Recoverin inhibits the phosphorylation of dark-adapted rhodopsin more than it does that of bleached rhodopsin: a possible mechanism through which rhodopsin kinase is prevented from participation in a side reaction

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In its resting state rhodopsin kinase is present in an inactive form and is activated after interaction with light-activated rhodopsin (Rho*). The activated rhodopsin kinase then phosphorylates Rho* but is also able to catalyse the phosphorylation of dark-adapted rhodopsin. A consequence of the latter behaviour of the activated kinase is that at low levels of bleach a large number of phosphoryl groups are incorporated per mol of Rho*. Recoverin- and Ca²⁺-dependent inhibition of rhodopsin kinase was found to be inversely related to the extent of bleaching; the lower the fraction of rhodopsin bleached, the greater the inhibition. The IC₅₀ of recoverin is approx. 1 µM at a 0.2% level of bleach and

about 5 µM in a fully bleached sample. The inhibitory effect of recoverin was studied separately on the phosphorylation of rhodopsin and Rho*. The formation of phosphorylated rhodopsin was inhibited 4.5-fold more strongly than that of phosphorylated Rho*. These results are interpreted to suggest that one of the roles of the recoverin-dependent regulation of the activity of rhodopsin kinase is to prevent the enzyme from participating in the unwanted phosphorylation of dark-adapted rhodopsin, directing it to fulfil its 'correct' function of quenching the transduction activity of Rho*.

INTRODUCTION

Light absorption by the visual receptor, rhodopsin, in retinal rod cells triggers a cascade process leading to the hydrolysis of cGMP. Since cation channels in the plasma membrane are kept open by cGMP, its hydrolysis causes the closure of the channels and results in membrane hyperpolarization. Under the aforementioned conditions, however, the Na⁺/Ca²⁺ exchanger continues to pump Ca²⁺ out of rod outer segments (ROS). Thus an additional result of the event is a decrease in [Ca²⁺], which is believed to be a signal for cell recovery. The cell is returned to the dark state by several processes including (i) restoration of the initial level of cGMP by guanylate cyclase which is activated at low [Ca²⁺] and (ii) down-regulation of the activated-receptor (Rho*) by phosphorylation catalysed by rhodopsin kinase ([1–5] and references therein). In addition to regulating the activity of guanylate cyclase, Ca²⁺ levels also influence the behaviour of rhodopsin kinase via a Ca²⁺-binding protein, recoverin [6–9], in bovine ROS or its counterpart, S-modulin, in the frog system [10].

The rhodopsin molecule contains several potential phosphorylation sites at its C-terminal domain, and up to nine [11] of these can be modified in ROS suspensions but only one *in vivo* [12]. Recent mechanistic studies have shown that rhodopsin kinase functions by a two-step process [eqn. (1)] in which it is first converted from an inactive into an active species by interaction with Rho* [13–15] and then this activated kinase can efficiently phosphorylate Rho*. However, following dissociation it is also able to catalyse the phosphorylation of certain synthetic peptides [13,14] and more significantly of dark-adapted rhodopsin (Rho) [15].

A profound consequence of the use by the activated rhodopsin kinase of Rho as a substrate is the incorporation, at low levels of bleach, of hundreds of phosphoryl groups per Rho* in suspensions containing intact ROS (a phenomenon dubbed 'high-gain' phosphorylation) [16] or tens of phosphoryl groups in reconstituted systems [15,17–22].

The Ca²⁺- and recoverin-dependent inhibition of rhodopsin kinase at different bleach levels, reported previously [21–23], has now been systematically investigated, and the time-dependence of the effect has been observed. In this paper we also report that, in the presence of Ca²⁺ and recoverin, the phosphorylation of Rho is inhibited more than is that of Rho*. These results suggest that the preferential inhibition may be used by the retinal rod cells to prevent rhodopsin kinase from participating in the unwanted phosphorylation of Rho, thus directing the enzyme to fulfil its essential physiological function of quenching the transduction activity of Rho*.

MATERIALS AND METHODS

[γ-³²P]ATP was purchased from Amersham, hydroxyapatite Bio-Gel HTP from Bio-Rad, and all other chemicals from either Sigma or Aldrich.

The Ca²⁺ buffer used was made from 26 mM CaCl₂ and 20 mM EGTA, pH 7.4, generating a final Ca²⁺ concentration of approx. 200 µM, with the free Ca²⁺ concentration being determined by a Ca²⁺-sensitive electrode [24]. The major buffer

Abbreviations used: Rho, dark-adapted rhodopsin (also unbleached rhodopsin); Rho*, bleached rhodopsin (also light-activated rhodopsin); Rho*-P, phosphorylated light-activated rhodopsin; Rho-P, phosphorylated rhodopsin; ROS, rod outer segments.

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used throughout the studies was 20 mM BTP, pH 7.4, containing 2 mM MgCl_2 , 0.1 mM EDTA, 1 mM benzamidine, 1 mM dithiothreitol and 0.2 mM PMSF (buffer A). The kinase extracts were generated in the same buffer but it also contained 280 mM NaCl and 0.04% Tween 80.

Retina from freshly slaughtered cattle were individually frozen in liquid nitrogen and stored at -70°C until required for generation of ROS, which was performed under dim red light. Rhodopsin kinase was extracted and purified from ROS employing a modified method of Pulvermüller et al. [25] and described in detail by us previously [26], with the rhodopsin kinase activity expressed as nmol of ^{32}P incorporated into receptor/min (6 to 12 units/ml of rhodopsin kinase extract were typically generated). Rhodopsin substrate required in phosphorylation assays was generated by washing ROS membranes with urea [27].

Recoverin

Recombinant myristoylated recoverin used in this work was prepared as described previously [22]. It should be noted that recoverin isolated from bovine retinae, in addition to possessing a myristoyl group, can contain other fatty acyl residues [28]; however, in this paper the name recoverin is used to designate the recombinant as well as the native forms.

Standard reconstituted rhodopsin phosphorylation assay

The standard reaction mixture of 50 μl contained urea-washed rhodopsin (5 μl ; 20 μM final concentration), $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5 μl ; 200 μM final concentration at approx. 10^6 c.p.m./nmol), Ca^{2+} buffer (5 μl), recoverin if present (10 μl) and rhodopsin kinase extract (25 μl ; minimum activity of 6 units/ml of extract). Samples were mixed, sonicated and incubated at 32°C before the introduction of light. Aliquots of volume 5 μl were taken at set times (see the Figure legends) and mixed with 5 μl of loading buffer (30 mM Na_2CO_3 , 1% SDS and 0.3% Bromophenol Blue) and subjected to SDS/PAGE (12% gels), with the relevant bands being excised and subjected to scintillation counting. Incorporations of 8000–24000 c.p.m. were generally obtained in light-activated samples, and 120–440 c.p.m. in the dark controls.

Ratios of Rho-P and Rho*-P formed at different levels of bleach

The methodology closely followed that described by us [15] and consisted of an initial incubation of 1 ml containing urea-washed rhodopsin (100 μl ; 25 nmol), $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (100 μl ; 500 μM final concentration; specific radioactivity 120000 c.p.m./nmol), rhodopsin kinase extract (550 μl ; minimum activity 10 units/ml of extract), Ca^{2+} /EGTA buffer (50 μl) and either recoverin (200 μl ; 4.4 μM) or buffer A. Each sample was exposed to calibrated light flashes to generate a known level bleaching (%), and the samples were then incubated in the dark at 32°C for 30 min, at which point a 20 μl aliquot was removed, mixed with 20 μl of loading buffer and analysed by SDS/PAGE (see above). The remainder of the sample was diluted to 4 ml with buffer A containing 100 mM NH_4OH to mop up released chromophore, and the sample pelleted at 50000 g for 15 min, before resuspending and washing the pellet twice in buffer A to remove unchanged ATP. The resultant pellets were mixed with 3 ml of 0.01 M imidazole, pH 7.4, containing 0.5% *N*-dimethyldodecylamine-*N*-oxide detergent (buffer B) and vigorously mixed and sonicated and then pelleted at 50000 g to remove non-solubilized material. The supernatant was divided into two, and one-half was supplemented with solubilized rhodopsin as a carrier (1 mg) and added directly to an hydroxyapatite column (7 cm $\times$ 1.2 cm

internal diameter; flow rate 0.33 ml/min). The second half of the sample, which acted as a control [15], was bleached and pelleted at 50000 g and the supernatant was supplemented with solubilized rhodopsin (1 mg) and added to a second hydroxyapatite column. The rhodopsin was eluted with a 0–300 mM phosphate gradient over 50 ml all in buffer B. Fractions were monitored for A_{500} and A_{280} to determine recovery of rhodopsin and its purity. Samples were then precipitated with trichloroacetic acid and counted by scintillation counting. Thus ^{32}P incorporation into both Rho and Rho* can be measured at any level of bleach [15], and from these values the partitioning of rhodopsin kinase between the phosphorylation of Rho and Rho* ($p\text{Rho}^*/p\text{Rho}$) can be expressed using the following equation:

$$\frac{p\text{Rho}^*}{p\text{Rho}} = \frac{{}^{32}\text{P into Rho}^*}{\text{{}^{32}\text{P into Rho}}} \times \frac{\text{amount of Rho in the sample}}{\text{amount of Rho}^* \text{ in the sample}}$$

Over the range of bleaching tested, i.e. 100 to 1%, this partitioning value was found to be almost constant (99 ± 21) and is assumed also to apply to very low bleaching levels. With this assumption, it is possible to use the $p\text{Rho}^*/p\text{Rho}$ ratio of 100 to calculate the number of phosphates incorporated into Rho/phosphate incorporated into Rho* at any level of bleach by the following equation:

$$\frac{\text{Phosphates in Rho}}{\text{Phosphates into Rho}^*} = \frac{100 - \% \text{ bleach}}{(p\text{Rho}^*/p\text{Rho}) \times \% \text{ bleach}}$$

This equation was used to generate the data for Figure 2.

RESULTS

In a reconstituted system consisting of urea-washed ROS membranes, rhodopsin kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, it was found that increased bleaching was accompanied by an increase in the overall incorporation of ^{32}P into the total receptor protein (Figure 1). However, when the data are expressed as ^{32}P

Figure 1 ^{32}P incorporation into receptor protein at various levels of bleaching

A reconstituted system containing urea-washed ROS membranes, rhodopsin kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was subjected to various levels of bleaching, incubated in the dark for 40 min and processed as described in the Materials and methods section. The data are expressed as total incorporation of ^{32}P into the receptor protein (●) and mol of ^{32}P incorporated/mol of Rho* (■). In the inset, the ● curve is enlarged to show the data at bleaching levels below 1%. Each data point represents the mean $\pm$ S.E.M. for three experiments.

Figure 2 Ratio of Rho-P and Rho*-P formed at different levels of bleach

Samples containing urea-washed rhodopsin, [γ - 32 P]ATP and rhodopsin kinase extract were subjected to graduated light flashes to generate known levels of bleaching (%). After a 30 min incubation period, samples were removed and assayed for total receptor phosphorylation (SDS/PAGE) and also Rho phosphorylation by hydroxyapatite chromatography analysis (see the Materials and methods section). ■, Experimentally determined values; —, theoretical profile at low levels of bleach.

Figure 3 Effects of various recoverin concentrations on rhodopsin phosphorylation at various bleach levels

Known concentrations of recoverin (0.4–20 μ M) were added to the standard rhodopsin-phosphorylation assay (see the Materials and methods section). Graded light flashes produced known levels of bleaching which are shown as follows: □, 100%; ◇, 6%; △, 1%; ○, 0.2% and 0.03%; the level of 32 P incorporation was determined. IC_{50} values were determined for each bleach data set and are shown in the inset. Each data point represents the mean $\pm$ S.E.M. for three separate experiments.

incorporation per Rho* (mol/mol), an inverse picture is obtained (Figure 1), showing a minimum incorporation at 100% bleach of approx. 2 32 P mol/Rho* and a maximum incorporation at 0.005% bleach of approx. 90 mol of 32 P/Rho*. The direct determination of the amounts of Rho-P and Rho*-P produced at bleaching levels of between 1 and 15% (Figure 2) showed that

Table 1 Inhibitory effects of recoverin on the formation of Rho-P and Rho*-P 3% bleach

Incubation mixtures of urea-washed rhodopsin, [γ - 32 P]ATP, rhodopsin kinase extract, Ca^{2+} buffer and, when present, recoverin (4.4 μ M) were subjected to a 3% bleach. Total receptor phosphorylation, Rho phosphorylation and Rho* phosphorylation were determined as in Figure 2. The results are the means $\pm$ S.E.M. for three separate experiments.

	— Recoverin	+ Recoverin
Total phosphate incorporated into receptor (nmol)	6.56 $\pm$ 0.61	2.54 $\pm$ 0.32
Phosphate incorporated into Rho* (nmol)	4.95 $\pm$ 0.46	2.37 $\pm$ 0.30
Phosphate incorporated into Rho (nmol)	1.61 $\pm$ 0.15	0.17 $\pm$ 0.02

the ratio of Rho-P/Rho*-P increased with decreased bleaching. When the data are extrapolated to lower levels of bleaching, using a numerical model (see the Materials and methods section), then at bleach levels below 1%, Rho-P is the predominant species. From the experiments of the type described in Figure 2, it can also be calculated that Rho is phosphorylated by rhodopsin kinase at a rate that is about 1% that of Rho*, hence Rho* is the favoured substrate of rhodopsin kinase.

The inhibitory effect of various concentrations of recoverin on rhodopsin kinase activity was studied at bleaching levels between 0.03 and 100%. Figure 3 shows that the IC_{50} of recoverin is shifted progressively to lower values with decreasing levels of bleach, the values being 0.8 μ M and 5.0 μ M at 0.03% and 100% respectively. Thus the higher the relative level of Rho in the system compared with Rho*, the more efficiently recoverin inhibits rhodopsin kinase.

The observations of Figure 3 led us to speculate that recoverin may be a more potent inhibitor of the phosphorylation of Rho than it is of its optimum substrate Rho*. This view was examined by studying the effect of recoverin on the formation of Rho-P and Rho*-P in incubation mixtures subjected to light flashes generating 3% Rho*. The level of bleaching was carefully selected from the systematic study in Figure 2 to ensure that sufficiently high levels of 32 P were incorporated into both the species of interest, i.e. Rho-P and Rho*-P, so that reliable assessment of the impairment of the labelling of these in the presence of recoverin could be made. Under the aforementioned conditions the incorporation of 32 P into the total receptor protein in the presence of 5 μ M recoverin was inhibited by approx. 62% (Table 1). However, when the phosphorylated mixture was fractionated, the formation of Rho*-P was found to be inhibited by 52% whereas that of Rho-P was inhibited by almost 90% (Table 1). These results show that, under the experimental conditions used, recoverin is about a 4.5-fold more potent inhibitor of the phosphorylation of Rho than of Rho*. The molecular basis of this selective inhibition is not yet known, but studies are under way to address this question.

Next, the time course of the inhibitory effect of recoverin at a concentration of 5 μ M was studied at two bleach levels, 0.2% and 100%. When the samples were analysed after 40 min, the phosphorylation of the low-bleached sample was inhibited by over 90% (Figure 4A) but that of the fully bleached sample was inhibited by only 50% (Figure 5A). More significant, however, is the observation that the inhibition displayed non-linear kinetic behaviour. From the initial rates the recoverin-dependent inhibition of rhodopsin phosphorylation during the first minute was found to be very similar for the low- as well as the high-

Figure 4 Kinetic profile for rhodopsin phosphorylation in the presence and absence of recoverin at 0.2% bleach

Employing the standard rhodopsin-phosphorylation assay (see the Materials and methods section) the time course for ^{32}P incorporation was followed over 60 min (**A**) for incubation mixtures containing no recoverin ($\square$) or $5\ \mu\text{M}$ recoverin ($\circ$) exposed to 0.2% bleach, and it was found that at the 60 min time point, recoverin had inhibited total ^{32}P incorporation by 91.5%. **B** focuses on the initial 2 min of the time course. The time courses were performed in duplicate. Values are means $\pm$ S.E.M. for three experiments.

bleached samples (Figures 4B and 5B). The fact that the full inhibitory effect of recoverin is not expressed in the 0.2% bleach sample during this initial period of the incubation possibly reflects the fact that during this phase the preferred substrate, Rho^* , is predominantly phosphorylated and it is only after Rho^* has been depleted that the phosphorylation of Rho becomes the main process that is more potently inhibited by recoverin.

The time-course study of Figures 4(A) and 5(A) provides a possible explanation for a contradiction in results reported in the literature [21,23]. Klenchin et al. [23], who found that recoverin had similar inhibitory effects at low and high levels of bleach, terminated their assays at about 2 min. Chen et al. [21], who found that the level of inhibition was dependent on the extent of bleaching, employed assay times of 40 min.

Finally, in view of a recent report that recoverin stimulated the activity of protein phosphatase 2A [29], which is involved in the dephosphorylation of Rho-P and $\text{Rho}^*\text{-P}$ [30–32], it was important to establish that the inhibition of phosphorylation observed in this work is not due to loss of ^{32}P by a dephosphorylation process. Accordingly, incubations involving

Figure 5 Kinetic profile for rhodopsin phosphorylation in the presence and absence of recoverin at 100% bleach

The experimental details and graphical notations are the same as in Figure 4 except that the samples were fully bleached (100%). The level of recoverin inhibition of total receptor phosphorylation at 60 min was found to be 41.5%. Each time course was performed in duplicate.

recoverin were performed in the presence of either a specific inhibitor of protein phosphatase 2A, okadaic acid [32], or a general phosphatase inhibitor, NaF [30]. In both cases no significant effect of these agents was noted (results not shown).

DISCUSSION

Regarding the physiological relevance of these findings, two specific features of the retinal rod cell need to be considered. First, the cell contains extremely high concentrations of rhodopsin, about 0.1 mM in human and 2.0 mM in frog ROS [33], and as a consequence rhodopsin is in massive excess (over 1000-fold) over rhodopsin kinase [34]. Secondly, under most physiologically relevant light conditions, only a fraction of the rhodopsin pool is present in a bleached state [35], which means that there is a very high ratio of Rho/Rho^* . Both these factors would appear to favour a situation where Rho phosphorylation would predominate over Rho^* . Previous studies, notably by Bownd's group [16] on 'high-gain' phosphorylation, together with the theoretical model emerging from our work [15,26], forecast the following consequences of such a scenario. When

considering a 1% bleach, equal numbers of Rho* and Rho molecules would be phosphorylated, whereas at 0.1% bleach the level of phosphorylation of Rho would be approximately 10 times greater than that of Rho* and at 0.005% bleach the potential phosphorylation of Rho would outstrip that of Rho* by 200:1. These numerical values, although not to be taken literally, highlight the fact that the ability of transiently activated rhodopsin kinase (see the Introduction) to phosphorylate the resting state of the receptor could lead to a gradual accumulation of Rho-P, thus depleting the cell of competent receptor molecules. The results described in this paper provide a possible mechanism to counter such an undesirable outcome by a Ca²⁺-plus-recoverin-dependent regulation of rhodopsin kinase, which prevents the enzyme from participating in the unwanted phosphorylation of Rho, leaving it to fulfil its 'correct' function of quenching the transduction activity of Rho* through its phosphorylation.

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