Activation of rhodopsin kinase

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The present study confirms our original assertion that peptides corresponding to the C-terminal sequence of rhodopsin are phosphorylated by rhodopsin kinase (RK), but only in the presence of photo-activated rhodopsin [Rho*, which is functionally equivalent to metarhodopsin II (Meta II)]. Under optimized conditions, the extent of peptide phosphorylation reached up to 60% that of Rho*. Rho* phosphorylation began to plateau within 15 min of the initiation of photolysis, whereas the peptide phosphorylation continued linearly for > 60 min. This lack of co-ordination in the phosphorylation of the physio-

logical (Rho*) and synthetic (peptide) substrates necessitated the present study, which showed that RK is activated for peptide phosphorylation not only by Meta II but also by Meta III, as well as by the phosphorylated derivatives of these species, but not by opsin. These results led to the conclusion that all the derivatives of opsin, which contain the Schiff base linkage with the all-*trans*-retinylidene moiety, retain the ability to activate RK.

Key words: metarhodopsin, opsin, retinyl, rhodopsin phosphorylation, Schiff base.

INTRODUCTION

The Ser/Thr phosphoryl transferase of retinal cell, rhodopsin kinase (RK) was the first member of a growing family of G-protein-coupled receptor kinases (GRKs) to be discovered and is classified as GRK1. β -Adrenergic-receptor kinase-1 and -2 (GRK2 and 3) are two other well-studied members of the family, whereas three related kinases have been deduced from cDNA sequences [1]. Like other members of the family, RK phosphorylates only the stimulated form of the visual receptor, which is produced by the action of visible light on rhodopsin [2–4]. In other cases, the receptors become substrates for their cognate GRK, following agonist occupation (see [1] and references therein). The effect of light on the 11-*cis*-retinylidene chromophore of rhodopsin ($\lambda_{\max} \approx 498$ nm) is complex, but at an ambient temperature and on a physiologically relevant time scale, it results in the production of a relatively long-lived intermediate metarhodopsin II (Meta II, $\lambda_{\max}$ 389 nm) [5]. The latter is the activated species (symbolized as Rho*) that first triggers the visual cascade and is then inactivated by phosphorylation catalysed by RK [6]. The extraordinary specificity of RK which allows it to act on Rho*, but neither on the dark-adapted rhodopsin nor on the apoprotein opsin, was originally explained by suggesting that the site of phosphorylation, the C-terminal region of the receptor, becomes accessible only after conformational changes accompanying bleaching (see [7] and references therein). Using peptides corresponding to the C-terminal region of rhodopsin it was then discovered that these were phosphorylated by RK, but only in the presence of catalytic amounts of Rho* [8,9] or a C-terminal truncated form of Rho* [10]. This led to the proposal that RK is normally present in an inactive resting state and is activated following interaction with Rho*. Two broad mechanisms for the activation of RK were considered [8,9]:

Equation (1) assumes that the activated form of the enzyme is present only within a Rho* · RK complex (see also [1,10]), whereas eqn (2) implies that RK may dissociate and independently exist as an activated entity.

Studies on the Rho*-dependent phosphorylation of synthetic peptide by RK have been extended to cover other aspects [11], e.g. the delineation of hierarchy of phosphorylation at different sites in Rho* [12]. Furthermore, it has been shown that GRK2 and GRK3 also phosphorylate synthetic peptides in the presence of the agonist-occupied form of their corresponding receptors [13,14]. Despite these sporadic observations, the activation hypothesis has been viewed with scepticism [15]. Here we establish optimal conditions for the Rho*-dependent phosphorylation of synthetic peptides and for the characterization of the form(s) of Rho* able to activate the kinase.

MATERIALS AND METHODS

Materials

[γ -³²P]ATP was obtained from Amersham, OptiPhase ‘Hisafe’ 3 liquid-scintillation-cocktail from Fison’s Chemicals and AG-1X2 (acetate form) anion-exchange media was from Bio-Rad (Richmond, CA, U.S.A.); all other chemicals were purchased either from Sigma or Aldrich.

The buffers used in this work were as follows: buffer A: 70 mM phosphate/2 mM MgCl₂/0.1 mM EDTA/2 mM dithiothreitol/1 mM benzamidine/0.2 mM phenylmethanesulphonyl fluoride (pH 7.4); buffer B: as in buffer A except that 70 mM phosphate was replaced by 20 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris propane); buffer C: 100 mM Tris/2 mM MgCl₂/1 mM EDTA/1 mM dithiothreitol (pH 7.4 at 37 °C).

Abbreviations used: RK, rhodopsin kinase (GRK1); GRK, G-protein-coupled receptor kinase; GRK2 and GRK3, β -adrenergic-receptor kinases-1 and -2; 11-mer Rho, ³³⁸SKTETSQVAPA³⁴⁸, residues 338–348 of the C-terminal of bovine rhodopsin; Meta II, III, metarhodopsin II, III; Rho*, bleached rhodopsin (considered to be equivalent to Meta II); ROS, rod outer segment; TFA, trifluoroacetic acid.

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Methods

Synthesis of 11-mer Rho ($^{338}\text{SKTETSQVAPA}^{348}$, residues 338–348 of the C-terminal of bovine rhodopsin) peptide (hereafter referred to as the peptide substrate)

The 11-mer Rho sequence, corresponding to the amino-acid sequence of residues 338–348 of the C-terminal tail of bovine rhodopsin and its phosphorylated derivative were synthesized as stated previously [11].

A general procedure for the study of the phosphorylation of Rho* and the peptide substrate by RK

Ovine rod outer segments (ROS), urea-washed ROS and RK were prepared and processed as described previously, except that both ROS and urea-washed ROS were finally taken up into buffer B (see [16] and references therein). Generally, the RK used had an activity of 7–18 units/ml (1 unit $\approx$ 2 μg of RK protein [17]). One unit of RK catalyses the incorporation of 1 nmol of ^{32}P /min from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in Rho*, isolated and analysed as phospho-opsin at 37 °C in the assay system described below. Typically, experiments were performed on samples of 300–450 μl .

A sample in a final volume of 450 μl consisted of 45 μl of urea-washed ROS (4.5 nmol), 45 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2.8 mM in the final solution; 2.5×10^5 c.p.m./nmol), 315 μl of RK (2.9 units (0.1 nmol) and 45 μl of peptide substrate in buffer A (2 mM final concentration). Samples were vortex-mixed, sonicated and incubated in the dark for 10 min at 37 °C (unless stated otherwise) and then exposed to continuous light for 60 min (unless stated otherwise), with identical foil-wrapped tubes serving as dark controls. During the course of the reaction samples were occasionally agitated and vortexed to prevent sedimentation of ROS membranes. Aliquots of 10 μl were removed at various time points and precipitated with 1 ml of 20% (w/v) aq. trichloroacetic acid. The pellet was centrifuged at 10000 g for 3 min and washed twice with 1 ml of 10% (w/v) aq. trichloroacetic acid containing 10 mM phosphate (10000 g for 3 min). The pellet was solubilized in 200 μl of formic acid and, after mixing with 1.5 ml of OptiPhase 'Hisafe' 3, subjected to scintillation counting. The accompanying phosphorylation of peptide substrate was monitored by quenching 50–80 μl of the incubation mixture in 300 μl of 7% (v/v) aq. acetic acid. Samples were centrifuged (10000 g for 3 min) and the supernatant (300 μl) passed down an AG-1X anion-exchange column (1.5 ml) equilibrated with 7% (v/v) aq. acetic acid to remove $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. For each sample, 6 ml of eluant was collected and lyophilized before being taken up into 800 μl of 0.1% (v/v) trifluoroacetic acid (TFA) in AnalaR water and the solution centrifuged (10000 g for 3 min) to remove contaminating AG-1X resin. A 300 μl aliquot was subjected to analytical reverse-phase-HPLC (C_{18} column) with a linear gradient of 0.1% aq. TFA and acetonitrile containing 0.1% TFA. Fractions of 1 ml were collected and 500 μl aliquots subjected to scintillation counting. Generally, > 95% of the counts loaded on to the column eluted at 37% acetonitrile, which corresponds to the position of the authentic 11-mer Rho phosphopeptide.

Phosphorylation of peptide substrate in a detergent-solubilized system was performed identically with that stated above with urea-washed ROS membranes replaced by urea-washed rhodopsin solubilized in 2% (w/v) dodecyl maltoside in buffer B (for preparation refer to [16]). Furthermore, the peptide work-up was identical with that for the membrane system mentioned above.

RK-catalysed phosphorylation with addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, at 0 and 30 min after the initiation of illumination

This was performed using the general protocol described above except that it was conducted at 20 °C for 120 min. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added to one sample at zero time, whereas another was supplemented with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ 30 min after the initiation of illumination.

Preparation of phosphorylated opsin

A mixture containing 150 μl of urea-washed ROS (6–9 nmol), 131 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (final concentration 2.5–3.5 mM with a specific activity of 2×10^5 – 4×10^5 c.p.m./nmol) and 1200 μl of RK (12–20 units) was thoroughly vortexed and sonicated in an ice-water bath (3 min) before incubation in the dark at 37 °C for 10 min. The sample was then exposed to continuous light for 30 min, with occasional agitation and vortexing to prevent sedimentation of ROS membranes. The reaction was stopped by terminating illumination and, in order to remove excess $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ from the phosphorylated opsin sample, was washed twice with 5 ml of buffer A and ultracentrifuged (240000 g for 10 min). The pellet was taken up into 50 μl of buffer A to give a final volume of 100 μl with a final concentration of approx. 300 μM . To determine the incorporation of ^{32}P into opsin, 10 μl aliquots were removed during the course of the reaction and subjected to trichloroacetic acid precipitation as discussed above. ^{32}P incorporation of ≥ 5 nmol of ^{32}P per nmol opsin was used for the generation of phosphorylated isorhodopsin as will be shown below. In order to generate non-phosphorylated opsin, an Eppendorf tube identical with that above, but with 1331 μl of buffer A replacing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and RK, was set up and processed in parallel with the above sample.

Regeneration with 9-*cis*-retinal to form phosphorylated isorhodopsin

A solution of 9-*cis*-retinal was prepared by the addition of 20 μl of Tween 80 in acetone (250 mg/ml) to 500 μg of 9-*cis*-retinal in an Eppendorf tube. The acetone was evaporated using a stream of nitrogen gas, 100 μl of AnalaR water was added and the concentration of aqueous 9-*cis*-retinal (λ_{max} 387 nm) was determined spectroscopically.

Approximately 2 M equivalents of aq. 9-*cis*-retinal was added to the phospho-opsin as discussed above and the sample incubated in the dark at room temperature for 1 h. Regeneration to give phosphorylated isorhodopsin was confirmed by monitoring the absorbance at 490 nm (λ_{max} of isorhodopsin) before, during and after incubation with 9-*cis*-retinal by the removal of 5 μl aliquots into 195 μl of 3% (w/v) *N,N*-dimethyl dodecylamine in buffer B. In order to confirm the absence of phosphatase activity during regeneration, the ^{32}P content of phospho-isorhodopsin was monitored by the removal of 5 μl aliquots for trichloroacetic acid precipitation as discussed above.

Phosphorylation of the peptide substrate in the presence of isorhodopsin or phosphorylated isorhodopsin

A 350 μl sample consisted of 245 μl of RK (2.3 units), 35 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (final concentration 2.8 mM; 3.4×10^5 c.p.m./nmol), 35 μl of peptide substrate in buffer A (final concentration 2 mM) and 35 μl of either phosphorylated isorhodopsin (5 nmol phosphate per nmol opsin) or isorhodopsin (both 10.5 nmol). The experiment was conducted as discussed in the General Procedure section above.

Rho* and peptide phosphorylation in the presence of NH_2OH

A 350 μl sample consisted of 245 μl of RK (2.5 units), 35 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (final concentration 3 mM: 8.6×10^4 c.p.m./nmol), 35 μl of urea-washed ROS (6.2 nmol), 17.5 μl of peptide substrate in buffer A (final concentration 2 mM) and 17.5 μl of aq. NH_2OH (final concentration 0.25 mM). An identical control was set up which contained buffer A in place of NH_2OH and identical foil-wrapped tubes served as dark controls. All samples were vortex-mixed, sonicated and incubated in the dark for 10 min at 20 °C before the introduction of continuous light. The incubation was conducted for 120 min at 20 °C and the other details are as described under the General Procedure section above.

Schiff base cleavage in ovine ROS membranes in the presence of NH_2OH

The Schiff base cleavage under the above conditions was studied in an independent experiment as follows: 1 ml of ROS membranes (12.5 nmol) in buffer C (pH 8.0 at 20 °C containing a final concentration of 0.25 mM NH_2OH) was incubated at 20 °C for 10 min in the dark with occasional agitation and vortexing to prevent sedimentation of ROS membranes. The sample was exposed to continuous light and 80 μl aliquots were removed at various times and added into 120 μl of an acid/detergent solution [3% (w/v) *N,N*-dimethyl-dodecylamine-*N*-oxide in 0.2 M aq. HCl; pH 2–3]. The Schiff base present was determined using the ratio of absorption at 440 nm [*N*-retinylidene opsin (NRO) which is formed from various Schiff base linked derivatives of rhodopsin under acid-denaturing conditions] and 380 nm (due to all-*trans*-retinal oxime; although the λ_{max} for this species is 365 nm under these conditions, this wavelength was selected for convenience). A standard curve correlating the percentage Schiff base cleavage in the presence of NH_2OH with the A_{440}/A_{380} ratio was constructed using a modification of the method of DePont et al. [18] as follows: 500 μl of ovine ROS membranes (62.5 nmol) was added to 4.5 ml of 3% (w/v) *N,N*-dimethyl-dodecylamine-*N*-oxide in buffer C (pH 8.0 at 20 °C) containing NH_2OH to give a final concentration of 12.5 μM rhodopsin and 10 mM NH_2OH . Two Eppendorf tubes containing 1 ml of this solution were incubated in the dark at 20 °C for 10 min. One of the tubes was then wrapped in tin foil and the other exposed to continuous light for 6 min. Unbleached and bleached samples were assumed to contain 100% and 0% Schiff base, respectively. Both samples were placed on ice immediately and processed in the dark. To Eppendorf tubes on ice, containing 120 μl of 0.2 M HCl, a total volume of 80 μl comprising a mixture of unbleached and bleached samples of rhodopsin containing the following compositions were added: 80, 70, 60, 50, 40, 30, 20, 10 and 0 μl of the unbleached solution and the remaining volume made up with Rho*. The A_{440}/A_{380} was plotted versus the calculated percentage Schiff base. The spectroscopic blank was identical with the experimental cell except that the 80 μl sample was replaced by 80 μl of 3% (w/v) *N,N*-dimethyl-dodecylamine-*N*-oxide in buffer C (pH 8.0 at 20 °C).

Phosphorylation of the peptide substrate in the presence of opsin

The experiment was conducted as in the General Procedure above except that in one experiment, urea-washed ROS was replaced by opsin (prepared as stated below).

Preparation of opsin

Urea-washed ROS (5.12 mg) in buffer B containing NH_2OH (10 mM final concentration) was exposed to continuous light

(30 min at 20 °C) and immediately centrifuged (10000 *g* for 3 min at 4 °C). The resultant pellet was washed in buffer B (3 ml) by repeated centrifugations (three times as above) in order to remove excess NH_2OH . Opsin was characterized using Schiff base content (as stated above except buffer C did not contain NH_2OH) and opsin concentration (using A_{280} and $\epsilon = 64000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [19], where 0% of the initial Schiff base was found to exist and a recovery of 79% of the starting amount of protein obtained.

RESULTS

The extent and time course of peptide substrate phosphorylation by RK

In order to demonstrate the Rho*-dependent phosphorylation of synthetic peptide substrates by RK beyond any reasonable doubt, it was important to establish conditions under which the differences between the phosphorylation levels in the dark and light were maximized. Therefore several improvements were introduced which included the use of larger amounts of the enzyme, higher specific activity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and exhaustive purification of the phosphorylated peptide so that it was completely free of any contaminating radioactivity. Figure 1 describes two types of experiments in which the phosphorylation of protein and peptide substrate was studied using Rho* either in ROS membranes or solubilized in dodecyl maltoside. In both systems, the phosphorylation levels of Rho* were identical, leading to the incorporation of 4 mol of phosphate per mol of receptor protein, and the bulk of the phosphorylation was complete within 15 min of the initiation of photolysis. In these and other experiments, the molar ratio of Rho/RK was 4.5:0.1 which, together with the stoichiometry of phosphate incorporation allows the estimation that during the overall conversion, each molecule of RK had turned over approx. 180 times. The Rho* phosphorylation was

Figure 1 Comparison of the light-dependent phosphorylation of Rho* and peptide substrate by RK using a detergent-solubilized or membrane system

The final ^{32}P incorporation into Rho* in both the detergent-solubilized and the membrane system (two upper curves) gave the same final incorporation of 4 nmol phosphate per nmol receptor protein. The dark control for Rho* ($\square$) and peptide substrate (∇) incorporated ^{32}P less than 5 and 1%, respectively compared with the experiment conducted in the light. A typical result of four independently performed experiments is shown.

quantified using the trichloroacetic acid precipitation method and showed that the control experiments, performed in the dark, incorporated less than 5% of the ^{32}P found in light. Since the Rho* phosphorylation is not a contentious issue, it was not deemed necessary to reduce further the background radioactivity by studying the incorporation specifically into receptor protein following separation by electrophoresis.

For peptide phosphorylation, the samples were rigorously purified by ion-exchange chromatography followed by reverse-phase HPLC and the time course of ^{32}P incorporation studied for up to 1 h. At that time, in the membrane system, the level of peptide phosphorylation had reached approx. 60% of the Rho* phosphorylation with less than 1% incorporation of ^{32}P in the control experiments performed in the dark. In the soluble system, however, the peptide phosphorylation was reduced in a time-dependent manner, being 66, 38 and 25% of the membrane system at 5, 10 and 30 min, respectively. Several factors may contribute to the impairment of peptide phosphorylation; one is the relatively facile hydrolysis of the Schiff base, in the detergent-solubilized system, producing opsin, which is not an activator of RK. It has been shown that the hydrolysis of the Schiff base linkage of Meta II is several times faster in dodecyl maltoside solution than in ROS membranes [20].

Meta II, Meta III and their phosphorylated forms activate RK for peptide substrate phosphorylation

In the membrane system, it was surprising that the phosphorylation of the peptide substrate continued linearly for more than 30 min, and longer in experiments not shown, after the phosphorylation of Rho* had been completed. This led to the investigation of the types of photointermediates of rhodopsin that are capable of activating RK for peptide substrate phosphorylation. Hence, we took advantage of our recent work [16] in which it was shown that the decay of Meta II into Meta III could be smoothly and accurately monitored spectroscopically at 20 °C in ovine membranes when the $t_{1/2}$ for both the decay of Meta II and the formation of Meta III was approx. 3.2 min, and at 30 min post-photolysis the equilibrium between Meta II and Meta III had been fully established. Phosphorylation experiments were now performed at 20 °C closely mimicking the spectroscopic conditions. Samples containing ovine ROS membranes and RK were bleached and one set supplemented with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ immediately and the other after 30 min. Figure 2 shows that the time-course profiles for the incorporation of ^{32}P into Rho* as well as the peptide substrate were parallel for both the sets. These experiments highlight that Meta III is as good a kinase substrate as Meta II and, more importantly, both Meta II and Meta III are equally effective in activating RK for catalysing the phosphorylation of a peptide substrate.

Next, the ability of the phosphorylated form of Meta II and Meta III to activate RK for catalysing peptide substrate phosphorylation was investigated. Samples of opsin or phosphorylated opsin (containing 5 nmol phosphate/nmol opsin) were prepared in parallel incubations and each regenerated with 9-*cis*-retinal to yield isorhodopsin and phosphorylated isorhodopsin, respectively. The choice of 9-*cis*-retinal was based on its commercial availability in a crystalline form, which ensured that identical amounts of the chromophore were used in the two regeneration experiments. Two sets of incubations were performed containing the peptide substrate, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, RK and either isorhodopsin or phosphorylated isorhodopsin. The photolysis of these should produce the 'same' Meta II and phosphorylated Meta II, which would be formed from the corresponding 11-*cis*-retinal-based proteins. In the incubation

Figure 2 Time-course profile of RK-catalysed phosphorylation at 20 °C in incubation supplemented with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at 0 and 30 min following the initiation of illumination

The results of three independent experiments $\pm$ S.D. are shown. The maximum incorporation of ^{32}P into Rho* and into the peptide substrate in the incubation supplemented with ATP at zero time is taken as 100%. In a typical experiment, 42.8 and 18 nmol of ^{32}P were incorporated into Rho* and the peptide substrate, respectively. ■, ●, Phosphorylation of Rho*; □, ○, peptide.

containing isorhodopsin, the expected incorporation of phosphate into the protein (at 60 min, 3.8 nmol phosphate per nmol receptor protein with dark controls containing less than 2% of that incorporated in the light experiment; Figure 3) as well as the peptide substrate was achieved. More significantly, however, the time course and the extent of peptide phosphorylation were the same whether the activation of RK was triggered by bleached isorhodopsin or bleached phosphorylated isorhodopsin. It is to be noted that in the experiment containing phosphorylated isorhodopsin, a further incorporation of 1.2 nmol phosphate per nmol receptor protein occurred. If, for the sake of argument, it is assumed that the latter incorporation involved the phosphorylation of a completely unphosphorylated population then the upper limit of this unphosphorylated isorhodopsin in the original sample will be less than 20% and not sufficient to activate RK maximally. We, therefore, deduced that the activation of RK for peptide phosphorylation in curve (V) of Figure 3 was promoted by the phosphorylated forms of Meta II/Meta III. The latter conclusion can also be drawn by a closer examination of the time-course curve in Figure 1, which shows that the bulk of Rho* phosphorylation occurred in the first 10 min following the initiation of photolysis. However, the peptide phosphorylation continued, almost linearly for 60 min; during most of this time the Rho* was predominantly present in its phosphorylated form. Hence, the activation of RK, in this phase, must have occurred using the phosphorylated form of Rho*. Here, it should be pointed out that contrary to common belief, the Schiff base hydrolysis in ROS membranes, at 20 °C, is a very slow process. In the kinetic experiments described in [16] (e.g. Figures 2B and 2C), there was no evidence of Schiff base hydrolysis for at least 40 min, which accounts for the fact that peptide phosphorylation, dependent on the activation of RK by photointermediates, was linear over the course of incubation. As stated above, the facile hydrolysis of the Schiff base in dodecyl maltoside may contribute to the fact that the peptide

Figure 3 Light-dependent phosphorylation of peptide substrate by RK in the presence of either isorhodopsin or phosphorylated isorhodopsin

Phosphorylation of peptide substrate in the incubation containing isorhodopsin (●) or phosphorylated isorhodopsin (◻). Phosphorylation of Rho* in the incubation containing isorhodopsin (●), at 60 min, 3.8 nmol phosphate per nmol receptor protein was incorporated, as in a parallel incubation containing rhodopsin) or phosphorylated isorhodopsin (○, at 60 min a further incorporation of 1.2 nmol phosphate per nmol receptor protein occurred). Dark controls were found to contain no more than 2% of the radioactivity incorporated in light. A typical result of this experiment is shown.

phosphorylation in this detergent decays with the passage of time (Figure 1).

Opsin does not activate RK for peptide substrate phosphorylation

Attention has already been drawn to the fact that the apoprotein opsin is not phosphorylated by RK [21–23]. Can opsin still act as an activator of RK? To address this question, in a pilot study, the phosphorylation reaction was performed in the presence of various concentrations of NH_2OH , in order to cleave the Schiff base linkage of Rho*. A concentration of 0.25 mM NH_2OH was then selected for critical experiments and three parameters were measured: (a) the cleavage of Schiff base, (b) protein phosphorylation and (c) peptide phosphorylation. The Schiff base decay curve in Figure 4 shows that the phosphorylation of Rho*, in the presence of 0.25 mM NH_2OH , commenced at a rate similar to that in the control experiment but was gradually arrested with increasing cleavage of Schiff base. Once the latter reaction was nearly complete, at approx. 30 min, no further Rho* phosphorylation was observed. The effect of cleavage is seen more clearly on the peptide phosphorylation because of its longer time course. Here, no significant ^{32}P incorporation occurred after 30 min when 86% of the Schiff base had been cleaved. The abolition of peptide phosphorylation, in the presence of NH_2OH therefore must be due to the inability of opsin, or for that matter, phosphorylated opsin, to serve as an activator of RK. Two possible explanations for the inhibition of phosphorylation in the presence of NH_2OH can be eliminated. First, it is not due to the inactivation of RK by NH_2OH , since the enzyme was found to be stable in the presence of the reagent for up to 1 h (results not shown). Secondly, the fact that the

Figure 4 Profile of RK-catalysed phosphorylation at 20 °C in the presence of NH_2OH

The maximum incorporation of 43.4 nmol of ^{32}P into Rho* and 34.3 nmol of ^{32}P into the peptide substrate at 120 min in the absence of NH_2OH is taken as 100%. Phosphorylation of Rho* (●) and the peptide substrate (□) as indicated above. A typical result of this experiment is shown. The decay of Schiff base was studied in a separate experiment performed similarly (○, 1 ml volume; 12.5 nmol rhodopsin), but not identically, to the above incubation as described in the Methods section.

Figure 5 Profile of RK-catalysed phosphorylation at 20 °C in the presence of opsin

The maximum incorporation of 25.2 nmol of ^{32}P into Rho* and 16.0 nmol of ^{32}P into the peptide substrate at 120 min in the presence of urea-washed ROS is taken as 100%. Phosphorylation of opsin (■), Rho* (●) and of the peptide substrate in the presence of either opsin (○) or Rho* (□) are as indicated above. A typical result of this experiment is shown.

phosphorylation of Rho*, in the presence of NH_2OH , was maintained during the initial phase at a rate similar to that in the control experiment shows that the phosphoryl transfer process itself is not affected by NH_2OH .

Evidence to rule out further the involvement of NH_2OH was provided by performing the phosphorylation of 11-mer Rho peptide in the presence of opsin. Figure 5 shows clearly that when compared with the parallel experiment containing urea-washed

ROS, in the presence of authentic opsin, there is a dramatic reduction in the ability of RK to phosphorylate either the peptide substrate or opsin. In addition, it should be noted that the phosphate incorporation in the dark samples, in the presence of opsin, was to a similar level: 4% for the peptide and 7% for opsin.

DISCUSSION

The experiments described above establish beyond doubt that peptide phosphorylation is a property of an activated form of RK and is formed by the interaction of the enzyme with a range of derivatives of Rho*, which retains the Schiff base linkage between the all-*trans*-retinylidene moiety and opsin. In a previous study [9], we had considered two broad mechanisms for the activation process in which the activated form of RK was either present only in the Rho* · RK complex [eqn (1)] or it could dissociate from the complex and exist as a freely diffusible species [eqn (2)]. Subsequently, preference was shown for the latter process [eqn (2)], because of the finding that RK when rapidly isolated from a suspension of Rho* and ATP was able to catalyse the phosphorylation of the dark-adapted form of rhodopsin [24], in a process known as 'high-gain' phosphorylation [25,26]. The latter conversion is considered to be a property of an activated form of RK.

Kinetic experiments reported in the literature [27] have shown that RK and Rho* form a tightly bound complex, the dissociation of which is progressively facilitated in the presence of ATP and, again, when these proteins become increasingly phosphorylated; RK by autophosphorylation and Rho* through conversion into multiphosphorylated-Rho*. A careful examination of the time-course profile in Figure 1 (filled circle), or the equivalent curves of Figures 2–4, shows that the rate of light-dependent peptide phosphorylation is not significantly impaired after 15 min of the initiation of incubation when both RK and Rho* are expected to be present in optimally phosphorylated forms. Under this condition of a large increase in the off-rate of RK, from the RK · Rho* complex, the peptide phosphorylation is maintained at the initial rate. This may be cited as evidence, albeit weak, in favour of the proposition that the catalyst, i.e. activated RK, can exist as a diffusible entity, as shown by eqn (2). Such a view is, however, questioned by the experiments of Rim et al. [15].

In conclusion, the cumulative evidence available to date clearly shows that RK (GRK1) and other GRKs, in particular GRK2 and GRK3 [13,14], are present in an inactive resting state and are activated only after interaction with the activated form of their cognate receptors. However, the intimate mechanistic details of this activation process still remain elusive.

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