Interaction between Photoactivated Rhodopsin and Its Kinase: Stability and Kinetics of Complex Formation†

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ABSTRACT: Rhodopsin phosphorylation is a key event in the deactivation of this G-protein-coupled receptor. Rhodopsin kinase mediates the reaction and requires specific cytoplasmic loop domains on rhodopsin, distinct from the sites of phosphorylation, for binding and activation. In this study, we characterize the formation of a stable complex between photolyzed rhodopsin in native washed disk membranes and its kinase. Centrifugation of the membranes after illumination and subsequent polyacrylamide gel electrophoresis demonstrates light-dependent binding of rhodopsin kinase to the membranes. A real-time monitor for the transition of the solubilized kinase into the bound state is provided by flash-induced light-scattering binding signals. The complex has the following characteristics: (i) the on-rate of the reaction rises in linear proportion to the concentrations of both the kinase and photoactivated rhodopsin; kinetic analysis yields a bimolecular rate constant of $k_{on} = 0.5–1\ \mu M^{-1}\ s^{-1}$. (ii) The dissociation constant of the complex is $0.3 < K_D < 0.5\ \mu M$ in the absence of ATP, but with ATP, it decreases by at least a factor of 10; however, phosphorylation of rhodopsin or (auto)phosphorylation of rhodopsin kinase leads to destabilization of the complex. (iii) In contrast to the binding of arrestin and transducin, the binding of rhodopsin kinase to photoactivated rhodopsin does not stabilize the metarhodopsin II photoproduct; however, rhodopsin kinase competes with the G-protein transducin for binding to photoactivated rhodopsin. Extrapolation of the kinetic parameters to cellular concentrations at room temperature suggests that free competitive binding of the kinase would strongly inhibit the G-protein activation process after a few hundred catalytic cycles.

In retinal rod disk membranes, photoactivated rhodopsin (R*)[1] interacts with the G-protein, transducin (G_t), and catalyzes nucleotide exchange in the G-protein. Active G_t stimulates the effector enzyme, a cyclic GMP-specific phosphodiesterase (PDE), resulting in lowering of the cyclic GMP concentration and transmission of a transduction signal. Besides G_t, a specific protein kinase and a regulatory protein (RK and arrestin, respectively) bind to R*. All three coupling processes are probably functional archetypes for the related G-protein systems. G_t activation is now understood as a multistep process (Bornancin et al., 1989; Bourne et al., 1988) that involves different types of interactions between R* and the G-protein and multiple cytoplasmic loop sites of R* (König et al., 1989, Kahlert et al., 1990; Franke et al., 1990; Hofmann & Kahlert, 1992). The initial collisional coupling between R* and G_t arises from a membrane-bound, dark binding state of the G-protein (Schleicher & Hofmann, 1987). By contrast, soluble arrestin couples to R* directly from solution. Both arrestin and G_t require an unusual conformation of R* (meta II), in which the Schiff base bond of the photoisomerized chromophore, *all-trans*-retinal, is deprotonated (Schleicher et al., 1989). Both arrestin and G_t undergo structural rearrangement during the binding process (Schleicher et al., 1989; Palczewski et al., 1991a).

A goal of the present study is to explore the mechanism of RK binding to R*. Previous work has suggested that the kinase is enzymatically activated by interaction with specific sites at the activated receptor (including a stretch at the third cytoplasmic loop) and that this step is different from the actual phosphorylation reaction at sites on the C-terminal extension of R* (Palczewski et al., 1991b). Kinetic parameters of the R*–RK complex are important with respect to the timing of the visual cascade. To limit the activation of the G_t pool on a disk membrane and to achieve a graded response, R* must be shut off in time. Receptor deactivation *in situ* takes 2 s at room temperature, as determined from light-scattering two-flash experiments and from the onset of the falling phase of the electrical response (Pepperberg et al., 1992). It is now recognized that the shut-off reaction involves the sequential interaction of R* with RK and arrestin (Wilden et al., 1986; Bennett & Sitaramayya, 1988; Palczewski et al., 1992a). RK-catalyzed phosphorylation of R* is required to trigger tight binding of arrestin (Schleicher et al., 1989; this study), which deactivates R* by blocking its interaction with the G-protein (Wilden et al., 1986). Arrestin binds to R* when only one or two of the multiple relevant sites have been phosphorylated (K. Palczewski, unpublished results), at a stage in which the direct effect of phosphorylation on the interaction with G_t is small (50% reduction of PDE activation at most; Miller et al., 1986). Thus, binding of arrestin is the actual quench step for R* in this deactivation mechanism, and the only role of the kinase is to operate the phosphorylation switch.

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[1] Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; G_t, G-protein of the rod cell transducin; LS, near-infrared light scattering; meta I, metarhodopsin I; meta II, metarhodopsin II; PDE, cyclic GMP phosphodiesterase; R*, photoactivated rhodopsin; RK, rhodopsin kinase; ROS, rod outer segment(s).

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It is known that the binding of arrestin to prephosphorylated R* is well in the subsecond range for extrapolated cellular conditions (Schleicher et al., 1989). This excludes the actual arrestin binding step from being rate-limiting for the 2-s reaction time of the overall shut-off process and draws attention to the reactions involved in R* phosphorylation. In this study, we document fast and stable binding of RK to R*. The measured reaction speed is high enough to exclude R*–RK formation from being rate-limiting for the overall shut-off reaction. The most interesting new information from this investigation is that G_t cannot bind when the kinase occupies R*. Together with the speed of binding, this opens the possibility that RK acts as a "pre-arrestin", which partially inhibits G_t activation by competition long before the enzymatically catalyzed phosphorylation enables arrestin to bind.

MATERIALS AND METHODS

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Merck (Darmstadt, Germany).

Protein Preparations. ROS were isolated under dim red light from fresh, dark-adapted bovine retinas (Papermaster, 1982). Rhodopsin, in the native disk membranes, was prepared by removing the soluble and membrane-associated proteins by repetitive washes with a low ionic strength buffer (washed disk membranes; Kühn, 1981). The membrane suspension in 10 mM piperazine-*N*,*N*′-(2-ethanesulfonic acid) buffer, pH 7.2, containing 130 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiotreitol, and 0.1 mM phenylmethanesulfonyl fluoride was stored at −80 °C until use. Phosphorylated opsin was prepared according to Wilden and Kühn (1982) and was used to prepare phosphorylated R by regeneration with 11-*cis*-retinal. Excess 11-*cis*-retinal was removed by incubation of the membranes with NH_2OH to form retinal oximes, and the membranes were washed, as described by Hofmann et al. (1992). Arrestin was purified as described (Palczewski et al., 1991c), and G_t was isolated from washed ROS by elution with GTP (Kühn, 1981). RK was purified as described by Palczewski et al. (1992b), with modifications. In order to minimize the concentration of the detergent Tween 80, the heparin–Sepharose column chromatography step was performed using 10 mM BTP buffer, pH 7.5, containing 0.004% Tween 80 (2.5-fold cmc) rather than 0.4% Tween 80. Unphosphorylated RK (termed γ-RK; Buczyłko et al., 1991) was eluted with 10 mM BTP, pH 7.5, containing 1 mM $MgCl_2$, 0.004% Tween 80, and 280 mM NaCl. This preparation yielded RK contaminated with an approximately equal amount of arrestin. Autophosphorylated kinase (termed α-RK; Buczyłko et al., 1991) was isolated by heparin–Sepharose column chromatography and eluted with ATP (Palczewski et al., 1992b), using 10 mM BTP buffer, pH 7.5, containing 1 mM $MgCl_2$, 100 mM KCl, 0.004% Tween 80, and 200 μM ATP. Approximately 140 μg of homogeneous RK was obtained from 100 freshly prepared bovine ROS. Before measurements, fractions containing RK were concentrated on a Centricon 30 (Amicon). Since Tween 80 and ATP could be coconcentrated by this procedure, a control sample containing Tween 80 and ATP without any protein was treated using the same procedure and applied instead of the kinase solution in control experiments. The purity of the kinase was assessed by SDS–PAGE.

RK Activity. Kinase activity was measured using urea-washed ROS membranes as a substrate (Palczewski et al., 1988). The reaction mixture contained 20 μM urea-washed ROS, 5 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP (100–500 cpm/pmol; Amersham) in 20 mM BTP buffer, pH 7.5. Phosphorylation was initiated by illumination in the presence of radioactive ATP.

SDS–PAGE. Electrophoresis was performed according to Laemmli (1970) using 12% acrylamide gels in a Bio-Rad minigel apparatus.

Protein Determinations. G_t concentration was determined using the Bradford method (Bradford, 1976); arrestin concentration was measured spectrophotometrically at 278 nm, assuming a molar absorption coefficient of $E^{0.1\%} = 0.638$ (Palczewski et al., 1992c) and a molecular mass of 45 275 Da (Shinohara et al., 1987). The concentration of the kinase was measured either spectrophotometrically at 280 nm, assuming a molar absorption coefficient of 70 000 mol^{-1} cm^{-1}, or using the Bradford method; R and R* concentrations were determined spectrophotometrically at 498 nm (Wald & Brown, 1953).

Spectrophotometry and Light Scattering. A two-wavelength spectrophotometer (Shimadzu UV300) was used to measure the differences in absorption at 380 and at 417 nm, the isosbestic point between meta I and in meta II (Schleicher et al., 1989). Photoactivation of R was induced by flash photolysis with green flashes (photoflash, filtered to 500 ± 20 nm). The mole fraction of R* in the sample was 20%. Light-scattering changes at an angular range of 16 ± 2° were monitored by a steady incident light beam in the near-infrared range (820 nm). Photoactivation of R was induced by a 500 ± 20-nm flash, and the mole fraction of R* in the sample was 35%. The scattering signal is interpreted as a gain of protein mass bound to the disk membranes and quantified as described for the binding of G_t (Schleicher & Hofmann, 1987) or arrestin (Hofmann et al., 1992). The control N-signal (the light-scattering reflection of R photoactivation) was subtracted from the original records [see Schleicher and Hofmann (1987) and the work cited therein].

RESULTS

RK Forms a Stable Complex with R.* Light-induced binding of the unphosphorylated form of RK (γ-RK; Buczyłko et al., 1991) to washed disk membranes was investigated using two methods of detection: (1) centrifugation (Figure 1A) and (2) flash-induced light-scattering (LS) binding signals (Figure 1B). In the first method, RK was mixed with ROS membranes in dim red light and incubated for 10 min at 30 °C. The membranes were pelleted by centrifugation, and both the supernatant and pellet were analyzed by SDS–PAGE. As shown in Figure 1A, RK is present only in the supernatant in the dark. Upon illumination, SDS–PAGE analysis shows that RK is found almost exclusively in the membrane fraction, indicating that binding of RK to ROS membranes is light-dependent. The second approach involved monitoring LS binding signals, which directly indicate the flash-induced transition of the kinase from a Tween-solubilized state to its membrane-bound state. To obtain the RK binding signal, the control N-signal (Figure 1B, signal c, LS from R photoactivation) is subtracted point by point from the original record (Figure 1B, signal b) to yield the resulting RK binding signal (Figure 1B, signal a). Note that, despite the presence of RK, R* should remain unphosphorylated in these experiments, due to the lack of ATP.

It has been demonstrated that the LS binding signal for G_t reflects a redistribution between a membrane-bound and a soluble state. The translocation of G_t to the membrane does not reflect the process of complex formation itself as indicated by the LS binding signal, but it is indirectly driven by complex

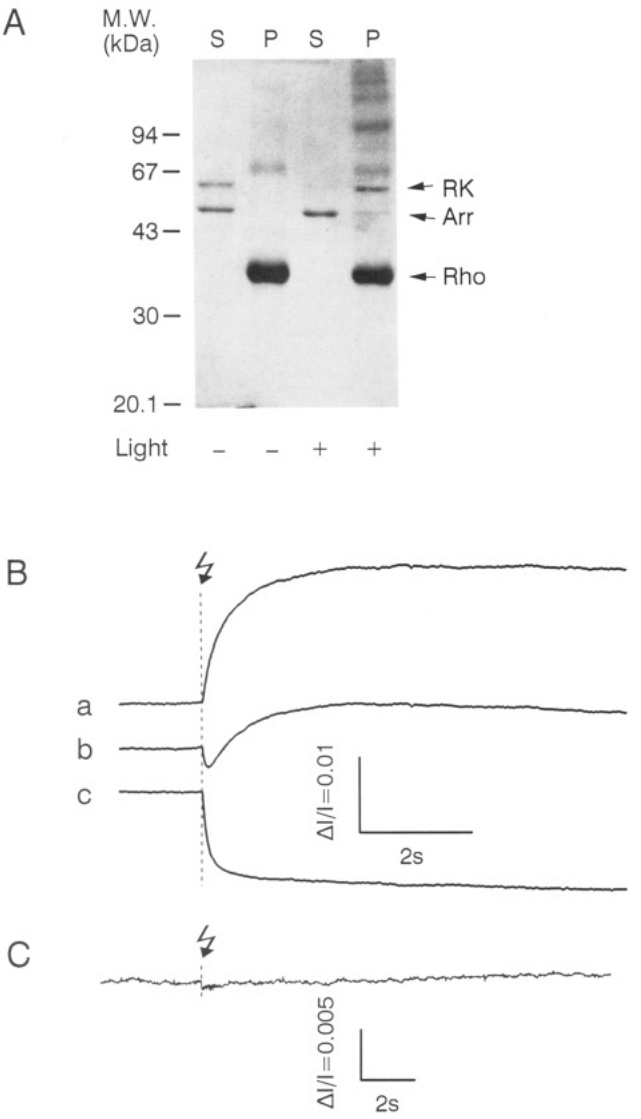

FIGURE 1: Light-dependent binding of RK to R*. (A) Centrifugation assay of the light-dependent binding of RK to R*. Under dim red light, washed ROS membranes (11 μL of 26 μM stock solution, see Materials and Methods) were mixed with γ-RK (γ-RK; Buczyłko et al., 1991; 33 μL in 10 mM BTP (pH 7.5), 100 mM KCl, 1 mM $MgCl_2$, and 0.004% Tween 80), containing approximately the same concentration of contaminating arrestin (see Materials and Methods), in 100 mM BTP buffer (pH 7). The final concentrations in the sample were 0.5 μM γ-RK, 5 μM R, and 0.0022% Tween 80 in a total volume of 60 μL. One sample was incubated in the dark (–light) for 10 min at 20 °C, while the other sample was illuminated under white light (+light) for 10 min at the same temperature. The ROS membranes were centrifuged at 15 000*g* for 10 min at 20 °C and washed twice with 60 μL 100 mM BTP buffer (pH 7.0). The first supernatant (labeled S) and the last pellet (labeled P) were analyzed using SDS–PAGE (Laemmli, 1970). (B) Flash-induced light-scattering (LS) binding signals of RK. Under dim red light, R (washed membranes) was incubated for 10 min with γ-RK (b) or without γ-RK (c) in 100 mM BTP buffer (pH 7) at 20 °C. The final concentrations in the sample were 5 μM R, 1 μM γ-RK, and 0.0043% Tween 80. Point by point subtraction of the control without γ-RK (N-signal, c) from the original record (b) yields the RK binding signal (a). Total volume was 300 μL, cuvette path length was 10 mm, and mole fraction of flash-excited R*/R = 35%. (C) LS binding signal under identical conditions as in B (trace a), but with 1 μM purified arrestin instead of RK; Tween 80 was maximally 0.016%. Note the extended scales.

formation between the membrane-bound G_t and R* (Schleicher & Hofmann, 1987; Heck & Hofmann, 1993). For the kinase, the fast binding mode and the absence of binding in the dark (Figure 1A) provide the first evidence that RK (at least in a Tween-solubilized form) binds directly to R* without intervening interaction with lipids. This is consistent with the observation that detergent-solubilized and affinity-purified R* is a good substrate for RK (Palczewski et al., 1988). With 35% R flash activation, 50% of the total kinase in the sample is bound to the membranes (Figure 1B), as calibrated by flashes which lead to complete kinase binding. The binding signal from R* and purified arrestin is immeasurably small (Figure 1C). Thus, the presence of arrestin in the γ-RK preparation (Materials and Methods, Figure 1A) does not affect the RK binding signal.

Saturation of the RK Binding Signal. The procedure used to characterize dissociation constants for R* with arrestin (Schleicher et al., 1989) and activated G_t with the PDE (Heck & Hofmann, 1993) was applied to determine the K_D of the RK–R* complex. At a constant concentration of R* and a varying kinase concentration, the LS signal amplitude increases and exhibits saturation, yielding the K_D for the complex (Figure 2). To fit the data, the mass action law is used, together with the conservation of the total amount of receptor (R) and kinase (RK):

$$[R^*\text{–}RK] = \frac{1}{K_D}([R^*]_{tot} - [R^*\text{–}RK])([RK]_{tot} - [R^*\text{–}RK]) \quad (1)$$

The solution is

$$[R^*\text{–}RK] = \frac{-b - \sqrt{b^2 - 4ac}}{2a} \quad (2)$$

where $a = 1$, $b = -(K_D + [R^*]_{tot} + [RK]_{tot})$, and $c = [R^*]_{tot}[RK]_{tot}$. Equation 2 was used to model the relative amplitudes ($\Delta I/I$) of the RK binding signal arising from the titration with $[RK]_{tot}$. A scaling factor (σ) relates the measured values to the corresponding concentration units ($\Delta I/I = \sigma[R^*RK]$). In the curves shown in Figure 2, K_D and σ were allowed to vary. The K_D obtained by employing this method for the complex between R* and γ-RK was estimated to be 0.5 μM (Figure 2A). When a fixed concentration of RK was reacted with varying [R*], the K_D was estimated to be 0.3 μM (Figure 2B). Thus, the K_D values for the two approaches are close (0.3 and 0.5 μM) but not identical. This indicates that the LS monitor is not strictly stoichiometric for one or both of the reaction components. Possible artifacts include a slight variation of the R* binding affinity with increasing concentrations in the membrane. However, the data provide an estimated K_D in the 0.3–0.5 μM range. The on-rate of kinase binding (reflected in the initial slopes of the binding signals) increases in direct proportion to either the kinase or the R* concentration. This finding further demonstrates that RK binds directly to R*, without intervening binding to membranes.

Bimolecular Rate Constant. The bimolecular rate constant for the formation of the complex between R* and RK (R*–RK) can be obtained from the initial slope of the binding signal. By definition (where RK represents kinase):

$$d[R^*\text{–}RK]/dt = k_{on}[R^*][RK] - k_{off}[R^*\text{–}RK] \quad (3)$$

The experiment in Figure 3A was performed with $[RK]_{tot}$ = 1 μM and $[R^*]_{tot} = 0.35[R]_{tot} = 1.8$ μM. The initial concentrations of the proteins immediately after the first flash on the fresh sample are $[R^*] = [R^*]_{tot}$, $[RK] = [RK]_{tot}$, and $[R^*\text{–}RK] = 0$. Since the binding signal can be calibrated by assuming that multiple flashes lead to virtually complete kinase

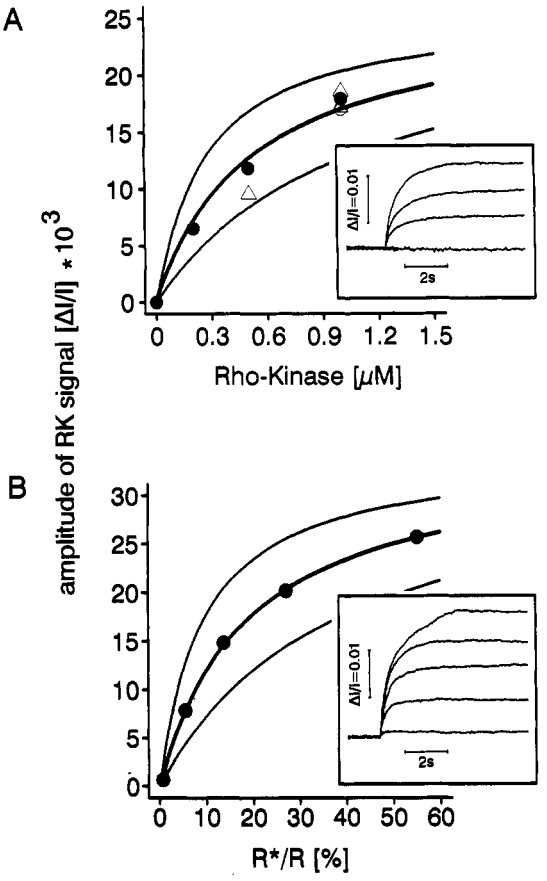

FIGURE 2: Titration of the RK binding signal. Flash-induced LS binding signals from washed ROS membranes, containing R and γ-RK, as a function of γ-RK or R* concentration. The analysis of the LS signals and the conditions of the experiment were described in the legend for Figure 1. (A) Saturation of the RK binding signal as a function of the γ-RK concentration. The plot shows final amplitudes of RK binding signals from a first flash on a fresh sample; ●, △, and ○ indicate the results obtained from different RK preparations. The best-fit curve (thick line) of the experimental data, calculated according to eq 2, yields a dissociation constant for the R*–RK interaction of K_D = 0.5 μM. The curves represent the theoretical fits for K_D = 0.25 (upper) or 1 μM (lower). Inset: Binding signals for 0, 0.25, 0.5, and 1 μM γ-RK, respectively. Measuring conditions: 5 μM R, flash excitation R*/R = 35%, 100 mM BTP buffer (pH 7), 20 °C; Tween 80 was maximally 0.0043%. (B) Saturation of the RK binding signal as a function of the mole fraction of R*. The best-fit curve (thick line) of the experimental data, calculated according to eq 2, yields a dissociation constant for the R*–RK interaction of K_D = 0.3 μM. The curves represent the theoretical fits for K_D = 0.15 (upper) or 0.6 μM (lower). Inset: RK signals with flash excitation of R*/R = 0.8%, 5.5%, 13.8%, 27.1%, and 55.2%. Measuring conditions were the same as in A, 1 μM γ-RK; Tween 80 was maximally 0.0077%.

binding, the concentration of kinase bound after 0.15 s was estimated to be 0.25 μM. Thus, 0.25 μM/0.15 s = (k_{on})(1.8 μM)(1 μM), or k_{on} = 1 $\mu M^{-1} s^{-1}$. The variation in k_{on} (0.5–1 $\mu M^{-1} s^{-1}$ in four determinations) correlates with the different detergent concentrations in the sample [0.0043% Tween 80 (2.7-fold cmc) and 0.016% (10-fold cmc) for the fastest and slowest recorded rates, respectively].

Phosphorylation of RK or R Weakens the Complex. Figure 3A shows RK binding signals for phosphorylated R* or R* and α- or γ-RK. Fast and tight binding between R* and RK was only observed when both the kinase and its substrate were unphosphorylated (Figure 3A, signal a). Small binding signals were seen for R* and α-RK (Figure 3A, signal c) and for prephosphorylated R* and γ-RK (Figure 3A, signal b). From previous work (Binder et al., 1990; Buczyłko et al., 1991), it is clear that RK does bind to R*, even when R* is

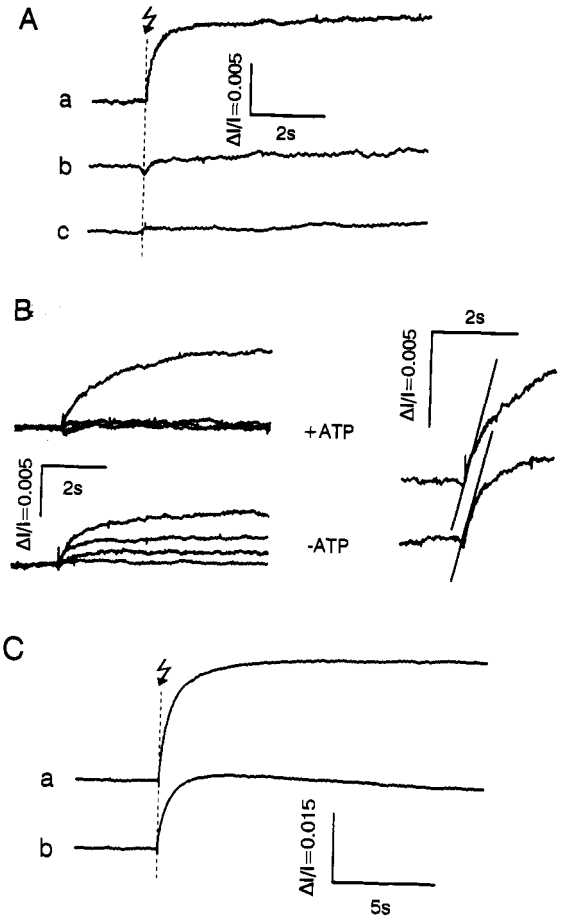

FIGURE 3: Effect of R* or RK phosphorylation on flash-induced light-scattering binding signals. (A) Flash-induced light-scattering (LS) binding signal from washed ROS membranes containing R and γ-RK (a). Washed ROS membranes containing phosphorylated R and γ-RK (b). Washed ROS membranes containing R and α-RK (c). The Tween 80 concentrations for traces a–c were maximally 0.016%, 0.011%, and 0.0054%, respectively. (B) LS binding signals from washed membranes containing R and γ-RK in the presence and absence of ATP (50 μM). On the left are LS binding signals obtained by a succession of flashes in the presence or absence of ATP. 35% of R was photoactivated by one flash, and 50 μM ATP was added before recording. The initial slopes obtained from traces of the LS binding signal (from the first flash) in the presence or absence of ATP are enlarged on the right. The Tween 80 concentration for Figure 3B was maximally 0.01%. The analysis of the LS binding signals and the buffer compositions were as in the legend for Figure 1. (C) Effect of sangivamycin, a nucleoside inhibitor of RK, on the RK binding signal. Binding signals from washed ROS membranes containing R and γ-RK in the presence of 10 μM the nucleoside inhibitor of RK, sangivamycin (Palczewski et al., 1992a) (a). Washed ROS membranes containing R and γ-RK (b). The analysis of the LS signals and buffer compositions were the same as in the legend for Figure 1. Maximal concentration of Tween 80 was 0.0043%.

phosphorylated, since more than one phosphate is incorporated into the activated receptor. However, the present results suggest that α-RK binds to R* with a much higher off-rate than does γ-RK.

ATP Stabilizes the Complex, Once Formed. In order to investigate the stability of the complex between R* and γ-RK in the presence of ATP, a succession of flashes was delivered (Figure 3B, left). The first flash evoked a relatively large LS binding signal, while the signals from successive flashes showed an abrupt decrease in the amplitude. In contrast, for the control sample without ATP, successive flashes led to a gradual decrease in the binding signal. The initial slope of the signal (expanded in Figure 3B, right) is identical in the presence and absence of ATP. We interpret these observations to mean

that there is a lower off-rate for the RK–(ATP)–R* complex than for the RK–R* complex. In the presence of ATP, RK forms a tight complex with its two substrates, ATP and R*, and one flash leads to almost complete translocation of the soluble kinase to the membranes. This hypothesis is strengthened by the LS binding signal recorded in the presence of an ATP analogue, sangivamycin. It was anticipated that, although this inhibitor blocks phosphorylation, it would not prevent formation of the R*–RK complex. Indeed, in the presence of sangivamycin, the first flash leads to the maximal level of the signal, which is identical to the sum of five flashes on a control sample (Figure 3C). These results support the conclusion that ATP or its analogue will tighten the complex between R* and RK. Direct measurements that involve the dissociation of RK are difficult to interpret since both R* and RK undergo phosphorylation and phosphorylated R* binds arrestin, which is present in our preparations.

Another type of inhibition is produced by hydroxylamine. This strong nucleophile induces the decay of meta II by forming retinal oximes with the photolyzed chromophore, resulting in the rapid dissociation of the complexes formed between R* and G_t (Hofmann et al., 1983) or between R* and arrestin (Hofmann et al., 1992). When up to 10 mM hydroxylamine was added to the sample, the RK binding signals obtained following a series of flashes were identical to the control (data not shown). Given the K_D of the complex with the kinase (Figure 2), R* is free of RK 50% of the time under these conditions. It is therefore unlikely that hydroxylamine is without effect because of reduced accessibility. This result suggests that the apoprotein opsin binds RK. The mechanism and timing of this interaction remain to be investigated.

Binding of RK Does Not Stabilize meta II. Both G_t and arrestin are known to yield an enhanced level of meta II after flash activation of disk membranes. This is explained by specific interaction and stabilization of meta II at the expense of its tautomeric forms, meta I and meta III. meta II is the form of R* in which the chromophore, *all-trans*-retinal, is still bound (to ^{296}Lys) but is deprotonated. The measurements were performed at pH 8.0 and 4 °C. In Figure 4A, signal a shows the stabilizing effect of G_t; under these conditions, a small control amount of meta II is formed in the absence of G_t. When G_t is added, extra meta II is formed because meta II, once formed, is stabilized by bound G_t and is hindered in its reaction back to meta I. In contrast to G_t, binding of RK shown in Figure 4A, signal b, does not produce a significant stabilization of meta II. Calculation according to Schleicher et al. (1989) with the parameter estimates above shows an expected level of enhancement by binding of a protein with the K_D of the kinase. It results in [extra meta II] = 0.3 μM. The enhancement in Figure 4A, signal b, if any, is much lower. When G_t and RK are present simultaneously, the kinase can compete quite effectively and suppress the stabilizing effect of the G-protein (Figure 4B, signal b). To enhance competition, the interaction with G_t was, in this case, weakened by GDP (Kahlert et al., 1990). This experiment confirms that the kinase interacts directly with the cytoplasmic surface of R and that the binding sites of G_t and RK, at least partially, overlap. This result is consistent with the inhibition of R* phosphorylation under conditions of R*–G_t complex formation (Pfister et al., 1983).

DISCUSSION

Rhodopsin–RK Complex. We found that a complex between R* and γ-RK is formed with micromolar affinity.

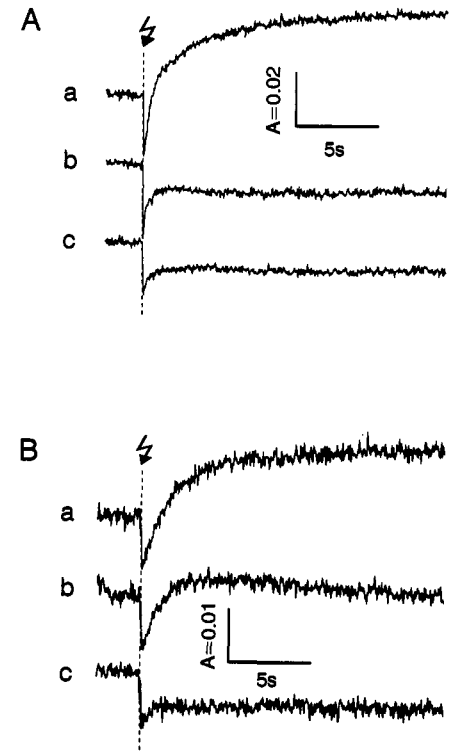

FIGURE 4: Effects of transducin and RK on the stability of meta II. (A) Formation of extra meta II (measured as described in Materials and Methods) induced by 1 μM G_t (a). Lack of extra meta II formation in the presence of 1 μM γ-RK (b). Control with R alone (c). The measurements were performed with 10 μM Rho/100 mM BTP buffer (pH 8) containing 10 mM NaCl, at 4 °C. Flash excitation, R*/R = 20%; cuvette path length, 2 mm, employing a dual wavelength flash photometer (see Materials and Methods). (B) Destabilization by RK of extra meta II formed with G_t. meta II formation with 1 μM G_t (a). Suppression of extra meta II formed with 1 μM G_t by the addition of 1 μM γ-RK (b). Control with rhodopsin alone (c). Samples contained 2 μM rhodopsin, 90 μM GDP, and buffer, and measurements were the same as for A. The maximal Tween 80 concentration was 0.0092%.

ATP (or its analogue) enhances the affinity of RK for R* before the enzyme expresses its phosphorylating activity. A fundamental difference between this interaction and the binding of transducin (G_t) or arrestin to the activated receptor is the lack of preference for binding to the meta II form of R* [reviewed in Hargrave et al. (1993)]. γ-RK avidly binds to meta II and also to the predecessor meta I before the key events of rhodopsin's activation as a receptor—deprotonation of the retinal Schiff base, proton uptake from the aqueous phase (Arnis & Hofmann, 1993), and a specific helix–loop conformation—have occurred. These results are consistent with the finding by Paulsen and Bentrop (1984) that R* is a substrate for the kinase under conditions favorable for meta I or meta II. Specific "steric trigger" events have been demonstrated that are linked to the formation of the meta I photoproduct and its possible equivalent in archaebacterial sensory R (Yan et al., 1991; Siebert, 1992). These events may be involved in the exposure of the binding domain of R* for RK. The most likely element to be operated on the surface of R* is a fragment of the third cytoplasmic loop, a domain implicated in kinase activation (Palczewski et al., 1991b). The binding state of R* for the γ-RK is not only expressed as early as in the meta I photoproduct but it is also present over minutes (Figure 1A). During that time, the interaction with the kinase remains at a stable equilibrium. For signal transduction, it is relevant how long one single given copy of the complex, once formed, remains stable before it dissociates

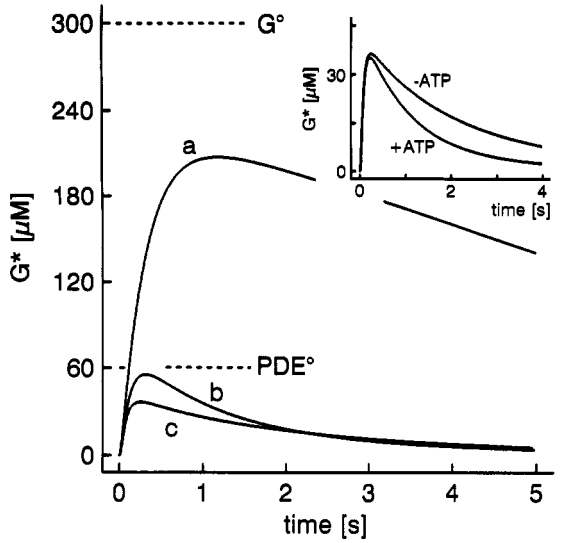

FIGURE 5: Computer simulation of transducin activation for different binding modes of RK. The curves describe the time course of transducin (G_t) in its active, GTP binding form (represented by G*). Rise and decay of G* result from the time-dependent activation of G_t by R* in the meta II form (MII), the decay by its endogenous GTPase, and the deactivation of MII. All simulations assume the same G* activation rate, the same MII deactivation rate, and the same total concentration of meta I, meta II, G_t, and effector (phosphodiesterase, PDE). They differ only in the assumed rate and K_D of RK binding. If meta II were deactivated without the binding of RK (line a), a large fraction of the total G_t pool would be activated with a surprisingly slow decay (see text). If it is assumed that RK competes with G_t for binding to R*, with the rate and dissociation constants determined in this study, a small fraction of the total G_t pool (G°) would be activated with a rapid falling phase (line b). The highest concentration of active G_t (peak of G* in the curve) does not exceed the total number of available effector binding sites (PDE°). The effect of ATP (inset and line c) was simulated by a 5 times lower K_D, as compared to the control (–ATP, identical to simulation c). The reaction model is given here as follows. Rate constants are given in $\mu M^{-1}\ s^{-1}$ for association, and s^{-1} for dissociation; in the formulae, G, MI, and MII are used instead of G_t, meta I, and meta II, respectively, and the reactions are considered to be at room temperature:

activation

$$1.\ \mathrm{MI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{MII} \qquad k_1 = 50,\ k_{-1} = 15$$

$$2.\ \mathrm{MII} + \mathrm{G} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{MII\text{–}G} \qquad k_2 = 15,\ k_{-2} = 0$$

$$3.\ \mathrm{MII\text{–}G} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{MII} + \mathrm{G}^* \qquad k_3 = 7000,\ k_{-3} = 0$$

deactivation

simulation a:

$$4.\ \mathrm{MII} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{MIIi} \qquad k_4 = 0.4,\ k_{-4} = 0$$

simulations b and c:

$$4.\ \mathrm{MII} + \mathrm{RK} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{MII\text{–}RK}$$

b: $k_4 = 0.5,\ k_{-4} = 0.2$

c: $k_4 = 1.0,\ k_{-4} = 0.5$

inset: identical to c (–ATP)

$k_4 = 1.0,\ k_{-4} = 0.1$ (+ATP)

$$5.\ \mathrm{MII\text{–}RK} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \mathrm{MIIi} + \mathrm{RK} \qquad k_5 = 0.4,\ k_{-5} = 0$$

$$6.\ \mathrm{G}^* \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \mathrm{G} \qquad k_6 = 1,\ k_{-6} = 0$$

Steps 1–3 are from known activation rates (Kahlert & Hofmann, 1991; Pugh & Lamb, 1993). The results are not very sensitive to these rates. In step 4, it is assumed that RK binds only to MII, but the curves are virtually the same if binding to MI is also allowed. Step 5 (for simulation a, step 4) describes in a short form the quasi-irreversible actual inactivation reaction; phosphorylation by bound γ-RK and fast binding of arrestin is replaced by a transition within 2.5 s of MII into inactive MII (MIIi). It is assumed that RK is released by this inactivation step. For simplicity, we did not introduce a new inactive RK form, but assumed that active RK is released; under the conditions, this does not influence the simulation. Step 6 is the inactivation of G* with 1 s (Vuong & Chabre, 1991). Assumed initial concentrations: [R*] = 0.3 μM, [G_t] = 300 μM, and [RK] = 30 μM.

in this on/off equilibrium. The estimation can be performed as follows: $K_D = k_{off}/k_{on}$, where k_{off} is the rate of spontaneous dissociation of the complex, and k_{on} is the rate of association (eq 3). When K_D = 0.5 μM and k_{on} = 0.5 $\mu M^{-1}\ s^{-1}$, the calculated k_{off} is 0.25 s^{-1}, meaning that the average lifetime of the R*–RK complex equals 4 s. This is a lower limit since rapid binding of ATP to the complex lowers its off-rate (Figure 3B). The calculation shows that spontaneous dissociation of the complex within the time frame set by the R* lifetime *in situ* (Pepperberg et al., 1988, 1992) would be a rather rare event. According to the hypothesis of Buczyłko et al. (1991), the postponed sequential phosphorylation of R* and autophosphorylation of RK will terminate the complex, thus illustrating why R* phosphorylation is needed in receptor quenching (Bennett & Sitaramayya, 1988; Palczewski et al., 1992a). The bimolecular rate constant of R*–RK formation is surprisingly high, higher than that of the arrestin–R* complex (Hofmann et al., 1992). The relative simplicity of the kinase interaction (expressed in the fact that it recognizes the presumably less complex early meta I form) may favor collisional coupling between R* and RK. It remains open whether the binding of the kinase to meta I, and even to opsin, is of physiological importance beyond this mechanistic implication. In principle, there are time windows in which only RK but not G_t or arrestin has access to R*.

Possible Implications for Signal Transduction. The rates found in this study may not occur under cellular conditions for several reasons, including the following: (i) the purified kinase *in vitro* was not in its native but in a solubilized state; (ii) kinetic parameters taken at micromolar concentration may not be extrapolated to the densely packed cellular compartment; and (iii) a chemical reaction scheme can never describe the stochastic process of activation by single R* molecules. However, it is striking that the most simple linear extrapolation to an assumed concentration of the kinase *in situ* (30 μM; Palczewski et al., 1992b) leads to an estimated time for R*–RK formation of approximately 10 ms. This is only 2 orders of magnitude slower than the average time it takes to associate R* with a copy of G_t in the early phase of activation of the G_t pool (Kahlert & Hofmann, 1991; Bruckert et al., 1992; Pugh & Lamb, 1993). As intuitively expected, inhibition of G_t activation at an early stage is indeed found by a computer simulation (Figure 5) based on a simple chemical reaction scheme. The remarkable result of the simulated response is that the total amount of activated G_t at peak would not exceed the available PDE sites to any degree. This reveals a potential mechanism for downregulating the active G_t pool in photoreceptor cells, which would precede phosphorylation and arrestin binding. It remains to be determined whether such a mechanism occurs physiologically. Recent investigations indicate that R phosphorylation depends on calcium-dependent feedback regulation (Kawamura, 1993). This raises the possibility that the interaction of RK with R* *in situ* is subject to a regulatory process that may delay its

effective inhibition of G_t activation to a time greater than that predicted here.

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