Calcium-bound recoverin targets rhodopsin kinase to membranes to inhibit rhodopsin phosphorylation

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Abstract In rod photoreceptor cells, Ca^{2+}-bound recoverin associates with disk membranes and inhibits light-dependent phosphorylation of rhodopsin. However, the functional significance of Ca^{2+}-induced membrane association of recoverin has not been fully evaluated. We found that Ca^{2+}-bound recoverin forms a complex with rhodopsin kinase preferentially at the membrane surface. Addition of increasing amounts of membranes promoted the membrane association of recoverin, and remarkably suppressed rhodopsin kinase activity. It was concluded that the Ca^{2+}-recoverin-rhodopsin kinase complex is stabilized by membrane association, leading to effective suppression of the kinase activity.

Key words: Recoverin; Ca^{2+}-binding protein; Photoreceptor cell; Rhodopsin; Rhodopsin kinase; G protein-coupled receptor kinase

1. Introduction

In vertebrate rod cells, a light signal initiates cGMP hydrolysis by the sequential activation of rhodopsin, transducin and cGMP phosphodiesterase. Light-induced cGMP hydrolysis disrupts Ca^{2+} influx through the cGMP-gated channel and lowers the intracellular Ca^{2+} concentration through the continued extrusion of cytosolic Ca^{2+} by the Na^+-Ca^{2+}/K^+ exchanger [1]. The light-induced decrease in Ca^{2+} concentration plays an important role in light adaptation of photoreceptor cells [2,3]. Frog S-modulin and bovine recoverin, new members of the EF-hand superfamily, mediate this role of Ca^{2+} [4] by inhibiting rhodopsin phosphorylation at high Ca^{2+} concentration [5,6]. The N-terminus of recoverin is heterogeneously acylated by one of four distinct fatty acids, C14:0, C14:1, C14:2 and C12:0 [7], by which recoverin is targeted to membranes in a Ca^{2+}-dependent manner [8,9]. Non-acylated 'soluble' recoverin is functionally active [10–12], and hence Ca^{2+}-induced membrane association might be unnecessary for the function, although *N*-myristoylation elevates recoverin activity [11]. Interestingly, the recoverin isoforms with distinct *N*-acyl groups showed a difference in maximal ability to inhibit rhodopsin phosphorylation, implying an important role of the membrane association of recoverin [13]. The present study was undertaken to see how the Ca^{2+}-dependent membrane interaction contributes to the recoverin function. Here we demonstrate that the membrane association of recoverin at higher Ca^{2+} concentration effectively quenches rhodopsin kinase activity by stabilizing the Ca^{2+}-recoverin-rhodopsin kinase complex at the disk membrane surface.

2. Materials and methods

2.1. Preparation of rod outer segment membranes and proteins

Rod outer segment (ROS) membranes were isolated from freshly dissected bovine retinas as described previously [14]. Recoverin and rhodopsin kinase (RK) were extracted from the membranes, and were purified to near homogeneity [13]. Meanwhile, the ROS membranes were washed with 5 M urea (urea-washed ROS membranes) for complete removal of soluble and peripheral proteins such as recoverin, RK and transducin [13]. Porcine brain βγ subunit (Gβγ) of GTP-binding regulatory protein (G protein) was prepared from Gs-enriched fractions, whereas β-adrenergic receptor (βAR) was partially purified from bovine lung membranes and then reconstituted in phospholipid vesicles composed of phosphatidylcholine, phosphatidylinositol and cholesterol (12:12:1) [15]. Bovine β-adrenergic receptor kinase (βARK) was expressed in Sf9 cells and purified as described previously [15].

2.2. Phosphorylation assay

Rhodopsin in urea-washed ROS membranes was exposed to orange light (>540 nm), and then phosphorylated by RK in a Ca^{2+}/EGTA buffer [13]. Under similar conditions, rhodopsin was phosphorylated by βARK in the presence or absence of Gβγ. On the other hand, the ligand-dependent phosphorylation of βAR by either βARK or RK was performed as follows. Prior to the reactions, RK was autophosphorylated with 50 nM unlabelled ATP for 2 h at 25°C to minimize ^{32}P labeling of RK which comigrates with ^{32}P-labeled βAR in SDS-PAGE [16]. Then, either βARK or autophosphorylated RK was incubated for 30 min at 25°C with βAR and recoverin in 50 mM Tris-HCl buffer (pH 7.9) containing 6 mM $MgCl_2$, 5.04 mM EGTA, 0.001%(w/v) Tween 80, 0.5 mg/ml ovalbumin, 10 μM (−)-isoproterenol and 5 μM [γ-^{32}P]ATP (in a total volume of 50 μl). The phosphorylation reaction was terminated by adding 950 μl of ice-cold EDTA solution (20 mM Tris-HCl, 30 mM EDTA, 5 mM EGTA and 250 mM NaCl; pH 7.9), and ^{32}P-labeled βAR in vesicles was pelleted by centrifugation (436000×*g* for 60 min at 4°C) to remove ^{32}P-labeled RK in the supernatant. The pelleted materials were dissolved in 50 μl of SDS-PAGE sample solution and subjected to the SDS-PAGE, followed by autoradiography.

3. Results and discussion

As demonstrated previously [13], the rate of rhodopsin phosphorylation catalyzed by RK was suppressed by recoverin to about 30% when Ca^{2+} concentration was increased from 0.1 μM to 1.7 μM (Fig. 1, solid bars). The experiments were performed in a reconstituted system composed of purified recoverin, RK and rhodopsin in urea-washed ROS membranes, indicating that Ca^{2+}-bound recoverin inhibits the functional interaction between photo-activated rhodopsin and RK. The Ca^{2+}-induced membrane association of recoverin suggests rhodopsin-recoverin interaction, but the following

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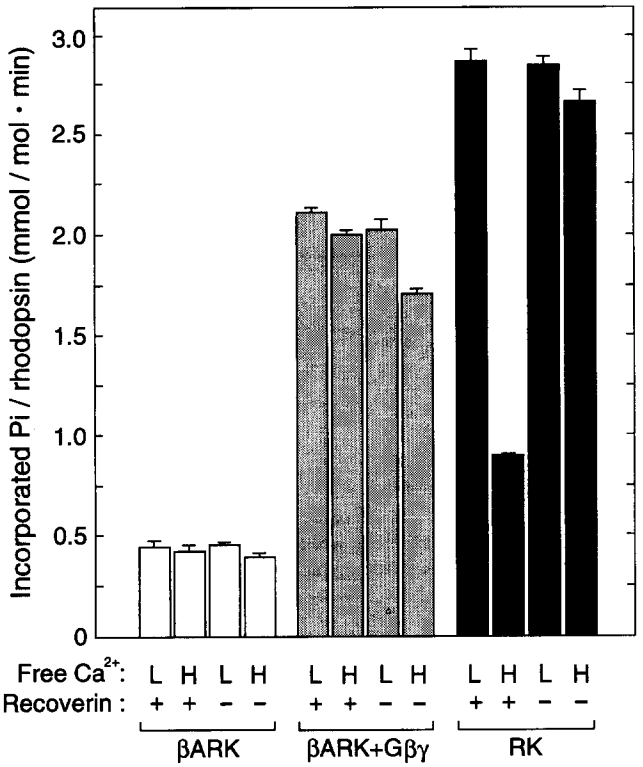

Fig. 1. Effects of Ca^{2+}/recoverin on the light-dependent rhodopsin phosphorylation by RK or βARK. In the presence (6 μM; +) or absence (−) of recoverin, rhodopsin (20 μM) in urea-washed ROS membranes was phosphorylated by 40 nM βARK without or with 400 nM Gβγ or by 16 nM RK at 0.1 μM (L) or 1.7 μM (H) free Ca^{2+}. The initial rates of phosphorylation represent the means ± range of variation of duplicate samples.

data argued against it. As shown in Fig. 1 (open bars), Ca^{2+}/recoverin had no effect on the rate of light-dependent rhodopsin phosphorylation by βARK which is another member of the G protein-coupled receptor kinases. Unlike RK, βARK is stimulated by Gβγ [17–19], but the Gβγ stimulated βARK activity was also unaffected by the addition of Ca^{2+}/recoverin (Fig. 1, stippled bars). By contrast, the rate of βAR phosphorylation catalyzed by RK was suppressed to 67% by recoverin in a Ca^{2+}-dependent manner (Fig. 2, solid bars). Again, the phosphorylation of βAR by Gβγ-stimulated βARK was insensitive to Ca^{2+}/recoverin (Fig. 2, stippled bars). These observations indicate that recoverin inhibits the receptor phosphorylation via specific interaction with RK. The difference in the inhibitory effect of recoverin on RK between the experiments (Fig. 1Fig. 2) can be ascribed to the lipid compositions of the receptor-containing membranes, i.e. native ROS membranes (Fig. 1) and artificial phospholipid vesicles (Fig. 2). The recoverin-membrane interaction strongly affects the degree of inhibition of the receptor phosphorylation, due to the stabilizing effect of membranes on the Ca^{2+}-recoverin-RK complex (see below).

Originally, it was reported that RK is present in cytosol fraction [20], but it associates with rhodopsin in membranes upon absorption of light. The specific interaction of recoverin with RK, both of which translocate between the cytosol and membranes, has raised an important question of how and where recoverin interacts with RK. To answer it, we investigated the effects of Ca^{2+} and recoverin on the distribution of RK between soluble and membrane fractions, which were separated from each other by centrifugation in the dark (without photo-activation of rhodopsin). In the absence of recoverin, the majority (70%) of RK activity was present in the supernatant at Ca^{2+} concentrations of both 0.1 and 1.7 μM (Fig. 3). By contrast, the addition of 1.0 μM recoverin at higher Ca^{2+} concentration (1.7 μM), markedly increased the amount of membrane-bound RK, and the ratio of the membrane-bound form reached up to ~80% on the addition of an excess amount of recoverin (6.0 μM). At lower Ca^{2+} concentration (0.1 μM), however, the excess recoverin resulted in no effect on the distribution (Fig. 3). Here it should be emphasized that, even at higher Ca^{2+} concentration, 80% of added recoverin (6 μM) was detected in the supernatant under the conditions employed (data not shown), while the majority (80%) of RK was in the membranes. Such a contrast in the distribution clearly indicates that the two events of interaction of Ca^{2+}-bound recoverin with membranes and with RK are closely related with each other, and perhaps cooperative: That is, RK has a higher affinity for membrane-bound recoverin than for the soluble form, or alternatively the affinity of Ca^{2+}-bound recoverin for membranes becomes much stronger

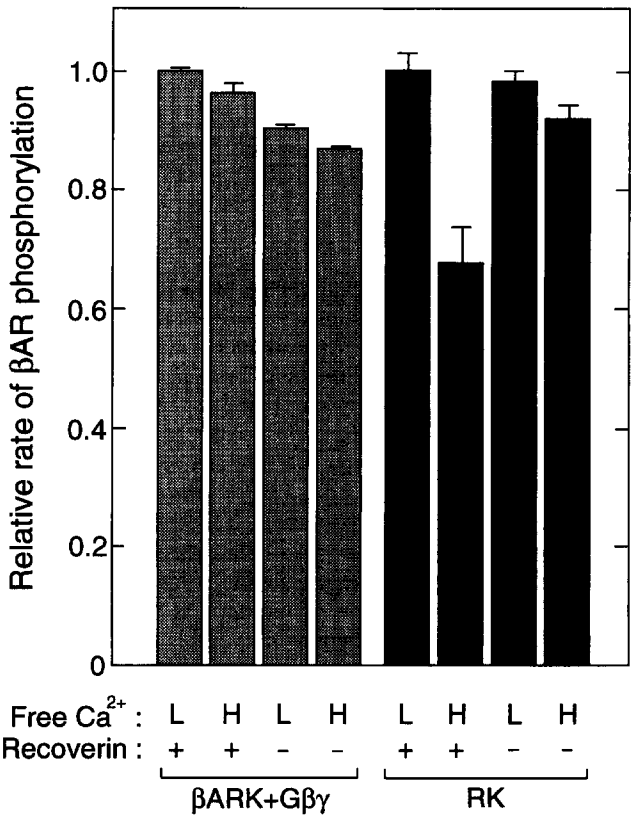

Fig. 2. Effects of Ca^{2+}/recoverin on the ligand-dependent βAR phosphorylation by RK or βARK. In the presence (6 μM; +) or absence (−) of recoverin, isoproterenol-stimulated βAR (3.6 nM) in phospholipid vesicles was phosphorylated by 8 nM RK or by 1.7 nM βARK with 25 nM Gβγ at 0.1 μM (L) or 1.7 μM (H) free Ca^{2+}. Rates of phosphorylation (means ± range of variation of duplicate samples) catalyzed by βARK were normalized to the rate at 0.1 μM free Ca^{2+} with recoverin, and the rates of phosphorylation catalyzed by RK were normalized independently. The observed rates of phosphorylation by RK and βARK at 0.1 μM free Ca^{2+} with recoverin were 2.25 ± 0.07 and 7.80 ± 0.02 mmol mol^{-1} min^{-1}, respectively.

when it is complexed with RK. In either case, we can conclude that the complex of Ca^{2+}-recoverin-RK is located preferentially at the membrane surface.

The importance of the membrane association of the complex for regulating RK activity was clearly shown by the following experiments, in which the amount of membrane-bound recoverin was varied in the mixture of the phosphorylation reaction. For this purpose, various amounts of boiled ROS membranes (see legend to Fig. 4) were mixed with a constant amount of unboiled membranes which served as the substrate for RK. In the absence of the substrate membranes, heat-denatured rhodopsin in the boiled membranes was not phosphorylated by RK at all (data not shown). The addition of the highest concentration of boiled membranes (0.76 mg/ml) to the substrate membranes slightly lowered the rate of rhodopsin phosphorylation in the absence of recoverin (Fig. 4A, open circles). By contrast, in the presence of 6 μM recoverin, rhodopsin phosphorylation was markedly inhibited by the addition of increasing amounts of boiled membranes in a dose-dependent fashion (Fig. 4A, closed circles). The inhibition was not attributed to the change in Ca^{2+} sensitivity of recoverin; i.e. the addition of boiled membranes enhanced the level of maximal inhibition, but had no effect on the Ca^{2+} sensitivity of the inhibition (Fig. 4B). Under the conditions of Fig. 4, the amount of membrane-bound recoverin increased in parallel with that of boiled membranes. However, 40% of the added recoverin remained in the supernatant at the highest concentration of boiled membranes (0.76 mg/ml), while the rate of rhodopsin phosphorylation catalyzed by RK was suppressed to less than 10% of the maximal rate even with smaller amounts of boiled membranes. These observations (Fig. 3Fig. 4), together with the finding that non-acylated recoverin is functionally active [10–12], indicate that RK activity is inhibited by the association with Ca^{2+}-bound recoverin, and that the complex of Ca^{2+}-recoverin-RK is stabilized by the membrane association, leading to efficient suppression of RK activity.

It is noteworthy that, at higher Ca^{2+} concentration, RK remains inactivated in the close vicinity of its substrate rhodopsin. The Ca^{2+}-dependent membrane localization of the complex of Ca^{2+}-recoverin-RK seems to have two important roles. First, at higher Ca^{2+} concentration, RK is effectively inhibited by the stabilizing effect of membranes on the com-

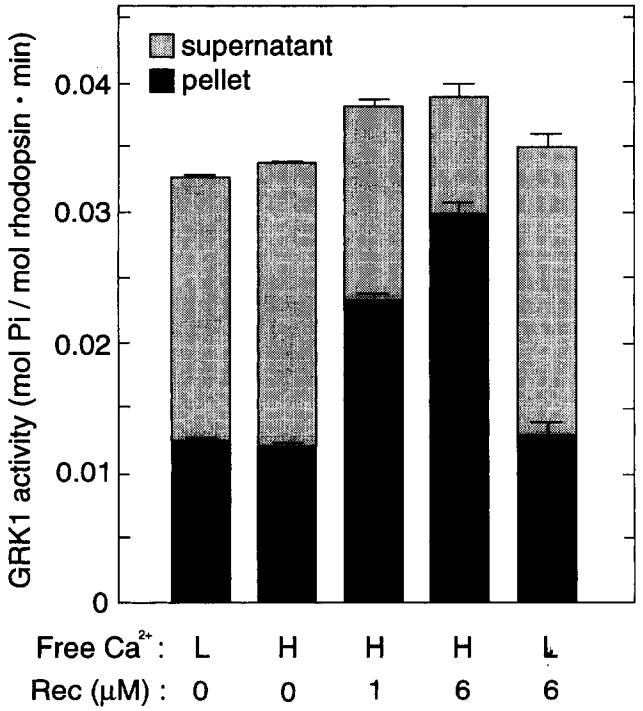

Fig. 3. Ca^{2+}/recoverin-dependent membrane association of RK. Urea-washed ROS membranes (10 μM rhodopsin) were mixed with 20 nM RK and various concentrations of recoverin in 50 μl of 50 mM Tris-HCl buffer (pH 7.9) containing 6 mM $MgCl_2$, 1 mg/ml ovalbumin, and 0.004% (w/v) Tween 80. Free Ca^{2+} concentration of the mixture was adjusted to 0.1 μM (L) or 1.7 μM (H). The mixtures were incubated in the dark at 25°C for 4 min, and then centrifuged at 39000×*g* for 10 min at 4°C. To estimate the amounts of RK partitioned into the membrane pellet and supernatant, the kinase activities of the two fractions were measured under equivalent conditions. That is, the supernatant was mixed with urea-washed ROS membranes (10 μM rhodopsin at a final concentration) in 50 mM Tris-HCl buffer (pH 7.9) containing 6 mM $MgCl_2$, 8 mM EGTA, 3.6 mM Ca^{2+}, 0.002% (w/v) Tween 80 and 200 μM [γ-^{32}P]ATP (in a total volume of 70 μl). Meanwhile the pelleted membranes were suspended in a buffer to give the reaction mixture with the same composition as above. Free Ca^{2+} concentration of all the mixtures was lowered to 10 nM to eliminate the inhibitory effect of recoverin on rhodopsin phosphorylation. The phosphorylation reaction was performed under room light as described previously [13], and the initial rates of the reactions were expressed as means ± range of variation of duplicate samples.

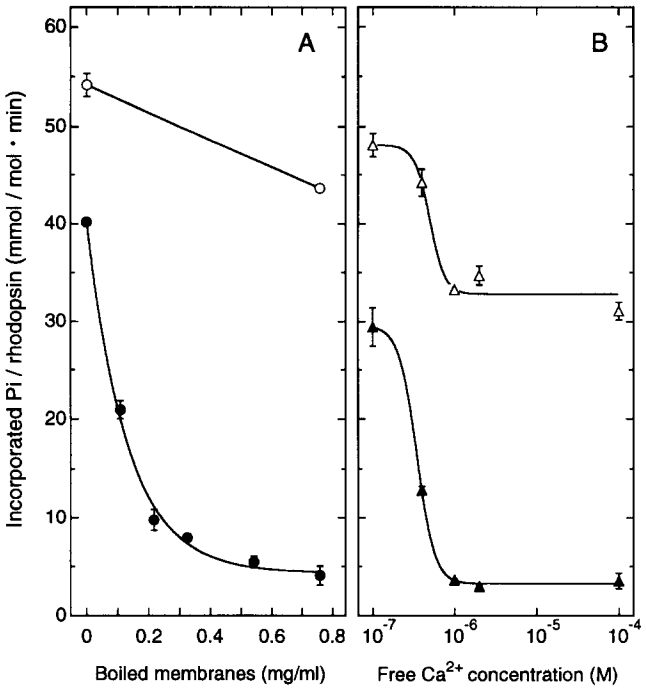

Fig. 4. Inhibition of rhodopsin phosphorylation by recoverin and boiled membranes. Boiled membranes were prepared by incubating urea-washed ROS membranes at 90°C for 10 min. Rhodopsin (1.0 μM) in urea-washed ROS membranes was phosphorylated under room light by 20 nM RK with (6 μM; closed circles) or without (open circles) recoverin. The reactions were performed with various concentrations of boiled ROS membranes at 1.7 μM free Ca^{2+} (A), or at various concentrations of free Ca^{2+} in the presence of 6 μM recoverin with (0.76 mg/ml; closed triangles) or without (open triangles) boiled membranes (B). The initial rates of the phosphorylation represent the means ± range of variation of duplicate samples. The amount of boiled membranes was expressed in terms of the concentration of rhodopsin which had been heat-denatured. The highest concentration of the membranes (0.76 mg/ml) corresponds to 20 μM rhodopsin in membranes.

plex. Second, when intracellular Ca^{2+} concentration falls, the location of the complex close to rhodopsin will help RK to attack photo-activated rhodopsin rapidly. The original work by Kühn [20] showed that RK was present mostly in the cytosol fraction in the dark, but we can speculate that the Ca^{2+} concentration in his experiment was so low that Ca^{2+}-free recoverin was unable to anchor RK to the membranes. The role of the Ca^{2+}-dependent shuttle of recoverin between the cytosol and membranes seems to escort RK, which stays at the membrane surface in the dark (high Ca^{2+}), but is released to the cytosol in the light (low Ca^{2+}) after phosphorylation of rhodopsin due to the effect of arrestin [21].

During the course of preparing this manuscript, the interaction between Ca^{2+}-bound recoverin and RK was reported by Chen et al. [11]. It was demonstrated that myristoylated recoverin inhibits rhodopsin phosphorylation 10-fold more effectively than non-acylated recoverin [11]. This observation is nicely explained by the stabilizing effect of membranes on the Ca^{2+}-recoverin-RK complex, since non-acylated recoverin has a reduced affinity for membranes [8,9]. Previously, we found that the magnitude of inhibition of rhodopsin phosphorylation was different among recoverin isoforms with distinct *N*-fatty acyl groups, and that they ranked in the same order of the hydrophobicity of the acyl groups [13]. The results are now understood as a consequence of the important role of the membrane association shown in the present study, on the assumption that the affinity of recoverin isoforms for membranes ranks in the same order of the hydrophobicity of the acyl groups. It should be noted, however, that the present results do not always indicate a direct interaction of the *N*-fatty acyl group with membranes. It is possible as well that the acyl group determines the affinity of Ca^{2+}-bound recoverin with its target, RK.

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