Contribution of Protein Kinase C to the Phosphorylation of Rhodopsin in Intact Retinas*

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The involvement of multiple kinases in the regulation of specific signaling pathways is a common cellular theme. For G protein-coupled pathways, two classes of kinases phosphorylate receptors: substrate-regulated G protein-coupled receptor kinases and second messenger-regulated kinases such as protein kinase A and protein kinase C (1). In the examples studied to date, phosphorylation by G protein-coupled receptor kinases is typically involved in homologous desensitization (the phosphorylation follows direct stimulation of the receptor), and phosphorylation by second messenger-regulated kinases is involved in heterologous desensitization (the phosphorylation is independent of the liganded state of the receptor and may be initiated by activation of the kinase by a separate receptor) (1, 2).

Phototransduction serves as the archetypal transduction pathway, and extensive studies with photoreceptor proteins have provided invaluable insight into the regulation of not only this pathway but numerous other G protein-coupled receptor signaling pathways as well (3–5). Activation of the heptahelical receptor, rhodopsin, by a photon of light results in activation of the G protein transducin, which in turn activates a cGMP phosphodiesterase. The ensuing drop in cGMP levels in photoreceptor outer segments causes hyperpolarization of the plasma membrane by closing cGMP-gated channels, thus converting a biochemical signal into an electrophysiological response (6). Phosphorylation of the light-activated receptor by rhodopsin kinase, followed by binding of arrestin, effectively quenches the signal. Ca2+ levels, which are lowered when the cGMP-gated channels close, regulate deactivation and recovery stages of phototransduction. Two targets of Ca2+ have been identified in photoreceptors: guanylate cyclases, which are responsible for restoring cGMP levels, are activated at low Ca2+ concentrations by calmodulin-like proteins (7), and the activity of rhodopsin kinase is inhibited by another Ca2+-binding protein, recoverin, at high concentrations of Ca2+ (8, 9).

Several reports over the past decade have implicated a role for protein kinase C in photoreceptor function; its presence in rod outer segments (10–12) and its phosphorylation of rhodopsin (13, 14), transducin (15), the γ subunit of the cGMP-phosphodiesterase (16), and arrestin (17) have been described. Protein kinase C is activated by the lipid second messenger diacylglycerol through binding of this ligand to a membrane-targeting domain (C1) that also binds phorbol esters (18, 19). Some isozymes of protein kinase C are sensitive to Ca2+, which binds to a separate membrane-targeting domain (C2) and thus also increases the enzyme’s affinity for membranes (18). Generation of diacylglycerol, the key activator of protein kinase C, has been reported to be stimulated by light in photoreceptors (20–23), consistent with a potential role for this kinase in phototransduction.

Evidence that the phosphorylation of rhodopsin by protein kinase C is physiologically relevant comes from the finding that hyperactivation of protein kinase C in intact retinas, by treatment with phorbol esters, alters the light-dependent phosphorylation of rhodopsin (24, 25). In vitro studies reveal remarkable similarities between the enzymology of phosphorylation of rhodopsin by protein kinase C and the phosphorylation of other G protein-coupled receptors by second messenger kinases (26). Most notably, phosphorylation is independent of the activation state of the receptor (13, 14) and results in decreased coupling of the receptor to G protein (13). Taken together, the in situ and in vitro data are consistent with direct modulation of rhodopsin

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by protein kinase C. Nonetheless, the extent of involvement of protein kinase C in rhodopsin phosphorylation in vivo remains to be established.

In the present study, specific inhibition of protein kinase C by calphostin C was used to determine the contribution of protein kinase C in the light-dependent phosphorylation of rhodopsin in intact frog retinas. We found that this kinase contributes to approximately 50% of the phosphorylation of rhodopsin. Furthermore, using phorbol esters to hyperactivate the kinase, we show that the kinetics of rhodopsin phosphorylation and dephosphorylation differ markedly, depending on whether protein kinase C or rhodopsin kinase activities dominate.

MATERIALS AND METHODS

Phorbol 12-myristate 13-acetate (PMA) and 4-α-phorbol 12-myristate 13-acetate (4-α-PMA) were purchased from Research Biochemicals International. sn-1-Palmitoyl-2-oleoylphosphatidylserine and sn-1,2-dioleoylglycerol were from Avanti Polar Lipids, Inc. Phenol red (free acid) was obtained from Mallinckrodt Inc., casamino acids from Difco, and calphostin C from Calbiochem. β-α-Glucose, HEPES, dithiothreitol (DTT), EGTA, hydroxyamine, and ATP were from Sigma. [32P]Orthophosphate (8,500–9,120 Ci mmol−1) and [γ-32P]ATP (3000 Ci mmol−1) were supplied by DuPont NEN. A protein kinase C inhibitor, peptide Ac-PKSSPKL-NH2 (27), was synthesized by the Indiana University Biochemistry Biotechnology Facility, Indiana University Medical School. Q-Sepharose Fast Flow, phenyl-Sepharose HR 5/5, and DEAE Sephadex were purchased from Pharmacia Biotech Inc. All other chemicals were reagent grade. Rat protein kinase C α from a baculovirus expression system was purified as described (28) and stored in a 50% glycerol. 2 This fraction was stored in 20 mm HEPES, pH 7.5, 1 mm EDTA, 5 mm EGTA, 1 mm DTT, 0.25% Triton X-100, 10 μg/ml leupeptin, and 50% glycerol. Rhodopsin kinase (bovine) was cloned into baculovirus, and the cytosolic fraction of infected SF-21 cells was used as the source of enzyme. 3 This fraction was stored in 20 mm HEPES, pH 7.5, 1 mm EDTA, 5 mm EGTA, 1 mm DTT, 0.25% Triton X-100, 10 μg/ml leupeptin, and 50% glycerol at −20 °C. Northern grass frogs (Rana pipiens), weighing 20–30 g, were purchased from Carolina Biological Supply Company.

Rhodopsin Phosphorylation in Situ—Freshly dissected retinas from dark-adapted (12–14 h) frogs were incubated individually in 1 ml of amphibian culture medium (ACM; 65 mM NaCl, 35 mM NaHCO3, 3 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 10 mM Na-HEPES (pH 7.3), 10 μM EDTA, 1 mm casamino acids, 10 mm β-2-

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1 The abbreviations used are: PMA, phorbol myristate acetate; ACM, amphibian culture medium; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
2 N. M. Greene, unpublished data.

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Fig. 1. Level of photoexcited rhodopsin resulting from exposure of dark-adapted intact frog retinas to different flash intensities. Intact frog retinas were exposed to increasing light levels, and the amount of photoexcited rhodopsin was measured as described under “Materials and Methods.” The arrow at a indicates the lower flash intensity used in the present study (Fig. 2 and Fig. 3, B and E); it results in the photoexcitation of 6% rhodopsin. The arrow at b indicates the higher flash intensity used in the present study (Fig. 2 and Fig. 3, C and F); it delivers 2.8 log units more light and results in the photoexcitation of 80% rhodopsin. Data represent mean ± S.E.
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Fig. 2. A, time course of rhodopsin phosphorylation in intact frog retinas as a function of illumination conditions. Retinas were incubated with [32P]orthophosphate under dim red light for 2 h and then subjected to different light stimuli. a, a flash of light that photoexcites 6% of the rhodopsin, followed by continued incubation under dim red light; b, a flash of light that photoexcites 80% of the rhodopsin, followed by continued incubation under dim red light; c, exposure to continuous room lighting (approximately 500 lux), which results in photoexcitation of 97% of the rhodopsin after 10 min. At the indicated times after the onset of illumination, 32P incorporation into rhodopsin was determined as described under “Materials and Methods.” Data represent the amount of 32P incorporation normalized to a constant amount of rhodopsin and are expressed relative to the maximal level of phosphorylation obtained after 10 min of continuous 500-lux illumination. Data from 15 separate experiments (n = 58), 12 separate experiments (n = 64), and 8 separate experiments (n = 34) were compiled for curves a, b, and c, respectively. B, determination of rhodopsin phosphorylation. The second of two procedures (as described under “Materials and Methods”) used to determine the phosphorylation of rhodopsin relied on the property of rhodopsin to oligomerize when heated in SDS-PAGE sample buffer (see “Materials and Methods”). Coomassie Blue-stained SDS-polyacrylamide gel (lanes 1 and 2) and phosphor image showing radioactivity (lanes 3 and 4) of a heated sample (lanes 1 and 3) and a sample kept at room temperature (lanes 2 and 4) are shown. The area of the gel analyzed is indicated by the boxes; the arrow indicates the position of rhodopsin. The heated sample contains little rhodopsin in this area and serves as the background level of protein and radioactivity,

300,000 × g, 10 min, 2 °C, in buffer containing 10 mM sodium phosphate, pH 7.3, 110 mM NaCl. Final pellets were solubilized in 80 μl of 50 mM sodium phosphate buffer, pH 7.0, containing 1% octylglucoside and incubated for 15 min on ice, and insoluble material was removed by centrifugation at 100,000 × g for 5 min, 2 °C. The A280/A436 absorbance ratios of supernatants were measured before and after centrifugation (λmax = 500 lux, 2 min) of rhodopsin in the presence of 20 mM hydroxylamine. Fig. 1 shows the amount of bleached rhodopsin in intact frog retinas calculated by this method as a function of flash intensity. Arrows indicate the two intensities used in the in situ phosphorylation experiments; setting the flash at 1/3 maximal power and using a 1.2-log unit neutral density filter bleached 6 ± 3% of the rhodopsin (arrow a), and an unfiltered flash set at 1/5 maximal intensity bleached 80 ± 6% of the rhodopsin (arrow b). Ten minutes of 500-lux continuous illumination bleached 97 ± 1% of the rhodopsin (not shown). Octylglucoside-solubilized rhodopsin from purified dark-adapted frog rod outer segments was illuminated under the same light regimes as intact retinas, and the amount of bleaching, as a function of light intensity, was determined as described above. Similar levels of bleaching resulted from illumination of intact retinas or solubilized rhodopsin from purified rod outer segments.

Protein Kinase C Assay—Activity of protein kinase C (0.6–3 nM) was measured in the presence of histone IIIS (200 μg/ml) or protein kinase C-selective peptide (Ac-FKKSFKL-NH2, 50 μg/ml) in 20 μl of 10 mM Tris-HCl buffer (pH 7.5–7.5 at 37 °C) containing 50–200 μM [γ-32P]ATP (0.1 Ci/mMol−1), 5 mM MgCl2, 0.1 mM bovine serum albumin, 1 mM DTT, lipid/detergent mixed micelles with 0.1% Triton X-100, 12.5 mol % phosphatidylserine (180 μM), 1.6 mol % dicetylphosphate, 24 μM, and either 100 μM CaCl2 or 1 mM EGTA for 5–8 min at 30 °C. Reactions were quenched and analyzed as described (28).

Phosphorylation of Rhodopsin in Vitro—Urea-stripped rod outer segment membranes were prepared from 30 frog retinas, as described (30), and stored in 20 mM Tris-HCl, pH 7.5, at 4 °C, 120 mM NaCl, 30 mM KCl, 2 mM MgCl2, 10% glycerol at −20 °C. The concentration of rhodopsin was 30–50 μg as assessed by its absorbance at 498 nm in 1% octylglucoside using an extinction coefficient of 40,600 M−1cm−1 (31).

Phosphorylation of rhodopsin by protein kinase C was determined as described above except that substrate was replaced with 0.5 μM rhodopsin in urea-stripped membranes and Triton X-100/lipid mixed micelles were omitted. In some experiments, 20–100 nM PMA was present. For calphostin inhibition studies, 0–1 μM of this inhibitor was included in phosphorylation mixtures that also contained 20 nM PMA to ensure full activation of protein kinase C. Phosphorylation by rhodopsin kinase was performed under similar conditions, except that protein kinase C was replaced with rhodopsin kinase and assays did not include PMA (PMA does not affect rhodopsin phosphorylation by rhodopsin kinase in vitro (14)). Reactions were quenched by the addition of 5 μl of a 5-fold concentrate of SDS-PAGE sample buffer, and samples were analyzed by SDS-PAGE (10% acrylamide). 32P incorporation into rhodopsin was assessed as described above for in situ experiments.

Isolation of Protein Kinase C from Frog Rod Outer Segments—Protein kinase C from rod outer segments was isolated following the procedure of Udovicenko et al. (32). Briefly, rod outer segments from 20 frog retinas were collected under dim red light, purified by two sequential sucrose gradients (29), and lysed in 10 mM Tris, pH 7.5, at 4 °C, 1 mM MgCl2, 2 mM DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml-1 pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml-1 sodium 1-ascorbate. Membranes were washed three times by centrifugation at 300,000 × g for 10 min in this buffer. Membranes were resuspended in buffer containing 10 mM Tris-HCl, pH 7.5, at 4 °C, 2 mM EGTA, 2 mM EDTA, 5 mM DTT, 1 μg/ml-1 leupeptin, 1 μg/ml-1 aprotinin, 1 μg/ml-1 pepstatin, 0.5 mM phenylmethylsulfonyl fluoride (buffer A) to extract protein kinase C, and sample was centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant, containing protein kinase C, was applied to a 50-μl DEAE-Sephacel column equilibrated with buffer A, and the column was washed with 2 ml of buffer A. Protein kinase C was eluted with a step gradient (50–1 ml steps in a 1-ml total volume) of 0–500 mM NaCl in buffer A, in 25 mM NaCl increments.
Phosphorylation of Rhodopsin by Protein Kinase C


time-dependent phosphorylation of rhodopsin after different illumination conditions. The data in Fig. 3 show the effect of PMA treatment on rhodopsin phosphorylation relative to that of untreated retinas (for these experiments, one retina from each frog was treated with PMA and the other with MeSO, the solvent for PMA). For all illumination conditions, the effects of phorbol esters were biphasic. In general, PMA first caused an increase in rhodopsin phosphorylation relative to controls; this increase was greatest at about 10–15 min after the onset of illumination. After this time, the relative difference in rhodopsin phosphorylation level between PMA-treated retinas and control retinas decreased, so that by approximately 30 min after the onset of illumination there was little difference. What was unique to each illumination condition was the magnitude of the phorbol ester effect. In response to the flash photoexciting 6% of the rhodopsin, PMA caused an approximately 20% increase in rhodopsin phosphorylation that was sustained for 20 min (Fig. 3, B and E). This increase was

As a first step, we characterized the light-dependent phosphorylation of rhodopsin initiated by three light treatments (Fig. 1): exposure to a filtered flash of light resulting in photoexcitation of approximately 6% of rhodopsin, exposure to a flash of light resulting in photoexcitation of approximately 80% of rhodopsin, and exposure to continuous room lighting (approximately 500 lux) resulting in photoexcitation of approximately 97% of rhodopsin. Fig. 2 shows the time-dependent increase in rhodopsin phosphorylation after treatment of intact frog retinas with these light regimes; these data are compiled from 35 separate experiments. All lighting conditions resulted in a rapid phosphorylation of rhodopsin, with a half-time of approximately 2 min. Relative to the phosphorylation resulting from photoexcitation of 97% of the rhodopsin, approximately 12 and 50% of the rhodopsin was phosphorylated following the two different flash intensities, which photoexcited 6 and 80% of the rhodopsin, respectively. Thus, conditions were established where the kinetics and extent of phosphorylation of control retinas, in the absence of any stimulation other than light, were characterized.

Previously we showed that PMA alters the phosphorylation of rhodopsin in intact rat retinas (24, 25). To understand better the effect of protein kinase C activation on rhodopsin phosphorylation, we undertook a systematic study of the time-dependent phosphorylation of rhodopsin after different illumination conditions. For these studies, we chose frog retinas because they have been well characterized in short term culture. They are more stable in culture medium than mammalian retinas, so that they are more suited to the present experiments, which require longer incubation periods than those of our previous studies (24, 25).

Fractions (50 μl) were collected and assayed for protein kinase C activity.

Calcium Concentration—Concentrations of free Ca^{2+} were calculated using a computer program kindly provided by Dr. Claude Klee that takes into account pH, Ca^{2+}, Mg^{2+}, K^+, Na^+, EGTA, EDTA, and ATP concentrations (33).

RESULTS

As a first step, we characterized the light-dependent phosphorylation of rhodopsin initiated by three light treatments (Fig. 1): exposure to a filtered flash of light resulting in photoexcitation of approximately 6% of rhodopsin, exposure to a flash of light resulting in photoexcitation of approximately 80% of rhodopsin, and exposure to continuous room lighting (approximately 500 lux) resulting in photoexcitation of approximately 97% of rhodopsin. Fig. 2 shows the time-dependent increase in rhodopsin phosphorylation after treatment of intact frog retinas with these light regimes; these data are compiled from 35 separate experiments. All lighting conditions resulted in a rapid phosphorylation of rhodopsin, with a half-time of approximately 2 min. Relative to the phosphorylation resulting from photoexcitation of 97% of the rhodopsin, approximately 12 and 50% of the rhodopsin was phosphorylated following the two different flash intensities, which photoexcited 6 and 80% of the rhodopsin, respectively. Thus, conditions were established where the kinetics and extent of phosphorylation of control retinas, in the absence of any stimulation other than light, were characterized.

We next studied how hyperactivation of protein kinase C, resulting from PMA treatment of retinas, altered the kinetics of rhodopsin phosphorylation. The data in Fig. 3 show the effect of PMA treatment on rhodopsin phosphorylation relative to that of untreated retinas (for these experiments, one retina from each frog was treated with PMA and the other with MeSO, the solvent for PMA). For all illumination conditions, the effects of phorbol esters were biphasic. In general, PMA first caused an increase in rhodopsin phosphorylation relative to controls; this increase was greatest at about 10–15 min after the onset of illumination. After this time, the relative difference in rhodopsin phosphorylation level between PMA-treated retinas and control retinas decreased, so that by approximately 30 min after the onset of illumination there was little difference. What was unique to each illumination condition was the magnitude of the phorbol ester effect. In response to the flash photoexciting 6% of the rhodopsin, PMA caused an approximately 20% increase in rhodopsin phosphorylation that was sustained for 20 min (Fig. 3, B and E). This increase was

Fig. 3. Effect of phorbol myristate acetate on rhodopsin phosphorylation in intact frog retinas. Frog retinas were incubated with [32P]orthophosphate under dim red light as in Fig. 2. A–D, relative effect of PMA. One retina from each frog was treated with 1 μM PMA, and the other retina served as a control. Retinas were incubated for 30 min and then either exposed to one of the light stimuli described in the legend to Fig. 2 or kept in the dark. 32P incorporation into rhodopsin was measured for retinas not exposed to light (A), retinas exposed to a flash resulting in photoexcitation of 6% of the rhodopsin (B), retinas exposed to a flash resulting in photoexcitation of 80% of the rhodopsin (C), and retinas exposed to 500-lux continuous illumination, resulting in photoexcitation of approximately 97% of the rhodopsin (D). Data are expressed as the level of rhodopsin phosphorylation (normalized to the amount of rhodopsin in each sample) of the PMA-treated retina relative to that of the control retina. They represent mean ± S.E. and were compiled from 6 (n = 12), 12 (n = 100), 13 (n = 104), and 5 (n = 26) separate experiments for A, B, C, and D, respectively. E–G, time courses of rhodopsin phosphorylation in control (solid lines) and PMA-treated (dotted lines) retinas. Curves a, b, and c from Fig. 2A are shown in E, F, and G, respectively, together with curves taken from the data in B, C, and D, respectively.

What was unique to each illumination condition was the magnitude of the phorbol ester effect. In response to the flash photoexciting 6% of the rhodopsin, PMA caused an approximately 20% increase in rhodopsin phosphorylation that was sustained for 20 min (Fig. 3, B and E). This increase was
followed by a marked decrease in the relative level of rhodopsin phosphorylation in PMA-treated retinas, such that by 60 min, it was 50% less relative to controls. In retinas exposed to the flash of light resulting in 80% photoexcitation of rhodopsin, PMA treatment resulted in 80% greater rhodopsin phosphorylation by 15 min, although the initial phosphorylation level in PMA-treated retinas was actually less (Fig. 3, C and F). As with the lower flash intensity, rhodopsin phosphorylation in PMA-treated retinas became progressively less relative to control with incubation times exceeding 15 min, except that at the higher flash intensity, the amount of phosphorylated rhodopsin in PMA-treated retinas was actually less (Fig. 3, C and F). No effect of PMA was detected in the absence of calphostin C, with 1 µM calphostin C having approximately 70% inhibition. Thus, calphostin C, like PMA, serves as a specific agent for examining protein kinase C effects.

As an alternative approach to studying the effect of protein kinase C on rhodopsin phosphorylation, we investigated the effect of a specific protein kinase C inhibitor, calphostin C, on rhodopsin phosphorylation. Calphostin C inhibits protein kinase C but not rhodopsin kinase in situ. Frog rhodopsin from urea-stripped membranes was incubated under room light with protein kinase C (open circles) or rhodopsin kinase (filled circles) and the indicated concentrations of calphostin C, as described under “Materials and Methods.” Data represent the amount of 32P incorporation into rhodopsin, expressed relative to the phosphorylation observed in the absence of calphostin C. The protein kinase C and rhodopsin kinase phosphorylation assays were performed under identical conditions except that 20 nM PMA and 1 µM Ca2+ were included in the protein kinase C assay; neither reagent affected the rhodopsin kinase assay under the conditions of these experiments. Data represent mean ± S.E.

Fig. 4. Calphostin C inhibits protein kinase C but not rhodopsin kinase in vitro. Frog rhodopsin from urea-stripped membranes was incubated under room light with protein kinase C (filled circles) or rhodopsin kinase (open circles) and the indicated concentrations of calphostin C, as described under “Materials and Methods.” Data represent the amount of 32P incorporation into rhodopsin, expressed relative to the phosphorylation observed in the absence of calphostin C. The protein kinase C and rhodopsin kinase phosphorylation assays were performed under identical conditions except that 20 nM PMA and 1 µM Ca2+ were included in the protein kinase C assay; neither reagent affected the rhodopsin kinase assay under the conditions of these experiments. Data represent mean ± S.E.

Concentrations up to 1 µM has no significant effect on other kinases (35). Fig. 4 shows that calphostin C specifically inhibits the protein kinase C-catalyzed phosphorylation of rhodopsin in vitro (filled circles), with no detectable inhibition of the rhodopsin kinase-catalyzed phosphorylation of rhodopsin (open circles). Protein kinase C was half-maximally inhibited by 400 nM calphostin C, with 1 µM causing approximately 70% inhibition. Note that this inhibition occurred in the presence of 20 nM PMA, which, without calphostin, results in full activation of the kinase; greater inhibition by calphostin would be expected at saturating concentrations of activator. In marked contrast, 1 µM calphostin C had no effect on rhodopsin kinase activity. Thus, calphostin C, like PMA, serves as a specific agent for examining protein kinase C effects.

Fig. 5 shows that treatment of intact retinas with 1 µM calphostin C resulted in a marked reduction in the light-dependent phosphorylation of rhodopsin. Compilation of data from four separate experiments revealed a 2-fold reduction in 32P incorporation into rhodopsin from calphostin-treated retinas relative to controls (as in the preceding experiments, one retina from each frog served as the experimental and the other as the control). These data reveal that at least 50% of the 32P incorporated into rhodopsin in response to continuous room illumination (97% bleach) depends on protein kinase C.

The foregoing data establish that protein kinase C is a major contributor to the phosphorylation of rhodopsin in vivo. To test whether frog rhodopsin serves as a direct substrate for protein kinase C, urea-stripped membranes from purified frog rod outer segments were incubated with recombinant protein kinase Cα and Mg2+-ATP. Fig. 6A shows that rhodopsin in these membranes was phosphorylated effectively by protein kinase Cα in a Ca2+-dependent manner. As reported for bovine rhodopsin (14), protein kinase C phosphorylated dark-adapted (stippled
Materials and Methods. $^{32}$P incorporation was determined from SDS-PAGE and phosphor images for dark-adapted (closed columns) or photoexcited (open columns) rhodopsin. Data represent the mean; error bars represent S.E. when it is larger than the symbol.

Fig. 6. A, protein kinase C phosphorylates dark-adapted and photoexcited rhodopsin in vitro. Frog rhodopsin (1.5 μM) from urea-stripped membranes was incubated with recombinant protein kinase Cα (PKCα; 5 nM) under the phosphorylation assay conditions described under "Materials and Methods." $^{32}$P incorporation was determined from SDS-PAGE and phosphor images for dark-adapted (closed columns) or photoexcited (open columns) rhodopsin, either in the absence or presence of 500 μM Ca$^{2+}$, as indicated. Data represent mean ± S.E. B, time course of phosphorylation of frog rhodopsin by protein kinase C. Photoexcited rhodopsin (200 nM) in urea-stripped membranes was phosphorylated by protein kinase Cα (5 nM) in the presence of γ-$^{32}$P]ATP (200 μM), Ca$^{2+}$ (500 μM), and PMA (100 nM), as described under "Materials and Methods." After the indicated intervals, aliquots of reaction mixture were analyzed for $^{32}$P incorporation into rhodopsin. Data represent the mean; error bars represent S.E. when it is larger than the symbol.

DISCUSSION

The present results establish that 1) protein kinase C is a major contributor to the phosphorylation of rhodopsin in intact frog retinas; 2) the kinetics of rhodopsin phosphorylation/depolymerization differ markedly, depending on whether or not protein kinase C is hyperactivated; and 3) frog rhodopsin is phosphorylated in vitro by protein kinase C.

Protein Kinase C Contributes Significantly to the Phosphorylation of Rhodopsin in Situ—Specific inhibition of protein kinase C by calphostin C provides a unique tool to evaluate the contribution of protein kinase C in the phosphorylation of rhodopsin in intact frog retinas. This inhibitor binds the phorbol ester-binding (C1) domain of protein kinase C and thus inhibits the regulation, rather the catalytic activity, of protein kinase C (34). This inhibitor was shown to have no effect on the activity of rhodopsin kinase in vitro, at concentrations causing about 70% inhibition of PMA-activated protein kinase C. It should be noted that inhibition of protein kinase C by this molecule is light-dependent and appears to be an oxidation step (37, 38). This property served an advantage in our experiments in that inhibition of protein kinase C was initiated by light exposure.

Inhibition of protein kinase C in retinas by calphostin C revealed that this kinase contributes to about half of the phosphorylation of rhodopsin under the conditions studied. Specifically, the extent of rhodopsin phosphorylation in intact retinas was decreased by approximately 50% in the presence of 1 μM calphostin C. Similar use of kinase-specific inhibitors to study the β-adrenergic signaling pathway suggested that protein kinase A and β-adrenergic receptor kinases each contribute 40–50% toward the desensitization of the β2-adrenergic receptor in intact A431 cells at high (70%) receptor occupancy (39). These conditions would be analogous to the high levels of rhodopsin photoexcitation in the our calphostin C experiments. In the β-adrenergic system, protein kinase A phosphorylation dominated over that of the G protein-coupled receptor kinase at ligand concentrations resulting in <10% receptor occupancy (39). More recently, the use of inhibitors and dominant negative kinase mutants revealed that protein kinase C and G protein-coupled receptor kinase 2 each contribute equally (about 40–50%) toward the phosphorylation of a different G protein-coupled receptor, the type 1 angiotensin II receptor, in 293 cells (40). Thus, the phototransduction pathway shares in common with at least two other G protein receptor transduction pathways, equal regulation of the receptor by a second messenger-regulated kinase and a G protein-coupled receptor kinase.

Effect of Phorbol Esters on Kinetics of Rhodopsin Phosphorylation in Intact Retinas—Previously we reported that hyperactivation of protein kinase C with phorbol esters had varying effects on the phosphorylation of rhodopsin in intact retinas. To resolve why under some conditions phorbol ester treatment
The progressive decrease in rhodopsin phosphorylation observed after 30 min postillumination in PMA-treated retinas could be accounted for by several mechanisms. First, the protein kinase C phosphorylation site could be more sensitive to dephosphorylation than the rhodopsin kinase site. At least in vitro, the primary sites of phosphorylation of bovine rhodopsin by protein kinase C and rhodopsin kinase differ (14), so that the phosphatase sensitivity of these sites could also differ (see further discussion below). Second, protein kinase C activation could stimulate phosphatase activity. Although Ser/Thr phosphatases directly regulate protein kinase C structure and function (42), the reverse has not been established; nonetheless, phorbol esters have been shown to indirectly modulate the activity of protein phosphatase 1 in intact cells (43). Third, selective binding of secondary proteins such as arrestin to receptor phosphorylated by rhodopsin kinase (44), but not protein kinase C, could decrease the phosphatase accessibility of the rhodopsin kinase-phosphorylated receptor. In this regard, arrestin has been shown to regulate β-adrenergic receptor phosphorylated by G protein-coupled receptor kinase but not protein kinase A (45). Whether or not protein kinase C regulates phosphatase activity in photoreceptors remains to be established.

The kinetics of the light-dependent phosphorylation and dephosphorylation of rhodopsin reported here for intact frog retinas are similar to in vivo data on frogs reported by Kühn (46). Kühn found that 20-min illumination of dark-adapted frogs resulted in a level of phosphorylation that was constant between 20 and 35 min after the onset of illumination and then decayed with a half time of approximately 30 min. Although these conditions are not identical to those in this report, the kinetics of phosphorylation/dephosphorylation observed under our conditions are remarkably similar.

The kinetics of light-dependent phosphorylation of rhodopsin have been examined more recently in mouse retinas as a function of phosphorylated residue (47). Two residues were found to be phosphorylated in vivo, Ser-334 and Ser-338. In striking contrast, only one of these sites, Ser-338, is a primary phos-
Phosphorylation site by rhodopsin kinase in vitro using bovine rhodopsin as the substrate; secondary phosphorylations occur in vitro at Ser-343 and Thr-336 (48–50). The novel site, Ser-334, can be phosphorylated to only 10% under appropriate pH and temperature conditions in vitro (51), suggesting that rhodopsin kinase is not the physiological regulator of this site. However, this site lies within the primary phosphorylation domain of protein kinase C, using bovine rhodopsin as the substrate (14). Interestingly, the kinetics of phosphorylation at both in vivo sites differ markedly. In response to three continuous flashes of light, both residues are phosphorylated equally; however, Ser-338 is phosphorylated and dephosphorylated significantly more rapidly compared with Ser-334. In contrast, continuous illumination results in significantly greater phosphorylation of Ser-334 than Ser-338, and both sites are dephosphorylated at comparable rates (47). Although the conditions in these in vivo experiments are clearly different from ours, it is noteworthy that in our experiments PMA caused a progressive increase in rhodopsin phosphorylation relative to the control, suggesting that phosphorylation of the protein kinase C site(s) lagged behind that of the rhodopsin kinase. The slower kinetics of the phorbol ester-stimulated phosphorylation would be consistent with regulation of the more slowly phosphorylated Ser-334 site by protein kinase C. Curiously, the dephosphorylation at Ser-334 is slow relative to that of Ser-338, yet PMA appeared to accelerate the dephosphorylation of rhodopsin. If Ser-334 is the protein kinase C site, the possibility that PMA activates a rhodopsin-directed phosphatase could account for the accelerated dephosphorylation in our experiments.

**Conclusion**—The use of a specific activator and a specific inhibitor of protein kinase C reveals that this kinase is a major contributor to the light-dependent phosphorylation of rhodopsin. Because the phosphorylation by protein kinase C is regulated by second messengers, rather than by substrate conformation, the protein kinase C-catalyzed phosphorylation of rhodopsin may be analogous to the heterologous regulation of other G protein-coupled receptors. Whether protein kinase C in photoreceptors is activated by signals separate from rhodopsin activation, perhaps arising from feedback pathways from elsewhere in the retina, remains to be explored.

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