho 3A6 antibody completely inhibits its binding. The 9′-Thr and 11′-Ser lie outside the primary binding domain of the rho 1D4 antibody, i.e., 1′–8′. Phosphorylation of these residues would be expected to have little, if any, effect on the binding of this antibody as observed.

This conclusion is supported in studies on the effect of phosphorylation on trypsin and *S. aureus* protease cleavage of the 1′–9′ and 1′–7′ peptides from the C-terminus of rhodopsin. Inhibition of cleavage by these enzymes as a function of phosphate incorporation (Figure 5) roughly parallels inhibition of rho 3A6 and rho 1C5 binding (Figure 3). The 9′-Thr residue lies in close proximity to both enzyme cleavage sites. Modification of this residue with a negatively charged phosphate may be expected to inhibit proteolytic cleavage as well as antibody binding. Synthetic C-terminal rhodopsin peptides phosphorylated at specific Ser and Thr residues would be useful in confirming which sites of phosphorylation affect antibody binding and proteolysis. Lack of a direct correlation of inhibition of antibody binding or proteolysis with phosphate incorporation, i.e., 60–70% inhibition at one phosphate per rhodopsin, supports previous studies suggesting multiple sites of phosphorylation (Wilden & Kühn, 1982).

Binding of the rho 1D4, rho 3A6, and rho 1C5 antibodies to the C-terminal region of rhodopsin has been observed to inhibit kinase-catalyzed phosphorylation of rhodopsin. These antibodies with similar binding affinities were equally effective in inhibiting this reaction (Figure 7). Inhibition of phosphorylation may arise either by the direct blocking of the phos-

phorylation sites by these antibodies and/or by inhibiting the binding of the kinase to rhodopsin due to the large size of the antibody. Support for the latter, i.e., steric inaccessibility of the kinase due to antibody binding, comes from an experiment in which another monoclonal antibody, rho 4B4, which is directed against the F_1–F_2 loop of rhodopsin (MacKenzie & Molday, 1982), was also found to inhibit phosphorylation as well as rho 3A6 antibody binding at the C-terminus of rhodopsin (unpublished results).

In conclusion, these monoclonal antibodies and specific proteases can be used to distinguish phosphorylated from nonphosphorylated rhodopsin, and therefore, they can serve as valuable probes for both in vitro and in vivo studies directed toward analyzing the molecular properties of the carboxyl-terminal segment of rhodopsin and elucidating the role of light-induced phosphorylation in vision and other cellular processes in ROS.

Registry No. Rhodopsin kinase, 54004-64-7; L-threonine, 72-19-5; L-serine, 56-45-1; trypsin, 9002-07-7; *Staphylococcus aureus* V-8 protease, 66676-43-5.

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Translational Diffusion of Lipids in Liquid Crystalline Phase Phosphatidylcholine Multibilayers. A Comparison of Experiment with Theory

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ABSTRACT: A systematic study of the translational diffusion of the phospholipid derivative *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) has been undertaken in liquid crystalline phase phosphatidylcholine bilayers by using the fluorescence recovery after photobleaching technique. This work was done with the intention of comparing the experimental results with the predictions of theoretical models for diffusion in membranes. The following is shown. (1) For NBD-PE, the dependence of the translational diffusion coefficient (D_t) upon the acyl chain length of the diffusant is not that predicted by continuum fluid hydrodynamic models for diffusion in membranes [Saffman, P. G., & Delbrueck, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3111–3113; Hughes, B. D., Pailthorpe, B. A., & White, L. R. (1981) *J. Fluid Mech. 110*, 349–372]. (2) Plots of D_t vs. $1/T$ (Arrhenius plots) are nonlinear in dilauroylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) bilayers where the acyl chain composition of the NBD-PE is matched with that of the host bilayer lipid. This suggests that a "free volume" model may be appropriate for the description of lipid diffusion in lipid bilayers. (3) In bilayers of phosphatidylcholines with saturated acyl chains at the same "reduced temperature", the magnitude of D_t follows the order distearoylphosphatidylcholine > DPPC > DMPC > DLPC. This is the inverse of what may be expected from the hydrodynamic model but is in agreement with the free volume in these bilayers. (4) A free volume model that takes into account the frictional drag forces acting upon the diffusing NBD-PE at the membrane–water interface and also at the bilayer midplane is shown to adequately describe the diffusion results for NBD-PEs in DLPC, DMPC, DPPC, and POPC bilayers in the liquid-crystalline phase.

The translational diffusion of lipids and lipid-like probe molecules in phospholipid bilayer membranes has been examined in several laboratories [for a review, see Vaz et al. (1982b)]. It has been experimentally verified that continuum

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