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Kinetics and Localization of the Phosphorylation of Rhodopsin by Protein Kinase C*

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Protein kinase C isolated from retina catalyzes the stoichiometric phosphorylation of bovine rhodopsin. Enzymological studies using receptor in rod outer segment membranes stripped of peripheral proteins reveal that the phosphorylation is independent of receptor conformation or liganded state; the half-time for phosphorylation of unbleached (dark-adapted) rhodopsin, bleached (light-activated) rhodopsin, and opsin (chromophore removed) is the same. The phosphorylation by protein kinase C is Ca^{2+} and lipid regulated; the K_m for Ca^{2+} decreases with increasing concentrations of membrane, consistent with known properties of Ca^{2+}-regulated protein kinase Cs. The K_m for ATP is 27 μM, with an optimal concentration for $MgCl_2$ of approximately 1 mM. The phosphorylation of rhodopsin by protein kinase C is inhibited by the protein kinase C-selective inhibitor sangivamycin. Proteolysis by Asp-N reveals that all the protein kinase C phosphorylation sites are on the carboxyl terminus of the receptor. Cleavage with trypsin indicates that Ser^{338}, the primary phosphorylation site of rhodopsin kinase, is not phosphorylated significantly; rather, the primary phosphorylation site of protein kinase C is on the membrane proximal half of the carboxyl terminus. The protein kinase C-catalyzed phosphorylation of rhodopsin is analogous to the ligand-independent phosphorylation of other G protein-coupled receptors that is catalyzed by second messenger-regulated kinases.

Second messenger-regulated kinases and substrate-regulated kinases provide two desensitizing mechanisms in the regulation of G protein-coupled receptors. Most notably, the β adrenergic receptor is desensitized at low ligand levels primarily by phosphorylation catalyzed by protein kinase A, and possibly C, and at high ligand levels by phosphorylation mediated by the β adrenergic receptor kinase (1). Recent evidence implicates both types of kinases in regulation of olfactory receptors (2, 3) and muscarinic acetylcholine receptors (4). For these signaling pathways, phosphorylation by two differently regulated kinases allows exquisite fine tuning of receptor function.

In phototransduction, it has been clearly established that a G protein-coupled receptor kinase, rhodopsin kinase, phosphorylates and deactivates light-activated rhodopsin (5, 6). In this pathway, absorption of a photon induces isomerization of the receptor's covalently bound ligand, 11-cis retinal, thus effecting a conformational change that exposes cytoplasmic surfaces on the receptor to allow interaction with transducin (6). These exposed surfaces also promote binding of rhodopsin kinase and subsequent phosphorylation on the receptor's carboxyl terminus. The primary phosphorylation sites by rhodopsin kinase *in vitro* have been recently identified as Ser^{338} and Ser^{343} (7–9). Phosphorylation on the carboxyl terminus decreases the interaction with transducin, an interaction that is effectively quenched when arrestin binds the polyphosphorylated carboxyl tail (10). Similar to other G protein-coupled receptor kinases, rhodopsin kinase displays strict specificity for the active conformation of the receptor.

Mounting evidence implicates phosphorylation by an additional kinase, protein kinase C, in the phosphorylation of rhodopsin. First, hyperactivation of protein kinase C in the intact retina, by treatment with phorbol esters, alters the phosphorylation of rhodopsin in a light-dependent manner (11, 12). Second, rhodopsin is phosphorylated by protein kinase C *in vitro* (11, 13). This phosphorylation has been shown to uncouple the receptor from transducin (13). Third, the allosteric activator of protein kinase C, diacylglycerol, has been shown by many groups to be produced in response to light (14–18). Phospholipase Cs have been biochemically isolated from and immunolocalized to photoreceptors (19, 20), and, more recently, cDNAs encoding 4 phospholipase Cβs with high homology to the *Drosophila norpA* gene (which encodes the phospholipase C involved in invertebrate phototransduction) have been identified (21). The sensitivity of rhodopsin phosphorylation to protein kinase C activators *in situ* and the light-dependent generation of diacylglycerol in rod outer segments support a role for protein kinase C in visual transduction.

This contribution provides a kinetic and structural analysis of the phosphorylation of rhodopsin by protein kinase C. Our data reveal that the phosphorylation of rhodopsin by protein kinase C is mechanistically similar to the phosphorylation of other G protein-coupled receptors by second messenger-regulated kinases. Most importantly, the phosphorylation is independent of the liganded state of the receptor, and the primary phosphorylation site, although on the carboxyl terminus, differs from that of the G protein-coupled receptor kinase.

EXPERIMENTAL PROCEDURES

Materials

ATP, leupeptin, phorbol myristate acetate (PMA),[1] trypsin (1.2×10^4 BAEE units mg^{-1}), and phenylmethylsulfonyl fluoride were purchased from Sigma. [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$) and chemiluminescence Ren-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L39909.

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[1] The abbreviations used are: PMA, phorbol myristate acetate; BAEE, N^a-benzoyl-L-arginine ethyl ester; DTT, dithiothreitol; MOPS, 3-[*N*-morpholino]propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

aissance reagents were supplied by DuPont NEN. Endoproteinase Asp-N (sequencing grade) and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from Boehringer Mannheim; octylglucoside was from Calbiochem. The ultrapure glycerol in which the protein kinase C was stored was supplied by U. S. Biochemical Corp. Q Sepharose Fast Flow and phenyl-Sepharose resins and phenyl Superose (HR 5/5) and Mono Q (HR 5/5) columns were obtained from Pharmacia Biotech Inc. The protein kinase C selective peptide substrate (FKKSFKL-NH_2; Ref. 22) was synthesized by the Indiana University Biochemistry Biotechnology Facility. A polyclonal antibody against opsin was generated as described (12). The 11-cis retinal was a generous gift from the National Eye Institute. All other chemicals were reagent grade.

Methods

Isolation of Rod Outer Segment Membranes—Rod outer segments were isolated from 60 dark-adapted bovine retinas using sucrose density gradient centrifugation as described previously (23). These were resuspended in 10 ml of 10 mM EGTA, 2 mM EDTA, 20 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride, 1 mM DTT, 20 mM Tris, pH 7.5, at 4 °C (lysis buffer) to effect osmotic lysis. The cytosolic and particulate fractions were separated by centrifugation at 245,000 × *g* for 45 min at 4 °C. The membranes were resuspended in 2 ml of lysis buffer and either urea-stripped or stored dark-adapted at −70 °C. The cytosol was stored in 50% glycerol and used as the source of rhodopsin kinase. All procedures were carried out under dim red light (Kodak filter 1).

Urea-Stripping of Rod Outer Segment Membranes—Membranes were stripped of peripheral proteins based on the procedure of Lee *et al.* (24). Briefly, dark-adapted membranes from 30 retinas (approximately 12 mg of rhodopsin) were centrifuged at 500,000 × *g* for 30 min at 4 °C. The pellet was resuspended in 1 ml of 4 M urea, 1 mM EDTA, 1 mM DTT, and 2 mM MOPS, pH 7.5, at 4 °C (Buffer A) and centrifuged again at 500,000 × *g* for 30 min at 4 °C. The wash with Buffer A was repeated for a total of 3 times. After the final centrifugation, the pellet was resuspended in 1 ml of 1 mM EDTA, 1 mM DTT, and 2 mM MOPS, pH 7.5, at 4 °C (Buffer B) and again washed 3 times in this buffer. The dark-adapted, stripped membranes were stored at −70 °C in 20 μM leupeptin and 20 mM HEPES, pH 7.5, at 4 °C; the rhodopsin concentration in this stock varied from 100 to 200 μM. The concentration of rhodopsin in octylglucoside-solubilized (1%) samples was determined spectrophotometrically at 500 nm using ϵ = 42,700 M^{-1} cm^{-1} (25). All procedures were carried out under dim red light (Kodak filter 1). The phospholipid/rhodopsin concentration was 90 ± 7 mol phospholipid/mol of rhodopsin as determined by phosphate analysis (26) of chloroform/methanol-extracted lipids (27).

Regeneration of Rod Outer Segment Membranes—Rhodopsin was regenerated based on the procedure of McDowell (28). Stripped membranes (50 μl of 100–200 μM rhodopsin) were incubated at 30 °C in 1 ml of solution containing 1 mM $MgCl_2$, 1 mM DTT, 20 mM HEPES, pH 7.5, at 30 °C, with 2 μl of 11-cis retinal (17.6 mM stored in 100% ethanol) in the dark overnight at 4 °C and then stored at −70 °C. The final concentration of ethanol in the regeneration tube was less than 1%.

Purification of Protein Kinase C—Protein kinase C was purified from 60 dark-adapted bovine retinas by sequential Q Sepharose, phenyl-Sepharose, and phenyl-Superose chromatography, essentially as described for purification of rod outer segment protein kinase C (29). Protein kinase C (typically 500 nM; 8 ml) was stored at −20 °C in 50% glycerol, 0.5 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, 100 mM KCl, 10 mM Tris, pH 7.4 (4 °C). In one experiment (see Fig. 2), protein kinase C was further purified by chromatography on a Mono Q column.

Phosphorylation Assay—Retinal protein kinase C (1–4 pmol) was incubated in a 64-μl reaction volume containing urea-stripped rod outer segment membranes (20 pmol of rhodopsin, unless otherwise stated) and phosphorylation initiated by the addition of 16 μl of a solution containing [γ-^{32}P]ATP (250 μM; 0.45 Ci $mmol^{-1}$ ATP) and $MgCl_2$ (25 mM) in 20 mM HEPES, pH 7.4. The final concentrations of species in a typical reaction mixture were as follows: 150 μM $CaCl_2$, 5 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP, 1 mM DTT, 100 μM leupeptin, 13 mM KCl, 25 μM EDTA, 25 μM EGTA, 6% glycerol, 20 mM HEPES, pH 7.5 (30 °C). For some experiments, rhodopsin, $MgCl_2$, ATP, or Ca^{2+} concentrations were varied as indicated in figure legends. In other experiments, 0.8 μl of PMA (100 nM in Me_2SO) was added to the reaction volume prior to addition of ATP and vortexed immediately. The final concentration of PMA (1 nM) corresponded to 0.003 mol % relative to the lipid. Experiments in which Ca^{2+} concentrations were varied were conducted in the presence of 525 μM EGTA to buffer the Ca^{2+}. Samples were incubated at 30 °C for 10 min unless indicated otherwise. Phosphorylations catalyzed by rhodopsin kinase (in rod outer segment cytosol or partially purified by phenyl-Sepharose chromatography) were carried out under the same conditions except that partially purified rhodopsin kinase replaced the protein kinase C, and 0.1 mM EDTA and 0.1 mM EGTA replaced the $CaCl_2$ in the assay to inhibit contaminating protein kinase C. Reactions were quenched by the addition of 25 μl of SDS-PAGE sample buffer (0.25 M Tris, 8% SDS, 40% glycerol, 0.008% bromphenol blue, 20% β-mercaptoethanol, pH 6.8), and the samples were analyzed by SDS-PAGE (10% acrylamide) followed by autoradiography (Kodak X-Omat film) or analysis on a Molecular Dynamics PhosphorImager. Rhodopsin bands were excised from gels, added to 2.5 ml of scintillation fluid (Biosafe II, Research Products International Corp.), and ^{32}P incorporation was detected by scintillation counting. Alternatively, reactions were quenched with 25 μl of 0.1 M cold ATP and 0.1 M EDTA and spotted on Whatman P-81 ion-exchange paper. The papers were washed 4 times in 0.4% (v/v) phosphoric acid and once in 95% ethanol and then added to 5 ml of scintillation fluid. Incorporation of ^{32}P into protein (>95% into rhodopsin) was detected by scintillation counting.

For the experiment shown in Fig. 4, the rate of phosphorylation of a protein kinase C selective peptide (FKKSFKL-NH_2; Ref. 22) was measured as described (30), except that rod outer segment membranes provided the lipid to stimulate protein kinase C rather than phosphatidylserine/diacylglycerol vesicles.

Generation of Opsin—Opsin was generated from rhodopsin as described by Hofmann *et al.* (31). Regenerated rod outer segment membranes (200 μl of 7 μM rhodopsin) were incubated for 30 min at room temperature under 150-watt lights with 200 μl of 200 mM NaCl, 20 mM hydroxylamine, and 20 mM HEPES, pH 7.5 (30 °C). The membranes were centrifuged at 500,000 × *g* for 10 min at 4 °C. The pellet was washed 3 times by resuspension in 200 μl of 100 mM NaCl and 10 mM HEPES, pH 7.5, at 4 °C and centrifugation. The final pellet was resuspended in 200 μl of 10 mM HEPES, pH 7.5, at 4 °C. Bleached rhodopsin was generated by exposure of rhodopsin to room light; phosphorylation reactions were initiated 10 min after light exposure.

Asp-N Proteolysis—Rod outer segment membranes were phosphorylated by protein kinase C or rhodopsin kinase, as described above, and the ^{32}P incorporation was inhibited by diluting the reaction 2.8-fold with 0.1 M EDTA and 0.1 M ATP (not radioactive). The membranes were centrifuged at 500,000 × *g* for 20 min to remove the chelator, DTT, and ATP and resuspended in 50 μM $CaCl_2$, 20 mM HEPES, pH 7.5 (30 °C), to a concentration of 0.30 μM receptor. The membrane-containing solution (0.25 μM receptor) was incubated with endoproteinase Asp-N (2.5 μg ml^{-1}) at 30 °C for 45 min. Proteolysis was stopped by the addition of SDS-PAGE sample buffer, and the samples were analyzed by SDS-PAGE (10% acrylamide) and autoradiography. To visualize the rhodopsin, the proteins were electrophoretically transferred to nitrocellulose and labeled with antibodies to opsin via incubation with horseradish peroxidase-conjugated IgG and detection by chemiluminescence (32).

Trypsin Proteolysis—Urea-stripped rod outer segment membranes were phosphorylated and centrifuged as described for the proteolysis with the endoproteinase Asp-N. The phosphorylated membranes were resuspended in 50 μM $CaCl_2$ and 20 mM HEPES, pH 7.5 (4 °C). Trypsin (2–20 μg ml^{-1}; 1.2 × 10^4 BAEE units mg^{-1}) was added to the solution (0.25 μM receptor) and incubated for 30 min at 30 °C. The proteolysis was stopped with SDS-PAGE sample buffer, and the samples were analyzed by SDS-PAGE (10% acrylamide), autoradiography, and Western blotting, as described for the Asp-N proteolysis.

Data Analysis—The dependences of the rate of protein kinase C-catalyzed phosphorylation of rhodopsin on Ca^{2+}, ATP, and rhodopsin concentrations were fit to the Michaelis-Menten equation or a modified Hill equation (33) using the program Grafit (34).

Free Calcium Determinations—Free calcium concentrations were calculated using a computer program provided by Claude Klee (35) that takes into account pH, Ca^{2+}, Mg^{2+}, K^+, Na^+, EGTA, and ATP concentrations.

RESULTS

Kinetic Analysis of the Phosphorylation of Rhodopsin by Protein Kinase C—Urea-stripped membranes containing unbleached rhodopsin, bleached rhodopsin, or opsin were incubated with [^{32}P]ATP in the presence or absence of retinal protein kinase C, Ca^{2+}, and PMA. The autoradiogram in Fig. 1*A* shows that protein kinase C catalyzed a Ca^{2+}-dependent phosphorylation of all three forms of rhodopsin (*lanes 4, 10,* and *16*). Addition of PMA had no significant effect on the Ca^{2+}-dependent incorporation of ^{32}P into rhodopsin (*lanes 5, 11,* and *17*), indicating that the membranes provided saturating con-

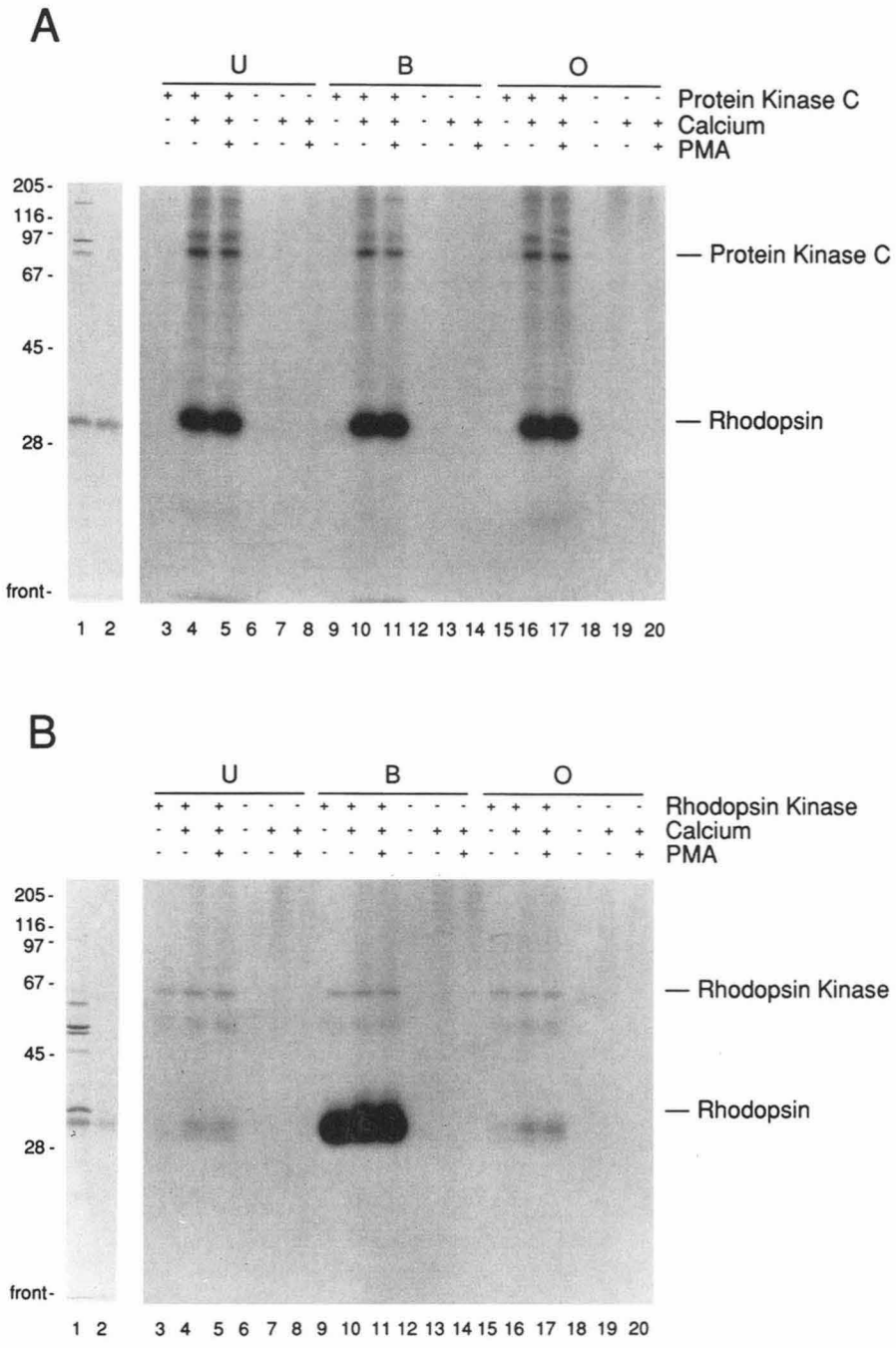

FIG. 1. **Phosphorylation of unbleached rhodopsin, bleached rhodopsin, and opsin by protein kinase C or rhodopsin kinase.** *A*, autoradiogram of unbleached rhodopsin (*U, lanes 3–8*), bleached rhodopsin (*B, lanes 9–14*), or opsin (*O lanes 15–20*) (275 nM receptor in all lanes), in urea-stripped rod outer segment membranes, incubated in the presence (*lanes 3–5, 9–11,* and *15–17*) or absence (*lanes 6–8, 12–14,* and *18–20*) of 40 nM retinal protein kinase C, 5 mM $MgCl_2$, and 50 μM ATP for 30 min at 30 °C. $CaCl_2$ (100 μM) or PMA (1 nM) was included as indicated. Receptor samples were prepared as described under "Methods." In addition to rhodopsin phosphorylation, autophosphorylation of protein kinase C is evident. Coomassie Blue-stained gel corresponding to the autoradiogram of *lanes 5* and *6*, showing rhodopsin and protein kinase C, is presented in *lanes 1* and *2*. *Panel B*, as in *A* except that rhodopsin kinase (10 nM) replaced protein kinase C.

centrations of diacylglycerol. In support of this, analysis of membranes revealed approximately 1 mol % diacylglycerol (data not shown), sufficient for maximal activity of protein kinase C (36). Autophosphorylation of protein kinase C, a good indicator of the intrinsic catalytic activity of protein kinase Cs (37), was also dependent on Ca^{2+} and was not additionally stimulated by phorbol esters (*e.g. lanes 4* and *5*), providing further support that the membranes provided an optimal environment for activation of this enzyme. No detectable ^{32}P was incorporated into receptor in the absence of added protein kinase C (*lanes 6–8, 12–14,* and *19–20*), indicating that the membranes had no associated kinase activity. Of particular importance was the lack of phosphorylation of bleached membranes (*lanes 12–14*), revealing that rhodopsin kinase was not associated with the membranes.

In contrast to the phosphorylation by protein kinase C, the phosphorylation catalyzed by rhodopsin kinase was specific for the bleached conformation of rhodopsin. Fig. 1*B* shows that unbleached rhodopsin (*lanes 3–5*) and opsin (*lanes 15–17*) were not substrates of rhodopsin kinase, whereas bleached rhodopsin was (*lanes 9–11*). The phosphorylation of bleached rhodopsin by rhodopsin kinase was not sensitive to Ca^{2+} (*lane 10*) or phorbol esters (*lane 11*) under the conditions of the experiment. Note that less than 0.1 μM recoverin was present in the reaction mixture, based on Coomassie staining of gels, and thus recoverin-dependent Ca^{2+} effects would not be expected (38).

Fig. 2 compares time courses for phosphorylation of unbleached rhodopsin, bleached rhodopsin, and opsin. For the concentrations of protein kinase C and receptor examined in this figure, the half-time of phosphorylation was approximately the same for all species of receptor: 57 ± 9 min for unbleached rhodopsin and opsin (weighted average ± S.E. for both sets of data) and 52 ± 3 min for bleached rhodopsin. The final stoichiometry for phosphorylation of bleached rhodopsin by retinal

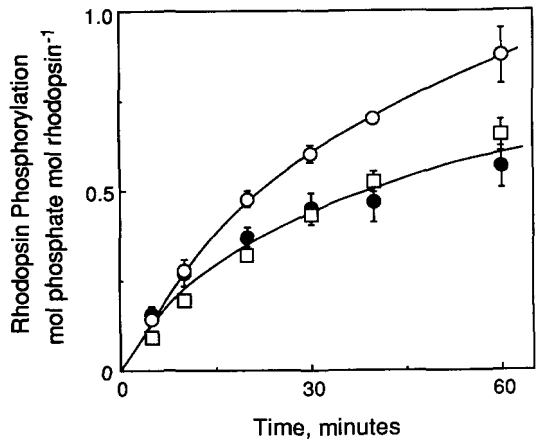

FIG. 2. **Time course of phosphorylation of unbleached rhodopsin (●), bleached rhodopsin (○), and opsin (□) by protein kinase C.** Receptor (275 nM) in urea-stripped rod outer segment membranes was incubated with retinal protein kinase C (40 nM) and 100 μM $CaCl_2$ for the indicated times at 30 °C. Data represent the mean ± S.E. of a triplicate assay; *lines* drawn are those predicted from the Michaelis-Menten equation.

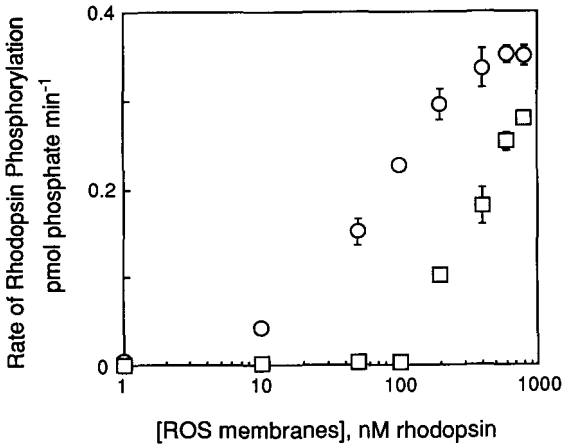

FIG. 3. **Dependence of rhodopsin phosphorylation on concentration of stripped rod outer segment membranes.** The initial rate of phosphorylation of bleached rhodopsin catalyzed by retinal protein kinase C (40 nM) (○) or rhodopsin kinase (2 nM) (□) was examined in the presence of increasing amounts of stripped rod outer segment membranes (1–800 nM rhodopsin). $CaCl_2$ (150 μM) was included in the protein kinase C incubation. Data represent the mean ± S.E. triplicates.

protein kinase C varied from 0.6 to 1.2 mol of phosphate/mol of receptor, depending on membrane preparation, and was typically 4-fold less than the maximal phosphorylation catalyzed by rhodopsin kinase (data not shown). In general, unbleached rhodopsin and opsin incorporated 80–100% of the phosphate incorporated by bleached rhodopsin. Addition of fresh protein kinase C to the reaction mixture after phosphate incorporation had plateaued did not result in a significant increase in phosphorylation, revealing that ^{32}P incorporation had plateaued because phosphorylation sites were no longer available. The receptor present in these experiments was quantitatively proteolyzed by endoproteinase Asp-N to generate a 32-kDa truncated form (see Fig. 7*B*). Because this protease cleaves the cytoplasmic (carboxyl) terminal tail of rhodopsin, all receptor molecules were oriented with their cytoplasmic surface exposed to the solution.

Fig. 3 shows the rate of rhodopsin phosphorylation catalyzed by protein kinase C or rhodopsin kinase as a function of increasing amounts of stripped rod outer segment membranes. Because the relative concentration of substrate in the membrane was the same for all rhodopsin concentrations (*i.e.* same rhodopsin/lipid ratio), a K_m for rhodopsin cannot be interpreted from these data (*i.e.* because of the reduction in dimensionality, once the kinase binds the first substrate, the local concentration of substrate near the kinase is the same for all conditions). However, the data do reveal that 1) protein kinase C has a higher affinity for rod outer segment membranes than does rhodopsin kinase and 2) under the conditions of these assays, 70 ± 2 nM rhodopsin (approximately 6 μM phospholipid) resulted in half-maximal activation of protein kinase C.

The Ca^{2+} dependence for the activation of retinal protein kinase C was measured for two substrates and as a function of membrane concentration. The concentration of Ca^{2+} resulting in half-maximal activity toward rhodopsin phosphorylation (Fig. 4*A*) or phosphorylation of a synthetic peptide (Fig. 4*B*, *open circles*) was the same (14 ± 3 μM or 14 ± 2 μM, respectively) in the presence of 32 μM lipid. Note that this concentration of lipid and rhodopsin (0.35 μM) result in stimulation of protein kinase C to 90% of its maximal rate (see Fig. 3). The identical Ca^{2+} requirement for phosphorylation of two different substrates is consistent with this cation allosterically modulating the membrane binding and catalytic activity of protein kinase C (39, 40) rather than substrate binding.

Fig. 4*B* shows that the K_m for Ca^{2+} decreased to 4.6 ± 0.3 μM when the concentration of rod outer segment membranes was increased to 320 μM lipid. The drop in K_m that occurred upon a 10-fold increase in lipid concentration reflects an increase in affinity of protein kinase C for these membranes. Mosior and Epand (40) showed that the apparent binding constant of conventional protein kinase Cs for membranes is linearly proportional to Ca^{2+} concentration between 100 nM and 0.5 mM Ca^{2+}. Because the binding of Ca^{2+} to protein kinase C depends on the total lipid concentration, mol % phosphatidylserine, and mol % diacylglycerol (39–41), the K_m for Ca^{2+} measured *in vitro* is relative to the specific assay conditions.[2] The Ca^{2+} requirement for phosphorylation of unbleached rhodopsin or opsin was not significantly different from that of bleached rhodopsin (not shown).

The K_m for ATP for the phosphorylation of bleached rhodopsin by protein kinase C was 27 ± 3 μM ATP (Fig. 5*A*). This number is similar to the K_m for ATP of known isozymes when measured using phosphorylation of synthetic peptides,[3] and slightly higher than the reported K_m for phosphorylation of histone (5–10 μM ATP, (42)). A concentration of 50 μM ATP was included in subsequent phosphorylation assays.

The dependence on Mg^{2+} for the phosphorylation of bleached rhodopsin by protein kinase C is presented in Fig. 5*B*. The maximal rate of phosphorylation of rhodopsin required 1–5 mM $MgCl_2$, with higher concentrations resulting in inhibition of the kinase. This stimulation is consistent with the Mg^{2+} requirements reported for the known Ca^{2+}-dependent protein kinase Cs; Burns and Bell (42) reported that protein kinase C α, βII and γ are half-maximally stimulated by 0.9, 0.6, and 0.7 mM Mg^{2+}, respectively. Subsequent phosphorylation assays were conducted in the presence of 5 mM $MgCl_2$.

Fig. 6 shows that sangivamycin selectively inhibited the phosphorylation of bleached rhodopsin catalyzed by protein kinase C compared with the phosphorylation catalyzed by rhodopsin kinase. Although this nucleoside analogue competes with ATP for the ATP-binding site, it displays significant se-

[2] As an example of high concentrations of one activator reducing the regulation by Ca^{2+}, rhodopsin reconstituted in membranes containing 95 mol % phosphatidylserine and 5 mol % diacylglycerol is phosphorylated by protein kinase C in the absence of Ca^{2+} (11).

[3] L. M. Keranen and A. C. Newton, unpublished data.

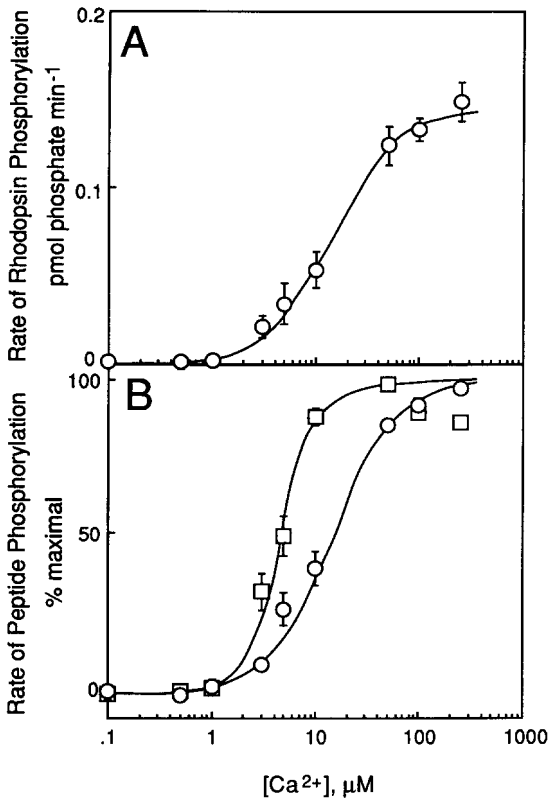

FIG. 4. **Ca^{2+}-dependence of retinal protein kinase C in the presence of two membrane concentrations.** *A*, the initial rate of phosphorylation of bleached rhodopsin (0.35 μM) catalyzed by retinal protein kinase C (10 nM) was examined in the presence of 0–250 μM free Ca^{2+} and stripped rod outer segment membranes resulting in a lipid concentration in the assay of 32 μM (○). Data represent the weighted average ± S.D. of two or three experiments, each in hextuplicate. *B*, The initial rate of phosphorylation of a protein kinase C-selective peptide, stimulated by 32 μM lipid (○) or 320 μM lipid (□) in rod outer segment membranes, was measured under the exact conditions described in *A* except that 50 μg ml^{-1} of the synthetic peptide were included in the assay. Data represent the weighted average ± S.D. of two experiments, each in hextuplicate. *Lines* drawn are those predicted from the modified Hill equation (33). All assays included 525 μM EGTA.

lectivity for protein kinase C's active site compared with that of other kinases (43). The phosphorylation catalyzed by protein kinase C was half-maximally inhibited by 13.4 ± 0.7 μM sangivamycin in the presence of 50 μM ATP; the IC_{50} for this concentration of ATP agrees with the K_i of 11 μM reported for Ca^{2+}-dependent protein kinase Cs (43). In contrast, rhodopsin kinase was considerably less sensitive to sangivamycin, with only 35 ± 1% inhibition observed in the presence of 100 μM inhibitor. Because rhodopsin kinase has a higher affinity for ATP than protein kinase C (K_m = 2 μM with 1 mM Mg^{2+} (44)), it may be less sensitive to inhibition by sangivamycin even though the K_i for interaction with sangivamycin may be similar. Inhibition of autophosphorylation of both kinases followed similar kinetics as inhibition of rhodopsin phosphorylation (data not shown), indicating that sangivamycin was inhibiting the intrinsic catalytic activity of each kinase rather than preventing substrate interaction.

Structural Analysis of the Phosphorylation of Rhodopsin by Protein Kinase C—In order to determine the domain of rhodopsin phosphorylated by protein kinase C, unbleached rhodopsin, bleached rhodopsin, and opsin were phosphorylated by protein kinase C and then treated with endoproteinase Asp-N. This protease cleaves bovine rhodopsin on the amino-terminal side of Asp^{330}, thus releasing a 19-residue peptide that contains all the hydroxyl residues on the carboxyl-terminal tail (45) (Fig. 7*A*). The autoradiogram in Fig. 7*B* shows that neither unbleached rhodopsin (*lane 2*), bleached rhodopsin (*lane 4*), or opsin (*lane 6*) had ^{32}P associated with them after removal of the carboxyl-terminal 19 residues. Similarly, Fig. 7*B* shows that bleached rhodopsin phosphorylated by rhodopsin kinase had no associated ^{32}P after removal of the carboxyl terminus (*lane 10*), as reported previously (45). Thus, protein kinase C modifies exclusively the carboxyl terminus of rhodopsin in its native membrane environment *in vitro*. This result is consistent with *in situ* phosphorylation data that revealed that rhodopsin is phosphorylated exclusively on the carboxyl tail in the presence or absence of phorbol esters (12).

Proteolysis with trypsin, which cleaves after Lys^{339} to release the carboxyl-terminal 9 residues (46), was used to further narrow down the domain phosphorylated by protein kinase C (Fig. 7*A*). Unbleached rhodopsin, bleached rhodopsin, or opsin were phosphorylated by protein kinase C or rhodopsin kinase and then treated with trypsin; 0.4 mol of phosphate were incorporated per mol of bleached rhodopsin for both kinases (the substoichiometric phosphorylation allowed determination of the initial phosphorylation domain). The Western blot in Fig. 7*C* shows that limited proteolysis by trypsin resulted in the formation of a fragment migrating with an apparent molecular mass 1 kDa smaller than the native enzyme, consistent with cleavage at Lys^{339}. The susceptibility of all three forms of protein kinase C-phosphorylated receptor to cleavage at Lys^{339} was similar (*e.g.* same amount of cleaved rhodopsin in *lanes 2, 6,* and *10*). Furthermore, the sensitivity to trypsin of the protein kinase C-phosphorylated receptor (*e.g. lanes 1–4*) was similar to that of nonphosphorylated receptor (approximately 50% proteolysis in the presence of 2 μg ml^{-1} trypsin); however, note that approximately 20% of the protein kinase C-phosphorylated receptor, but not unphosphorylated receptor, was resistant to proteolysis at the highest trypsin concentration. Thus, phosphorylation by protein kinase C did not affect the accessibility of Lys^{339} to trypsin for the majority of the receptor population. Ohguro *et al.* (8) have shown that phosphorylation at the adjacent Ser^{338}, the primary phosphorylation site by rhodopsin kinase, inhibits proteolysis at Lys^{339}. The autoradiogram in Fig. 7*C* shows that rhodopsin phosphorylated by protein kinase C had significant ^{32}P associated with it after cleavage at Lys^{339} (*e.g. lanes 4, 8,* and *12*). In contrast, bleached rhodopsin phosphorylated by rhodopsin kinase was significantly less sensitive to proteolysis by trypsin (*lanes 17–20*). Importantly, any cleaved rhodopsin from the sample phosphorylated with rhodopsin kinase was not radioactive (*lane 20*). The inability of trypsin to cleave receptor phosphorylated at Ser^{338} indicates that the primary phosphorylation site of protein kinase C is not Ser^{338}; protein kinase C-phosphorylated receptor is cleaved at Lys^{339}. Rather the primary phosphorylation site is on the carboxyl terminus between residues 330 and 337. Some phosphorylation on the trypsin-sensitive domain is also catalyzed by protein kinase C as well as minor phosphorylation at Ser^{338} (based on insensitivity of approximately 20% of the phospho-rhodopsin to proteolysis at Lys^{339}).

DISCUSSION

Table I compares kinetic and structural parameters for the phosphorylation of rhodopsin by protein kinase C and by rhodopsin kinase. The most striking difference in the phosphorylation by the two kinases is that protein kinase C does not discriminate between receptor conformations or liganded state, whereas rhodopsin kinase phosphorylates only bleached rhodopsin (5). A second important difference is the regulation of the two kinases; protein kinase C is regulated by a second messenger, whereas rhodopsin kinase is regulated by the con-

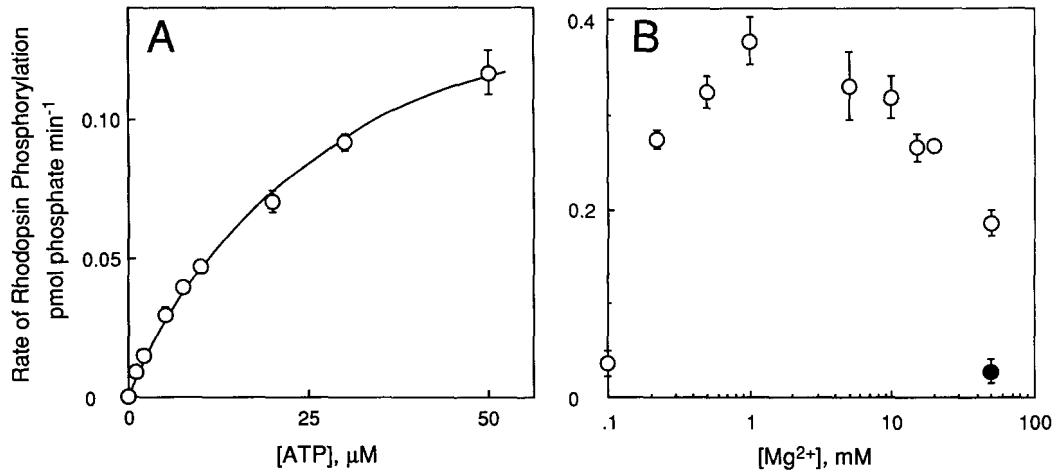

FIG. 5. **Dependence of the rate of rhodopsin phosphorylation catalyzed by retinal protein kinase C on ATP and Mg^{2+}.** *A*, bleached rhodopsin in stripped rod outer segment membranes (275 nM receptor) was incubated with retinal protein kinase C (25 nM), 100 μM $CaCl_2$, 5 mM $MgCl_2$, and 0–50 μM ATP for 10 min at 30 °C. Data represent the mean ± S.D. of an experiment in triplicate. The *line* drawn is that predicted from the Michaelis-Menten equation. *B*, phosphorylation reactions were as described in *A* except that the ATP concentration was 50 μM and the $MgCl_2$ concentration was varied from 0–50 mM. The *solid circle* represents activity in the absence of $CaCl_2$. Data represent the mean ± S.E. of an experiment in triplicate.

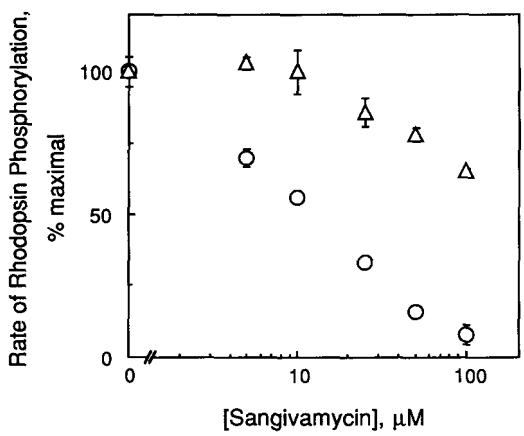

FIG. 6. **Sangivamycin selectively inhibits rhodopsin phosphorylation catalyzed by retinal protein kinase C.** The initial rate of rhodopsin phosphorylation was measured in the presence of 50 μM ATP, 10 mM $MgCl_2$, and 0–100 μM sangivamycin. Phosphorylation was catalyzed by 20 nM retinal protein kinase C (in which case 330 μM $CaCl_2$, 0.12 mM EDTA, and 0.12 mM EGTA were included in the reaction mixture) or 8 nM of rhodopsin kinase (in which case 0.12 mM EDTA and 0.12 mM EGTA were included in the reaction mixture; similar data were obtained in the presence of 330 μM $CaCl_2$ (not shown)). Data represent the mean ± S.D. of an experiment in triplicate.

formation of its substrate. The most important similarity in the effects of the two kinases is that both catalyze a desensitizing phosphorylation of the visual receptor. Because both kinases have similar consequences on rhodopsin function, the advantage of having two different kinases would be if each were dominant under different conditions (47), perhaps allowing rhodopsin to be regulated under a much broader range of illumination as well as by a heterologous pathway.

Ca^{2+} and Lipid Regulation—The protein kinase C in rod outer segments that catalyzes the phosphorylation of rhodopsin is a member of the Ca^{2+}-regulated protein kinase Cs (29). *In situ* phosphorylation studies have revealed that protein kinase C will only phosphorylate rhodopsin in retinas that have been exposed to some light (12), yet Ca^{2+} levels in rod outer segments drop upon illumination. This seeming discrepancy may be explained by the nature of the allosteric regulation of protein kinase C by lipid and Ca^{2+} (36). Specifically, the concentration of Ca^{2+} required for activation decreases with increasing concentrations of lipid (see Fig. 4), similar to other allosterically regulated proteins such as calcineurin, where the requirement for Ca^{2+} decreases with increasing concentrations of another allosteric regulator, calmodulin (48). Indeed, the concentration of membrane is so high in rod outer segments (approximately 200 mM phospholipid (49)) that consideration of the reported binding constants of conventional protein kinase Cs for lipid reveals that most of the protein kinase C will be membrane-bound if diacylglycerol is present but the Ca^{2+} concentration is below the K_d of protein kinase C for the cation. Specifically, Mosior and Epand (40) have shown that the apparent association constant for Ca^{2+}-regulated protein kinase Cs for membranes containing 20 mol % phosphatidylserine and 1 mol % diacylglycerol is 30 M^{-1} in the presence of physiological ionic strength and Ca^{2+} concentrations below the K_d. Given that the apparent association constant is equal to the ratio of membrane-bound protein kinase C to free protein kinase C divided by the total lipid concentration (see Ref. 50), then an apparent association constant of 30 M^{-1} and lipid concentration of 0.2 M, 86% of the protein kinase C would be membrane-bound. With more diacylglycerol, the fraction membrane-bound would be even greater. Thus, the association constant of protein kinase C for membranes containing 1 mol % diacylglycerol and physiological amounts of phosphatidylserine is sufficiently high in the complete absence of Ca^{2+} that membrane-binding, and hence activation, can occur independently of Ca^{2+}. What is more relevant is that the apparent association constant in the absence of diacylglycerol is over 2 orders of magnitude lower (39, 41, 50), indicating that no significant amount of protein kinase C would be membrane-bound in the absence of diacylglycerol. Thus, given the high membrane density in rod outer segments, the key to the regulation of rhodopsin phosphorylation by protein kinase C *in situ* is likely to depend on whether or not diacylglycerol has been produced.

Phosphorylation Domain—Both rhodopsin kinase and protein kinase C modify exclusively the carboxyl-terminal tail of rhodopsin. However, the stoichiometry of the phosphorylation and the sites phosphorylated by the two kinases differ. Protein kinase C catalyzes the incorporation of one mol of phosphate/mol of receptor, whereas rhodopsin kinase catalyzes the incorporation of up to 9 mol of phosphate/mol of receptor *in vitro* (51), although recent evidence suggests that only 3 of these are physiologically relevant (8). Second, sensitivity to proteolysis

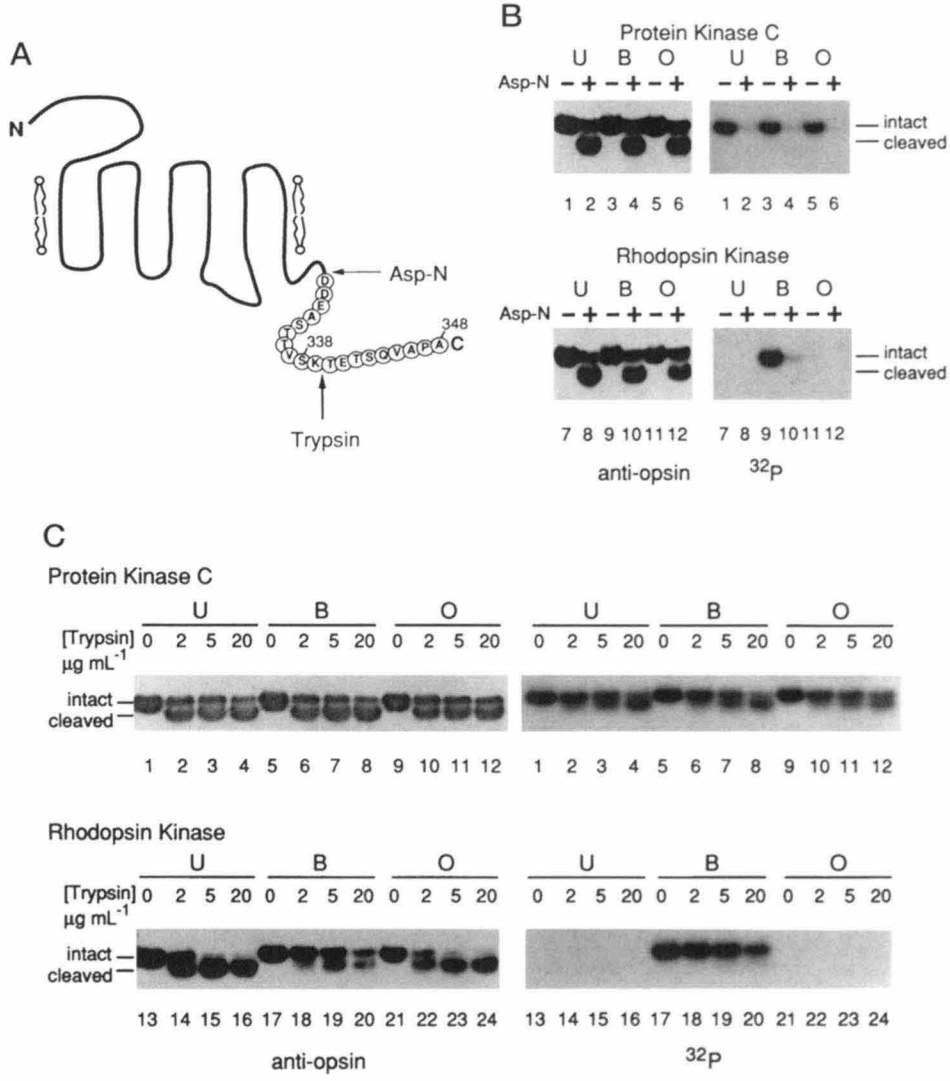

FIG. 7. **Partial proteolysis to identify the domain of rhodopsin phosphorylated by protein kinase C.** *A*, Cartoon representation of bovine rhodopsin showing sites of cleavage on the carboxyl terminus catalyzed by endoproteinase Asp-N and trypsin. *B*, Western blot (*panels* on *left*) and corresponding autoradiogram (*panels* on *right*) of unbleached rhodopsin (*U*), bleached rhodopsin (*B*), or opsin (*O*) phosphorylated with protein kinase C (*lanes 1–6*) or rhodopsin kinase (*lanes 7–12*) and then treated with endoproteinase Asp-N for 30 min at 30 °C. The receptor phosphorylated by protein kinase C incorporated 0.2 mol of phosphate/mol of receptor; the bleached rhodopsin phosphorylated by rhodopsin kinase incorporated 0.4 mol of phosphate/mol of receptor. *C*, Western blot (*panels* on *left*) and corresponding autoradiogram (*panels* on *right*) of unbleached rhodopsin (*U*), bleached rhodopsin (*B*), or opsin (*O*) phosphorylated with protein kinase C (*lanes 1–12*) or rhodopsin kinase (*lanes 13–24*) and then treated with the indicated concentration of trypsin for 30 min at 30 °C. All receptor species incubated with protein kinase C, and bleached rhodopsin incubated with rhodopsin kinase, incorporated 0.4 mol of phosphate/mol of receptor.

TABLE I

Comparison of biochemical parameters describing the phosphorylation of rhodopsin by protein kinase C and rhodopsin kinase

	Protein kinase C	Rhodopsin kinase
Specificity	Unbleached/bleached/opsin	Bleached
Calcium	Increases membrane affinity	Not required
ATP, K_m [a]	27 ± 3 μM	5 μM (44)
Magnesium, optimal	1–5 mM	1–10 mM (56)
Phosphorylation site	Carboxyl tail, not Ser^{338}	Carboxyl tail, 1° site Ser^{338} (7–9)
Stoichiometry	1 phosphate/rhodopsin	Multiple phosphates/rhodopsin
Effect of phosphorylation	Uncoupling from transducin (13)	Uncoupling from transducin in the presence of arrestin (10)
Regulation	Lipid second messenger	Substrate conformation

[a] Measured in the presence of 10 mM Mg^{2+}.

by trypsin indicates that the primary phosphorylation site of rhodopsin kinase, Ser^{338} (7–9), is not modified significantly by protein kinase C. Rather, most of the phosphate incorporated by reaction with protein kinase C is between Asp^{329} and Val^{337}. Phosphoamino acid analysis by Kelleher and Johnson (13) indicated that protein kinase C phosphorylated rhodopsin primarily on Thr residues; 2 such residues are present in the stretch from 330 to 337.

Identification of the major phosphorylation site by protein kinase C as being on the amino-terminal half of the carboxyl tail supports *in situ* findings. Hyperactivation of protein kinase C in the intact rat retina by treatment with phorbol esters results in an increase in the phosphorylation of rhodopsin exposed to a brief flash of light (11, 12). Proteolytic digests of receptor phosphorylated *in situ* revealed that the increased phosphorylation resulting from phorbol ester treatment occurred on a trypsin-resistant (11) but Asp-N-sensitive (12) domain. Thus, protein kinase C modifies the same domain of rhodopsin *in situ* and *in vitro*.

Phosphorylation by Rhodopsin Kinase and Protein Kinase C—The phosphorylation of rhodopsin by rhodopsin kinase or protein kinase C may have the same functional consequence: deactivation of the receptor. Phosphorylation by rhodopsin kinase reduces the rate of light-dependent coupling to transducin and hence the activation of the phosphodiesterase (52), an inhibition that is enhanced upon binding of arrestin (10). Similarly, a report by Kelleher and Johnson (13) and our own preliminary data (53) indicate that rhodopsin phosphorylated

by protein kinase C couples to transducin with a reduced rate. The inhibition of G protein-coupling as a result of phosphorylation on the membrane-proximal half of the C terminus is consistent with a report that a synthetic peptide of this domain of the receptor interacts directly with transducin (54). Whether arrestin binding is promoted by the protein kinase C-catalyzed phosphorylation remains to be examined. For other G protein-coupled receptors, phosphorylation by G protein-coupled receptor kinases requires binding of arrestin-like proteins for maximal desensitization, whereas the uncoupling resulting from phosphorylation by second messenger-regulated kinases is unaffected by arrestin-like proteins (55).

The phosphorylation of rhodopsin by protein kinase C is analogous to the heterologous phosphorylation of other G protein-coupled receptors. Notable similarities are as follows. 1) The phosphorylation by protein kinase C is independent of receptor conformation, similar to the ligand-independent phosphorylation of the β adrenergic receptor by protein kinases A or C or the ligand-independent phosphorylation of the muscarinic acetylcholine receptor by protein kinase C. This contrasts with the ligand-dependent homologous phosphorylation catalyzed by G protein-coupled receptor kinases. 2) One mol of phosphate is incorporated per mol of receptor, similar to the phosphorylation of only one or two sites of the β adrenergic receptor by protein kinases A or C, and contrasting with the multiple phosphorylations catalyzed by G protein-coupled receptor kinases. 3) The phosphorylation catalyzed by protein kinase C is regulated by a second messenger, diacylglycerol; the heterologous phosphorylation of the β adrenergic receptor is also stimulated by a second messenger, cAMP. This contrasts with the regulation of G protein-coupled receptor kinases by substrate conformation. 4) The major phosphorylation site by protein kinase C differs from that of rhodopsin kinase, although it is also on the carboxyl terminus. Protein kinases A and C modify the β adrenergic receptor on the carboxyl tail of this receptor (as well as on the third cytoplasmic loop), but on a residue proximal to the membrane span that is not a phosphorylation site by G protein-coupled receptor kinases.

Phosphorylation of rhodopsin by two differently regulated kinases may allow rhodopsin to respond to a much broader range of stimuli. Whether protein kinase C is activated directly in response to light on the photoreceptor cell or whether its activation arises heterologously from activation of another signaling pathway remains to be determined.

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