

# Phosphorylation of the Regulator of G Protein Signaling RGS9-1 by Protein Kinase A Is a Potential Mechanism of Light- and $\text{Ca}^{2+}$ -Mediated Regulation of G Protein Function in Photoreceptors<sup>†</sup>

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**ABSTRACT:** In vertebrate photoreceptors, photoexcited rhodopsin interacts with the G protein transducin, causing it to bind GTP and stimulate the enzyme cGMP phosphodiesterase. The rapid termination of the active state of this pathway is dependent upon a photoreceptor-specific regulator of G protein signaling RGS9-1 that serves as a GTPase activating protein (GAP) for transducin. Here, we show that, in preparations of photoreceptor outer segments (OS), RGS9-1 is readily phosphorylated by an endogenous Ser/Thr protein kinase. Protein kinase C and MAP kinase inhibitors reduced labeling by about 30%, while CDK5 and CaMK II inhibitors had no effect. cAMP-dependent protein kinase (PKA) inhibitor H89 reduced RGS9-1 labeling by more than 90%, while dibutyryl-cAMP stimulated it 3-fold, implicating PKA as the major kinase responsible for RGS9-1 phosphorylation in OS. RGS9-1 belongs to an RGS subfamily also including RGS6, RGS7, and RGS11, which exist as heterodimers with the G protein  $\beta$  subunit  $\text{G}\beta 5$ . Phosphorylated RGS9-1 remains associated with  $\text{G}\beta 5\text{L}$ , a photoreceptor-specific splice form, which itself was not phosphorylated. RGS9-1 immunoprecipitated from OS was in vitro phosphorylated by exogenous PKA. The PKA catalytic subunit could also phosphorylate recombinant RGS9-1, and mutational analysis localized phosphorylation sites to Ser<sup>427</sup> and Ser<sup>428</sup>. Substitution of these residues for Glu, to mimic phosphorylation, resulted in a reduction of the GAP activity of RGS9-1. In OS, RGS9-1 phosphorylation required the presence of free  $\text{Ca}^{2+}$  ions and was inhibited by light, suggesting that RGS9-1 phosphorylation could be one of the mechanisms mediating a stronger photoresponse in dark-adapted cells.

Many cell surface receptors mediate downstream signaling via heterotrimeric G proteins (1). In vertebrate photoreceptors, capture of a photon by rhodopsin promotes its interaction with the G protein transducin (Gt),<sup>1</sup> causing it to bind GTP and to dissociate into the GTP-bound  $\alpha$  subunit ( $\text{G}\alpha\text{t}$ ) and  $\text{G}\beta\gamma$  complex. In its GTP-bound form  $\text{G}\alpha\text{t}$  activates its effector enzyme, cGMP phosphodiesterase (PDE), by sequestering its inhibitory subunit, PDE $\gamma$ . The resulting reduction in the cGMP concentration leads to closing of cGMP-

gated cation channels and cell hyperpolarization, which ultimately sends the signal to the brain (2).

Photoreceptor recovery is carried out by several molecular mechanisms, at least some of which are mediated by the drop in intracellular free  $\text{Ca}^{2+}$ , which occurs due to the light-induced closure of the cGMP-gated cation channel. For example, reduction of  $\text{Ca}^{2+}$  leads to stimulation of guanylate cyclase that restores the cGMP pool and activates rhodopsin kinase that quenches the active state of rhodopsin (3–6). Inactivation of the phototransduction cascade also occurs at the G protein level, via the photoreceptor-specific regulator of G protein signaling (RGS), RGS9-1. Similar to other RGS proteins (7–10), RGS9-1 directly binds to  $\text{G}\alpha\text{t}$  and acts as a GTPase activating protein (GAP) (11). RGS9-1 is sufficient for GAP activity, but the effector subunit PDE $\gamma$  significantly enhances the rate of GTP hydrolysis (12, 13). The role of RGS9-1 in supporting the rapid kinetics of photoresponse has been demonstrated by a number of biochemical (11–14) and physiologic experiments (15), as well as the recent studies of RGS9-1 knockout mice (16).

Regulators of G protein signaling constitute a diverse family of proteins having a characteristic 120 amino acid RGS domain that is responsible for their GAP activity (8, 10, 17–20). In addition, RGS9-1 has three other domains, namely, the GGL (G protein  $\gamma$ -like domain), the DEP (disheveled-Egl-10-pleckstrin) domain, and the C-terminal domain. The  $\text{G}\gamma$ -like (GGL) domain, also found in RGSs 6,

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<sup>1</sup> Abbreviations: GAP, GTPase activating protein; RGS, regulator of G protein signaling; PDE $\gamma$ ,  $\gamma$  subunit of cGMP phosphodiesterase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; CaMK II, calmodulin-activated protein kinase II; GGL, G protein  $\gamma$ -like domain; OS, photoreceptor outer segments; DEP, disheveled-Egl10-pleckstrin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; GST, glutathione S-transferase; Gt, G protein transducin;  $\text{G}\alpha\text{t}$ , transducin  $\alpha$  subunit; GRK 1, G protein coupled receptor kinase 1.

7, and 11, is responsible for interaction with the G protein  $\beta 5$  subunit ( $G\beta 5$ ) (21, 22). In photoreceptors,  $G\beta 5$  is represented by its splice version,  $G\beta 5L$ , which is longer by 42 amino acids than  $G\beta 5$  (23). Similar to its nonphotoreceptor homologues RGS7 and RGS6 (24, 25), RGS9-1 cannot be purified apart from  $G\beta 5L$  (26, 27). It exists exclusively as a dimer with  $G\beta 5L$ , apparently due to the proteolytic degradation of the unassociated monomer subunits (16, 27). Although it is clear that dimerization is important for their stability in cells, the function of the  $G\beta 5$  moiety in the RGS- $G\beta 5$  dimer is not fully understood. For RGS7,  $G\beta 5$  has been shown to inhibit  $G\alpha o$ -RGS7 binding (21). At the same time, RGS7, RGS6, and RGS11 dimers with  $G\beta 5$  have been shown to possess GAP activity toward  $G\alpha o$ , but it has not been compared to that of monomeric full-length RGSs 6, 7, and 11 (22, 28). Such a comparison was done recently for RGS9-1 by Wensel and colleagues, who showed that  $G\beta 5L$  reduces GAP activity of recombinant RGS9-1, but in the presence of PDE $\gamma$ ,  $G\beta 5L$  stimulated the GAP activity (13). This finding is in accord with the observations that, in cell-based assays,  $G\beta 5$  appeared to stimulate RGS7 activity, perhaps because of the presence of the G protein effectors (27, 29, 30). The role of the third distinct domain of RGS9-1, DEP, remains unknown although it is present in a number of other proteins involved in signal transduction (31). The unique C-terminal domain of RGS9-1 appears to be involved in its membrane association, but this has not yet been established (32).

Initial studies of RGS proteins showed that they are ubiquitously expressed, have very high potency as GAPs, and low specificity toward the  $G\alpha$  subunits (18, 33). This raised the question of how G proteins can deliver their signals at all and led to the idea that RGS proteins themselves must be regulated (7). Indeed, studies confirmed this notion for some of the RGS proteins, revealing several mechanisms including changes in transcription levels (34), subcellular localization (35, 36), and phosphorylation (37, 38). While this paper was in preparation, Hu and colleagues reported that RGS9-1 is phosphorylated at Ser<sup>475</sup> by a PKC-like kinase (32).

Here, we report that RGS9-1 is phosphorylated in OS by endogenous protein kinase A. Our data show that this phosphorylation occurs at two sites, Ser<sup>427</sup> and Ser<sup>428</sup>, is regulated by light and calcium, and results in reduction of RGS9-1 GAP activity. On the basis of these observations, we put forward a model for the regulation of RGS9-1 function by the light–dark cycle. In light, RGS9-1 is a potent GAP, causing rapid transducin inactivation and photoreceptor recovery. In the dark, when rising concentration of Ca<sup>2+</sup> and cAMP in the cell activates PKA (and other kinases), RGS9-1 is phosphorylated, and its GAP activity is reduced, which results in a longer lifetime of active transducin and stronger photoresponse.

## MATERIALS AND METHODS

**Materials.** Photoreceptor outer segments (OS) were prepared from frozen bovine retinas using the stepwise sucrose density gradient method as described in detail previously (24, 39). Antibodies against RGS9 and  $G\beta 5$  were described previously (21, 27). Monoclonal antibody against RGS9 was a generous gift from Dr. K. Palczewski (University of

Washington). Bovine RGS9-1 cDNA was provided by Dr. T. Wensel (Baylor College of Medicine). Secondary antibodies were obtained from Jackson Immunologicals. Protein kinase inhibitors, PKA catalytic subunit, and nucleotides were from Calbiochem and NEN Life Science Products Inc., respectively. Dibutyl- $\gamma$ -cAMP and protease inhibitor cocktail used were from Boehringer Mannheim.

**Labeling of OS with [ $\gamma$ -<sup>32</sup>P]ATP and RGS9-1 Immunoprecipitation.** Purified OS (100  $\mu$ M rhodopsin) were incubated in 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM MgCl<sub>2</sub>, and 0.05% Triton X-100 with 15  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP at 37 °C under dim red light, unless otherwise specified. OS were then lysed in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5 mM ATP, 80 mM *N*-octyl  $\beta$ -D-glucopyranoside, and 1 $\times$  protease inhibitor cocktail and centrifuged, and the supernatant was used for immunoprecipitation. Immunoprecipitation was carried out using affinity-purified anti-RGS9 antibody and protein A–Sepharose as described previously (27). The obtained fractions were resolved by SDS–PAGE. For autoradiography, the gels were either dried, stained, and exposed to film or first transferred to nitrocellulose membrane, which was dried, and exposed to film (Kodak BioMax MR). In the latter case, the filters were subsequently rehydrated in 20 mM Tris, pH 7.6, 50 mM NaCl, and 0.3% Tween-20 and probed with anti-RGS9 and anti- $G\beta 5$  antibody as described earlier (27).

**Mutagenesis, Expression, and Purification of Recombinant RGS9-1.** Putative PKA phosphorylation sites were identified using the PROSITE database of protein families and domains (40). The extended RGS domain (amino acids 276–431) was PCR amplified and subcloned into expression vector pGEX-KG. Substitutions of Ser<sup>400</sup>, Ser<sup>427</sup>, and Ser<sup>428</sup> were introduced by PCR and confirmed by sequencing. The glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* strain BL21 and purified on glutathione–Sepharose 4B (Pharmacia) using a standard protocol. The purity of proteins was tested by SDS–PAGE and found to be more than 95%. Protein concentrations were determined using the Advanced Protein Assay Reagent (Cytoskeleton Inc.) and verified by resolving the proteins with known standard concentrations of BSA on SDS–PAGE, stained with Coomassie Blue, and densitometric scanning.

**In Vitro Phosphorylation of Proteins by PKA.** Purified GST fusion proteins (0.5  $\mu$ g) were incubated for 5 min at 30 °C with 50 units of the PKA catalytic subunit in 50  $\mu$ L of labeling buffer (25 mM HEPES, pH 7.4, 5 mM MgCl<sub>2</sub>, 5 mM EGTA, 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP). The proteins were precipitated with 100  $\mu$ L of ethanol at –20 °C for 1 h and resolved by SDS–PAGE, stained with Coomassie to confirm equal loading, dried, and exposed to an X-ray film. In vitro phosphorylation of the native RGS9-1– $G\beta 5L$  complex was carried out following its immunoprecipitation from OS. The protein A beads with the immobilized complex were washed and resuspended in the labeling buffer (volume 50  $\mu$ L), and the reaction was allowed to proceed with or without added PKA for 5 min at 30 °C with periodic mixing of the beads. The resin was then washed twice with the same buffer and eluted with SDS–PAGE sample buffer, and the proteins were analyzed by autoradiography and Western blot.

**GAP Activity Assay.** Single turnover transducin GTPase assays were carried out as described (41). Briefly, for kinetic experiments, illuminated urea-washed membranes (15  $\mu$ M

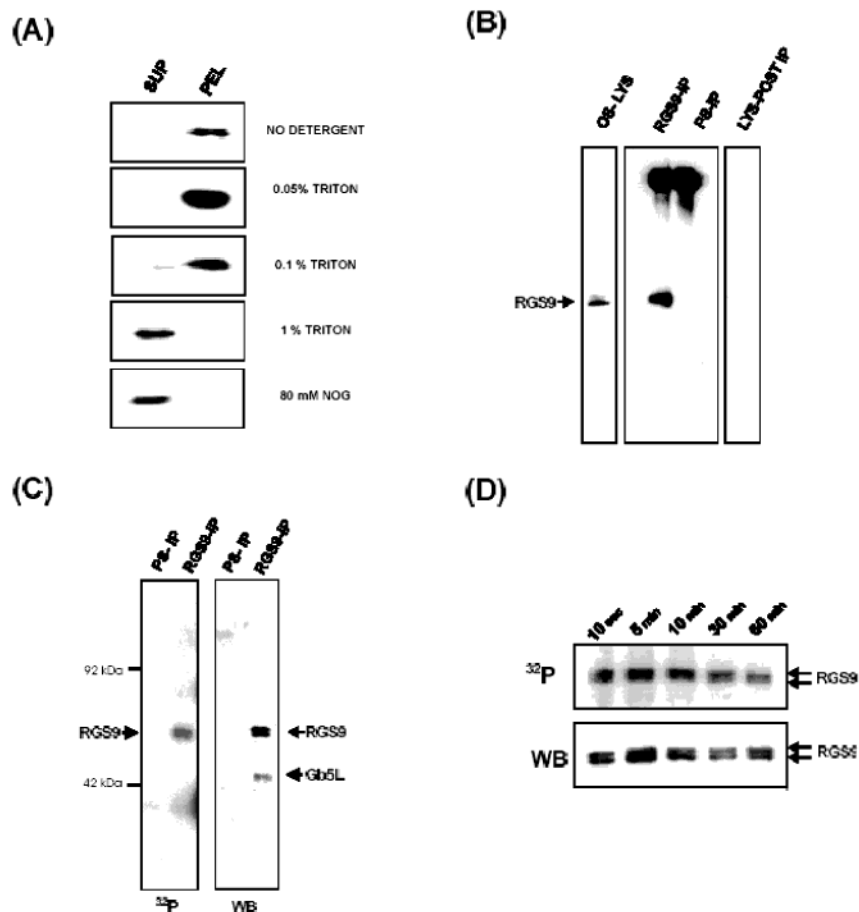


FIGURE 1: Phosphorylation of RGS9-1 in OS. (A) Distribution of RGS9-1 in permeabilized OS. OS were incubated in a buffer containing the indicated concentrations of Triton X-100 or 80 mM *N*-octyl  $\beta$ -D-glucopyranoside in light. After 30 min, the samples were centrifuged, and the distribution of RGS9-1 between the supernatant (SUP) and the pellet (PEL) was compared by Western blot with the anti-RGS9 antibody. At 0.05% Triton X-100 concentration, 100% of RGS9-1 remains membrane-bound. (B) Immunoprecipitation of RGS9-1 from OS extract. Shown is a Western blot analysis of the following fractions: the total soluble OS lysate (OS LYS), eluate from the anti-RGS9 antibody bound protein A beads (RGS9-IP), preimmune serum bound protein A beads (PS-IP), and the immunodepleted lysate (LYS-POST IP). (C) Immunoprecipitation of RGS9-1 from radiolabeled OS. OS were incubated in a buffer with 0.05% Triton X-100 in the presence of [ $\gamma$ - $^{32}\text{P}$ ]ATP under dim red light, lysed with 80 mM *N*-octyl  $\beta$ -D-glucopyranoside, and immunoprecipitated with anti-RGS9 antibody (RGS9-IP) or preimmune serum (PIS-IP) as described in Materials and Methods. Fractions were resolved by SDS-PAGE, transferred to a filter, and exposed to film. The left panel ( $^{32}\text{P}$ ) shows the autoradiograph of the immunoprecipitated material. The right panel (WB) shows the same filter probed with antibodies to RGS9 and G $\beta$ 5L. (D) Kinetics of RGS9-1 phosphorylation. OS were labeled for increasing periods of time (10 s, 5 min, 10 min, 30 min, and 60 min) under dim red light, and RGS9-1 was immunoprecipitated. The immunoprecipitates were analyzed by autoradiography (top panel,  $^{32}\text{P}$ ) and Western blotted with the anti-RGS9 antibody (lower panel, WB). The stoichiometry of phosphorylation was 0.1 mol of phosphate/mol of RGS9-1. In the experiment shown, proteins were resolved by SDS-PAGE under nonreducing conditions, and RGS9-1 was detected as a doublet.

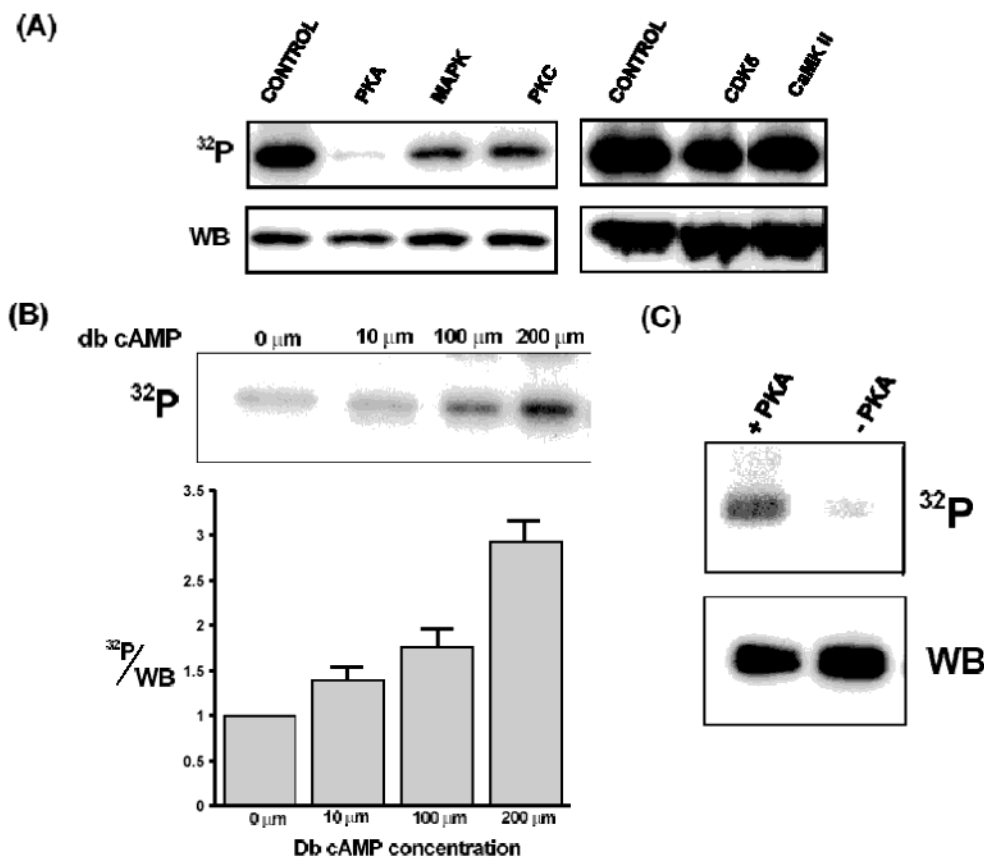
rhodopsin) were mixed with 1  $\mu\text{M}$  purified transducin and 3  $\mu\text{M}$  RGS9-1 GST fusions. The reaction was started by the addition of 100 nM [ $\gamma$ - $^{32}\text{P}$ ]GTP (ICN) and stopped at various times using 5% perchloric acid. The amount of [ $^{32}\text{P}$ ]P $_i$  released from hydrolyzed GTP was determined by the activated charcoal assay. The first-order rate constants for GTP hydrolysis ( $k_{\text{inact}}$ ) were obtained by fitting the data to single exponentials. Protein titration experiments were performed as described above except that the RGS9-1-GST fusion proteins were added at 5 s after the addition of [ $\gamma$ - $^{32}\text{P}$ ]GTP, and the reaction quenched at 10 s with 5% perchloric acid (11). The GTP hydrolysis rate constant was determined according to the equation  $K_{\text{inact}} = -(\ln[(\% \text{ of GTP unhydrolyzed at 10 s})/(\% \text{ of GTP unhydrolyzed at 5 s})])/5 \text{ s}$ .

## RESULTS

*RGS9-1 Is Phosphorylated in Photoreceptor Outer Segments by an Endogenous Ser/Thr Kinase.* To permit the entry

of [ $\gamma$ - $^{32}\text{P}$ ]ATP, we permeabilized OS with low concentrations of the nonionic detergent Triton X-100. Permeabilization and labeling were carried out in the same reaction to ensure minimal change in the status of OS proteins. This resulted in phosphorylation of several OS proteins, whereas without permeabilization, no labeling was detected. Treatment with 0.05% Triton X-100 was sufficient for [ $\gamma$ - $^{32}\text{P}$ ]ATP entry, while the membrane localization of RGS9-1 and G $\beta$ 5L proteins was not altered (Figure 1A). Since a number of OS proteins were radiolabeled, the analysis of the phosphorylation status of RGS9-1 required its immunoprecipitation from the lysates. Figure 1B shows that the anti-RGS9 antibody specifically and quantitatively binds RGS9-1 from the OS extract. No RGS9-1 was detected in the preimmune serum immunoprecipitate or the OS lysate immunodepleted by the RGS9-specific antibody. Immunoprecipitation of the [ $\gamma$ - $^{32}\text{P}$ ]ATP-labeled extract with anti-RGS9 antibody resulted in specific absorption of a distinct  $\gamma$ - $^{32}\text{P}$ -labeled protein with





**FIGURE 2:** Identification of the RGS9-1 kinase (A) Effect of protein kinase inhibitors on RGS9-1 phosphorylation in OS. OS were preincubated with specific kinase inhibitors, permeabilized, labeled with [ $\gamma$ - $^{32}\text{P}$ ]ATP for 5 min under dim red light, and lysed, and RGS9-1 was immunoprecipitated. The immunoprecipitate was analyzed by autoradiography ( $^{32}\text{P}$ ) and Western blot using the anti-RGS9 antibody (WB). Shown is a representative of several experiments in which the following inhibitors were used: PKA—H89 at 10  $\mu\text{M}$ , MAPK—SB203580 at 50  $\mu\text{M}$ , PKC—calphostin C at 0.5  $\mu\text{M}$ , CDK5—roscovitine at 50  $\mu\text{M}$ , and CaMK II—KN93 at 10  $\mu\text{M}$ . Inhibitors were added to the labeling reaction from 100 $\times$  stocks, and 1% DMSO was used as control. (B) Stimulation of RGS9-1 phosphorylation by dibutyryl-cAMP. OS were preincubated with increasing concentrations of the PKA activator dibutyryl cAMP (10, 100, and 200  $\mu\text{M}$ ) and then labeled with [ $\gamma$ - $^{32}\text{P}$ ]ATP under dim red light. OS were lysed, RGS9-1 was immunoprecipitated, and its phosphorylation was determined by autoradiography ( $^{32}\text{P}$ ). The amount of  $\gamma$ - $^{32}\text{P}$  in the RGS9-1 band was normalized to the amount detected by Western blotting. The band intensities were determined by densitometric scanning (Sicon image analysis software). Values obtained at 0  $\mu\text{M}$  cAMP were equated to 1, and the relative increase in phosphorylation for two separate experiments is represented in the histogram. (C) RGS9-1 was first immunoprecipitated from OS under dim red light, and then the beads with the bound antibody-antigen complex were incubated with or without the purified catalytic subunit of PKA. Following the reaction, RGS9-1 was eluted from the beads with SDS-PAGE sample buffer, resolved by SDS-PAGE, transferred to a filter, dried, and exposed to film ( $^{32}\text{P}$ ). The amount of RGS9-1 present on the beads and eluted was verified by probing the same filter with the anti-RGS9 antibody (WB).

a molecular mass identical to that of RGS9-1 ( $\approx 56$  kDa), as confirmed by Western blot (Figure 1C). Elution of the antibody-bound protein complexes from the protein A beads required denaturing conditions, so along with RGS9-1, these fractions contained rabbit immunoglobulins. To avoid the interference from IgG chains, we analyzed these samples under nonreducing conditions. Under these conditions, RGS9-1 behaved as a distinct doublet, with both bands equally phosphorylated (Figure 1C,D). Under reducing conditions, RGS9-1 was detected as a single protein band. The identity of the immunoprecipitated 56 kDa phosphoprotein as RGS9-1 was also confirmed using the anti-RGS9 monoclonal antibodies (42) (data not shown). Immunoprecipitation of RGS9-1 was accompanied by quantitative coprecipitation of G $\beta$ 5L, indicating that phosphorylation of RGS9-1 does not result in dissociation of the RGS9-1–G $\beta$ 5L complex. G $\beta$ 5L itself was not phosphorylated (Figure 1C). In accord with our previous results (27), no G $\alpha$ t was detected in the immunoprecipitates regardless of RGS9-1 phosphorylation status (data not shown). To assess the kinetics of the RGS9-1 phosphorylation reaction in OS, they were

permeabilized and labeled with [ $\gamma$ - $^{32}\text{P}$ ]ATP for increasing periods of time in the dark and lysed, and RGS9-1 was immunoprecipitated. We found that labeling of RGS9-1 was rapid, reaching its maximum level in less than 10 s, and remained constant for over 1 h (Figure 1D). Under these conditions, the stoichiometry of labeling was approximately 0.1 mol of phosphate/mol of RGS9-1. This rather low stoichiometry could be the result of some of the RGS9-1 in OS being already phosphorylated.

To determine the specificity of RGS9-1 phosphorylation (Ser/Thr or Tyr), an SDS gel containing RGS9-1 phosphate was treated with 1 N NaOH. This procedure results in the specific removal of phosphate on Ser/Thr residues from the protein (43). Upon this treatment, RGS9-1 was retained in the gel, but the radiolabel was lost, indicating that it is phosphorylated on Ser/Thr residue(s). In accord, an anti-phosphotyrosine antibody did not detect RGS9-1 phosphorylation (data not shown).

**The Main RGS9-1 Kinase Is Protein Kinase A.** To determine the kinase(s) responsible for RGS9-1 phosphorylation, OS were labeled in the presence of specific protein

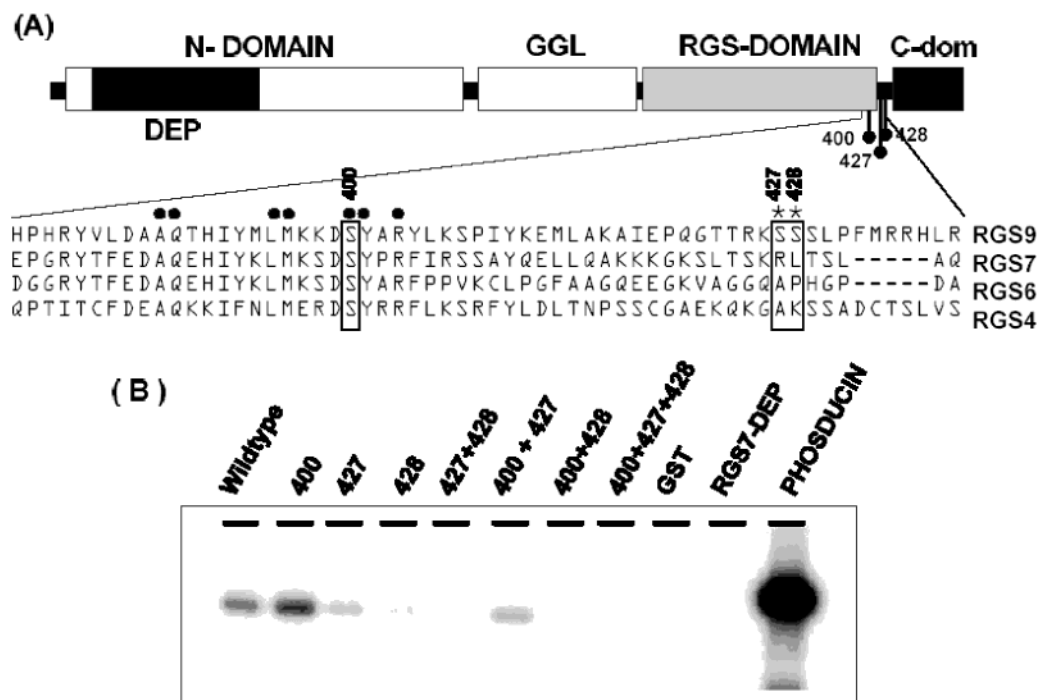


FIGURE 3: Phosphorylation of extended RGS9-1 domain and its mutants with purified PKA. (A) Putative PKA phosphorylation sites on RGS9-1. Shown is the structure of RGS9-1, with the DEP, GGL, RGS, and C-terminal domains highlighted. The location of putative PKA site Ser residues 400, 427, and 428 is indicated. The portion of the RGS9-1 sequence with the phosphorylation sites is aligned with the corresponding regions of RGS7, RGS6, and RGS4 to highlight the unique location of Ser<sup>427</sup> and Ser<sup>428</sup> in RGS9-1. The positions of the most conserved residues are denoted by black circles above the RGS9-1 sequence. (B) In vitro phosphorylation of recombinant RGS9-1 and its mutants. The recombinant extended RGS domain (wild type) and putative phosphorylation site Ser/Ala mutants were generated, expressed, and purified as described under Materials and Methods. They were then incubated with the PKA catalytic subunit and [ $\gamma$ -<sup>32</sup>P]-ATP, precipitated, resolved by SDS-PAGE, and analyzed by autoradiography. GST fusion of the RGS7 DEP domain and recombinant phosducin were used as negative and positive controls, respectively, for the PKA-mediated phosphorylation.

kinase inhibitors (Figure 2A). The concentrations of these inhibitors were much higher than their reported IC<sub>50</sub> values (H89, IC<sub>50</sub> = 50 nM; SB203580, IC<sub>50</sub> = 600 nM; calphostin C, IC<sub>50</sub> = 50 nM; roscovitine, IC<sub>50</sub> = 200 nM; KN93, IC<sub>50</sub> = 370 nM). CDK5 and CaMK II inhibitors did not alter RGS9-1 phosphorylation. Protein kinase C and MAPK inhibitors reduced labeling by approximately 30%. In contrast, PKA inhibitor H89 had a major effect, decreasing RGS9-1 phosphorylation by more than 90% (Figure 2A). Further supporting the role of OS PKA in RGS9-1 phosphorylation, the PKA activator dibutyryl-cAMP stimulated phosphorylation of RGS9-1 in a concentration-dependent manner (Figure 2B). To find out if PKA is directly responsible and sufficient for RGS9-1 phosphorylation, we immunoprecipitated the RGS9-1-G $\beta$ 5L complex from OS and incubated it with the PKA catalytic subunit in the presence of [ $\gamma$ -<sup>32</sup>P]ATP. This reaction resulted in PKA-dependent labeling of RGS9-1, confirming it to be a substrate for PKA (Figure 2C). G $\beta$ 5L in the complex was not labeled in the reaction (data not shown). As shown in Figure 3, the recombinant RGS9-1 fragment is also a substrate for PKA. Taken together, these results strongly indicate that PKA is the major RGS9-1 kinase that phosphorylates it in OS and in vitro.

**RGS9-1 Ser<sup>427</sup> and Ser<sup>428</sup> Are the Sites for PKA-Mediated Phosphorylation.** Analysis of the RGS9-1 sequence using the PROSITE database of protein families and domains identified residues Ser<sup>400</sup>, Ser<sup>427</sup>, and Ser<sup>428</sup> as the putative PKA phosphorylation site residues located either within the predicted RGS domain that spans amino acids 290–410 of

RGS9-1 or immediately downstream (Figure 3A). Among these, Ser<sup>400</sup> is conserved among the RGS protein family, whereas Ser<sup>427</sup> and Ser<sup>428</sup> appear to be unique. We expressed the extended RGS domain (267–431) in *E. coli* as a GST fusion protein and found that this construct is a substrate for the catalytic subunit of PKA in an in vitro phosphorylation reaction (Figure 3B). Next, we created Ser/Ala mutants of the putative phosphorylation sites and analyzed them using the in vitro phosphorylation assay. Replacing Ser<sup>400</sup> did not affect labeling, whereas mutations of Ser<sup>427</sup> or Ser<sup>428</sup> substantially reduced phosphorylation. Substitution of both Ser<sup>427</sup> and Ser<sup>428</sup> for Ala residues abolished labeling of the RGS9-1 construct. Since this construct has GAP activity (see below), the lack of labeling cannot be attributed to protein denaturation, and therefore Ser<sup>427</sup> and Ser<sup>428</sup> are the PKA phosphorylation sites on RGS9-1. Comparison of phosphorylation of the Ala<sup>400</sup>/Ser<sup>427</sup>/Ser<sup>428</sup>, Ala<sup>400</sup>/Ala<sup>427</sup>/Ser<sup>428</sup>, and Ala<sup>400</sup>/Ser<sup>427</sup>/Ala<sup>428</sup> mutants showed that Ser<sup>428</sup> was more readily phosphorylated by PKA than Ser<sup>427</sup>. Phosphorylation of constructs containing both Ser residues was higher than the sum of the phosphate incorporated in the mutants where only one of the sites was available, suggesting that the initial phosphorylation of one site might potentiate phosphorylation of the other. Such a synergism has been noted earlier for other PKA substrates, for example, cardiac troponin I on Ser<sup>24</sup> and Ser<sup>23</sup> (44).

**Effect of Ser<sup>427</sup> and Ser<sup>428</sup> Mutations on the RGS9-1 GAP Activity.** Replacement of Ser residues with Ala makes the sites unavailable for phosphorylation, whereas Glu is known to mimic the phosphorylated residues. To investigate the

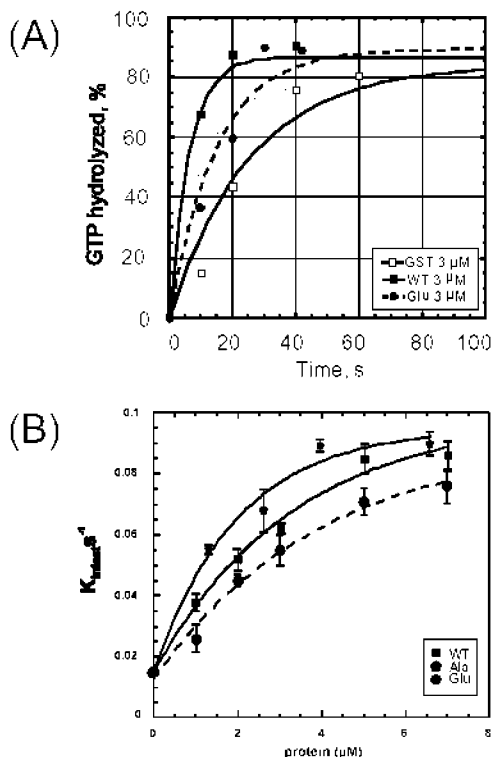


FIGURE 4: Effect of Ser<sup>427</sup> and Ser<sup>428</sup> mutations on the GAP activity of RGS9-1. The extended RGS domain of RGS9-1 representing the wild type (filled square), the Ala<sup>427,428</sup> (closed pentagon), and the Glu<sup>427,428</sup> (filled circle) were expressed in *E. coli* as GST fusion proteins and tested for their GAP activity toward transducin. The data were obtained and fit as described in Materials and Methods. (A) Effect of RGS9-1 on the kinetics of GTP hydrolysis at a fixed RGS concentration (3  $\mu$ M). Aliquots from the GTPase reactions were taken at the indicated times, and the amount of  $\gamma$ -<sup>32</sup>P produced was determined as described in Materials and Methods. For control of the intrinsic rate of GTP hydrolysis, GST was added (open square). (B) Dose-response from added RGS. Measurements were taken, as described in Materials and Methods, at exactly 5 s after addition of RGS. Data are from three independent experiments. The amount of RGS9-1 protein added to the reaction was determined as described in Materials and Methods.

functional effect of PKA-mediated RGS9-1 phosphorylation, we compared the GAP activity of the wild-type recombinant RGS9-1 domain to that of the double mutants, Ala<sup>427</sup>/Ala<sup>428</sup> and Glu<sup>427</sup>/Glu<sup>428</sup>. Figure 4 shows that the acceleration of GTP hydrolysis by transducin elicited by the Glu<sup>427</sup>/Glu<sup>428</sup> RGS9-1 mutant was lower than that elicited by the wild-type protein or the Ala<sup>427</sup>/Ala<sup>428</sup> mutant. The positive effect of the substitutions of the Ser residues for Ala on the GAP activity of RGS9-1 was rather unexpected. This could be due to a basal phosphorylation of wild-type RGS9-1 in *E. coli*, which would decrease its GAP activity, or due to the fact that the two side chains of the Ser residues are important for RGS9-1 structure and activity. It is noteworthy that the differences between the wild type and mutants cannot be attributed to a fluctuation of protein concentration used. To account for the differences in GAP activity, the concentration should be different by at least 2-fold, which would have been detected upon quantification of the proteins by SDS-PAGE and densitometry. In summary, the results of mutational analysis indicate that phosphorylation of Ser<sup>427</sup> and Ser<sup>428</sup> can attenuate the GAP activity of RGS9-1.

**Regulation of RGS9-1 Phosphorylation.** To investigate the factors regulating RGS9-1 phosphorylation, we first com-

pared labeling of RGS9-1 in the dark-adapted and illuminated OS and found that phosphorylation was drastically reduced in light (Figure 5). This was in sharp contrast to phosphorylation of rhodopsin, which, as expected, was phosphorylated in light (data not shown). Active phosphorylation of rhodopsin in light resulted in the incorporation of only  $\approx 10\%$  of the total [ $\gamma$ -<sup>32</sup>P]ATP under the incubation conditions. This suggests that available ATP levels did not limit RGS9-1 phosphorylation in light. A major consequence of the light stimulus is a rapid decrease in free Ca<sup>2+</sup> levels from approximately 500 to 50 nM (3). To determine if Ca<sup>2+</sup> could be involved in modulation of RGS9-1 phosphorylation, we tested the effect of the known Ca<sup>2+</sup> chelator EGTA. In the presence of 5 mM EGTA, RGS9-1 labeling was undetectable, suggesting that free Ca<sup>2+</sup> is required for RGS9-1 phosphorylation (Figure 5).

## DISCUSSION

The photoreceptor system must adapt to changes in light intensity as well as respond to fast movement of objects across the visual field. This role calls for molecular mechanisms capable of mediating rapid activation and inactivation of the phototransduction cascade. At least some of these mechanisms are based on protein phosphorylation by several protein kinases present in OS and occur at different steps in the phototransduction pathway. Rhodopsin is phosphorylated by GRK1 and PKC (5); phosducin is a major substrate of PKA (45, 46), and the  $\alpha$  subunit of transducin (47, 48) and PDE $\gamma$  (49, 50) were also shown to be substrates for phosphorylation. A key molecular event regulating the kinetics of photoresponse is stimulation of transducin's GTPase activity by the RGS9-1-G $\beta$ 5L complex. The goal of this work was to test if the RGS9-1 in this complex is itself the subject for regulation by protein kinases.

**RGS9-1 Is Phosphorylated by PKA at Residues Ser<sup>427</sup> and Ser<sup>428</sup>.** Our results demonstrate rapid phosphorylation of RGS9-1 by an endogenous Ser/Thr kinase (Figure 1), and the following lines of evidence indicate that the main RGS9-1 kinase in OS is protein kinase A. First, PKA inhibitor H89 significantly inhibited RGS9-1 phosphorylation (>90%), whereas other inhibitors have smaller (PKC and MAPK) or no effect (CaMK II and CDK5) (Figure 2A). Second, the PKA activator dibutyryl-cAMP stimulated RGS9-1 phosphorylation (Figure 2B). Third, the purified PKA catalytic subunit phosphorylated RGS9-1 in the native RGS9-1-G $\beta$ 5L complex immunoprecipitated from OS extracts (Figure 2C), as well as the recombinant extended RGS domain in vitro (Figure 3B). Fourth, mutations of the two putative PKA sites Ser<sup>427</sup> and Ser<sup>428</sup> abolished phosphorylation of the extended RGS domain by externally added PKA catalytic subunit (Figure 3B). Taken together, these observations provide strong circumstantial evidence that RGS9-1 is phosphorylated by PKA at Ser<sup>427</sup> and Ser<sup>428</sup>.

Our data also show that other protein kinase(s) participate in RGS9-1 phosphorylation. Addition of H89 to the phosphorylation reaction did not result in a 100% inhibition of RGS9-1 labeling in OS, whereas EGTA completely blocked it, indicating that a Ca<sup>2+</sup>-dependent mechanism is involved. PKA is only indirectly regulated by Ca<sup>2+</sup>, which can stimulate production of cAMP via the Ca<sup>2+</sup>-calmodulin-adenylate cyclase pathway. In contrast, PKC $\alpha$ , the predominant PKC isoform present in OS,<sup>2</sup> is directly regulated by

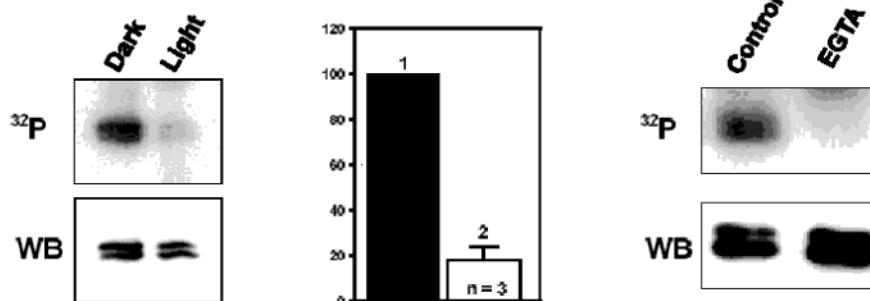


FIGURE 5: Effect of light and EGTA on RGS9-1 phosphorylation. Phosphorylation of RGS9-1 in OS was carried out for 10 min under dim red light (Dark) or ambient light (Light). Outer segments were lysed, RGS9-1 was immunoprecipitated, and its phosphorylation was detected by autoradiography ( $^{32}\text{P}$ ). The amount of RGS9-1 in the immunoprecipitate was verified by Western blot (WB) (left panel). The histogram shows data from three separate experiments, with phosphorylation levels in the dark equated to 100% (filled) and the relative phosphorylation in light accordingly calculated (open). RGS9-1 in OS was similarly phosphorylated in the dark with or without 5 mM EGTA and, following immunoprecipitation, analyzed by autoradiography and Western blot.

$\text{Ca}^{2+}$ . In accord, we found that the PKC inhibitor calphostin C notably reduces RGS9-1 phosphorylation, indicating that PKC could be the other kinase involved in RGS9-1 phosphorylation in OS. The significance of the negative effect of the MAP kinase inhibitor is not clear, as the presence of MAP kinase in OS has not been reported. One obvious possibility is that this inhibitor affects a different kinase(s). Recently, Hu et al. have found that RGS9-1 is phosphorylated by an endogenous kinase and, using mass spectrometry, identified Ser<sup>475</sup> as the phosphorylation site (32). On the basis of the effects of  $\text{Ca}^{2+}$  and a PKC inhibitor, they defined the RGS9-1 kinase as PKC-like (32). The difference between our two studies are the OS preparations (intact permeabilized OS versus sheared membranes) and the specific conditions used in the phosphorylation assays. The combined evidence indicates that RGS9-1 can be phosphorylated on at least three sites, Ser<sup>427</sup>, Ser<sup>428</sup>, and Ser<sup>475</sup>. Analysis of the role of each site will be important for understanding the phosphorylation-mediated regulation of RGS9-1 function.

**Effect of Phosphorylation on RGS9-1 Functional Activity.** The GAP activity for transducin is the only functional role that has been established for the RGS9-1–G $\beta$ 5L complex both in vitro and in vivo. The positions of Ser<sup>427</sup> and Ser<sup>428</sup> near the RGS box suggested their role in regulation of the RGS9-1–transducin interaction, and therefore we investigated the effect of phosphorylation on the GAP activity of RGS9-1. So far, similarly to Hu and colleagues (32), we also did not achieve a stoichiometric phosphorylation of RGS9-1 in vivo or the extended RGS domain in vitro (data not shown). The stoichiometry of phosphorylation of the extended RGS domain in vitro was only on the order of 0.1 mol of phosphate/mol of protein and hence it could not be used in the GAP assays. To circumvent this problem, we relied upon the use of the recombinant RGS9-1 fragment where the Ser residues are substituted either for Ala, making them unavailable for phosphorylation, or for Glu residues known to mimic Ser-phosphate. The Ser<sup>427,428</sup>/Glu<sup>427,428</sup> mutant displayed a reduced GAP activity compared to wild-type RGS9-1, indicating that phosphorylation at these sites attenuates GAP activity. To our surprise, we also found that the Ala mutations resulted in a stronger GAP activity. It is tempting to speculate that the region around Ser<sup>427,428</sup> is a flexible hinge between the RGS and C-terminal domains

(Figure 3); the Ala substitutions stabilize RGS9-1 in a highly active state whereas phosphorylation (and the Glu<sup>427,428</sup> mutation) makes it a less efficient GAP. Indeed, as demonstrated by Wensel's laboratory, RGS9-1 function is subject to remarkably complex regulation by its multiple domains as well as G $\beta$ 5L and PDE $\gamma$  (13). Both the negative effect of the Glu<sup>427,428</sup> mutation and the positive effect of the Ala<sup>427,428</sup> mutation appear to be small in magnitude; however, additional data support the significance of the results presented here. We tested the GAP activity of our mutants by an alternative method, measuring the rate of decay of GTP-induced fluorescence of another G protein, G $\alpha_i$  (51). Compared to the single turnover [ $\gamma$ - $^{32}\text{P}$ ]GTP hydrolysis, this technique provides superior time resolution because the data are recorded in real time. The results showed a similar pattern, with the Ala<sup>427,428</sup> mutation marginally increasing and Glu<sup>427,428</sup> mutation reducing GAP activity.<sup>3</sup> Furthermore, the unpublished data of Hu and colleagues indicate that incubation of OS with ATP results in a diminished GAP activity although there was no direct correlation of this effect with phosphorylation of Ser<sup>475</sup> (32). At the moment, it is not clear if the subtle effect of RGS9-1 phosphorylation that we detected here implies that the contribution of this mechanism in fine-tuning of photoresponse is indeed small. One can speculate that, because of other mechanisms, dark adaptation might not require a major reduction of the lifetime of transducin's GTP-bound state. However, intuitively it seems that RGS9-1 phosphorylation should have a more profound effect, particularly because in vivo only a portion of RGS9-1 molecules might become phosphorylated. The low magnitude of the effect we observe is likely to be due to the limitations of our reagents and assays and perhaps will be more prominent in the context of the full-length RGS9-1 and/or the entire RGS9-1–G $\beta$ 5L complex. A more drastic reduction of the GAP activity might also require mutation of all three (or more) sites. Although mimicking of phosphorylation by glutamate is a well-established approach, it is still possible that, in RGS9-1, the introduced Glu residues inhibit GAP activity with less efficiency than the more bulky Ser-phosphates. It should also be noted that the effects of phosphorylation on GAP activity of other RGS proteins were also small, but they were significantly augmented by additional factors (38, 52). For example, the 10–

<sup>2</sup> N. Balasubramanian, unpublished data.

<sup>3</sup> H. Zhong and R. Neubig, unpublished results.



15% decrease in RGS7 GAP activity following its phosphorylation by PKC increased 5-fold upon 14-3-3 binding to the RGS7-phosphate (52). Thus it is possible that an additional factor present in OS could distinctly enhance the magnitude of modulation of RGS9-1 GAP activity by protein kinase mediated phosphorylation.

**Regulation of RGS9-1 Phosphorylation.** The light-mediated inhibition of RGS9-1 phosphorylation prompted us to test  $\text{Ca}^{2+}$  as the mediator of this effect. We found that total RGS9-1 phosphorylation was inhibited by excess EGTA. These results are in general agreement with Hu and colleagues, who using alternative approaches found phosphorylation of Ser<sup>475</sup> to also be stronger in the dark and dependent on  $\text{Ca}^{2+}$  (32). Because more than 90% of RGS9-1 labeling in OS is H89 sensitive and hence PKA mediated, our observations show that light and EGTA affect PKA-mediated phosphorylation at Ser<sup>427</sup> and Ser<sup>428</sup> as well. These findings strongly indicate that RGS9-1 phosphorylation is physiologically relevant, and we propose a working model in which phosphorylation of RGS9-1 represents a novel mechanism of dark adaptation. In the dark (high  $\text{Ca}^{2+}$ ), RGS9-1 kinases are active, RGS9-1 is phosphorylated, and its GAP activity is lower, increasing the lifetime of the transducin-GTP complex, which contributes to the stronger photoresponse in the dark. One of these kinases is PKA, which can be activated by  $\text{Ca}^{2+}$  through the  $\text{Ca}^{2+}$ -calmodulin-mediated stimulation of OS adenylate cyclase and a resulting increase in cAMP level. Another kinase, which is activated by  $\text{Ca}^{2+}$  directly, might be the PKC-like kinase described by Hu et al. (32). In light,  $\text{Ca}^{2+}$  concentration drops and RGS9-1 is dephosphorylated, becoming a more potent GAP that inactivates the G protein faster. Our experiments were done on permeabilized OS that should be unable to extrude  $\text{Ca}^{2+}$  in light as the intact cells. This suggests the existence of an additional mechanism that inhibits RGS9-1 phosphorylation in light in a  $\text{Ca}^{2+}$ -independent manner. For example, the light-induced association of RGS9-1 with GTP-bound  $\alpha$  transducin and PDE $\gamma$  could reduce its ability to be phosphorylated. The true sequence of molecular events is likely to be more complicated, as regulation of other basic mechanisms such as RGS9-1 dephosphorylation remains unexplored. Unraveling these mechanisms will require additional experimentation using biochemical and physiologic approaches.

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