Phosphorylation of Rhodopsin by Protein Kinase C in Vitro*

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Calculated for rhodopsin kinase. Rhodopsin phosphorylation was a reduced ability to stimulate the light-dependent rhodopsin activation of \(^{32}\)P
guanosine 5'-O-(thiotriphosphate) binding to transducin, the GTP-binding regulatory protein present in ROS. Properties of the calcium-stimulated interaction of protein kinase C with membranes and in vitro phosphorylation of intrinsic proteins are discussed based upon the findings.

Photoreceptor excitation involves absorption of a photon by rhodopsin which triggers a set of events resulting in a membrane voltage change. The sensitivity of the photoreceptor becomes reduced after illumination, a phenomenon referred to as adaptation or attenuation (1, 2). Phosphorylation-dependent regulation of rhodopsin function has been identified. We have now succeeded in demonstrating calcium-dependent phosphorylation of rhodopsin in vitro and have resolved this activity from the previously described rhodopsin kinase that phosphorylates only bleached rhodopsin in a calcium-independent manner (3–7). The calcium-dependent rhodopsin-phosphorylation activity was protein kinase C, which we have purified from ROS1 (18). Protein kinase C is present in ROS in approximately the same abundance as that calculated for rhodopsin kinase. Rhodopsin phosphorylation by protein kinase C was irreversible with nothing but required calcium. Protein kinase C phosphorylation of rhodopsin reduced rhodopsin's ability to activate transducin, the postulated rate-limiting step in photoreceptor excitation (19–24).

These results suggest a mechanism whereby rhodopsin function could be regulated in a calcium-dependent manner that could be amplified during visual excitation by changes in phosphatidylinositol breakdown and calcium mobilization, processes which are documented in the photoreceptor ROS (8–10, 12–15).

MATERIALS AND METHODS

Preparation of ROS Membranes—ROS were isolated from frozen dark-adapted bovine retinae. After thawing in 20 mM Tris-HCl, pH 7.4, 1 mM CaCl\(_2\), 5 μg/ml leupeptin, 0.3 unit/ml aprotinin, 8.7 μg/ml phenylmethylsulfonyl-fluoride (buffer A), and 45% (w/w) sucrose the rod segments were disrupted by several passes through a 50-ml syringe. ROS were isolated by flotation in a SW 28 rotor centrifuged at 25,000 \( \times \) g for 20 min. The ROS were collected and diluted in buffer A before being layered over a step gradient of 25 and 35% (w/w) sucrose in buffer A and centrifuged at 100,000 \( \times \) g for 20 min. The pellets were resuspended in 2 ml of 200 mM Na-Hepes, pH 8.0, 20 mM EDTA/50 mM retine to extract extrinsic membrane proteins, pelleted, and repeated 2 times. The extracts were pooled and dialyzed against 20 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1 mM DTT.

The pellets containing rhodopsin were recovered and represent 11.3 mg of rhodopsin and 50 mg of total protein. Stripped ROS membranes were prepared from this preparation by the method of Yamaizaki et al. (25). The final preparation of stripped ROS yielded 8.8 mg of rhodopsin and 25 mg of total protein from 50 retinas. The stripped ROS were stored at a concentration of 50 mg rhodopsin in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM MgCl\(_2\), 0.5 mM DTT at –80 \(^\circ\)C.

Purification and Assay of Protein Kinase C—Protein kinase C was purified by sequential chromatography on phenyl-Sepharose and DEAE-cellulose (18). Protein kinase C was assayed essentially as described by Vilgrain et al. (36).

Phosphorylation Assays—Specific conditions for phosphorylation assays are given in the figure legends. Incorporation of \(^{32}\)P from \(\gamma\)-\(^{32}\)P]ATP into rhodopsin was quantitated by Cerenkov counting of phosphopeptide profile on 15% acrylamide-SDS gels.

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The abbreviations used are: ROS, rod outer segments; protein kinase C, calcium/phospholipid-dependent protein kinase; PMA, 12-O-tetradecanoyl phosphorol 13-acetate; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography; EGTA, ethylenediaminetetraacetic acid; GTPyS, guanosine 5'-O-(thiotriphosphate); EGF, epidermal growth factor.
of Phosphopeptides—Stripped ROS were phosphorylated with either rhodopsin kinase or protein kinase C. The membranes were dissolved in SDS and reduced with DTT overnight at 25°C followed by alkylation with iodoacetamide (28). Samples were then electrophoresed on 10% acrylamide SDS gels and autoradiographed for 60 min to locate the rhodopsin band. The band was excised, washed for 40 min with distilled H2O to remove SDS, and then digested with three 50 μg/ml tosylphenylalanil chloride (tosylphenylalanyl chloromethyl ketone–trypsin aliquots in 3 ml of 50 mM NH4HCO3, pH 8.4, at 37°C for 24 h. Eighty-five per cent of the label in the rhodopsin band was released from the gel, and the pooled fractions were lyophilized and then resuspended in 1% trifluoroacetic acid. Samples were chromatographed on a C18 reverse phase HPLC column (Waters Bondapak, 3.9 mm × 30 cm) equilibrated in 0.1% trifluoroacetic acid and 0.05% triethylamine. Half-ml fractions were collected during a 0–45% acetonitrile gradient followed by a 100% methanol wash of the column. Fractions were analyzed by Cerenkov counting.

Phosphoamino acid analysis of the [35S]arachidonic acid analysis of the [35S]phosphopeptides was performed by partial acid hydrolysis for 1 h at 110°C in 6 N HCl followed by thin layer electrophoresis as described by Hunter and Sefton (29).

**Light-dependent Rhodopsin Activation of [35S]GTPγS Binding to Transducin—** Transducin was purified according to Baehr et al. (30). [35S]GTPγS (0.01–2.0 μM) binding to transducin as described in the legend to Fig. 6 was saturable and to a single high affinity site of Kd = 0.05 μM. Binding of [35S]GTPγS in the absence of rhodopsin was less than 2% of that observed in the presence of rhodopsin. Rhodopsin-stimulated [35S]GTPγS binding was dependent upon light and temperature. No binding was detectable if purified transducin was absent from the reaction mixture.

**RESULTS**

Isolated ROS contain the necessary components for light-activated phosphodiesterase stimulation as well as the phosphodiesterase activity that modifies bleached rhodopsin. All of the known components, except for rhodopsin, in these reactions are extrinsic membrane proteins which can be quantitatively stripped from the ROS membranes. Rhodopsin is an intrinsic membrane protein whose structure has been recently defined (31, 33). The rhodopsin-regulated phosphodiesterase activation, as well as the rhodopsin-phosphorylating activities, can be reconstituted simply by mixing the stripped ROS membranes containing rhodopsin with the extrinsic membrane proteins. Fig. 1A shows the results of such an experiment demonstrating that the extrinsic membrane protein fraction (referred to as extract) reconstitutes the light-dependent phosphorylation of rhodopsin. If ROS membranes are prepared in the presence of calcium and the extrinsic membrane proteins exhaustively stripped with chelator, dialyzed, and subsequently reconstituted with the stripped ROS in the presence of 200 μM CaCl₂, phosphorylation is observed in the absence of light and further enhanced by rhodopsin bleaching (Fig. 1B). Rhodopsin kinase is obviously present in the extract, but no rhodopsin phosphorylation is observed in the dark in the absence of added calcium (Fig. 1A, lane 3). Rhodopsin kinase readily phosphorylates bleached rhodopsin and does not require calcium for its activation (3–7). These facts indicate that the calcium-dependent rhodopsin phosphorylation observed in Fig. 1 was not due to the presence of bleached rhodopsin in the dark-adapted ROS, because rhodopsin kinase would readily phosphorylate the bleached photopigment. The simplest interpretation of the calcium-dependent rhodopsin phosphorylation was the presence in the extract of two rhodopsin-phosphorylating activities. An alternative explanation for the finding was a calcium-dependent inhibition of either a phosphatase or even an ATPase. It seemed unlikely that phosphatase activity was being inhibited by calcium because the light-dependent rhodopsin phosphorylation in the absence of calcium was stable for long incubation periods at room temperature (not shown), indicating the rhodopsin phosphorylation was not readily reversed with these conditions. Similarly, increasing the ATP levels up to 8-fold did not enhance rhodopsin phosphorylation in dark-adapted ROS suggesting ATP hydrolysis was not a problem in the protocol described in Fig. 1.

For these reasons attention was turned toward identifying specific kinases in the extract in addition to the previously characterized rhodopsin kinase. Table I shows the soluble extract from ROS membranes contained significant calcium- and phospholipid-dependent histone H1-phosphorylating activity. This activity was dependent on the simultaneous presence of calcium and a mixture of phosphatidylyserine and diacylglycerol. The concentration of calcium required in order to obtain maximal histone phosphorylating activity was greatly reduced in the presence of the phorbol ester FMA (18). As described elsewhere (18) we have purified this histone kinase activity from the ROS extract and demonstrated it correlates with the presence of an 85-kDa protein on SDS-acrylamide gels and has the properties of protein kinase C.

Preparation of ROS membranes in the absence of calcium resulted in the loss of protein kinase C activity that could be stripped by EDTA during the removal of extrinsic membrane proteins and resulted in the loss of the calcium-dependent light-independent rhodopsin phosphorylation observed in Fig. 1. These observations suggested that the protein kinase C might be responsible for the calcium-dependent phosphorylation of rhodopsin.

It should be noted that protein kinase C-dependent phosphorylation of intrinsic membrane proteins such as the recep-
Rhodopsin Phosphorylation

Extrinsic proteins associated with the isolated ROS membranes were solubilized using 20 mM EDTA and after dialysis assayed for histone H1 kinase activity. Varying amounts of CaCl2 were added to the incubation mixture and free calcium concentrations added were estimated using a computer-assisted program shown were based upon 10-min incubations, but phosphorylation concentrations of calcium or PMA alone was significantly less 200 μM DAG). Incubation mixtures were solubilized using either unbleached or bleached rhodopsin, and the ability of the EGTA-eluted fractions to phosphorylate rhodopsin occurred in the absence of illumination, but essentially identical results were obtained using bleached rhodopsin. The binding of protein kinase C to the ROS membranes occurred when the free calcium added was raised above 0.1 μM and was complete at 2 μM. The calcium concentration required for rhodopsin phosphorylation (Fig. 4B) was similar to that for inducing translocation, consistent with the notion that interaction of protein kinase C with the membrane activated the enzyme in a calcium-dependent mechanism. The strong correlation of the supernatant loss in phosphatidylserine and diacylglycerol-dependent histone phosphorylation with the increased rhodopsin phosphorylation of the particulate fraction also indicates that protein kinase C was responsible for both activities. Presumably, the protein kinase C binding involved an anionic phospholipid such as phosphatidylserine and possibly diacylglycerol in the ROS membranes.

Fig. 5 demonstrates two independent peptide-mapping strategies to characterize the rhodopsin phosphorylation sites for protein kinase C relative to the well characterized sites phosphorylated by rhodopsin kinase (3–5, 27, 37, 38). Short phosphorylation periods were used with both kinases, and the concentration of each kinase was adjusted so that similar amounts of 32P were incorporated into rhodopsin. These conditions were chosen so that the results might provide information regarding the preferred serine and threonine residues on the rhodopsin molecule. Light-dependent phosphorylation was performed in the presence of added EGTA to ensure that calcium-dependent phosphorylation was completely absent. The calcium-dependent protein kinase C phosphorylation of rhodopsin was performed in the dark. The autoradiographs in Fig. 5A show that the light-dependent kinase activity was specific for rhodopsin. Calcium-dependent protein kinase C phosphorylation of ROS membrane proteins was somewhat less specific. The preferred substrate, however, was rhodopsin which is labeled much more intensely than any other band. Previous experiments indicated the labeled band at about 68 kDa was actually a rhodopsin dimer (not shown). After phosphorylation the membranes were washed and treated with Staphylococcus aureus V8 protease. Labeled peptides were then analyzed on polyacrylamide-SDS gels. V8 protease has been demonstrated to cleave membrane-bound rhodopsin specifically at positions Glu229 and Glu341 (27, 37). Residue Glu229 resides in one of the three predicted cytoplasmic loops of rhodopsin (27, 31, 32), and Glu341 is 7 residues from the carboxyl terminus (27, 31). As judged by Coomassie Blue staining the V8 cleavage of rhodopsin was similar after phosphorylation by the two kinases (not shown). Labeled phosphate could not be detected in the largest V8-generated peptide (met-Glu229) after rhodopsin phosphorylation by either kinase, even after autoradiography for up to 2 weeks. For light-dependent phosphorylation by rhodopsin kinase this finding is in agreement with previous work (27, 37, 38) that

### TABLE I

| Assay condition | Calcium | Histone kinase activity |
|-----------------|---------|-------------------------|
| PS/DAG | μM | dpm/25 μl/10 min |
| – | 3,827 ± 392 |
| – | 3,762 ± 200 |
| + | 3,592 ± 116 |
| + | 0.05 | 106,087 ± 5,510 |
| + | 0.2 | 206,710 ± 6,806 |
| + | 100 | 290,550 ± 6,811 |
| + | 600 | 264,437 ± 11,503 |

2 R. J. Davis, G. L. Johnson, D. J. Kelleher, J. K. Anderson, J. E. Mole, and M. F. Czech, manuscript in preparation.
FIG. 2. Purification of protein kinase C by calcium- and phosphatidylserine-dependent hydrophobic chromatography. One ml of dialyzed extract prepared from ROS membranes was brought to 0.035 mM phosphatidyserine, 3 mM MgCl₂, and 3 mM CaCl₂ and then applied to a 1-ml phenyl-Sepharose Cl-4B column equilibrated in 20 mM Tris-HCl, pH 7.4, 0.1 mM CaCl₂, 1 mM DTT. The column was then washed with 10 ml of equilibration buffer (arrow A). Protein kinase C activity was eluted using 20 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM DTT (arrow B). One ml fractions were collected, and 10-μl aliquots were assayed for histone H1 kinase activity with an excess of calcium over the chelator and in the presence or absence of phosphatidylserine and diaclylglycerol (PS/DAG). Thirty-μl aliquots of each fraction were then assayed for their ability to phosphorylate rhodopsin. Stripped ROS membranes devoid of measurable kinase activity were used as substrate. Reactions were performed for 10 min at 30 °C either with constant illumination (+hu) or in the dark (−hu). The final reaction mixture (150 μl) contained 2.5 μg of membrane protein, 20 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 0.5 mM DTT, 0.33 mM EGTA, 12 μM [γ-32P]ATP (8000 dpm/pmol) in the presence or absence of 1 mM CaCl₂. Reactions were terminated by additions of SDS and electrophoresed on 10% acrylamide gels. Autoradiographs represent 3-h exposures.

![Graph](image)

Fractions were assayed for histone H1 from 0 to 40. Incorporation into histone H1 from 0 to 40 is shown on the graph. The graph shows the fraction number on the x-axis and the incorporation of [γ-32P]ATP on the y-axis.

FIG. 3. PMA stimulation of rhodopsin phosphorylation at a limiting calcium concentration. Five microliters of the dialyzed extract was incubated (5 min at 30 °C) with stripped ROS membranes (2.5 μg) in a mixture containing 40 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 530 μM EDTA, 200 μM EGTA, 0.5 mM DTT, 10 μM [γ-32P]ATP in the presence (lanes 1 and 3) or absence (lane 2) of 100 μM CaCl₂. The samples represented by lanes 1 and 3 also contained 0.2 μM PMA. The reaction was stopped with SDS and the samples electrophoresed as described for Fig. 1.

![Image](image)

Fig. 3. PMA stimulation of rhodopsin phosphorylation at a limiting calcium concentration. The figure shows the phosphorylation of rhodopsin in the presence of PMA and calcium. The phosphorylation was measured by autoradiography after electrophoresis. The figure indicates that phosphorylation is stimulated by PMA and calcium.

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with stripped ROS membranes for 30 min at 4°C in the dark. The
phospholipid-dependent histone kinase activity eluate from the phenyl-Sepharose column (Fig. 2) were incubated
protein kinase C to ROS membranes. Pooled fractions of the EGTA
varying amounts of CaCl2. The samples were then centrifuged for 2
min and the pellets and supernatants separated in the dark. Phos-
pholipid-dependent histone kinase activity (■■■■) was measured
in the supernatants and expressed as the per cent of control. Control
activity (1.6 × 10^4 dpm/50 µl/min) was determined using supernatant
from samples originally incubated without added CaCl2. The pellets
were resuspended to 150 µl in 40 mM Tris-HCl, pH 7.4, 10 mM MgCl2,
253 µM CaCl2, 33 µM MnCl2, 330 µM EDTA, 0.5 mM DTT, and 10 µM [γ-32P]ATP
(7000 dpm/pmol). After incubation for 5 min at 30°C in the dark the
reaction was stopped with SDS and the samples electrophoresed on
10% acrylamide-SDS gels. Note that all phosphorylation reactions
were in the presence of a saturating concentration of added calcium.

A reaction mixture in a final volume of 500 µl contained 20 mM Tris-
HCl, pH 7.4, 900 µM MgCl2, 200 µM EGTA, 100 µM EDTA, stripped
ROS membranes (3.2 µg), 100 µl of protein kinase C eluate, and
varying amounts of CaCl2. The samples were then centrifuged for 2
min and the pellets and supernatants separated in the dark. Phos-
pholipid-dependent histone kinase activity (■■■■) was measured
in the supernatants and expressed as the per cent of control. Control
activity (1.6 × 10^4 dpm/50 µl/min) was determined using supernatant
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10% acrylamide-SDS gels. Note that all phosphorylation reactions
were in the presence of a saturating concentration of added calcium.

The 32P incorporation into rhodopsin was determined by Cerenkov
counting of the excised 39-kDa rhodopsin band and is expressed as
per cent of control (■■■■). The control activity (9.5 × 10^3 dpm
incorporated into rhodopsin in 5 min/ROS pellet) was determined
using pellets from samples originally incubated with a saturating
concentration of free calcium (112 µM). B, calcium concentration
dependence of rhodopsin phosphorylation. Stripped ROS and the
EGTA-eluted protein kinase C fraction from the phenyl-Sepharose
column were incubated as described above, and after 30 min at 4°C
in the dark 10 µl [γ-32P]ATP (2000 dpm/pmol) was added and the
samples were incubated for 5 min at 30°C. The reaction was stopped
with SDS and the samples electrophoresed on 10% acrylamide-SDS
gels and autoradiographed.

Peptide mapping of phosphorylated rhodopsin. A, rhodopsin was phosphorylated in a light-dependent manner using rhodopsin kinase with EGTA in excess of CaCl2. Alternatively protein kinase C was used to phosphorylate rhodopsin in darkness and in the presence of CaCl2. The kinase preparations were those resolved by phenyl-Sepharose chromatography as shown in Fig. 2. Phosphate incorporation/mol of rhodopsin was 0.046 mol for rhodopsin kinase and 0.051 mol for protein kinase C. After digestion of rhodopsin with V8 protease as described under "Materials and Methods" the samples were analyzed on 15% acrylamide gels. The gels were stained with Coomassie Blue to visualize the characteristic V8 digestion pattern of membrane-associated rhodopsin (27) and then autoradiographed to visualize the 32P phosphopeptides. B, rhodopsin which had been phosphorylated as described above was reduced, alkylated, and electrophoresed on a 10% acrylamide-SDS gel. The rhodopsin band was visualized by autoradiography, excised from the gel, washed in H2O followed by 30 mM ammonium bicarbonate, and digested for 24 h with trypsin. Phosphopeptides were analyzed by reverse phase HPLC as described under "Materials and Methods." Three 32P-phosphopeptide peaks were identified at 18, 22, and 26% acetonitrile. Recovery of labeled radioactivity from the C18 column was approximately 50%. Phosphoamino acid analysis was performed on the peak fractions.

FIG. 5. Peptide mapping of phosphorylated rhodopsin. A, rhodopsin was phosphorylated in a light-dependent manner using rhodopsin kinase with EGTA in excess of CaCl2. Alternatively protein kinase C was used to phosphorylate rhodopsin in darkness and in the presence of CaCl2. The kinase preparations were those resolved by phenyl-Sepharose chromatography as shown in Fig. 2. Phosphate incorporation/mol of rhodopsin was 0.046 mol for rhodopsin kinase and 0.051 mol for protein kinase C. After digestion of rhodopsin with V8 protease as described under "Materials and Methods" the samples were analyzed on 15% acrylamide gels. The gels were stained with Coomassie Blue to visualize the characteristic V8 digestion pattern of membrane-associated rhodopsin (27) and then autoradiographed to visualize the 32P phosphopeptides. B, rhodopsin which had been phosphorylated as described above was reduced, alkylated, and electrophoresed on a 10% acrylamide-SDS gel. The rhodopsin band was visualized by autoradiography, excised from the gel, washed in H2O followed by 30 mM ammonium bicarbonate, and digested for 24 h with trypsin. Phosphopeptides were analyzed by reverse phase HPLC as described under "Materials and Methods." Three 32P-phosphopeptide peaks were identified at 18, 22, and 26% acetonitrile. Recovery of labeled radioactivity from the C18 column was approximately 50%. Phosphoamino acid analysis was performed on the peak fractions.

reaction were confirmed by mixing the two 32P-labeled rhod-
opsin digests and demonstrating co-elution of the peptides
in peaks I, II, and III (not shown). Peak I was predominantly
labeled in the rhodopsin kinase reaction. In contrast, peak II
was predominant in the protein kinase C reaction, but signif-
ificant phosphorylation was also observed in peaks I and III.
Phosphoamino acid analysis of each peak indicated P-Ser in
peak I, primarily P-Thr with small amounts of P-Ser in peak
II, and a mixture of P-Ser and P-Thr in peak III for the
phosphopeptides derived from both the light-dependent and
calcium-dependent phosphorylation protocols.

The combined results in Fig. 5 of exhaustive trypsin
digestion of rhodopsin and V8 protease digestion of membrane-
associated rhodopsin indicate the phosphorylation domains
are similar for the two kinase reactions. The preferred phos-
phorylation sites on rhodopsin, however, appear to be differ-
ent in the two kinase reactions. Our findings are consistent
with the extensive work of others (37-40) that the phos-
phorylation sites for rhodopsin kinase map to the carboxy-
terminal region of rhodopsin.

An important observation during the course of these exper-
iments was that protein kinase C phosphorylates rhodopsin
in the absence of light (Fig. 5). This suggests that the car-

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Boxyl-terminal phosphorylation sites on rhodopsin are exposed on the cytoplasmic surface of the rhodopsin molecule in the absence of bleaching. The conformational change occurring in the rhodopsin molecule upon photon absorption stimulates both the binding and activation of rhodopsin kinase. This mechanism might serve to target rhodopsin kinase to bleached rhodopsin, rather than to expose the phosphorylation sites. A similar targeting mechanism involving conformational changes in rhodopsin is not used for protein kinase C since its activation is independent of light. Two mechanisms described to occur in vivo could function to activate protein kinase C and amplify the covalent modification of rhodopsin beyond that observed with the bleached molecules and rhodopsin kinase. First, it has been proposed that light induces a translocation of calcium from intradiscal sites to the surface of discs (8, 9). Second, light has been demonstrated to stimulate the breakdown of phosphatidylinositol which will result in the generation of diacylglycerol (12). Both mechanisms could increase the binding of protein kinase C to membranes and stimulate its activity. Because these mechanisms are activated by light in vivo they could provide the necessary targeting specificity for protein kinase C binding to membrane domains near the sites of rhodopsin bleaching for amplification of the adaptation signal.

In support of this hypothesis, previous work by other laboratories has demonstrated that ROS preparations containing rhodopsin kinase activity phosphorylate rhodopsin upon bleaching and that the phosphorylated rhodopsin has a diminished ability to activate the retinal GTP-binding protein transducin (39, 40). The mechanisms and enzymes mediating these responses in vivo are not clearly defined; however, calcium and changes in phosphatidylinositol metabolism appear important in the in vivo adaptive responses. The peptide mapping shown in Fig. 5 indicated that both rhodopsin kinase and protein kinase C will phosphorylate sites within the carboxyl-terminal domain of rhodopsin. This result predicts that similar functional changes might be seen in rhodopsin's ability to activate transducin. To test this prediction, control and protein kinase C-phosphorylated rhodopsin was used in measurements of light-dependent rhodopsin stimulation of [35S]GTPyS binding to the a subunit of purified transducin. Conditions were chosen so that rhodopsin was limiting in the activation of [35S]GTPyS binding to transducin (Fig. 6). The absence of added Mg-ATP prevented the stimulating and activating effects of protein kinase C phosphorylation of rhodopsin and was used as a control to show that the purified protein kinase C preparation did not contain an inhibitor of transducin activation. Similarly, calcium in the absence of protein kinase C was without effect. When protein kinase C was incubated with the stripped ROS in the presence of calcium and Mg-ATP, there was a 40–45% decrease in the ability of rhodopsin to activate [35S]GTPyS binding to transducin. However, part of this inhibition was observed with protein kinase C and calcium alone, in the absence of added Mg-ATP. Using the conditions described in Fig. 6 about 0.15 mol of 32P, was incorporated per mol of rhodopsin, and this value correlates well with the decrease in transducin activation attributable to phosphorylation which was approximately 20 ± 5% (average ± S.D., N = 4 experiments). The reduction in rhodopsin activation of transducin in the absence of ATP appeared related to protein kinase C binding to the membranes. If the protein kinase C bound to sites near rhodopsin, it could sterically inhibit transducin binding. This is the simplest explanation for this observation (see below), and a similar observation has been made for rhodopsin kinase-inhibiting transducin activation in the absence of phosphorylation. Nonetheless, there is a positive correlation with rhodopsin phosphorylation and a decreased ability of light absorption to activate [35S]GTPyS binding to transducin.

High concentrations of protein kinase C (0.8–1.2 units/ml, where 1 unit is defined as 1 nmol of phosphate transferred to histone H1/min) were required in the experiments shown in Fig. 6 when 25–50 nm rhodopsin was included in the reaction mixture. The high protein kinase C to rhodopsin ratios were required to observe sufficient phosphorylation to measure significant decreases in transducin activation, similar to the problems reported for rhodopsin kinase using similar protocols. An initial unexpected observation was that phosphorylation incubations for longer times did not increase the incorporation of phosphate into rhodopsin. As shown in the inset of Fig. 7, rhodopsin phosphorylation was rapid and plateaued after approximately 5 min. Addition of EGTA to release the membrane-bound protein kinase C indicated it was still active as measured by its ability to phosphorylate soluble histone H1 in the presence of phosphatidylerine, diacylglycerol, and additional calcium. In fact, the histone phosphorylation was linear for greater than 20 min (not shown). This finding indicated that protein kinase C was still functional, and the “turn-off” of rhodopsin phosphorylation was not due to kinase denaturation. Furthermore, the phosphorylation was stable and no evidence for a contaminating phosphatase was observed.

Fig. 7 also characterizes the stoichiometry of the protein kinase C phosphorylation of rhodopsin intrinsically associated with disc membranes. For all concentrations of protein kinase C and membrane-associated rhodopsin used, the phosphorylation reaction reached a plateau within 5–8 min, and further addition of ATP was without effect. With increasing protein kinase C, the stoichiometry of rhodopsin phosphorylation increased with higher stoichiometries obtained as the ratio of protein kinase C/rhodopsin increased. Similar characteristics of rhodopsin phosphorylation by rhodopsin kinase have been recently described by Sitaramayya and Liebman (41). They used “rhodopsin kinase-enriched membranes” and found that with 22% bleaching the stoichiometry of phosphorylation was 0.07 mol of phosphate/mol of bleached rhodopsin. As the percentage of bleached rhodopsin was decreased the stoichiometry of phosphorylation of bleached rhodopsin increased. The functional consequence of decreasing the percentage of bleaching was to effectively raise the ratio of rhodopsin kinase to bleached rhodopsin, similar to the change in ratio of protein kinase C to total rhodopsin observed in Fig. 7. The properties of rhodopsin phosphorylation by the two kinases, therefore, appears both qualitatively and quantitatively similar.

The majority of studies characterizing protein kinase C have utilized mixtures of phosphatidylerine, diacylglycerol, and calcium to phosphorylate substrates that have generally been soluble, such as histone H1. Under these conditions, a unit of protein kinase C transfers 1 nmol of phosphate/min. However, the consensus from several studies suggests that protein kinase C targets are usually membrane-associated proteins and that the generation of diacylglycerol in the membrane activates protein kinase C associated with the membrane. This is obviously different from the phosphorylation of a soluble protein in the presence of high concentrations of phospholipids. Table II indicates that 1 unit of protein kinase C measured using the standard histone H1 assay will transfer 1–4 pmol of phosphate/min to rhodopsin on average during a 5-min phosphorylation assay. To date, we are aware of only two other intrinsic membrane proteins that have been shown to be substrates for protein kinase C with functional
to ROS membranes and phosphorylates rhodopsin. Protein kinase C is, therefore, expressed at about the same level as rhodopsin kinase (42) in the ROS. The phosphorylation sites on rhodopsin are near the carboxyl terminus for both kinases. Thus, the functional consequences of rhodopsin phosphorylation by either kinase are predicted to be similar. This is, in fact, what is observed in that the catalytic activation of transducin is diminished when rhodopsin is phosphorylated. These findings make protein kinase C a strong candidate for an amplifying mechanism to regulate rhodopsin activity, since it is now thought that adaptation in the photoreceptor involves calcium and changes in phosphatidylinositol metabolism (12–17).

Recently, a 48-kDa protein, identified as the 48K protein which probably corresponds to the retinal S antigen, has been shown to bind to ROS in a light-dependent manner similar to rhodopsin kinase (43–45). The 48K protein appeared to have enhanced binding to bleached phosphorylated rhodopsin. It is possible that the 48K protein could also bind to unbleached phosphorylated rhodopsin and further uncouple its ability to activate transducin. Alternatively, the 48K protein may require the bleached conformation of rhodopsin and could be involved in the regeneration of the photopigment. The ability to isolate phosphorylated unbleached rhodopsin now allows this question to be addressed specifically.

The finding that protein kinase C phosphorylated residues...
commonly recognize such residues (46). The EGF receptor phosphorylation site at Thr<sup>644</sup> for protein kinase C is within a very basic sequence on the cytoplasmic domain near the membrane-spanning region for the protein (47). Few other membrane proteins have been determined to be substrates for protein kinase C so no consensus sequences are apparent. Like other protein kinases, however, secondary and tertiary structural determinants are probably very important for protein kinase C recognition (48). In this regard, the protein kinase C phosphorylation site is obviously close to the membrane. Interestingly, of the three predicted cytosolic loops and carboxyl-terminal tail for rhodopsin, the greatest conservation is within the first loop structure (31-33, 49, 50). Six of the 12 amino acids are conserved between <i>Drosophila</i> and bovine rhodopsins, and two of the six conserved residues are basic. No acidic residues are present on this loop in any of the rhodopsins whose sequence has been determined. If basic residues are indeed important for protein kinase C recognition it is easily seen how the carboxyl-terminal phosphorylation sequences could be in close proximity to the basic residues in the first cytoplasmic loop. Furthermore, the conservation in sequence of this first loop indicates it must be important for rhodopsin function and regulation. We have succeeded in developing antisera to synthetic peptides that bind to the cytoplasmic domains of rhodopsin which will allow us to address this problem directly using site-directed probes and purified kinases.

The final issue our results address is the mechanism of protein kinase C regulation of membrane proteins. Since it is assumed that diacylglycerol is important in activation and regulation of protein kinase C (51, 52), then protein kinase C probably is activated <i>in vivo</i> when associated with a membrane. The breakdown of phosphatidylinositol is one mechanism to generate diacylglycerol and is thought to be involved in the regulation of protein kinase C (51, 52), Nishizuki and co-workers (53) and studies in our laboratory<sup>e</sup> have demonstrated that protein kinase C bound to plasma membranes does not readily phosphorylate soluble substrates such as histone H1. The calcium-dependent binding and activation of protein kinase C in the presence of membranes is very different from the phosphatidylinerine and diacylglycerol mixtures used to stimulate protein kinase C phosphorylation of soluble proteins. The apparent tight binding of protein kinase C to the membrane appears to actually sequester the enzyme in a localized region. The consequence of such a sequestration appears to be to limit the access of available substrates for phosphorylation. This could serve to target the enzyme and allow specificity of regulation for an enzyme that appears, <i>in vitro</i>, to have broad substrate recognition (51, 53). Inactivation of protein kinase C would require additional metabolism of membrane components so that the stability of the enzyme-calcium-membrane complex would be reduced allowing the enzyme to dissociate. In the cell, this process would be predictably fast, and as long as a stimulus exists that generates diacylglycerol and/or calcium redistribution the enzyme could cycle by binding to the membrane followed by its release. In the ROS, for example, light might serve as the stimulus. In isolated membranes the ability to metabolize the membrane components required for the targeting and cycling of protein kinase C is apparently lost. In the presence of calcium protein kinase C is tightly bound to the membrane and remains sequestered until the calcium is chelated. The consequence of such sequestration in the presence of calcium is an apparently low turnover number for protein kinase C bound to mem-

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**TABLE II**

| Substrate       | Time course<sup>a</sup> | Protein kinase C activity |
|-----------------|--------------------------|---------------------------|
|                 |                          | pmol/min/unit             |
| EGF receptor<sup>b</sup> | 2                        | 0.3-0.6                   |
| Transferrin receptor<sup>c</sup> | 8                        | 1.6                       |
| Rhodopsin       | 5                        | 1-4                       |
| Histone         | >60                      | 1000                      |

<sup>a</sup>Time at which a plateau for maximal phosphorylation is obtained.
<sup>b</sup>Ref. 34.
<sup>c</sup>Davis et al., manuscript in preparation (Footnote 2).

near the carboxyl terminus of rhodopsin and probably within the serine- and threonine-rich phosphorylation domain on the amino-terminal side of Lys<sup>399</sup> is consistent with the reported site specificity of protein kinase C which appears to

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<sup>d</sup>D. J. Kelleher and G. L. Johnson, unpublished observation.
branes in vitro. Although a relatively low turnover number would be predicted for a targeting mechanism that involves binding of an enzyme to membrane components that are required for activation, in vivo protein kinase C would be predicted to cycle as the membrane constituents like diacylglycerol are metabolized and the enzyme is released from the membrane and becomes available for binding to new sites in the continued presence of an appropriate stimulus.

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