Novel Mechanism for the Activation of Rhodopsin Kinase: Implications for other G Protein-Coupled Receptor Kinases (GRK's)[†]

Kevin R. Dean and Muhammad Akhtar*

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton S016 7PX, U.K.

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ABSTRACT: ATP, its nonhydrolyzable analogue, AMP-PNP, and albumin were found to promote the dissociation of rhodopsin kinase from rod outer segments (ROS) containing photoactivated-rhodopsin (Rho*). These features were embodied in a protocol for the recovery of rhodopsin kinase from incubations containing ROS which had been subjected to a wide range of treatments. It was found that the supernatants recovered from mixtures containing ATP, rhodopsin kinase, and photolyzed ROS membranes catalyzed a Rho*-*in*dependent peptide phosphorylation as well as dark-phosphorylation of rhodopsin. The activities of this activated kinase in the two aforementioned assays were 7−8% of the maximum intrinsic activity found in appropriate standard assays (i.e., light-stimulated phosphorylation of rhodopsin and Rho*-dependent peptide phosphorylation). The activated kinase reverted to its inactive resting-state in a time dependent fashion, giving a $\tau_{1/2}$ of decay of ∼2 min. The intrinsic activity of kinase as measured by the standard assay, however, remained constant during this decay period. No positive evidence was found to suggest that the interconversion activated kinase ↔ inactive kinase occurred by a phosphorylation event. Cumulatively, the results show that the interaction of rhodopsin kinase·ATP complex with Rho* leads to the formation, presumably due to the reorganization of the protein structure, of a soluble active kinase species which reverts to the inactive resting state in a time-dependent fashion.

Rhodopsin kinase is a prototypical member of an expanding family of G protein-coupled receptor kinases (GRK's) (Inglese et al.,1993) which down-regulate the stimulated forms of receptors by phosphorylations (Dohlman et al., 1991; Stryer 1991; Palczewski & Benovic, 1991). In the case of rhodopsin kinase, its target is the light-stimulated form of rhodopsin (Bownds et al., 1972; Kühn et al., 1973; Frank & Buzney, 1975) (symbolized as Rho* and considered to be equivalent to metarhodopsin II) which is multiply phosphorylated at several Ser/Thr residues located at its C-terminal (Sale et al., 1978; Wilden & Kühn, 1982; Thompson & Findlay, 1984). The initial sites of phosphorylation in bovine rhodopsin are Ser-343 and Ser-338, as was deduced from several independent studies (Brown et al., 1992; Pullen et al., 1993; McDowell et al., 1993; Ohguro et al., 1993; Papac et al., 1993), and the sequence of subsequent phosphorylations from studies using synthetic peptides has been predicted (Pullen & Akhtar, 1994).

A pointer to the mechanism which enables the GRK's to discriminate against the resting states of their cognate receptors and act only on the stimulated forms was originally provided by experiments in which it was found that certain peptides corresponding to the C-terminal domain of rhodopsin were effectively phosphorylated by rhodopsin kinase only in the presence of Rho* (Fowles et al., 1988; Brown et al., 1992). These findings were confirmed in subsequent studies for the rhodopsin system (Palczewski et al., 1991) and also extended to β-adrenergic receptor kinase whose activity for the phosphorylation of peptides was greatly increased in the presence of the agonist-occupied form of β-adrenergic receptor (Kameyama et al., 1993; Kim et al., 1993).

The work on the Rho*-dependent peptide phosphorylation led us to postulate that rhodopsin kinase normally exists in an *in*active resting state and is only activated following interaction with Rho*. This proposed model provided an explanation for an intriguing phenomenon dubbed "high-gain phosphorylation" originally observed by Miller and Paulsen (1975), Miller et al. (1977), Sitaramayya and Liebman (1983), and Aton (1986) and extensively investigated by Bownd's group (Binder et al., 1990). It was found that, at exceptionally low bleaches, several hundred phosphoryl groups from ATP were incorporated into the receptor protein per photolyzed rhodopsin (Rho*). Since rhodopsin contains a maximum of only nine potential phosphorylation sites at its C-terminal (Wilden & Kühn, 1982; Aton et al., 1984), it appeared that under the conditions of low bleach not only Rho* but also dark-adapted rhodopsin is phosphorylated. Direct experimental proof for this was provided by our study (Dean & Akhtar, 1993) made possible by the development of a method which allowed the separation of phosphorylated rhodopsin from phosphorylated opsin (derived from phosphorylated Rho*).

Cumulatively, the above findings led to the proposal of Scheme 1 which envisages that rhodopsin kinase, initially present in an inactive state, is activated following interaction with Rho* (Fowles et al., 1988; Brown et al., 1992; Dean & Akhtar, 1993). Once activated, it catalyzes the phosphorylation of its preferred substrate Rho* but is also able to phosphorylate the dark-adapted receptor, i.e., rhodopsin, as well as synthetic peptides. Two broad mechanisms for the

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* To whom correspondence should be addressed: Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton, SO16 7PX, U.K. Telephone: (+44)(0)1703 594323. Fax: (+44)(0)1703 594459. E-mail: M.Akhtar@soton.ac.uk.

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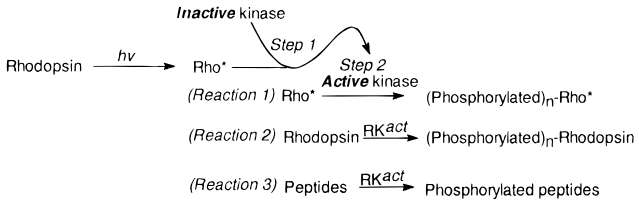
Scheme 1: Proposed Two-Step Model for Rhodopsin Kinase Activation and Catalysis[a]



[a] The two-step process for the rhodopsin kinase reaction involves the initial interaction of the inactive kinase with Rho* (step 1) to generate the active kinase (RK^{act}), and this species can then catalyze the phosphorylation of a number of substrates (step 2: reactions 1, 2 and 3).

activation of rhodopsin kinase were considered: one of these assumes (eq a)

$$\text{Rho}^* + \text{inactive kinase} \rightarrow \text{Rho}^*\cdot\text{inactive kinase} \rightarrow \text{Rho}^*\cdot\text{active kinase} \quad \text{(a)}$$

$$\text{Rho}^* + \text{inactive kinase} \rightarrow \text{Rho}^*\cdot\text{inactive kinase} \rightarrow \text{Rho}^*\cdot\text{active kinase} \rightarrow \text{Rho}^* + \text{active kinase} \quad \text{(b)}$$

that the activated form of the enzyme is present only within a Rho*−rhodopsin kinase complex, while the other (eq b) proposes that rhodopsin kinase may dissociate and independently exist as an activated entity.

At present, the latter scenario (eq b) is the only one amenable to experimental scrutiny, and in this paper we describe approaches toward such a goal, reporting on the isolation of a soluble form of rhodopsin kinase that catalyzes a Rho*−*in*dependent peptide phosphorylation and also the dark phosphorylation of rhodopsin.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP and RPN-6 Hyperfilm MP were purchased from Amersham, while plastic backed cellulose plates were from Eastmann-Kodak (Rochester, NY) and analytical grade AG1-X2 anion exchange material was supplied by Bio-Rad (Richmond, CA). 5′-Adenylimidodiphosphate tetralithium salt (AMP-PNP) was obtained from Calbiochem, while all other chemicals were purchased from either Sigma or Aldrich.

The following buffers were also routinely prepared and used: buffer A was 20 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane (BTP), 2 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT), pH 7.4, buffer B was 5 mM BTP, 2 mM $MgCl_2$, 1 mM DTT, and 0.04% Tween 80, pH 7.4, buffer C was as buffer A but contained 125 mM NaCl and 0.004% Tween 80, and buffer D was as buffer A but contained 280 mM NaCl and 0.004% Tween 80. All the buffers also contained 1 mM benzamidine and 0.1 mM phenylmethanesulfonylfluoride (PMSF).

Preparation of ROS and Rhodopsin Kinase. Bovine eyeballs were obtained from freshly slaughtered cattle from H. M. Bennet, Funtley (Hampshire, U.K.), and the retinas were dissected out under red light at 4 °C and used to prepare rod outer segments (ROS) as well as urea-washed ROS as described previously (Dean & Akhtar, 1993). The preparations were stored at −20 °C in buffer A.

The purification of rhodopsin kinase was performed by a modification of a published procedure (Pulvermüller et al., 1993) in which ROS (containing 40 mg of rhodopsin) were extracted in alternating high salt buffer (buffer A but also containing 500 mM NaCl and 0.04% Tween 80) and low salt buffer (buffer B). The combined extracts (30 mL) were diluted with water to give 125 mM NaCl and 0.04% Tween and loaded onto a heparin agarose column (i.d. 1.25 cm; bed volume 20 mL; flow rate 30 mL/h). After being washed with three bed volumes of buffer C, the kinase was eluted with buffer D, and the kinase activity was usually found in six fractions of 1 mL volume each. Standard kinase assays employing urea-washed ROS as the substrate were performed as described below, and fractions containing greater than 6 units of rhodopsin kinase activity/mL were deemed suitable for further use. (One unit of kinase activity is defined as the activity that catalyzes the incorporation of 1 nmol of ^{32}P/min into light-stimulated rhodopsin under the assay conditions described below.) In some cases fractions from the column were concentrated using Centricon-30 concentrators (Anachem) to give up to 11−12 units of kinase activity/mL. Rhodopsin kinase usually had an activity of 180−240 units/mg of protein and when stored in the elution buffer (buffer D) at 0 °C retained 90% of the activity for 5 days; after this period there was a rapid deterioration of activity. SDS−PAGE analysis of a typical preparation showed that about 40% of the protein was associated with an ill-resolved doublet at M_r ∼65 000 which cross-reacted with an antibody raised against a peptide corresponding to the C-terminal sequence of rhodopsin kinase. The enzyme was present predominantly in a nonphosphorylated form and incorporated 1.8 mol of ^{32}P/mol of rhodopsin kinase, calculated using a specific activity of 550 units/mg for the homogeneous enzyme (Palczewski et al., 1992).

Peptide and Phosphopeptide Synthesis. A peptide containing the sequence of the last 11 amino acids of the C-terminal of bovine rhodopsin, ^{338}SKTETSQVAPA^{348}, which has been shown to be an optimal substrate for the Rho*-dependent phosphorylation by rhodopsin kinase (Brown et al., 1992) was synthesized by the standard Merrifield solid-phase method (Barany & Merrifield, 1979). The peptide has been shown to be phosphorylated by rhodopsin kinase at Ser-343, and an authentic sample of phosphopeptide phosphorylated at this position was synthesized by the method previously described form this laboratory (Pullen et al., 1993). The phosphopeptide gave the expected M+1 ion at 1198 (theoretical, 1198).

Standard Assays of Rhodopsin Kinase

Light-Stimulated Phosphorylation of Rhodopsin. The assay mixture in a final volume of 50 μL was constituted from urea-washed ROS (50 μg; 5 μL of a 250 μM stock solution), 3 mM [γ-^{32}P]ATP (50 000−120 000 cpm/nmol of ^{32}P; 5 μL of 30 mM stock solution) and the rhodopsin kinase sample (40 μL). After sonication and vigorous mixing the samples were incubated at 32 °C in continuous light, and 10 μL samples were removed at 0 and 10 min and following trichloroacetic acid precipitation analyzed for radioactivity by scintillation counting (Dean & Akhtar 1993).

Rho-Dependent Peptide Phosphorylation.* The incubation mixtures in a final volume of 150 μL contained urea-washed ROS (150 μg), 3 mM [γ-^{32}P]ATP (120 000−200 000 cpm/nmol of ^{32}P), 3 mM peptide (10 μL of a 45 mM stock solution), and rhodopsin kinase (110 μL, over 0.7 units).

After incubation at 32 °C in continuous light for 1 h, the reaction was terminated by the addition of 7% acetic acid (300 μL), the mixture was centrifuged at 12000*g*, and the supernatant was loaded onto a 1 mL AG1-X2 column. The peptide was eluted in 3.5 mL of 7% acetic acid, when the bulk of the unreacted ATP was retained on the column. After freeze-drying, the sample was dissolved in 150 μL of 0.1% TFA, and a 40 μL aliquot was loaded onto a cellulose thin layer plate (TLE) and subjected to high-voltage electrophoresis using pyridine/acetic acid/water (1:10:89), pH 3.5, at 7 °C at 1000 V for 2 h (Brown et al., 1992). Peptide spots were detected by ninhydrin staining, and phosphopeptide was visualized by exposure to RPN-6 Hyperfilm for between 5 and 6 days. For quantitation of the phosphopeptide band, it was scraped and analyzed by scintillation counting.

Aliquots (50 μL) from the remaining peptide sample were analyzed in duplicate on a C-18 analytical reverse-phase column, using a 0−80% acetonitrile gradient (containing 0.1% TFA) run over 22 min. Nonradioactive synthetic phosphopeptide (100 μg) was included as an internal marker. The eluent was collected in 0.4 mL fractions and scintillation counted. When present, the radioactivity was found solely under the marker phosphopeptide peak. The two methods of analysis gave excellent quantitative agreement on the incorporation of ^{32}P into phosphopeptide.

The Extractability of Rhodopsin Kinase. The recovery of rhodopsin kinase from ROS membranes was investigated under various conditions as follows. A final volume of 100 μL contained rhodopsin kinase (80 μL, above 0.5 unit), urea-washed ROS (100 μg of rhodopsin), and various concentrations of ATP or AMP-PNP or albumin. In some incubations albumin and one of the two nucleotides were present together. Control samples were maintained in the dark while the others were bleached for 30 s under a 150 W light source, ensuring a greater than 90% bleaching of rhodopsin (Dean & Akhtar, 1993). The ROS membranes were then pelleted by centrifugation at 436000*g* for 5 min on a Beckmann TLX benchtop ultracentrifuge. The supernatants were removed, and 40 μL aliquots were immediately used in standard rhodopsin kinase assays to determine the degree of light-stimulated rhodopsin phosphorylation. A scaled up experiment to the one described above had an incubation volume of 300 μL and contained rhodopsin kinase (240 μL, above 1.5 units), urea-washed ROS (300 μg of rhodopsin), albumin (50 μg), and when present 3 mM ATP. Once centrifuged, the supernatant was used in standard rhodopsin kinase assays to determine the level of light-stimulated rhodopsin phosphorylation (40 μL of the recovered supernatant) and Rho*-dependent peptide phosphorylation (110 μL of the recovered supernatant). Mention should, however, be made of the fact that the supernatant removed from experiments incubated without [γ-^{32}P]ATP were supplemented with the latter to bring the final concentration to 3 mM in the final assay mixture (100 000−120 000 cpm/nmol ^{32}P), while appropriate amounts of ATP were added to ATP/kinase extracts to offset dilution effects and obtain a final concentration of 3 mM with the same specific activity as above. Control experiments were always performed in parallel in which rhodopsin kinase was mixed with ATP, AMP-PNP, and or albumin but without ROS, and aliquots of these supernatants were used to assay for the light-stimulated phosphorylation of rhodopsin or Rho*-dependent peptide phosphorylation. The activities so obtained were taken as 100% and used to quantify the recoveries of rhodopsin kinase from the aforementioned experiments containing ROS membranes.

The Generation of Activated-Rhodopsin Kinase and the Measurement of Its Activity

Typically in a final volume of 300 μL, incubation mixtures contained albumin (50 μg), urea-washed ROS (300 μg of rhodopsin), rhodopsin kinase (240 μL, 1.5−2.2 units of activity), and 3 mM [γ-^{32}P]ATP. Samples were either bleached to the extent of greater than 90% (see above) or left in the dark. These incubations will be referred to as "activation step" incubations and were subjected to centrifugation at 436000*g* (as above), and the supernatants were recovered and immediately used in several separate experiments. A 25 μL aliquot of this activation step supernatant was also incubated in the light and then analyzed on SDS−PAGE to determine the level of rhodopsin contamination (see below).

Dark Phosphorylation of Rhodopsin. In a final volume of 100 μL, the assay mixture contained urea-washed ROS (100 μg of rhodopsin), the activation step supernatant (80 μL), and unlabeled ATP to give a final concentration of 3 mM [γ-^{32}P]ATP (120 000−200 000 cpm/nmol). Samples were incubated in the dark for 30 min, and then 25 μL aliquots of the incubation mixture were mixed with 10 μL of loading buffer (30 mM sodium carbonate, 1% SDS, and 0.3% bromophenol blue). Thirty microliter samples of the latter mixture were subjected to 12% SDS−PAGE, and the gels, following drying, were visualized for radioactivity by exposure to RPN-6 Hyperfilm for 2−3 days. The radioactive bands corresponding to the position of phosphorylated opsin were excised and the levels of incorporation of ^{32}P determined by scintillation counting. An identical aliquot of the activation step supernatant was also used in the standard light-stimulated rhodopsin phosphorylation assay (see above) to determine the optimal activity of the kinase which was taken as 100%.

Rho-Independent Peptide Phosphorylation.* In a total volume of 175 μL, the activation step supernatant (140 μL) was immediately mixed with the peptide and unlabeled ATP to give 3 mM peptide and 3 mM [γ-^{32}P]ATP (120 000−200 000 cpm/nmol of ^{32}P). Incubations were left in the light for 1 h at 32 °C before removing a 25 μL aliquot for determining the levels of rhodopsin contamination in the activation step supernatant (see below), and the remaining incubation (150 μL) was terminated by the addition of 300 μL of 7% acetic acid. This sample was then analyzed by TLE and HPLC to ascertain the levels of peptide phosphorylation (see above). Another aliquot of the activation step supernatant was used to determine the optimal activity of the kinase in the standard Rho*-dependent peptide phosphorylation assay.

The Examination of the Activation Step Supernatant for Contamination with Rhodopsin. The 25 μL aliquot from either the activation step supernatant in the case of dark phosphorylation of rhodopsin studies or the assay step incubation in the case of the Rho*-independent peptide phosphorylation studies was subjected to 12% SDS−PAGE analysis, and, following drying, the gel was viewed by autoradiography. In order to detect low levels of radioactivity which will be associated with the contaminant phospho-

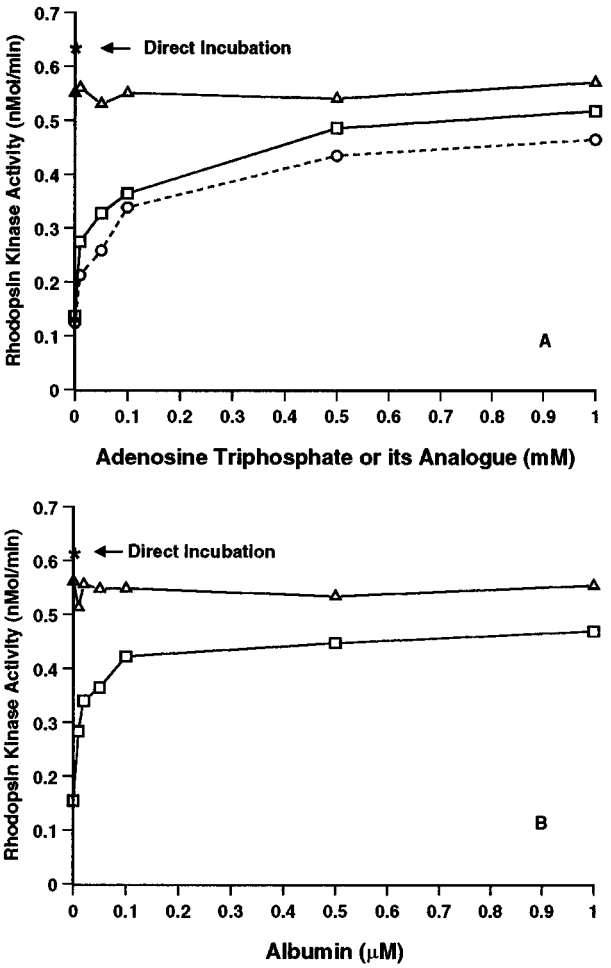


FIGURE 1: Effect of ATP, its nonhydrolyzable analogue (AMP-PNP), and albumin on the recovery of rhodopsin kinase from ROS. In a final volume of 100 μL, samples contained rhodopsin kinase (0.5−0.7 units), urea-washed ROS (100 μg of rhodopsin), and various concentrations of ATP (□, panel A), AMP-PNP (○, panel A) or albumin (□, panel B). The samples were either bleached or left in the dark and then were centrifuged at 436000*g* for 5 min. The activity of rhodopsin kinase in the resulting supernatant was determined using the standard assay involving the light-stimulated phosphorylation of rhodopsin. Panels A and B represent a typical data set with the traces (△) representing the recovery of the kinase from unbleached controls while the activity of the kinase that had *not* been preincubated is shown by (*).

rylated opsin, RPN-6 Hyperfilm was exposed for up to 14 days. The position of rhodopsin kinase was revealed by the ^{32}P incorporated into the enzyme during incubation with[γ-^{32}P]ATP. The position of phosphorylated opsin was marked by the inclusion of at least one sample from an incubation mixture which was designed to produce ^{32}P-phosphorylated opsin.

RESULTS

Recovery of Rhodopsin Kinase from ROS Membranes Subjected to Various Treatments. A prerequisite for the elucidation of the mechanism implied in eq b is the availability of a reliable protocol for the recovery of rhodopsin kinase from ROS membranes subjected to various treatments. Figure 1A,B shows that when a sample of rhodopsin kinase was mixed in the dark with ROS membranes and the supernatant recovered by rapid centrifugation at 436000*g*, it contained up to 85% of the original kinase activity. In the experiments described in this paper no attempt was made to obtain the remaining enzyme adhering to ROS membranes by further extractions, since such an operation was incompatible with the projected use of the recovered enzyme. In contrast to the good recovery of the enzyme in the dark, when a similar mixture of rhodopsin kinase and ROS membranes was first bleached and then centrifuged, less than 40% of the enzyme was present in the supernatant, thus confirming earlier findings that rhodopsin kinase binds to Rho* (Kühn, 1978; Buczylko et al., 1991; Pulvermüller et al., 1993). Figure 1A shows that when a mixture containing rhodopsin kinase and ROS membranes was bleached in the presence of ATP, the kinase was recovered in good yields at ATP concentrations above 0.5 mM. Similar observations have been made previously and the facile recovery of the kinase from bleached membranes in the presence of ATP was attributed to the fact that under the aforementioned conditions the two proteins become phosphorylated, which may decrease their mutual affinity due to electrostatic repulsion caused by the phosphate groups (Buczylko et al., 1991). The next series of experiments, however, cast doubt on such an explanation and show that bleaching a mixture of ROS membranes and rhodopsin kinase in the presence of a nonhydrolyzable analogue of ATP, AMP-PNP, also resulted in a good recovery of the kinase (Figure 1A). In these experiments the enzyme was assayed in a mixture containing 0.4 mM final concentration of the analogue and 3 mM ATP. At this concentration of the analogue, the phosphorylation reaction was inhibited by less than 10%; the level of inhibition reached 35% when the analogue concentration was comparable to that of ATP. The simplest explanation of these results is that the binary complex composed of rhodopsin kinase·ATP, or rhodopsin kinase·AMP-PNP, has lower affinity for Rho* than has the uncomplexed enzyme. The subtle and weak nature of the interaction suggested by the AMP-PNP experiment encouraged us to search for other conditions which may cause the dissociation of rhodopsin kinase from the rhodopsin kinase·Rho* complex. Figure 1B shows that such a purpose is easily achieved by the inclusion of relatively low concentrations of albumin in the incubation mixtures. Thus 75−85% of rhodopsin kinase was routinely recovered in supernatants from mixtures of rhodopsin kinase and Rho* when the molar ratio of rhodopsin/albumin was 10:1. The data indicate that rhodopsin kinase binds to Rho* through weak protein−protein interactions which are readily disrupted by albumin.

In the next series of experiments albumin was included in all incubations, and the activity of the recovered rhodopsin kinase was assayed using the light-stimulated phosphorylation of rhodopsin and also for the Rho*-dependent peptide phosphorylation (Fowles et al., 1988; Brown et al., 1992). Under the conditions used in the present work, the phosphorylation of the peptide was about 11% that of rhodopsin. High recoveries of both these activities were obtained when rhodopsin kinase was incubated with ROS membranes under a wide range of conditions, and, in all the incubations of Figure 2, the recovered kinase displayed similar activity ratios for the two substrates: thus, in the presence of AMP-PNP or albumin the behavior of the rhodopsin kinase was not adversely affected. No losses of activities of the recovered kinase were observed when these supernatants were used in the two assay systems after leaving at room temperature for 10 min (data not shown). The significance of this observation will become clear later.

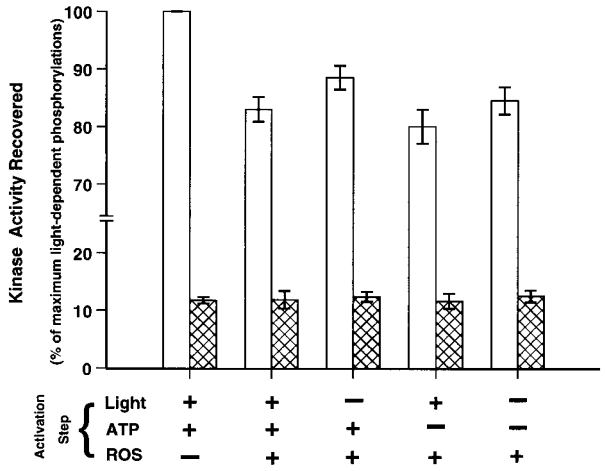


FIGURE 2: Recovery of rhodopsin kinase from bleached and dark-adapted ROS in the presence of albumin with or without ATP. The incubation in a final volume of 300 μL contained rhodopsin kinase (1.3−2.2 units), urea-washed ROS (300 μg of rhodopsin), albumin (50 μg, 2.7 μM), and when present 3 mM [γ-^{32}P]ATP. The light-exposed samples as well as the dark controls were centrifuged at 436000*g*, and the recovered supernatants (40 μL aliquot) were used for light-stimulated rhodopsin phosphorylations (open bars) or (110 μL aliquot) for Rho*-dependent peptide phosphorylations (hatched bars) as described in the Experimental Procedures. Identical profiles were obtained when the supernatants were assayed after being stored for 10 min at room temperature. When normalized to the same amount of rhodopsin kinase used in the standard assays, the peptide phosphorylation during a 60 min incubation period is ≈11% that of rhodopsin phosphorylation over 10 min. The conditions to which the kinase was exposed, prior to assay, are indicated in the chart below the figures.

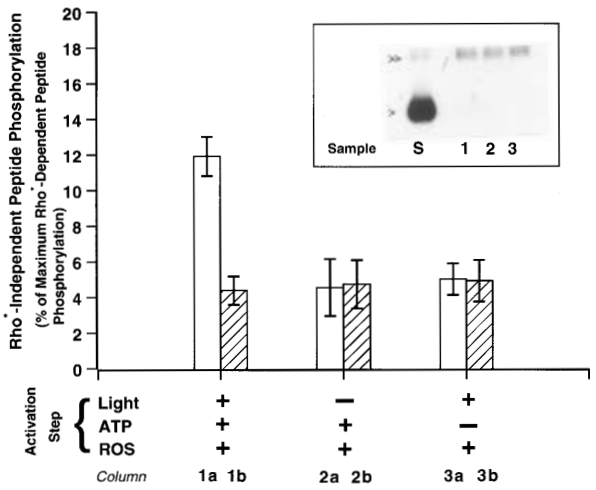


FIGURE 3: Generation of activated rhodopsin kinase that catalyzes a Rho*-independent peptide phosphorylation. The activation step incubations were performed as described in the legend to Figure 2 in the presence of albumin (50 μg, 2.7 μM) and 3 mM [γ-^{32}P]-ATP, in which mixtures were either bleached (0.5min) or kept in the dark and then centrifuged at 436000*g*. An aliquot (110 μL) of each supernatant was used in the Rho*-dependent peptide phosphorylation assay to quantify the recovery of the kinase, and a 140 μL aliquot was used in a final volume of 175 μL for Rho*-independent peptide phosphorylation. The latter incubation was performed in light for 1 h. Thereafter, a 25 μL aliquot was removed for PAGE analysis (see inset), and the remaining was used to quantify the level of peptide phosphorylation by two methods, TLE and HPLC (see Experimental section). The incorporations are normalized using the Rho*-dependent peptide phosphorylation as 100%, and the results from five independent experiments are presented with standard deviations. Open bars show the incorporations when the supernatants were used immediately after the recovery and the hatched bars after the supernatants were stored for 10 min. The variable features of the activation step incubations are indicated below the columns. (Inset) The 25 μL aliquots of the assay step supernatants were subjected to 12% SDS−PAGE analysis, and the gel was viewed by autoradiography. The position of the phosphorylated rhodopsin kinase (≫) and phosphorylated opsin (>) are marked. Lanes 1, 2, and 3 are supernatants from the incubations of columns 1, 2, and 3, respectively, while lane S is the standard sample of ^{32}P-phosphorylated opsin.

An Activated-Rhodopsin Kinase Species Catalyzing a Rho*-Independent Peptide Phosphorylation. The assurance provided by the proceeding experiments that similar levels of rhodopsin kinase are recovered from albumin-containing incubations subjected to different treatments led to the design of experiments for the study of the formation of an activated form of rhodopsin kinase. The mechanism of Scheme 1 predicts that, once activated by interaction with Rho*, the activated form of rhodopsin kinase should promote a Rho*-independent peptide phosphorylation (reaction 3, Scheme 1) and also the phosphorylation of rhodopsin in a dark reaction (reaction 2, Scheme 1). The methodology used in the following experiments consisted of a two-step protocol: the generation of the activated species and the measurement of its activity. Rhodopsin kinase was incubated with ROS membranes in the presence or absence of ATP and the mixtures either exposed to light for 30 s or maintained in the dark and then spun at 436000*g* for 5 min. Aliquots of the supernatants were then used to investigate the Rho*-independent peptide phosphorylation and the levels of the phosphorylation determined by two analytical procedures, involving cellulose thin layer electrophoresis (TLE) and HPLC. Data from five such experiments are combined in Figure 3 and show that the kinase recovered from ROS membranes exposed to light in the presence of ATP promoted significant levels of Rho*-independent peptide phosphorylation (column 1a) compared to its dark counterpart (column 2a) or the supernatant obtained from the bleached mixture without ATP (column 3a). The three supernatants were also used to study the Rho*-dependent peptide phosphorylation, and it was found that identical amounts of rhodopsin kinase had been recovered in all the three samples. Quantitatively, the data in Figure 3 show that the Rho*-independent phosphorylation by the supernatant from bleached ROS membranes in the presence of ATP was about 12% of its maximum activity found in the standard Rho*-dependent peptide phosphorylation assay (column 1a, Figure 3), while the supernatant from the dark control or the sample without ATP gave 4−5% of the activity found in the Rho*-dependent peptide phosphorylation assay (column 3a, Figure 3). Subtracting the dark control sample from Rho*-independent peptide phosphorylation thus generated a value of 7−8%.

In another set of experiments (Figure 3, columns 1b, 2b, and 3b, hatched bars) supernatants were used for Rho*-independent peptide phosphorylation after being stored at room temperature for 10 min when the level of peptide phosphorylation catalyzed by the supernatant recovered from the mixture of rhodopsin kinase and ROS membranes exposed to light in the presence of ATP was reduced to dark control levels (compare columns 1a with 1b). That the loss of Rho*-independent peptide phosphorylation was not due to the denaturation of rhodopsin kinase was shown by measuring its intrinsic activity for Rho*-dependent peptide phosphorylation at both time 0 and 10 min, which was found to be unaltered, as could be predicted from the systematic study performed in connection with Figure 2.

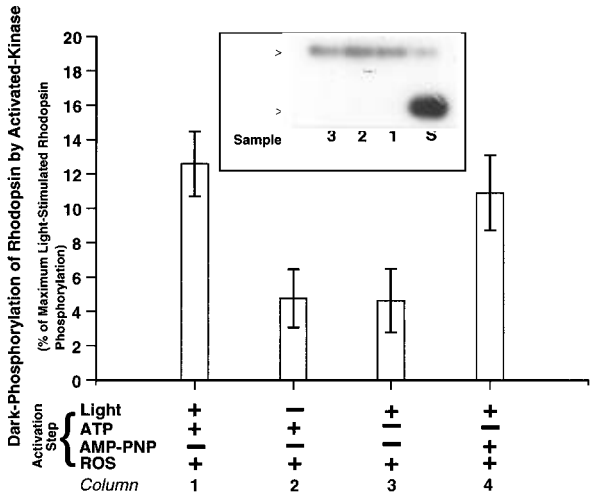


FIGURE 4: Dark phosphorylation of rhodopsin by the activated rhodopsin kinase: requirements for ATP or its nonhydrolyzable analogue. In a final volume of 300 μL, samples contained rhodopsin kinase (1.3−2.2 units of activity), urea-washed ROS (300 μg of rhodopsin), albumin (50 μg), and when present either 3 mM [γ-^{32}P]-ATP or 0. 5 mM AMP-PNP or no nucleotide. Samples were bleached (0.5 min) or kept dark adapted. An aliquot (80 μL) of the recovered supernatant from each incubation was immediately mixed with ROS (100 μg of protein) supplemented as appropriate to give 3 mM final concentration of [γ-^{32}P]ATP and assayed for dark phosphorylation. Another 40 μL of the supernatant was used for the standard light-stimulated rhodopsin phosphorylation. Six independent experiments are normalized using light-stimulated rhodopsin phosphorylation as 100%, and the variable features of the activation step incubations are indicated below the bar chart. (Inset) Aliquots of the supernatant from the activation step were incubated in the light for 30 min to enable the detection of rhodopsin contamination, if present, through phosphorylation (lane 2 was supplemented with 3 mM [γ-^{32}P]ATP). The samples were analyzed as in Figure 3 (inset); rhodopsin kinase (upper band) and phosphorylated opsin (lower band). Lanes 1, 2, and 3 correspond to the experiments of columns 1, 2, and 3, respectively. Lane S is a standard sample of ^{32}P-phosphorylated opsin. The supernatant from the experiment of column 4 was analyzed to show the absence of rhodopsin contamination, but the pictorial evidence is not included since it was part of a different gel.

The Dark Phosphorylation of Rhodopsin by the Activated Rhodopsin Kinase. The other behavior of the activated rhodopsin kinase predicted by the mechanism of Scheme 1 is the phosphorylation of rhodopsin in a dark reaction (reaction 2, Scheme 1), hereafter referred to as the dark phosphorylation of rhodopsin. This was studied using the experimental regime of Figure 3, in which mixtures of rhodopsin kinase and ROS membranes were bleached in the absence or presence of [γ-^{32}P]ATP, with the inclusion of relevant dark controls. In consonance with the results obtained using the Rho*-independent peptide phosphorylation assay, the dark phosphorylation of rhodopsin was catalyzed only by the supernatant obtained from a mixture in which rhodopsin kinase and ROS membranes were exposed to light in the presence of ATP. Interestingly, AMP-PNP, a nonhydrolyzable analogue of ATP, was also effective giving 85% of the activity found with ATP (Figure 4, column 4). In the absence of ATP or the analogue and without light, the activities of the supernatants for the dark phosphorylation of rhodopsin were found to be similar and comparable to urea-washed ROS containing rhodopsin kinase incubated in the dark. Under the conditions used in these experiments, the dark phosphorylation of rhodopsin by the activated rhodopsin kinase was about 8% of the intrinsic activity displayed by the same sample for the light-stimulated phosphorylation of rhodopsin in the standard assay when the dark control value was subtracted (column 1 − column 2, Figure 4), within experimental error the same percentage of the intrinsic activity which is shown by similar samples in the peptide phosphorylation assays (Figure 3).

Assessment of Rhodopsin Contamination in Supernatants Containing the Activated Rhodopsin Kinase. The main concern in this study was whether the Rho*-independent peptide phosphorylation and the dark phosphorylation of rhodopsin was due to the contamination of the supernatant with a rhodopsin-like species which could maintain the rhodopsin kinase in an activated state during incubations. In order to critically scrutinize such a scenario, the following measures were introduced. (1) In all incubations containing ATP, radioactivity was added at the outset so that the phosphorylation of rhodopsin kinase and Rho* leads to the incorporation of ^{32}P into these proteins. Aliquots of the supernatants were then incubated in the light to ensure that rhodopsin, if present, is maximally labeled as a result of light-stimulated phosphorylation. SDS−PAGE analysis (Figure 3, inset, and Figure 4, inset) of such samples followed by autoradiography revealed the presence of a band corresponding to the position of phosphorylated rhodopsin kinase while none was seen in the position of phosphorylated opsin, as marked by electrophoresing an authentic sample of ^{32}P-labeled phosphorylated opsin in a parallel lane (Figure 3, inset, column S). The data from only those experiments in which no rhodopsin was detected by the aforementioned method are included in the results of Figures 3 and 4. For quantitation these bands were excised and subjected to scintillation counting. In a typical analysis 2300 dpm were found in the kinase band (representing the incorporation of 1.8 mol of ^{32}P/mol of rhodopsin kinase) and 20−30 dpm in the position of the opsin: similar levels of ^{32}P incorporation were found in the two regions from the supernatants containing the activated rhodopsin kinase and its dark counterpart. This radiochemical approach independently confirmed that the yield of rhodopsin kinase was identical whether the enzyme was recovered from dark or light incubations containing ROS and ATP. Furthermore, similar low amounts of radioactivity were present in the phosphorylated opsin regions in the two types of supernatants, indicating it to be due to background noise rather than being associated with a Rho*-like species. (2) What is the likely consequence if the low radioactivity in the opsin region of the gel above represents a genuine contamination with Rho*? It was found that under the conditions used in the experiment described above the conventional light-stimulated phosphorylation of rhodopsin resulted in the incorporations of 5.0 mol of ^{32}P/mol of rhodopsin in 10 min. Using this value, the radioactivity found in the opsin region of the gel above represents the presence of a maximum of 0.09 pmol of Rho* in the 80 μL aliquot of supernatant, which when mixed with 2.5 nmol of rhodopsin in the assay step incubation (see Experimental Procedures) gives an equivalent of ∼0.004% Rho* in the assay mixture. The dark phosphorylation of rhodopsin was studied in the presence of various amounts of freshly bleached rhodopsin, and the data allowed the conclusion to be drawn that, to simulate the ^{32}P incorporation found in column 1 (Figure 4), the level of contamination would need to be more than 100-fold greater than estimated by us. (3) Finally, if the Rho*-independent peptide phos-

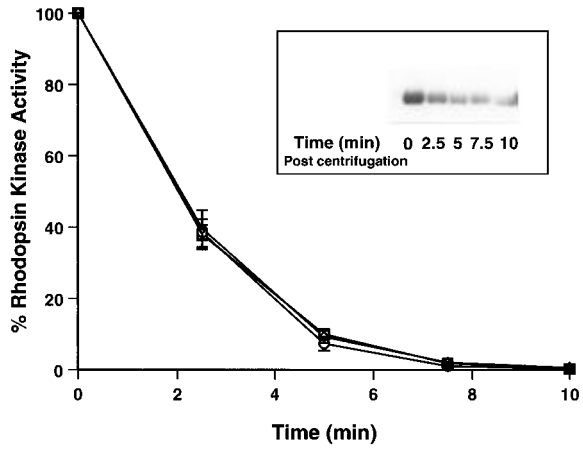


FIGURE 5: Time course of the decay of the activated rhodopsin kinase activity for the dark phosphorylation of rhodopsin. In a final volume of 300 μL, samples contained rhodopsin kinase (1.3−2.2 units of activity), albumin (50 μg), urea-washed ROS (300 μg of rhodopsin), and one of the following: 3 mM [γ-^{32}P]ATP (expt 1), 500 μM AMP-PNP (expt 2), or 3 mM [γ-^{32}P]ATP + 200 nM okadaic acid (expt 3). The supernatants were recovered after bleaching or dark adaptation (see Experimental Procedures). At various times postcentrifugation (0, 2.5, 5, 7.5, and 10 min) 40 μL aliquots were removed, mixed with urea-washed ROS, and incubated for 30 min to measure the dark-phosphorylation of rhodopsin. Data from three independent experiments are plotted with ± standard deviation (expt 1, □; expt 2, ○; expt 2, ◇). Each time point has been corrected by reference to controls in which the activation step incubations were kept in the dark and the recovered supernatants assayed for the dark phosphorylation of rhodopsin. The data are normalized by taking the dark phosphorylation of rhodopsin catalyzed by the supernatant at 0 time as 100%. (Inset) Autoradiogram of ^{32}P-phosphorylated opsin produced in experiment 1 using supernatants removed at times 0, 2.5, 5, 7.5, and 10 min postcentrifugation.

phorylation found in lane 1 (Figure 3) was due to the activating effect of contaminant rhodopsin-like species, then all the samples would have produced similar levels of peptide phosphorylation since these incubations were conducted in the light. This is seen not to be the case.

The Decay Half-Life of the Activated-Rhodopsin Kinase. The transient nature of the activated rhodopsin kinase signaled by the experiments performed on the Rho*-independent peptide phosphorylation (compare columns 1a and 1b, Figure 3) was investigated in further detail using the more convenient assay involving the dark phosphorylation of rhodopsin. Activated kinase was generated as in previous experiments from a mixture of rhodopsin kinase and ROS membranes and bleaching in the presence of ATP, and samples of this recovered kinase, at various times postcentrifugation (0, 2.5, 5, 7.5, and 10 min), were used to study the dark phosphorylation of rhodopsin. The level of phosphorylation was quantified following SDS−PAGE analysis and the autoradiograph of one such gel (Figure 5, inset) shows the expected level of ^{32}P incorporation into the phosphorylated opsin band when the recovered kinase was used immediately after centrifugation (i.e., the 0 min time sample). However, there was a gradual decline in the level of radioactivity with increasing time between the removal of the supernatant and its use in the assays; the sample at 10 min postcentrifugation produced only background levels of phosphorylation. The data from several independent experiments of the type in Figure 5 (inset) were combined and plotted in Figure 5, generating a $\tau_{1/2}$ of about 2 min for the decay of the activity of the activated rhodopsin kinase. The same decay $\tau_{1/2}$ for the conversion of the activated rhodopsin kinase into inactive resting state was obtained when the preceding experimental regime was performed but with okadaic acid being present in the activation step as well as at the assay stage. Okadaic acid is an inhibitor of protein phosphatase, notably protein phosphatase 2A which is involved in the dephosphorylation of phosphorylated rhodopsin/opsin (Palczewski et al., 1989; Fowles et al., 1989; King et al., 1994) as well as phosphorylated rhodopsin kinase (Buczylko et al., 1991). The activated rhodopsin kinase was also generated using bleached ROS membranes and AMP-PNP, and the assay of the recovered supernatant for the dark-phosphorylation of rhodopsin at various times postcentrifugation gave the same profile as obtained using the activated-kinase generated in the presence of ATP, showing almost identical $\tau_{1/2}$ of ≃2 min. Once again it must be emphasized that the supernatants used in these experiments were free from contamination with rhodopsin-like species as established by SDS−PAGE analysis and possessed identical intrinsic activities when assayed for light-stimulated phosphorylation of rhodopsin.

DISCUSSION

The main thrust of the present work was to study the light and rhodopsin dependent formation of an activated rhodopsin kinase as shown in Scheme 1. The activated kinase can exist either in association with Rho* (eq a) or as a free entity (eq b), and in this work these alternatives were examined. For this purpose a method by which rhodopsin kinase could be recovered in reproducibly high yields from ROS membranes subjected to various treatments was developed.

We have now shown that the interaction of rhodopsin kinase·ATP or rhodopsin kinase·AMP-PNP complex with Rho* leads to the formation of an activated enzyme, which, in accordance with the prediction of the model of Scheme 1, catalyzes a Rho*-independent peptide phosphorylation (reaction 3, Scheme 1) and also the dark phosphorylation of rhodopsin (reaction 2, Scheme 1). At room temperature the activated enzyme has a decay half-life of ∼2 min. Under the conditions used in the present study, about 10 min is required for the separation of rhodopsin kinase from ROS membranes and the subsequent use of the recovered supernatants in various assays. We estimate that for the first 5 min of this procedure the interaction between Rho* and the kinase are maintained, and therefore during this period rhodopsin kinase is continually being activated. The decay of the activated kinase must then begin, but its extent at 0 °C has not been quantified. The Rho*-independent peptide phosphorylation, by the activated kinase so recovered, was found to be ≃7−8% of its maximal activity as determined by the Rho*-dependent phosphorylation assay (Figure 3). It is gratifying to note that the activated kinase also gave a similar activity ≃8% for the corresponding dark phosphorylation of rhodopsin compared to its intrinsic activity as measured by the conventional assay involving the light-stimulated rhodopsin phosphorylation. Both these quoted values have had their equivalent dark control samples subtracted (e.g., column 1 − column 2, Figure 3).

The levels of phosphorylation under the aforementioned conditions were 3 times those in control experiments with various omissions, and their extent could be conveniently quantified. No attempt was therefore made to reduce the

time taken for the recovery of the kinase from the activation mixtures. The paramount consideration in the present study was the complete sedimentation of ROS membranes and ensuring that the supernatant was removed carefully without contamination from rhodopsin; therefore, the speed of the operation was regarded not to be important. Three lines of experimental evidence have been cited in Results to indicate that the dark phosphorylation of rhodopsin and the Rho*-independent phosphorylation described in the paper are not due to the involvement of Rho* contamination in the supernatant containing the activated kinase. However, in view of the intrinsic importance of the observation, the question will need to be scrutinized using alternative approaches.

The molecular basis of the activation process was investigated, with the main focus being on the phosphorylation of rhodopsin kinase since such a modification underpins the regulation of the activities of many proteins involved in signalling. It was found that the phosphoryl groups introduced into rhodopsin kinase in the autophosphorylation reaction were conserved during the catalytic turnover of the enzyme. Similarly, studies with the ^{32}P-labeled kinase recovered from the activation mixture showed that the time-dependent decay of activity was not accompanied by any loss of ^{32}P (data not shown). The findings, when taken in conjunction with the fact that the requirement of ATP for the optimal activation of rhodopsin kinase could be partially fulfilled by its nonhydrolyzable analogue 5′-adenylimidodiphosphate, indicate that the phosphorylation of rhodopsin kinase by an external donor and the subsequent dephosphorylation of the resulting species are not involved in the interconversion of the activated rhodopsin kinase and its inactive resting form.

The simplest interpretation of our results is that the interaction of rhodopsin kinase·ATP binary complex with Rho* leads to the formation of an activated enzyme that exists in a free-form and is recoverable as a soluble entity as required by eq b. Such a possibility was originally foreshadowed by the experiment in which it was found that at low bleaches the phosphorylation of dark-adapted rhodopsin by the activated kinase, generated *in situ*, could be demonstrated in detergent solutions at high dilutions (Dean & Akhtar, 1993). This finding was suggested to be most readily explicable by invoking the participation of a free form of the activated kinase rather than Rho*·kinase complex as implied by eq a.

The results described in this paper when taken in conjunction with earlier studies provide a straightforward explanation for the phenomenon known as "high-gain" phosphorylation (Binder et al., 1990) which involves the incorporation of several hundred phosphate groups into the receptor protein per Rho* at low bleaches. According to the present study, this is the consequence of the formation of a readily diffusible form of the activated kinase that promotes the phosphorylation of its preferred substrate, Rho*, but is also able to phosphorylate the dark-adapted rhodopsin, albeit poorly. A numerical model has been developed which rationalizes the observation that the phosphorylation of dark-adapted rhodopsin is most readily noticeable at low bleaches because of the overwhelming preponderance of rhodopsin over Rho* under those conditions (Dean & Akhtar, 1993).

The nature of the molecular events which participate in the activation $\rightleftarrows$ deactivation of rhodopsin kinase are not revealed by this work, though certain obvious options have been eliminated and the following new theoretical possibilities highlighted. First, the inactive and active states of the kinase may arise by an intramolecular covalent rearrangement, in which a preexisting group, for example a phosphoryl moiety, migrates from one position in the protein to another. This possibility involving a phosphoryl moiety, however, is rendered less likely by the recent work of Palczewski et al. (1995), in which it was found that mutants of rhodopsin kinase which could not be phosphorylated were still functionally active. The second possibility entails an association−dissociation process involving another species present either in urea-washed ROS membranes or in the incubation mixture. The modulation of the activities of certain G protein-coupled receptor kinases, notably β-adrenergic receptor kinase 1 and 2 (GRK2 and GRK3), by $\beta\gamma$ subunits of a G protein (Pitcher et al., 1992; Kameyama et al., 1993; Inglese et al., 1993), and also by lipids (DebBurman et al., 1995) have been observed in previous studies. The final scenario involves a transient protein reorganization of the kinase·ATP complex following interaction with Rho* which gives rise to an active conformation of the enzyme that then reverts to the inactive resting state in a time-dependent manner. To various extents, all these options are novel, but the last mechanism, if found to operate in the case of rhodopsin kinase, as far as we are aware, will be unprecedented.

To conclude, the understanding of the mechanism through which GRK's are activated by interaction with the activated form of their receptors has been brought one step further by this study on rhodopsin kinase described in this paper and challenging new problems have been posed.

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