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Recoverin mediates the calcium effect upon rhodopsin phosphorylation and cGMP hydrolysis in bovine retina rod cells

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Abstract

Rhodopsin phosphorylation and in consequence cGMP hydrolysis in bovine rod outer segments are Ca^{2+} dependent in the presence of ATP. The level of rhodopsin phosphorylation decreases and the lifetime of active phosphodiesterase increases when the free [Ca^{2+}] is raised from < 1 nM to about 1 μM; in both cases the half-maximal effect was observed at 140–170 nM of free Ca^{2+}. Antibodies to recoverin reverse both effects at high [Ca^{2+}] but have no influence at low [Ca^{2+}]. We conclude that the Ca^{2+} effects observed are mediated by recoverin which inhibits rhodopsin kinase at a high Ca^{2+} level.

Key words: Photoreception; Phosphorylation; Rhodopsin; Rhodopsin kinase; Recoverin; Bovine rod

1. Introduction

Activation of the cascade Rh–transducin–PDE in retina rod cells in response to photon absorption causes a decrease in the cytoplasmic level of the photoreceptor second messenger, cGMP, a closure of the cationic channels in the ROS plasma membrane and as a result hyperpolarization of the membrane (see [1,2] for reviews). One more important event accompanying the channel closure is a fall in the cytoplasmic free Ca^{2+} level [3] which is thought to control the visual molecular machinery at several points. One of them is guanylate cyclase which is activated at low Ca^{2+} concentrations [4–7]. Originally a novel Ca^{2+}-binding protein, recoverin [5,6,8], was suggested [5,6] to mediate the Ca^{2+} effect upon the enzyme but it is now clear that recoverin performs as yet unknown function [7,9]. cGMP-gated channels in the ROS plasma membrane are sensitive to Ca^{2+} whose effect upon the channels is mediated by calmodulin [10]. Rh phosphorylation was also suggested to be a point of the Ca^{2+} action [11]. However, a physiological role of the effects observed was in doubt [12].

Recently a novel Ca^{2+}-binding protein, S-modulin, was supposed to mediate the Ca^{2+} action upon Rh phosphorylation and in consequence cGMP hydrolysis in frog RS [13,14]. However, to reveal the Ca^{2+} effect upon both reactions it was essential to add S-modulin [14] or recoverin [15] to the ROS suspension whereas in the absence of exogenous Ca^{2+}-binding proteins the frog ROS did not manifest the Ca^{2+} sensitivity. Thus it cannot be excluded that addition of the proteins merely mimicked the action of a real endogenous mediator.

In the preceding paper [7] we found a Ca^{2+}-sensitive complex of recoverin with an unidentified protein, presumably RK, and suggested that this enzyme is a functional target for recoverin. In the present work we attempted to answer the question whether recoverin is a mediator of the Ca^{2+} effect upon Rh phosphorylation and in consequence upon light-dependent cGMP hydrolysis in bovine ROS. Since the approach using an exogenous protein is not sufficient to be sure that recoverin actually serves as the mediator we used antibodies to recoverin and found that the antibodies modulate the Ca^{2+} effects upon cGMP hydrolysis and Rh phosphorylation in the bovine ROS suspension.

2. Experimental

ROS prepared from fresh or frozen bovine retinas [16] under dim red light were frozen in liquid nitrogen and stored at −70°C. The Rh concentration was determined by the difference in the optical densities of the ROS suspension at 500 nm before and after illumination taking ε = 42,000 [17]. The Coomassie blue binding method [18] was used to determine protein concentrations. Purified recoverin was obtained as described in [7]. The preparations referred to as 'recoverin-specific antiserum' and 'control serum' were obtained from sera of immunized and control rabbits, correspondingly, by ammonium sulphate precipitation at 50% saturation with subsequent desalting, the desalted samples were stored in 50% (v/v) glycerol at −20°C; the sera specificity was tested with ELISA [19]. Monospecific antibodies to recoverin were further obtained as described in [5,8].

PDE was assayed at 30°C by recording the pH change during cGMP hydrolysis [20] triggered by a test flash (Rh*/Rh = 10^{-4}). The reaction mixture (0.5 ml) contained 20 mM HEPES, pH 7.6, 100 mM KCl, 2 mM $MgCl_2$, 0.45 mM GTP, 4 mM cGMP, 1 mM EGTA, ROS membranes prepared in the dark (10 μM Rh); $CaCl_2$ additions (none, 0.5, 0.7, 0.8, 0.9, 1.0 mM) were used to obtain the following free Ca^{2+} concentrations

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Abbreviations: ROS, rod outer segments; Rh and Rh*, 'dark' and bleached rhodopsin, respectively; RK, rhodopsin kinase; cGMP, 3′,5′-cyclic guanosine monophosphate; PDE, cGMP-phosphodiesterase.

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(nM): < 1, 45, 104, 178, 399, 5600 [21]. If indicated, recoverin-specific or control serum (1 mg per sample) was also present. ATP (5 μM) was added into the samples (unless otherwise indicated) 50 s prior to the test flash. The initial velocity of cGMP hydrolysis after the test flash was normalized to the velocity after full bleaching. The exponential process of PDE turnoff after the test flash was quantified with the characteristic time, τ.

Rh phosphorylation was carried out at 25°C in the following mixture (30 μl): 75 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 0.5 mM GTP, 1 mM EGTA, ROS membranes (10 μM Rh) prepared in the dark; $CaCl_2$ was added as shown in the PDE assay (see above); if indicated, also monospecific antibodies to recoverin, control serum or bovine serum albumin (10 μg/ml each) were present. Immediately after illumination (Rh*/Rh = 3×10^{-2}) 10 μl of 15 μM [γ-^{32}P]ATP (1000–2000 cpm/pmol) were added to start the reaction which was stopped 20 s later by adding 200 μl of 50 mM ATP + 20 mM EDTA. The samples were centrifuged (10,000 × g, 15 min), the pellet was washed with the mixture of 10% trichloroacetic acid and 10 mM H_3PO_4 to remove the excess of radioactive ATP [22]. SDS-PAGE [23] of the pellet in 12.5% gel and 0.1% SDS followed by Coomassie blue staining and autoradiography were used to control ^{32}P-incorporation into the ROS membranes; the main portion of the radioactivity was found in Rh.

3. Results and discussion

One can see from Fig. 1 that the course of cGMP hydrolysis in the bovine ROS suspension is changed when free [Ca^{2+}] in the ATP-containing reaction mixture increases: the PDE turnoff time, τ, rises from ~8.5 s to ~20 s when [Ca^{2+}] is changed from < 1 nM to ~400 nM; K_{50} of the effect is equal to 150–170 nM. The τ value in the absence of ATP (see Fig. 1) and the normalized initial velocity of cGMP hydrolysis independent of the presence of ATP (data not shown) were poorly sensitive to calcium. The level of ^{32}P-incorporation into Rh in a bovine ROS suspension decreases as [Ca^{2+}] is raised (Fig. 2); the half-maximal effect is revealed at 140–150 nM free [Ca^{2+}] which is close to the K_{50} value in the case of cGMP hydrolysis (see Fig. 1).

It follows from Table 1 that the dependence of the

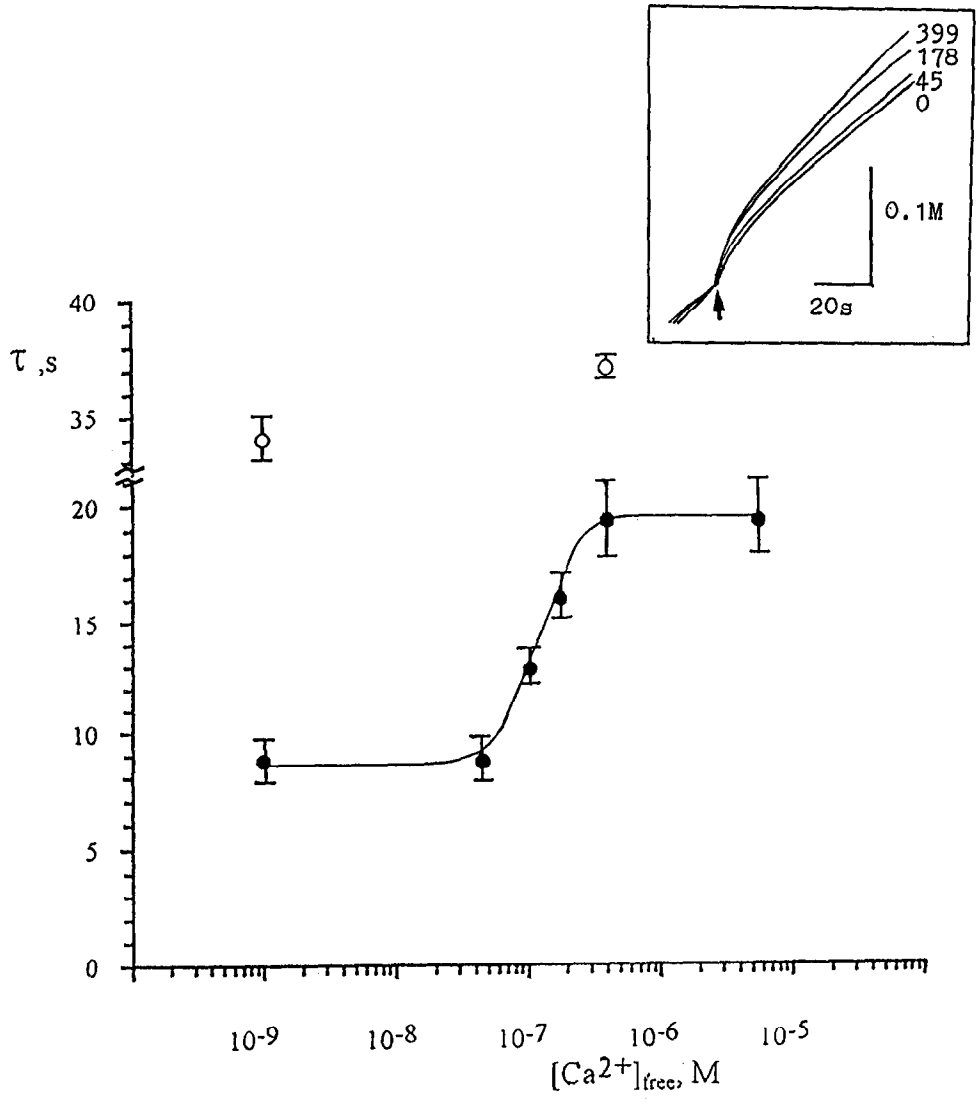

Fig. 1. Dependence of the characteristic time of the PDE turnoff on free Ca^{2+} concentration. τ was calculated from pH traces after the test flash at different Ca^{2+} concentrations in the presence (filled circles) or absence (open circles) of ATP. Points represent the average values (n = 3–4) with S.D. bars, for one of two different ROS preparations. Insert exemplifies the records at the shown free Ca^{2+} concentrations (nM) in the presence of ATP; the test flash is indicated by an arrow.

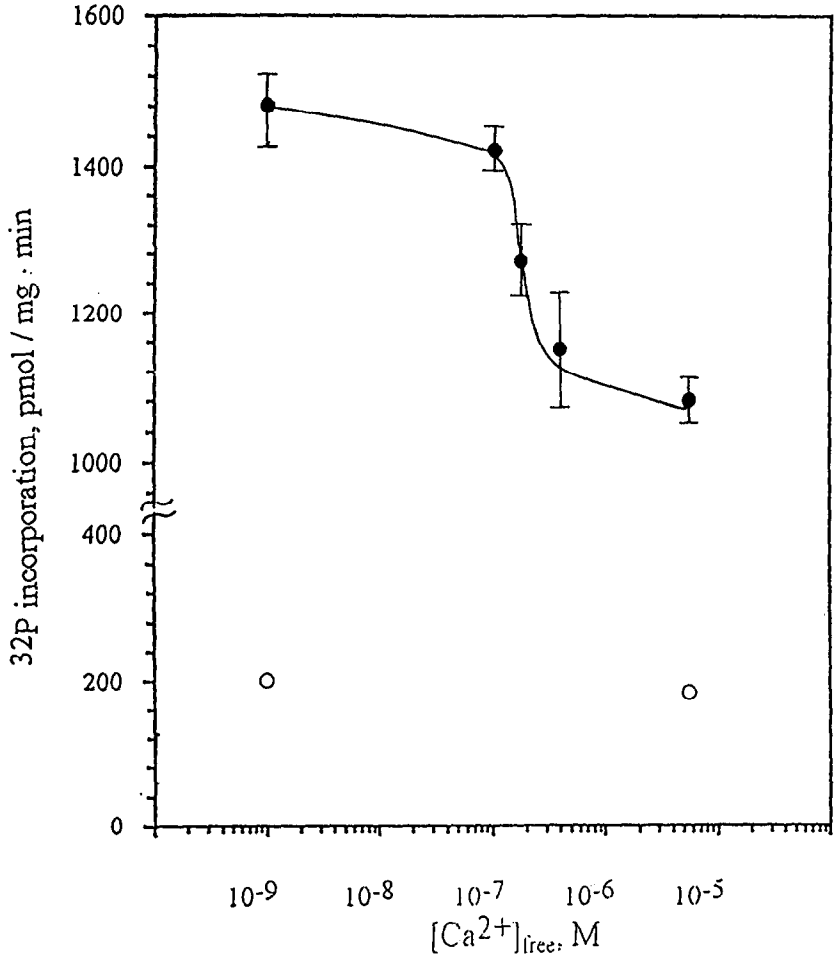

Fig. 2. Dependence of Rh phosphorylation on free Ca^{2+} concentration. The dark level of ^{32}P incorporation is shown by open circles. Points represent the average values ($n = 2$) with S.D. bars, for one of three different ROS preparations.

PDE turnoff time on $[Ca^{2+}]$ is fully suppressed by the recoverin-specific antiserum which reduces τ at high $[Ca^{2+}]$ but has no effect at low $[Ca^{2+}]$; the dependence is conserved in the presence of the control serum. (Notice that the control serum preparation itself causes the τ increase irrespective of $[Ca^{2+}]$.)

Thus one may conclude that: (i) the PDE turnoff time is sensitive to Ca^{2+} in the presence of ATP; (ii) the cation effect upon cGMP hydrolysis is mediated by recoverin; and (iii) recoverin prolongs the active PDE lifetime at high $[Ca^{2+}]$. The two latter conclusions follow from the antiserum ability to diminish τ at high $[Ca^{2+}]$ as a result of blocking the recoverin action.

Table 1 shows that Rh phosphorylation dependence

Table 1
Blocking of the Ca^{2+} effect upon cGMP hydrolysis and Rh phosphorylation by preparations of antibodies to recoverin

	cGMP hydrolysis*				Rh phosphorylation**			
	τ (s) in the presence of		Calcium effect		^{32}P incorporation in the presence of		Calcium effect	
	Low $[Ca^{2+}]$	High $[Ca^{2+}]$	Δ	%	Low $[Ca^{2+}]$	High $[Ca^{2+}]$	Δ	%
Control serum	12.5 ± 1.5	17.5 ± 0.5	5.0	100.0	2050 ± 60	1480 ± 90	570	100.0
Antibodies to recoverin	11.0 ± 0.0	11.0 ± 0.0	0.0	0.0	2120 ± 25	2080 ± 40	40	7.0

*Data are presented as means ($n = 2$–4) ± S.D. at low (< 1 nM) and high (~ 400 nM) free $[Ca^{2+}]$ in the presence of ATP. Recoverin-specific antiserum was used as a preparation of antibodies.

**Data on ^{32}P incorporation into ROS membranes (pmol/mg Rh·min) are presented as means ($n = 2$) ± S.D. at low (< 1 nM) and high (5.6 μM) free $[Ca^{2+}]$. Monospecific antibodies to recoverin were used. For further details, see section 2.

on [Ca^{2+}] is not revealed in the presence of monospecific antibodies to recoverin which fully reverse the inhibitory effect of high [Ca^{2+}] upon Rh phosphorylation but have no effect at low [Ca^{2+}]. The Ca^{2+} sensitivity of Rh phosphorylation persists in the presence of control serum (see Table 1) or bovine serum albumin in place of control serum (not shown). (The control serum preparation and bovine serum albumin cause some non-specific inhibition of Rh phosphorylation irrespective of [Ca^{2+}] used.)

Therefore, it may be concluded that recoverin: (i) mediates the Ca^{2+} effect upon Rh phosphorylation which determines (mainly or exclusively) the active PDE lifetime sensitivity to Ca^{2+}; and (ii) acts as an inhibitor of Rh phosphorylation at high [Ca^{2+}] but not as an activator of the reaction at low [Ca^{2+}]. The latter conclusion follows from the ability of recoverin-specific antibodies to stimulate Rh phosphorylation at high [Ca^{2+}] and their inability to act at low [Ca^{2+}] when the maximum phosphorylation level is observed.

It should be noted that in our experimental conditions a light-dependent phosphorylation of Rh consisted of two components: the first one discussed above was sensitive to recoverin-specific antibodies and was inhibited at high [Ca^{2+}] and the second one, which was insensitive to the antibodies and was not suppressed at micromolar [Ca^{2+}] (see Table 1). The first component was usually lost on keeping the frozen ROS, and the presence of the second component may be ascribed to a loss in the Ca^{2+}- and recoverin-sensitivity of RK in the ROS preparations. The presence of the second component can also be the result of a relatively low recoverin concentration in the ROS suspension as compared with native rods, although it cannot be excluded that the existence of two different forms of RK or functioning of an additional kinase(s) is a cause of the Rh phosphorylation picture described.

In principle two radically different hypothetic mechanisms of the recoverin inhibitory action upon Rh phosphorylation can be considered. (i) At high [Ca^{2+}] recoverin binds both to the ROS membrane [24,25] and RK [7]; as a result, the latter is anchored to the membrane and becomes incapable of interacting with Rh. At low [Ca^{2+}] RK is detached from the anchor and phosphorylates the substrate. Since recoverin is capable of binding to delipidated Rh [26], the latter itself can serve as an anchor providing that the binding site is remote from the site of phosphorylation. However, this may be the membrane lipids and/or a protein(s) different from Rh. (ii) The RK activity is inhibited as a result of recoverin binding to Rh thus hindering the enzyme–substrate interaction and/or to RK thus truly inhibiting the enzyme. At present we are carrying out experiments which could help us to choose between these hypothetical variants or to confirm both of them.

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