Structural and Enzymatic Aspects of Rhodopsin Phosphorylation*

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Photoactivated rhodopsin (Rho*) is phosphorylated near the C terminus at multiple sites, predominantly at Ser334, Ser338, and Ser343. We systematically examined the sites of phosphorylation upon flash activation of Rho in rod outer segment (ROS) homogenates. Addition of an inhibitory antibody against rhodopsin kinase (RK) lowered phosphorylation at Ser334, Ser338, and Ser343, without changing the ratio between phosphorylation sites. In contrast, no effect of protein kinase C was detected after stimulation (by a phorbol ester), inhibition (with H7), or reconstitution of protein kinase C with purified ROS membranes. The stoichiometry and the ratio between different phosphorylation sites in purified Rho were also reproduced using RK, purified to apparent homogeneity from ROS or from an insect cell expression system. Thus, we conclude that light-dependent phosphorylation of Rho is mediated primarily by RK. Depalmitoylation of Rho at Cys322 and Cys323 altered the conformation of the C terminus of Rho, as observed by phosphorylation by casein kinase I, but did not affect phosphorylation by RK. The sites of phosphorylation were influenced, however, by the presence of four conserved amino acids at the C terminus of Rho. The accumulation of phosphorylated Ser338 observed in vivo could result from slower dephosphorylation of this site as compared with dephosphorylation of Ser338 and Ser343. These data provide a molecular mechanism for the site-specific phosphorylation of Rho observed in vivo.

G protein-coupled receptors, a large family of topologically homologous proteins, share several characteristics that include seven transmembrane α-helical segments and two domains, extra- and intracellular. Intracellular loops are involved in the interaction with G proteins, G protein-coupled receptor kinases, and arrestins, while extracellular fragments are important for protein folding and, in some cases, for the binding of large polypeptide hormones or calcium (reviewed by Baldwin (1994)). In addition, most of G protein-coupled receptors are co- and/or post-translationally modified by glycosylation at the N-terminus and by palmitoylation and phosphorylation on the cytoplasmic surface. Among this group of proteins, Rho possesses a special status because of its high expression in retinal rod photoreceptors and strict cellular compartmentalization to ROS, the precision in its activation by light, and its rapid physiological response (Hargrave and McDowell, 1992).

In rod photoreceptor cells, during transition from active Rho* to inactive opsin, the receptor assumes three relatively stable conformations: Meta I, II, and III (Baumann and Reinheimer, 1973). Meta II binds to and activates hundreds of photoreceptor-specific G proteins (transducins), thus initiating the signal-amplifying cascade of reactions (Hargrave et al., 1993). This activated state of Rho, along with Meta I, serve as substrates for RK (Paulsen and Bentrop, 1983; Pulvermüller et al., 1993), a major protein kinase in ROS. The dissociation of RK from Rho* is followed by the binding of p44 and/or arrestin, thereby preventing continuous G protein activation (Wilden et al., 1986; Palczewski et al., 1994b). Studies in vitro have shown that Rho* is phosphorylated at multiple Ser and Thr residues. Depending on the experimental conditions, the initial sites of phosphorylation were identified at Ser334 and Ser343 (McDowell et al., 1993; Ohguro et al., 1993; Papac et al., 1993), prior to multiple phosphorylation. The binding of arrestin to Rho* and the reduction of the photolyzed chromophore, all-trans-retinal, by membrane-bound retinol dehydrogenase limit the high stoichiometry of phosphorylation (Ohguro et al., 1994b). In vivo analysis however, stands in sharp contrast with in vitro studies, because only monophosphorylated species were found at Ser334, Ser343, and Ser343 (Ohguro et al., 1995). Kawamura (1994) and, subsequently, other investigators (Gorodovikova et al., 1994a, 1994b) proposed that RK is under a calcium mediated inhibition through a retina-specific calcium-binding protein (Dizhoor et al., 1991; Lambrecht and Koch, 1991; Polans et al., 1991). PKC was also proposed to phosphorylate Rho in a light-independent manner in a reconstituted system (Greene et al., 1995) or in the whole retina when PKC was hyperactivated by a phorbol ester (Newton and Williams, 1993).

To obtain additional insight into the structural and functional aspects of Rho phosphorylation, we systematically tested whether RK, PKC, or another kinase is responsible for phosphorylation of flash-activated Rho in ROS homogenates and for effects of Rho palmitoylation on the stoichiometry and selectivity of Rho phosphorylation. We also examined the role of the C-terminal four amino acids in the specificity of phosphoryla-

The abbreviations used are: ROS, rod outer segment(s); BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane; PKC, protein kinase C; PrP, protein phosphatase; Rho, rhodopsin; Rho*, photolyzed rhodopsin; RK, rhodopsin kinase; transducin, G-protein of the rod cell; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; fc, footcandles; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; PMA, phorbol 12-myristate 13-acetate; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiprazine.
tion, the effect of phosphorylation on accumulation of phosphoryl-
ated species of Rho, and the effect of calcium on Rho phos-
phorylation/dephosphorylation in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials

Bovine ROS were prepared from fresh bovine retinas (Papamaker, 1982). To eliminate endogenous kinases and other soluble proteins, ROS were treated with 5 mM urea (Shichi and Somers, 1978) and ex-
tensively washed with 10 mM BTP buffer, pH 7.5, containing 100 mM NaCl, 5 mM adenosine, and 200 mM KCl. The phosphorylated and unphos-
phorylated species of Rho, and the effect of calcium on Rho phos-
phorylation and dephosphorylation of Rho* were studied using fresh ROS
homogenates. Under dim red illumination, fresh ROS were suspended
for the catalytic subunit of PKC (ω-soymone) was a gift from Dr. Michael Walsh (University of Calgary). An anti-RK antibody (GS19; IgG class) was purified from antisera of rabbits immu-
nized with a peptide encompassing the N-terminal region of RK (Pal-
czewski et al., 1993) by protein G-Sepharose column chromatography
according to the manufacturer’s protocol (Pharmacia Biotech Inc.). Pu-
ficated casein kinase I was a gift from Dr. Paul Graves (Indiana Univer-
sity), and PrP inhibitor-I (Inh-I) was a gift from Dr. T. Ingebritsen
(Iowa State University). 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine
(70 μM) was purchased from ICN Pharmaceutics. Phorbol myristoyl ace-

Chemicals

Fisher Scientific.

Digestion of Protein Samples

Full-length Rho, as well as the catalytic subunit of PKC (ω-soymone) was a gift from Dr. Michael Walsh (University of Calgary). An anti-RK antibody (GS19; IgG class) was purified from antisera of rabbits immu-
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(70 μM) was purchased from ICN Pharmaceutics. Phorbol myristoyl ace-

Dephosphorylation of Rho*—Palmitoylated groups were partially removed from Rho without the hydrolysis of chromophore, by treating urea-washed Rho with 1 mM hydroxylamine HCl, pH 7.5, at 30 °C for 2 h (Morrison et al., 1993). The membranes were extensively washed with 10 mM Hepes buffer, pH 7.5, containing 100 mM NaCl. Mixtures of depalmitoylated, monopalmitoy-

Carboxymethylation

Membranes were extensively washed with 100 mM sodium borate
buffer, pH 8.0, before modification of Lys and Cys residues with acety-
laric acid was washed twice with the same solution; the pellet was dissolved
in 300 μl of a standard phosphorylation buffer composed of 200 mM BTP
buffer, pH 7.5, containing 60 mM KCl, 20 mM NaCl, 0.5 mM γ-[32P]ATP (300 cpm/pmol), 5 mM MgCl2, 0.1 mM CTP, 0.1 mM GTP, 0.4 mM EDTA,
and 0.16 mM CaCl2 (calculated free Ca2+ 0.16 mM, 250 mm, Vydac) was
employed for the catalytic subunit of PKC (ω-soymone) was a gift from Dr. Michael Walsh (University of Calgary). An anti-RK antibody (GS19; IgG class) was purified from antisera of rabbits immu-
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(70 μM) was purchased from ICN Pharmaceutics. Phorbol myristoyl ace-

ROS (Palczewski, 1993).

Expression of RK in Insect Cells—The full-length Stul/BamHI frag-
ment of RK (1847 base pairs; Lorenz et al., 1991) was subcloned into a baculovirus transfer vector, pVL1393 (Pharmingen). pVL-RK (3 μg) and helper virus carrying a lethal deletion (0.5 μg) were co-transfected into insect Sf9 cells (2 × 10^7) (in vitrogen) in a 60-mm tissue culture dish


counteracted on a C18 reverse phase column (2.1 mm, 100 mM NaCl in the same buffer. The fractions containing RK activity were combined and mixed with 2 μg of PrP 2A, dialyzed overnight
against 10 mM BTP buffer, pH 7.5, containing 0.4% Tween 80 and 1 mM
benzamidine (1 liter), and loaded onto a heparin-Sepharose column (1 × 5 cm) equilibrated with the same buffer. After the column was washed with 10 mM BTP buffer, pH 7.5, containing 0.4% Tween 80, 1 mM MgCl2, and 125 mM NaCl, RK (≥98% pure, −300 μg) was eluted with ATP in 10 mM BTP buffer, pH 7.5, containing 0.4% Tween 80, 1 mM MgCl2, and 100 mM NaCl (for details see Palczewski et al., 1992) and Palczewski
(1993).  

Reconstruction

ROS were treated with 5M urea (Shichi and Somers, 1978) and ex-
tensively washed with 10 mM BTP buffer, pH 7.5, containing 0.4% Tween 80, 1 mM MgCl2, and 125 mM NaCl (for details see Palczewski et al., 1992) and Palczewski
(1993).
the digestion, the truncated Rho in the membranes was extensively washed with 10 mM BTP buffer, pH 7.5, containing 100 mM NaCl and 5 mM MgCl₂, by repeated pelleting of the membranes and suspending in the same buffer. Rho truncated at Glu³⁴¹ or Lys³³⁹ was prepared by the method described by Palczewski et al. (1991). Briefly, urea-washed Rho (19.3 mg) was digested with TPCK-treated trypsin (0.96 mg, Worthington), or S. aureus V8 (0.19 mg, Boehringer Mannheim) for 1 h at room temperature. The digestion was quenched by soybean trypsin inhibitor (9.6 mg) or 5 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride, respectively. After the digestion, the truncated Rho in membranes was extensively washed with 10 mM BTP buffer, pH 7.5, containing 60 mM NaCl, 20 mM KCl, 5 mM MgCl₂, 0.4 mM EDTA, 0.16 mM CaCl₂, 0.1 mM GTP, and 0.1 mM cGMP (calculated free Ca²⁺ concentration, 0.05 μM) at 30°C for 5 min in the dark after a flash that bleached ~15% of Rho. The phosphorylation antigens were separated by the addition of 250 mM potassium phosphate buffer, pH 7.2, containing 200 mM EDTA, 5 mM adenine, 100 mM KF and 200 mM KCI (300 μl). Phosphorylated and unphosphorylated Rho C-terminal peptides were obtained by proteolysis with endoprotease Asp-N (Palczewski et al., 1991). The percent of the total phosphorylation incorporated at specific sites of monophosphorylated or multipe phosphorylated forms was calculated from the radioactive elution profiles of the peptides subdigested with trypsin as described by Ohguro et al. (1994b).

**TABLE I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Multiple phosphorylated Rho</th>
<th>%</th>
<th>%</th>
<th>%</th>
<th>%</th>
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<tbody>
<tr>
<td>ROS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROS + GS19</td>
<td></td>
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<td></td>
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<tr>
<td>ROS + H7</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>ROS + PMA</td>
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<tr>
<td>ROS + 4α-PMA</td>
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<td></td>
<td></td>
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<tr>
<td>RK + Rho</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>rRK + Rho</td>
<td></td>
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</table>

For reconstitution, urea-washed Rho was phosphorylated with purified homogeneous RK, isolated from ROS (Palczewski, 1993). For reconstitution, urea-washed Rho was phosphorylated with purified recombinant RK (rRK), isolated from High Five insect cells (Fig. 1). All experiments were performed in triplicate.

**RESULTS**

In vitro and in vivo studies reveal that light-dependent Rho phosphorylation occurs at the C-terminal Ser³³⁴ and Ser³³⁸, and Ser³⁴³ (Ohguro et al., 1993, 1994b, 1995; McDowell et al., 1993; Papac et al., 1993). The specificity and extent of phosphorylation could result from 1) reactions catalyzed by two or more protein kinases, 2) different states of palmitoylation of Rho at the C terminus, 3) dynamically altering conformations of Rho* that RK has phosphorylated at different sites, 4) random catalysis by RK; 5) selective phosphatase activity, or 6) metabolism of all-trans-retinal.

**Is Rho* Phosphorylated by Protein Kinases Other Than...
RK?—All phosphorylation sites on opsin were identified within the C-terminal D_{330}-A_{348} peptide by digesting with trypsin and analyzing by the mass spectrometric methods described by Ohguro et al. (1994). Under standard conditions, ROS were exposed to a single flash, and multiple phosphorylated (61.3 ± 3.9%) and monophosphorylated (38.7%) forms were observed (Table I). The dominant, singly phosphorylated species were modified at either Ser_{338} or Ser_{343}, whereas only 14.3 ± 0.7% of the monophosphorylated forms were phosphorylated on Ser_{334}. The reaction was not limited by the amount of RK or affected by dilution, because similar results were obtained after flashes that bleached 5% or 10% of Rho, and for Rho concentrations ranging from 1 to 10 mg/ml (data not shown).

Although the extent of phosphorylation was decreased by the inclusion of a specific anti-RK antibody (Palczewski et al., 1993) that inhibits secondary phosphorylation of Rho*, the ratio among initial phosphorylation sites was not changed (Table I). Similar results were observed with H7, a potent inhibitor of PKC (Hidaka et al., 1984) and a weak inhibitor of RK (Palczewski et al., 1990). Active and inactive phorbol myristoyl acetate isomers (PMA and 4-α-PMA) inhibited phosphorylation of Rho* to a similar degree, most likely by perturbing the membrane structure, rather than affecting PKC.

Purified Rho (urea-washed ROS lacking endogenous kinase

![Figure 2](image-url)
activity) was phosphorylated by homogeneous RK, purified from ROS or from an insect cell expression system (Fig. 1) at all three Ser residues (Table I), as in ROS homogenates. Minor variability in the initial sites of phosphorylation may relate to the presence of T80 in preparations of RK. Rho purified in dodecyl-β-maltoside on concanavalin A-Sepharose (Litman, 1982), was also phosphorylated by homogeneous RK at these three Ser residues (data not shown). No phosphorylation of Rho was detected by recombinant PKC (α-isoenzyme) or a constitutively active form of PKC (Allen et al., 1994), despite high specific radioactivity of [γ-32P]ATP (more than 1000 cpm/pmol) and high enzymatic activities of PKCs toward myelin basic protein (2 nmol of phosphate transferred/reaction/10 min). These data show that Rho* in ROS homogenates is phosphorylated at different C-terminal Ser residues exclusively by RK, but not by PKC or any other protein kinases.

Does Palmitoylation Affect Rho Phosphorylation?—Dipalmitoylation of Cys322 and Cys323 residues rigidly attaches the C-terminal region of Rho to disk membranes (Ovchinnikov et al., 1988; Papac et al., 1992; Moench et al., 1994), modifying accessibility of this region for the interaction with soluble proteins. Before we could analytically dissect the phosphorylation sites on depalmitoylated Rho, we had to solve a difficult technical problem. The usual depalmitoylation protocol employs 1 M dodecyl-β-maltoside on concanavalin A-Sepharose (Litman, 1982), producing a mixture of di-, mono-, and depalmitoylated Rho. Consequently, to identify the form of Rho from which a phosphorylated fragment is derived, the peptide should contain a “palmitoylation tag.” To separate depalmitoylated peptide from mono- and dipalmitoylated forms, phosphorylated opsins was acetylated to block Lys residues, carboxymethylated to block free Cys residues, and digested with trypsin at Arg314. The peptides heterogeneous palmitoylated at Cys322 and Cys323 were separated on a C8, pH-stable, HPLC column to depalmitoylated, monopalmitoylated, and dipalmitoylated Asn315–Ala348 peptides (Fig. 2A, panels a and b, and d). The identity of these peptides was confirmed by partial or complete depalmitoylation, Edman degradation, and matrix-assisted laser desorption mass spectrometry.

To identify phosphorylation sites, a mixture of partially depalmitoylated Rho (Morrison et al., 1991) was phosphorylated with purified RK. The distribution of phosphate on di-, mono-, and depalmitoylated tryptic Asp315, Ala348 peptides was analyzed by further digestion of individual species with endoprotease Asp-N. Di-, mono-, and unphosphorylated Asp315, Ala348 mixture of peptides were separated to individual phosphorylated components by HPLC chromatography in the presence of heptafluorobutyric acid (Ohguro and Palczewski, 1995). Di- and monophosphorylated peptides were further digested with S. aureus V8. No differences in phosphorylation for di- and depalmitoylated Rho were observed (Fig. 2B). Similar results were obtained for monopalmitoylated Rho* (data not shown). Phosphorylation was also not altered for depalmitoylated Rho after modification of Cys322 and Cys323 by carboxymethylation or S-sulfenylsulfonylation (negative charged groups), or carboxamidomethylation (neutral groups). Importantly, neither the rate of dephosphorylation by catalytic or holo-PrP 2A nor the binding of arrestin was affected by depalmitoylation (data not shown).

In contrast, casein kinase I phosphorylated depalmitoylated Rho/opsin decisively faster than Rho*, while Rho was not phosphorylated (Fig. 3). As determined by mass spectrometric analysis and proteolytic fragmentations, the phosphorylation was restricted to the C-terminal region of Rho, which contains a consensus sequence for casein kinase I, Asp-Asp-Glu-Ser334 (Agostinis et al., 1989).2 Apparently, depalmitoylation removed some conformational or accessibility constraints to casein kinase I.

![Diagram](image)

**Fig. 3.** Phosphorylation of depalmitoylated Rho but not palmitoylated Rho by casein kinase I. Urea-washed Rho or depalmitoylated urea-washed Rho (100 μg) was phosphorylated by casein kinase I (1 μg) at 30 °C in the dark or under 150-watt illumination. At each indicated time, an aliquot was precipitated with 10% trichloroacetic acid, and the stoichiometry was determined by 32P counting (indicated by parentheses) was estimated by radioactive profiles in trypsin subdigestion of the monophosphopeptide (peak a in Fig. 4A). Ratios described in the table were calculated by peak heights of each components and/or radioactive profiles. Experiments were performed in duplicate. All Rho preparations were phosphorylated to overall stoichiometry of 0.24–0.3 Pi/Rho.

**TABLE II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Multiple phosphorylated Rho</th>
<th>Monophosphorylated Rho</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>RK + Rho</td>
<td>39</td>
<td>60</td>
</tr>
<tr>
<td>RK + Gin344 Rho</td>
<td>11</td>
<td>89 (11)</td>
</tr>
<tr>
<td>RK + Glu345 Rho</td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>RK + Lys335 Rho</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

2 Phosphorylation at the first site led to subsequent phosphorylation that was confined to the C terminus. This sequential phosphorylation stemmed from formation of a new consensus sequence that involved phospho-Ser/Thr (Litchfield et al., 1990). Small amounts of highly heterogeneous phosphorylated Rho did not allow precise identification of the phosphorylation sites.
Among the opsins family of proteins, the sequence of the C-terminal four amino acids is highly conserved and mutations within this region have been frequently associated with retinitis pigmentosa (Macke et al., 1995). These observations suggest that this tetrapeptide may contribute to a conformation important for phosphorylation. To test this hypothesis, three Rho proteins truncated at Gln344, Glu341, and Lys339 were phosphorylated by RK, and the C-terminal peptides purified by HPLC after digestion with endoproteinase Asp-N. For each truncated Rho, multi-, mono-, and unphosphorylated peptides was further separated by HPLC using heptafluorobutyric acid (Ohguro et al., 1995), and then monophosphorylated peptides were rechromatographed under standard conditions on a C18 column. The radioactive profiles showed that C-terminal truncation reduced multiple phosphorylation (Table II). A single major monophosphorylated peptide (peak a, Fig. 4A) was obtained from Gln344 Rho, whereas two species of monophosphorylated peptides (peaks b and c and peaks d and e) were purified from Glu341 Rho and Lys339 Rho, respectively (Fig. 4A). MS/MS spectra of the monophosphorylated peptides a–e identified the major phosphorylation sites of Gln344 Rho (Ser338, minor at Ser334), Glu341 Rho (Ser334, Ser338), and Lys339 Rho (Ser334, Thr336) (Table II). These data demonstrate that the C-terminal conformation has a strong influence on the interaction with RK and may regulate the sites of phosphorylation within Rho*.

Does Rho Phosphatase Preferentially Dephosphorylate Specific Sites on Phosphorylated Rho?—Addition of a generic PrP inhibitor, KF, increased the extent of phosphorylation in ROS suspensions, presumably by preventing dephosphorylation, while an activator of a latent form of PrP 2A (Cai et al., 1995), Co2+, accelerated dephosphorylation (Fig. 5A). Addition of extra PrP 2A affected only minimally the phosphorylation level at 5 min, but accelerated the dephosphorylation of opsin, particularly at Ser343 (Fig. 5B). These differences in the preferred sites of opsin dephosphorylation may be a factor in the accumulation in vitro of species phosphorylated at Ser334 or Ser338.

How Do Calcium and Recoverin Affect Rho Phosphorylation in Vitro?—At micromolar concentrations of free calcium, recoverin has been reported to inhibit RK activity in a reconstituted system (Kawamura, 1993; Kawamura et al., 1994). We confirmed these results and found that phosphorylation of Rho* is typically inhibited by 10–20% in physiological range of free calcium (−50–600 nM; Gray-Keller et al. (1994)), without changes in the sites of phosphorylation (data not shown). We also found that recoverin/Ca2+ activated PrP 2A holoenzyme, but not the catalytic subunit or calmodulin (Fig. 6). The activation was less than that by poly-Lys. In contrast, dephosphorylation of phosphorylase a with holo-PrP 2A was unaffected by
recovery, while it was stimulated by poly-Lys (data not shown). These data suggest that the calcium effect mediated by recoverin is specific for the holo-enzyme and phosphorylated opsin.

Do Different Background Illuminations Affect In Vivo Phosphorylation of Mouse Rho?— To decipher the effect of calcium on Rhophosphorylation in vivo, we tested the level of phosphorylation using light-adapted mice. The idea is that continuous background illumination closes the cation channels (Lagnado and Baylor, 1992) and, in turn, lowers the internal free calcium in photoreceptor outer segments. Background illumination at 8 fC led to phosphorylation of Ser\(^{334}\) and Ser\(^{338}[/sup]/Ser\(^{343}\) at similar levels of \(-3.3\) and \(-3.1\%\) of total Rho (Table III). A single intense flash produced a modest increase in the phosphorylation of Ser\(^{334}\) and a more significant increase at Ser\(^{338}\)/Ser\(^{343}\). The latter increase is tempered as the background illumination is increased, and consequently free calcium decreased. These data suggest that as calcium is lowered, further phosphorylation in response to flash of light was decreased, in contrast to observed in vitro effects of recoverin on Rho phosphorylation.

**DISCUSSION**

Phosphorylation of Rho*: One or Many Protein Kinases— Results of Rho phosphorylation in vivo (Ohguro et al., 1995) suggested that upon illumination, Ser\(^{334}\), Ser\(^{338}\), and Ser\(^{343}\) are phosphorylated. All three sites were phosphorylated using purified native or recombinant RK and native ROS disk membranes stripped of endogenous protein kinases, or highly purified Rho. Furthermore, a specific RK-inhibitory antibody did not change the ratio of phosphorylation sites in ROS homogenates.
Methods we used to identify phosphorylated sites reconcile these findings? The reason could be inherent in the monophosphorylated species are detected in vivo readily observed (Wilden, 1995; Wilden and Kühn, 1982; Aton, 1995). Indeed, constitutive active or recombinant isoforms of PKCs did not produce any significant effect. In previous studies, the site(s) of PKC phosphorylation was not identified; however, it was confined to the C-terminal region of Rho phosphorylated by RK (Greene et al., 1995). It is worth noting that this region lacks, even loosely defined, a consensus sequence for PKC (Kennelly and Krebs, 1991), and that phosphorylation described in previous reports was performed with Rho at low nanomolar concentrations (Kelleher and Johnson, 1986; Newton and Williams, 1993), which may produce highly fragmented forms of membrane vesicles. If the sites of phosphorylation by PKC are identical to those modified by RK, based on the amounts of enzymatic activities, the contribution of PKC would be identical to those modified by RK and different preparations and concentrations of Rho and RK. 

### Table III

<table>
<thead>
<tr>
<th>Conditions Flash</th>
<th>Monophosphorylated Rho</th>
<th>Rho (non-phosphorylated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ser334</td>
<td>Ser270/Ser343</td>
</tr>
<tr>
<td>Dark</td>
<td>0%</td>
<td>(100.0)%</td>
</tr>
<tr>
<td>Dark +</td>
<td>4.5%</td>
<td>(88.2)%</td>
</tr>
<tr>
<td>8 fc</td>
<td>3.1%</td>
<td>93.6%</td>
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<tr>
<td>8 fc +</td>
<td>3.5%</td>
<td>76%</td>
</tr>
<tr>
<td>16 fc</td>
<td>3.3%</td>
<td>76.3%</td>
</tr>
<tr>
<td>16 fc +</td>
<td>4.1%</td>
<td>88.7%</td>
</tr>
<tr>
<td>32 fc</td>
<td>5.8%</td>
<td>86%</td>
</tr>
<tr>
<td>32 fc +</td>
<td>6.6%</td>
<td>85.1%</td>
</tr>
</tbody>
</table>

* Data are from previous studies (Ohguro et al., 1995).

No evidence was found for involvement of PKCs in Rho phosphorylation, as an activator or an inhibitor; addition of purified constitutive active or recombinant α isoforms of PKCs did not produce any significant effect. In previous studies, the site(s) of PKC phosphorylation was not identified; however, it was confined to the C-terminal region of Rho phosphorylated by RK (Greene et al., 1995). It is worth noting that this region lacks, even loosely defined, a consensus sequence for PKC (Kennelly and Krebs, 1991), and that phosphorylation described in previous reports was performed with Rho at low nanomolar concentrations (Kelleher and Johnson, 1986; Newton and Williams, 1993), which may produce highly fragmented forms of membrane vesicles. If the sites of phosphorylation by PKC are identical to those modified by RK, based on the amounts of enzymatic activities, the contribution of PKC would be identical to those modified by RK (Palczewski, 1993; Newton, 1993). It is unlikely that our preparations of ROS lost PKC, because they retained soluble proteins such as arrestin, and they were prepared without buffering calcium, conditions that should promote association of PKC to ROS membranes. It should be noted that electrophysiological experiments with intact Gecko ROS to which several different inhibitors of PKC, including H7, H8, staurosporin, or chelerythine, were introduced, did not affect electrical light responses. We conclude that it is not necessary to implicate any protein kinase other than RK in the phosphorylation of Rho.

Rho Phosphorylation: Mono- or Multiple Phosphorylated Forms—Mono- or Multiple Ser and Thr residues at the C-terminal region of Rho, cone pigments, or other G protein-coupled receptors are considered hallmarks for multiple phosphorylation (Baldwin, 1994; Hargrave and McDowell, 1992; Premont et al., 1995). Indeed, in vitro multiple phosphorylation of Rho was readily observed (Wilden, 1995; Wilden and Kühn, 1982; Aton et al., 1984; Aton, 1986; Adamus et al., 1993); however, only monophosphorylated species are detected in vivo. How does one reconcile these findings? The reason could be inherent in the methods we used to identify phosphorylated sites in vivo, and multiple phosphorylation could be an artifact of in vitro biochemical procedures. Our analysis of in vivo phosphorylation relies on an isolation of ROS membranes that requires 10–20 s (after the flash) before further phosphorylation/depolymerization is inhibited, potentially sufficient time for rapid dephosphorylation of multiply phosphorylated Rho. Alternatively, intensive bleaches could overproduce Rho before it decays to opsin, and this may not be phosphorylated by RK to high stoichiometry.

Arguments against these suppositions are based on evidence that dephosphorylation of Rho is a slow process in vitro and in vivo (minutes) (Palczewski et al., 1989a, 1989b; Fowles et al., 1989; King et al., 1994; Kühn, 1974; Ohguro et al., 1995). Prerequisites to dephosphorylation include binding of p44/5222, and removal of arrestin (Hofmann et al., 1992) and reduction of photolyzed chromophore by retinal dehydrogenase (Palczewski et al., 1994a) to remove arrestins (Palczewski et al., 1998b). In addition, slow decay of Meta II (τ½ = 16 s in frog at 30 °C, and even slower release of all-trans-retinal) (Baumann and Reinheimer, 1973) and accumulation of all-trans-retinal in the first minutes following illumination (Zimmerman, 1974), suggest that dephosphorylation occurs on a time scale of minutes, rather than seconds. Finally, the minimum estimated enzymatic activity of RK in vivo would predict the introduction of 3 P i/Rho during 3 s after a bleach that generated 0.4% Rho, well within the detection limit of our methods. A single phosphorylation is sufficient for the quenching of phototransduction (Bennett and Sitaramayya, 1988), and a prolonged, low intensity illumination (3 h) did not produce multiply phosphorylated species at detectable levels in the mouse retina. The multiple phosphorylation of Rho could be an artifact of biochemical procedures that uses very dilute ROS preparations with compromised membrane structures, intense bleaches without proper recycling of the visual chromophore, and long phosphorylation time courses.

At intense bleaches in vivo, less than half of the Rho is phosphorylated (Ohguro et al., 1995), suggesting that some steric hindrance, such as the binding of arrestins, prevents phosphorylation of each Rho molecule. This would be consistent with a report that only ~25% of Rho is accessible to transducin (Fung, 1983). This mechanism could limit phosphorylation of Rho at high levels of illumination but allow each Rho to be phosphorylated, at low levels of illumination; however, as ROS membranes are fragmented and arrestins are removed or diluted in vitro, this accessibility could be enhanced.

Requirements for Rho Phosphorylation: Kinase and Rho Conformations—Why is Rho phosphorylated in a heterogeneous manner at these three Ser residues? During photobleaching of Rho, three stable intermediates, Meta I, II, and III, are substrates for RK (Paulsen and Bentrup, 1983), and all appear to be phosphorylated at similar sites (Ohguro et al., 1994b). Upon binding of RK to Rho, the enzyme becomes more active either by simple proximity to the C-terminal region (Palczewski et al., 1991), or by actual activation of RK by Rho (Brown et al., 1993; Pullen et al., 1993; Dean and Akhtar, 1993). We believe that the proximity of the C-terminal peptide, despite its poor affinity for RK (Palczewski et al., 1998c), is sufficient to account for the increased catalysis (Fowles et al., 1988; Palczewski et al., 1991). A weak interaction between the C-terminal peptide of Rho and the active site of RK would promote a more random phosphorylation at the three sites.

Analysis of phosphorylation of C-terminally truncated forms of Rho (Ohguro et al., 1993, 1994b, 1995) or corresponding peptides (Fig. 7) (Pullen and Akhtar, 1994) revealed that the four terminal amino acids (VAPA) are critical in the selectivity of phosphorylation. Multiple phosphorylation appears to be sequential (as described previously by Ohguro et al. (1993))

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3 P. B. Detwiler, unpublished results.

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4 Note that minor variabilities between the initial phosphorylation sites were observed in previous studies (Ohguro et al., 1993, 1994b; Papae et al., 1993; McDowell et al., 1993; Palczewski et al., 1995). It is probable that they are a consequence of experimental conditions, including different time courses of phosphorylation, illumination regimes, and different preparations and concentrations of Rho and RK.
with a neighboring hydroxyl-containing amino acid being next to the primary site of phosphorylation. Interestingly, Sung et al. (1994) discovered that truncation of Rho at Gin344, as found in retinitis pigmentosa, prevented phosphorylation at Ser343 (Fig. 7), and produced 15% longer time-to-peak in the electrophysiological responses. The complete truncation of the C terminus at Gly329 leads to more prolonged responses, suggesting that Rho phosphorylation is needed for normal inactivation of the phototransduction cascade (Chen et al., 1995b). Phosphorylation at different sites may have important physiological consequences in serving different inactivation pathways of Rho.

Dephosphorylation of Rho—Dephosphorylation of Rho is a slow process in biochemical assays and in vivo (Palczewski et al., 1989a, 1989b; Fowles et al., 1989; King et al., 1994). In this paper, we investigate dephosphorylation of Rho in ROS homogenates supplemented with purified PrP2A (Fig. 5). We found that dephosphorylation occurs in the following order: Ser334 > Ser338 > Ser343. Considering continuous inactivation as a collection of many small flashes, one might predict an accumulation of phosphorylation at slowly dephosphorylating Ser334, as compared to Ser338/Ser343. Indeed, in vivo studies show that Ser334 remains phosphorylated longer after illumination than Ser338/Ser343 (Ohguro et al., 1995), in agreement with the proposed explanation.

Rho Phosphorylation: Calcium and Recoverin—A mammalian rod photoreceptor of the retina contains 10–15 Rho molecules that participate in signal transduction. Under low bleaching conditions Rho activation causes the photoreceptor cell to undergo hyperpolarization of the plasma membrane. However, in most daytime activities, our rod cells are either saturated or operate in a desensitized mode due to Ca2+-dependent adaptation to background illumination (Lagnado and Baylor, 1993). It has been proposed that the adaptation processes are moderated, in part, by a calcium-binding protein, recoverin, which inhibits RK activity when in a complex with Ca2+ (Kawamura, 1993). The data in Fig. 6 indicate that recoverin stimulates Rho phosphatase activity. Others have reported interactions of recoverin with immobilized opsins used for its purification (Dizhoor et al., 1991; Lambrecht and Koch, 1991), binding to RK (Gorodovikova et al., 1994a, 1994b; Chen et al., 1995a), and interference with transducin activation. In these studies, an attractive hypothesis, interaction of recoverin with RK (Kawamura et al., 1993), was investigated indirectly in vivo (Table III). In response to adaptation to background illumination, the photoreceptor cells lower their endogenous free calcium. In light-adapted animals, we found that a flash produced a decrease in Rho phosphorylation, rather than the increase expected if RK inhibition by recoverin was abolished. These data suggest that the recoverin effects may play only a minor role in a complex regulation of Rho phosphorylation in vivo.

These studies emphasize the importance of correlating in vitro and in vivo studies. From a combination of both approaches, we interpret Rho phosphorylation as a marker of conformational change in Rho* and RK, and we suggest that heterogeneity of phosphorylation may serve many different physiological functions during light responses in rod photoreceptors.

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REFERENCES

K. P. Hofmann, unpublished results.