

Rhodopsin phosphorylation in bovine rod outer segments is more sensitive to the inhibitory action of recoverin at the low rhodopsin bleaching than it is at the high bleaching

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Received 19 March 1997

Abstract Recoverin, a calcium-binding protein, is supposed to have rhodopsin kinase as a target in the retinal rod cell. In the present work, we show that efficiency of recoverin as an inhibitor of rhodopsin phosphorylation in bovine rod outer segments is inversely proportional to the level of rhodopsin bleaching. These results, together with the data obtained previously in a reconstituted system (Senin et al. (1997) *Biochem. J.* 321, 551–555), allow us to hypothesize that recoverin might be responsible for a Ca^{2+} -dependent regulation of the kinase *in vivo*, preventing it from participating in the phosphorylation of unbleached rhodopsin.

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Key words: Photoreception; Phosphorylation; Rhodopsin; Rhodopsin kinase; Recoverin; Calcium-binding protein; Retinal rod cell

1. Introduction

The phosphorylation of bleached rhodopsin (Rho^*) by rhodopsin kinase (RK) is commonly accepted to be responsible for the desensitization of the visual receptor, Rho^* [1]. The overall process of the Rho^* phosphorylation consists of two stages [2–6]: initially the kinase, present in an inactive state in the absence of Rho^* , is converted into an active state (RK^*) by interaction with Rho^* ; then Rho^* which contains 9 potential sites for phosphorylation [7] is phosphorylated by RK^* . It should be stressed that the kinase after being activated by Rho^* also is capable of phosphorylating the non-bleached rhodopsin (Rho), although Rho is a much poorer substrate of the enzyme than is Rho^* [4]. Due to the ability of RK^* to phosphorylate Rho, the phosphate incorporation into rhodopsin, calculated per Rho^* , can reach tens in reconstituted systems [4,8–10] or even hundreds in suspensions of electropermeabilized rod outer segments (ROs) [11]. However, the physiological function of this phenomenon called ‘high gain rhodopsin phosphorylation’ [11] remains a mystery.

In the reconstituted system the Ca^{2+} -binding protein, recoverin [12,13], which is supposed to have RK as the target [14–16], inhibits high gain phosphorylation at high Ca^{2+} concentrations [8,10]. Besides there are indications that this high gain reaction is sensitive to Ca^{2+} in suspensions of electropermeabilized ROs, being more active at nM than at mM Ca^{2+} levels (in review [17]).

Our recent work [10] has suggested that high gain phosphorylation is, merely, a consequence of an unwanted side-reaction, the conditions for which are created by the huge excess of Rho over Rho^* , in ROS membranes, at low levels of illumination that corresponds to the normal working regime of the retinal rod. Using a reconstituted system, we have found in [10] that recoverin inhibits the phosphorylation of Rho more effectively than it does that of Rho^* and suggested that the preferential inhibition may be used *in vivo* to prevent the kinase from participating in a side-reaction of the Rho phosphorylation. Now, in developing the work [10], we have compared the sensitivity of Rho^* and Rho phosphorylation to recoverin in ROS suspensions, i.e. under conditions which are closer to the *in vivo* situation than is the reconstituted system investigated earlier.

2. Materials and methods

$[\gamma\text{-}^{32}\text{ATP}]$ was purchased from the Physicoenergetical Institute (Russia), all other chemicals were obtained through ‘Sigma’.

ROs prepared from frozen retinae under dim red light [18] were frozen in liquid nitrogen and stored at -70°C . Rhodopsin concentration was determined by the difference in the optical densities of the samples at 500 nm before and after illumination in the presence of cetyltrimethyl ammonium bromide taking $\epsilon = 42\,000$ [19]. Recombinant myristoylated recoverin [9] was used as a recoverin source; the protein concentrations were estimated by absorbance at 280 nm taking $\epsilon = 36\,400$ [20]. SDS-PAGE was performed as described in [21]. $[\text{Ca}^{2+}]_i$ was determined by using Ca^{2+} -sensitive electrode [22].

RK, endogenously present in ROS aliquots, was assayed as described in [10] at 30°C in the reaction mixture (50 ml) containing 20 mM Tris-HCl (pH 7.4), 140 mM NaCl, 2 mM MgCl_2 , 500 μM $[\gamma\text{-}^{32}\text{ATP}]$ (2.5×10^4 cpm/nmol), 100 μM GTP, 10 μM rhodopsin in the content of ROS membranes (the corresponding concentration of endogenous RK in the sample could be estimated as 0.01–0.1 μM [23]), 1 mM EGTA or 1 mM EGTA+1.26 mM CaCl_2 ($[\text{Ca}^{2+}]_i < 1$ nM or 200 μM respectively); 0–20 μM recombinant myristoylated recoverin (as indicated) and 50 mM NaF. The reaction was initiated by a light flash (percentage of rhodopsin bleaching as indicated) and the addition of ATP 30 s after the flash. At times shown in the figure legends aliquots were taken from the mixture and mixed with the SDS-PAGE sample buffer to stop the reaction. After SDS-PAGE of the samples, zones of rhodopsin were cut out and ^{32}P incorporation was estimated by Cherenkov counting in plastic tubes; the counting level was 250–30 000 cpm per sample from which the ‘dark’ level (80–150 cpm) was subtracted in all cases. The data represented in Figs. 1–3 and Table 1 are means $\pm$ S.D. of triplicate assays carried out on three occasions.

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Abbreviations: Rho, unbleached rhodopsin; Rho^* , bleached rhodopsin; Rho-P, phosphorylated unbleached rhodopsin; Rho^* -P, phosphorylated bleached rhodopsin; RK, non-activated rhodopsin kinase; RK^* , activated rhodopsin kinase; ROS, rod outer segment; $[\text{Ca}^{2+}]_i$, free Ca^{2+} concentration

3. Results

The initial increase of the ^{32}P incorporation into rhodopsin in ROS suspensions is followed by its dephosphorylation which is more pronounced at 0.2% (Fig. 1A) than at 100% (Fig. 1B) rhodopsin bleaching; the protein phosphatase inhibitor, NaF [24] fully arrests the dephosphorylation in both cases. Since the recoverin effect upon RK (but not upon the phosphatase [25]) has been our only interest in this work, all the subsequent experiments on the recoverin effect upon rhodopsin phosphorylation in the ROS suspension were performed in the presence of NaF so that the rhodopsin phosphorylation would be determined by the kinase and not affected by the phosphatase.

In the ROS suspension used in the present work, the maximal ^{32}P incorporation into rhodopsin (calculated per mole Rho^*) was equal to 1.4 and 35 at 100% and 0.2% rhodopsin bleaching, respectively (see Fig. 1); since the maximal incorporation in one Rho^* molecule may not exceed 9 [7], it is clear that at least 26 P of 35 incorporated at the low bleaching represented the formation of the Rho-P . Direct quantitative determination of the $\text{Rho-P/Rho}^*\text{-P}$ ratio in the reconstituted system [10] has shown that at $<1\%$ rhodopsin bleach, Rho-P

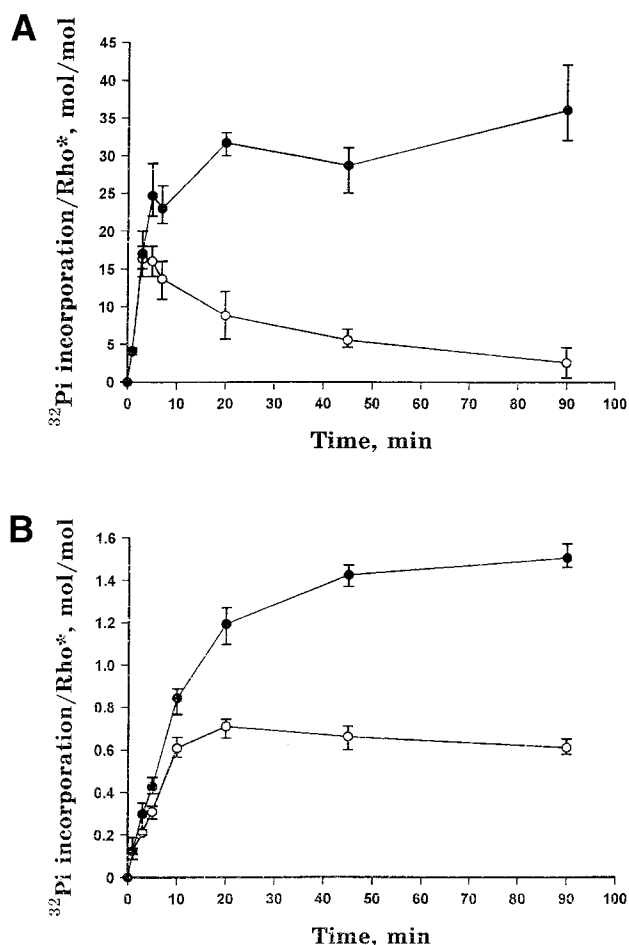


Fig. 1. Time dependence of the level of ^{32}P incorporation into rhodopsin in ROS suspensions at 0.2% (A) and 100% (B) rhodopsin bleaching in the presence (●) or in the absence (○) of 50 mM NaF. $[\text{Ca}^{2+}]_i = 200 \mu\text{M}$; for other details of this and the following experiments (Figs. 2 and 3 and Table 1) see Section 2.

Table 1

Comparison of the half-times ($t_{1/2}$) of rhodopsin phosphorylation in ROS suspensions at the high (100%) and the low (0.2%) rhodopsin bleaching

Conditions	bleaching, %	$t_{1/2}^a$ (min)	Recoverin effect ^b
5 μM recoverin			
–	100	14 ± 3.5	
+	100	11 ± 4.0	1.3
–	0.2	12 ± 2.0	
+	0.2	1 ± 0.4	12.0

The level of ^{32}P incorporation into rhodopsin in ROS suspensions was measured in reaction mixtures (see Section 2), containing 50 mM NaF and 200 μM $[\text{Ca}^{2+}]_i$, in the presence or in the absence of recoverin at two levels of rhodopsin bleaching as indicated in the table.

^a $t_{1/2}$ is referred to as the reaction time whereby the total level of ^{32}P incorporation into rhodopsin is equal to 1/2 of the maximal one.

^b'Recoverin effect' is referred to as the ratio of $t_{1/2}$ in the absence and in the presence of recoverin.

was the main product whereas at a bleach level of $>5\%$, $\text{Rho}^*\text{-P}$ was almost the only phosphorylated species in the system. Although the data obtained in the reconstituted system cannot be directly applied to ROS suspensions they allow us, if only qualitatively, to assume that in the suspension at bleaches much lower than 1% Rho-P predominates over $\text{Rho}^*\text{-P}$; as for rhodopsin bleaches about 100%, the main (or the only) phosphorylated species is represented by $\text{Rho}^*\text{-P}$. An additional argument in favour of this assumption follows from the similarity of the plots ' ^{32}P incorporation into rhodopsin vs. rhodopsin bleaching' in the ROS suspension (Fig. 2) and in the reconstituted system (compare with Fig. 1 in [10]): in both cases the half-maximal incorporation manifests at about 1% bleaching.

The following experiments indicate that in a ROS suspension, recoverin inhibits phosphorylation of Rho more effectively than it does that of Rho^* . First, the recoverin addition caused an increase in the inhibition of the ^{32}P incorporation into rhodopsin which was more pronounced at the low rhodopsin bleaching than at high bleaching (not shown). (Without recoverin addition, the level of ^{32}P incorporation into rhodopsin at $[\text{Ca}^{2+}]_i = 200 \mu\text{M}$ was approximately 25% lower than that at $<1 \text{ nM}$ $[\text{Ca}^{2+}]_i$ which could be explained by the presence of 'endogenous' recoverin in the ROS suspension [16] and possibly by the direct inhibitory action of the high Ca^{2+} concentration upon rhodopsin phosphorylation). Second, the plot ' ^{32}P incorporation into rhodopsin vs. recoverin concentration' was shifted towards lower recoverin concentrations as the rhodopsin bleaching decreased from 100% to 0.2% (Fig. 3): the corresponding values for the half-maximal inhibitory concentration of recoverin were about 7 and 2.5 μM . Third, a comparison of the recoverin effect on the time course of Rho^* and Rho phosphorylation in the presence of Ca^{2+} (Table 1) revealed a particularly striking difference: at the high bleaching the effect was only 1.3-fold (the reaction half-time was 14 ± 3.5 and 11 ± 4 min in the absence and in the presence of 5 μM recoverin respectively) whereas at the low bleaching a 12-fold effect was manifested ($t_{1/2}$ decreased from 12 ± 2 min to 1 ± 0.4 min as a result of recoverin addition).

Therefore, one may conclude that rhodopsin phosphorylation in bovine ROS suspensions is more sensitive to the inhibitory action of recoverin at low rhodopsin bleachings, i.e. at the conditions under which Rho predominates over Rho^* .

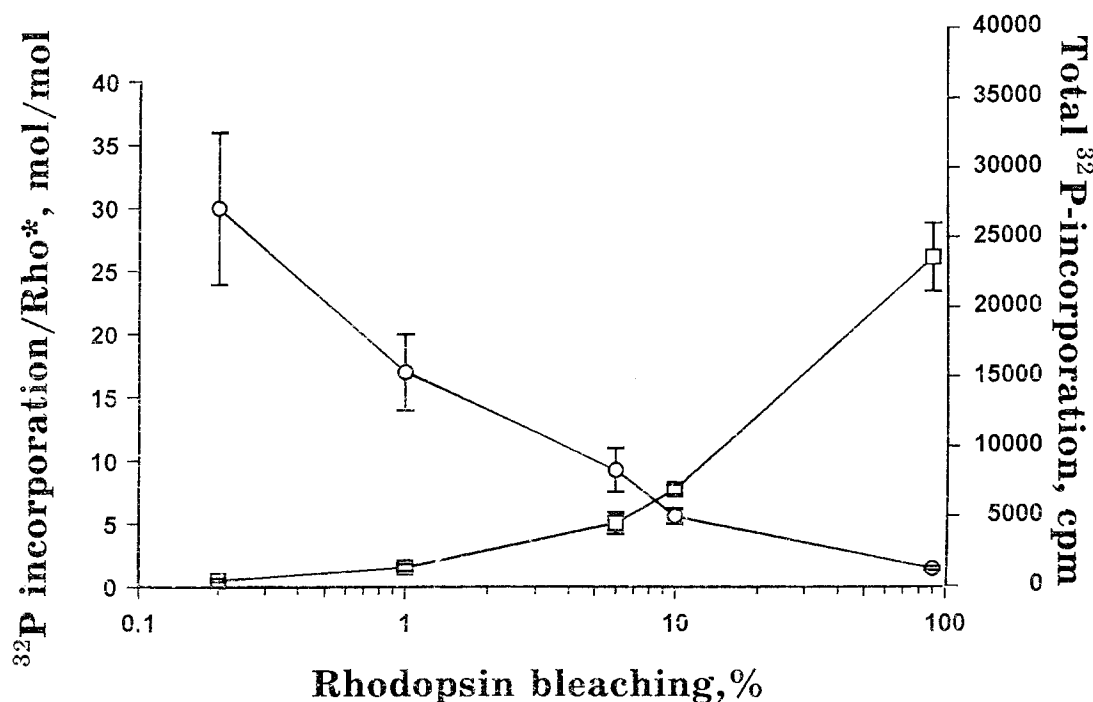


Fig. 2. Dependence of the total (□) and the relative (○) ^{32}P incorporation into rhodopsin on rhodopsin bleaching in the ROS suspension. 50 mM NaF and 200 μM $[\text{Ca}^{2+}]_f$ were present, the reaction time was 40 min.

4. Discussion

The retinal rod cell can function as a quantum counter (in review [26]). Such a high sensitivity is reached due to the packing of rhodopsin molecules in the rod light antenna, ROSs to the unprecedented – for receptor proteins – concentration in the range 0.1–1 mM (in review [27]). This should unavoidably create a problem for the Rho* turn-off mechanism since the key enzyme involved in the process, RK will need to find and phosphorylate a few Rho* molecules in the presence of the vastly larger number (an order of 10^6) of non-

bleached receptors. Thus the possibility exists that RK, in spite of its preference for bleached rhodopsin compared to unbleached rhodopsin [4], may phosphorylate Rho in addition to Rho*. Indeed such a reaction does occur in vitro at low Ca^{2+} concentrations [8,10,17] and is responsible for the phenomenon dubbed as high gain rhodopsin phosphorylation [11]. In isolated frog retina and in the retina of living frogs, however, under physiological light conditions only 1–2% of the total rhodopsin pool is phosphorylated [28]. If high gain rhodopsin phosphorylation observed in vitro also operated in vivo then a much higher pool of phosphorylated rhodopsin will be expected to be present (cf. [10]). This not being the case suggests the existence of a mechanism in vivo which prevents RK from the wasteful phosphorylation of Rho directing the kinase to fulfil its 'correct' function of Rho* desensitization. The results of this and our recent work [10] suggest that recoverin could serve such a role.

Our working hypothesis on the in vivo role of recoverin is as follows. *In the dark*: Rho* and hence RK* are absent; RK is in a complex with recoverin due to high $[\text{Ca}^{2+}]_f$ in the ROS cytoplasm. *Upon illumination*: Rho* appears and initiates the signal transduction; cytoplasmic $[\text{Ca}^{2+}]_f$ decreases which favors the dissociation of the RK–recoverin complex; Rho* now binds RK and converts it into RK* which phosphorylates Rho*; the appearance of RK* creates conditions for Rho phosphorylation; recoverin competes with Rho for RK* (it is suggested that $[\text{Ca}^{2+}]_f$ is high enough for this) and prevents RK* from catalyzing the Rho phosphorylation.

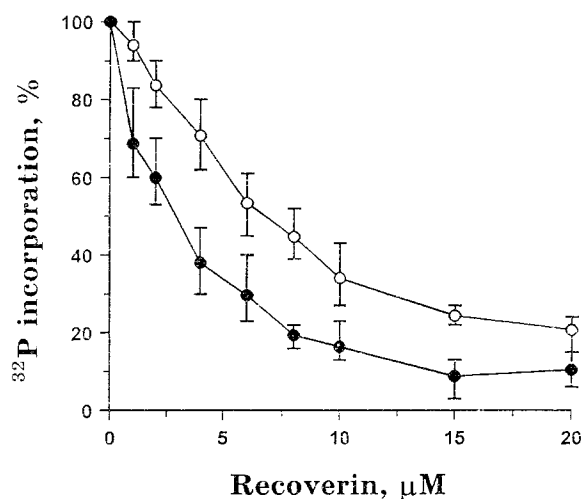


Fig. 3. Dependence of ^{32}P incorporation into rhodopsin in ROS suspensions on recoverin concentration at 100% (○) and 0.2% (●) rhodopsin bleaching. 50 mM NaF and 200 μM $[\text{Ca}^{2+}]_f$ were present, the reaction time was 40 min. The incorporation obtained in the absence of recoverin is taken as 100%.

Acknowledgements: We are indebted to Dr. Kevin R. Dean and Nina McCarthy for helpful discussions, to Sergey V. Shulga-Morskoy for participating in the preparation of recoverin and to Irina P. Vorobjkina, Dr. Larissa L. Adlina and Maureen Smith for their excellent assistance. This work was supported in part by grants from the Royal Society of Great Britain, the Russian Foundation for Basic Research

(N 94-04-11673), the 'International Projects' of the Russian State Committee of Science and Technology, the International Soros Science Education Programme (I.S.S.) and the Medical Research Council (UK).

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