

# Phosphorylation of recoverin, the calcium-sensitive activator of photoreceptor guanylyl cyclase

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Received 15 October 1991

**Recoverin, a new calcium binding protein from bovine rod photoreceptor cells, activates guanylyl cyclase below a free calcium concentration of 200 nM. We show here that recoverin is phosphorylated by an endogenous kinase and Mg-ATP at the same decreased calcium concentration. The calcium-dependent activation of guanylyl cyclase is enhanced in the presence of ATP. We suggest that phosphorylation of recoverin reinforces the stimulation of guanylyl cyclase at decreased calcium concentrations.**

Photoreceptor; Guanylyl cyclase; Recoverin; Phosphorylation; Light adaptation

## 1. INTRODUCTION

Visual excitation in vertebrate photoreceptors is mediated by a light-triggered enzyme cascade that leads to the hydrolysis of the internal transmitter guanosine 3',5'-cyclic monophosphate (cGMP). Subsequent closing of the cGMP-gated cation channels prevents the entry of calcium ions into the cell. Continuous extrusion of calcium via a Na/Ca,K exchanger results in the lowering of the cytoplasmic calcium concentration (for recent reviews see [1–3]). The intracellular calcium level drops from a dark value of 300–400 nM [4–6] to a value near or below 100 nM [4]. The termination of the enzyme cascade and the re-synthesis of cGMP by the rod guanylyl cyclase (GC) results in the restoration of the dark state. GC is cooperatively activated at low calcium concentrations by a novel calcium binding protein, named recoverin [7–9]. The binding/unbinding reaction of calcium to recoverin and the activation of GC occurs between 100–300 nM free calcium [7–9]. The decrease in the concentration of calcium and the activation of GC are part of a negative-feedback loop that controls recovery of the light response and light adaptation in vertebrate photoreceptors [10–12].

Phosphorylation of illuminated rhodopsin (Rho\*) by rhodopsin kinase and subsequent termination of Rho\*-transducin coupling by arrestin are an important part

of the terminating reactions [13–15]. Other phosphorylation reactions in photoreceptors are catalyzed by a protein kinase C [16–18] and cyclic nucleotide-dependent protein kinases [19–21]. Autophosphorylation of rhodopsin kinase was proposed to change the affinity of rhodopsin kinase to rhodopsin [22]. The physiological significance of most phosphorylation reactions is still a matter of discussion. We report here the phosphorylation of recoverin which strictly depends on the free calcium concentration. We further show that the calcium-dependent activation of GC is enhanced in the presence of ATP.

## 2. MATERIALS AND METHODS

Rod outer segments (ROS) were prepared from fresh bovine eyes according to a standard protocol [23] with some modifications [8]. The GC assay and the use of calcium buffers were exactly as described previously [8]. Recoverin was isolated as published [8] with one change in the protocol: extracts of soluble ROS proteins obtained after bleaching whole ROS were not denatured by a heat treatment. Instead, soluble proteins were fractionated on a HiLoad 16/60 Superdex 75 prep grade FPLC gel filtration column (Pharmacia) in a buffer containing 50 mM NaCl and 20 mM Tris-HCl, pH 8.0. Fractions containing recoverin were pooled and further purified on a MonoQ anion exchange column.

Extracts of soluble ROS proteins were obtained from dark kept ROS in a buffer containing 10 mM Tris-HCl pH 8.01, 1 mM DTT and 0.5 mM PMSF by centrifugation at  $263\,000 \times g$  for 20 min. Protein phosphorylation was performed by incubating these extracts (1.5–2 mg protein/ml) for 30 min at 30°C with a phosphorylation buffer containing: 75 mM  $\text{Na}_2\text{HPO}_4$ , 25 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.2, 12.5 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{MgCl}_2$ , 0.1 mM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (1  $\mu\text{Ci}$ ) in 0.1 ml. Phosphorylation was terminated by denaturation in 0.1 ml SDS sample buffer (3.2%, w/v SDS, 2% v/v 2-mercaptoethanol, 100 mM Tris-HCl, pH 7.6, 25% w/v glycerol, 0.02% w/v Bromophenol blue) at 40°C for 20 min. In experiments where the influence of membranes was tested we incubated purified ROS (6–8 mg rhodopsin/ml) with the phosphorylation buffer. These reactions were terminated by adding ice-cold EDTA

**Abbreviations:** ROS, rod outer segments; cGMP, guanosine-3',5'-cyclic monophosphate; GC, guanylyl cyclase; PDE, cGMP phosphodiesterase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography.

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(5 mM) and ATP (10 mM). Samples were centrifuged (20 min,  $40\,000 \times g$ ,  $4^\circ\text{C}$ ), the pellet (membrane fraction) was resuspended and denatured in SDS sample buffer, the supernatant (soluble fraction) was again centrifuged (20 min,  $40\,000 \times g$ ,  $4^\circ\text{C}$ ) and diluted 1:1 with SDS sample buffer. Experiments with endogenous photoreceptor protein kinase C were performed according to [18]. Different concentrations of free calcium were adjusted by different Ca/EGTA ratios as described previously [8]. Soluble and membrane fractions were analyzed by SDS-PAGE [24]. The gel was exposed to a Kodak X-omat X-ray film overnight at  $-80^\circ\text{C}$ . Quantitation of  $^{32}\text{P}$  incorporation into recoverin was determined by cutting the recoverin protein band from a gel. The gel was homogenized in scintillation fluid containing tissue solubilizer and counted on a scintillation counter.

### 3. RESULTS AND DISCUSSION

Recoverin was phosphorylated when the free calcium concentration in the phosphorylation buffer was lowered from  $>500$  nM to 4 nM (Figs. 1B and 2). Significant phosphorylation occurred below 200 nM free calcium (Figs. 2A and B), which is exactly the range to which the cytoplasmic calcium drops after illumination. Thus phosphorylation and the calcium-dependent activation of GC by recoverin occurs at the same free calcium concentration (Figs. 2 and 3). No influence on the phosphorylation pattern of recoverin was observed in the presence of cAMP and phospholipids. Presence of ROS membranes during the phosphorylation assay and illumination of ROS did not influence the phosphorylation of recoverin. No autophosphorylation was detected. High concentration of cGMP (1 mM) inhibited the phosphorylation at 20 nM free calcium. Although magnesium is needed at an equimolar concentration to ATP, variation of its concentration between 1–12.5 mM

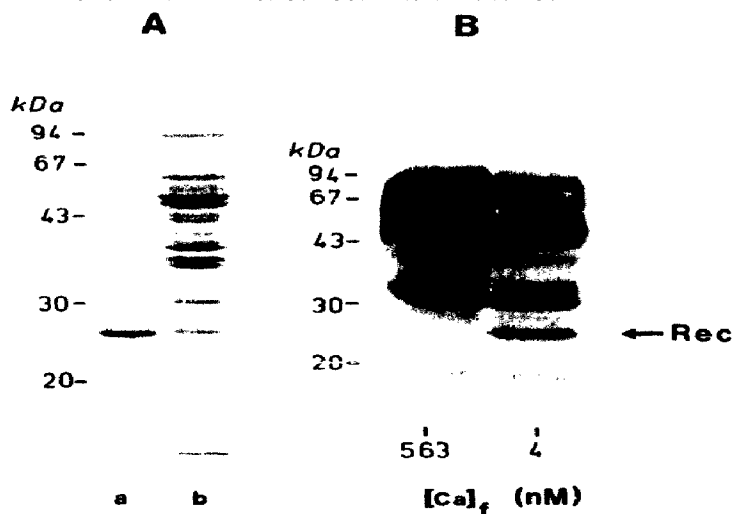


Fig. 1. A: SDS PAGE analysis on a 12.5% acrylamide gel. (Lane a) purified recoverin (3  $\mu\text{g}$ ); (lane b) extract of soluble ROS proteins (15  $\mu\text{g}$ ). Protein concentration was determined according to [28]. B: Autoradiogram of  $^{32}\text{P}$ -phosphorylated soluble ROS proteins. Extracts of ROS proteins obtained from dark-adapted ROS were incubated in phosphorylation buffer with and without calcium. Samples were analyzed as in Materials and Methods. Arrow indicates band of recoverin (Rec).

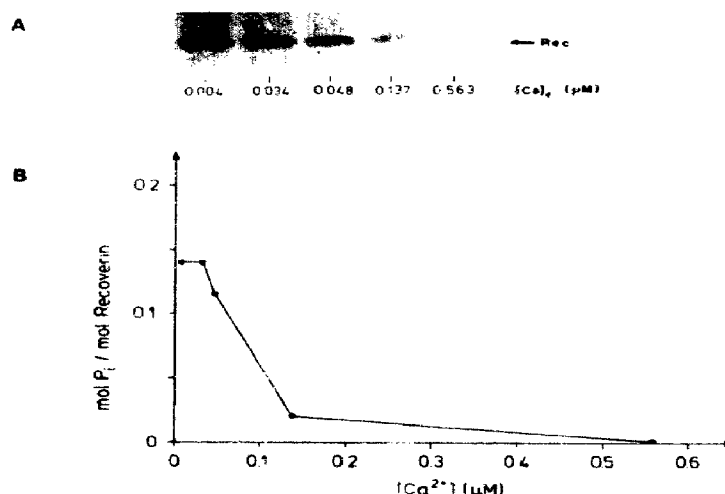


Fig. 2. Calcium-dependent phosphorylation of recoverin. A: Autoradiogram of  $^{32}\text{P}$ -phosphorylated recoverin. Samples were incubated with our phosphorylation buffer at the indicated free calcium concentration. B: Phosphate incorporation into recoverin determined from cut gel pieces. The corresponding autoradiogram is shown in A.

had no influence on the phosphorylation of recoverin. The phosphorylation level of recoverin was determined by counting the  $^{32}\text{P}$  content of cut gel pieces. We estimated a phosphate incorporation of 0.15–0.25 mol  $\text{P}_i$  per mol recoverin (4–5 determinations) assuming that 100% of endogenous recoverin was present in an extract of soluble ROS proteins (Fig. 1A, lane b) obtained from dark adapted ROS and taking a ratio of recoverin to rhodopsin of 1:100. This calculation is the lowest possible estimation of phosphate incorporation. Since recoverin is not completely removed from ROS after one extraction step (Lambrecht and Koch, unpublished observation) and the estimated molar ratio to rhodopsin

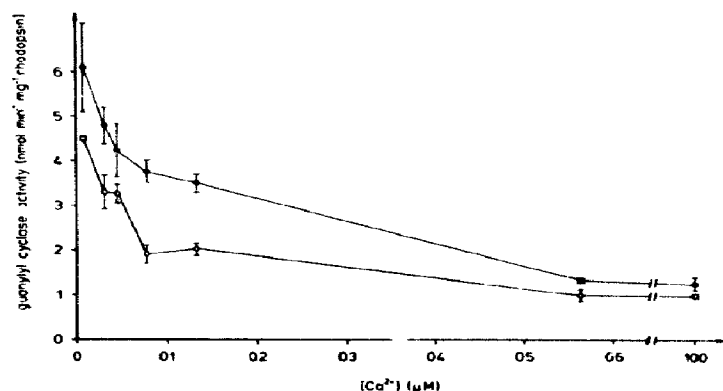


Fig. 3. Effect of ATP on the calcium-dependent activation of guanylyl cyclase. Whole dark-adapted ROS were assayed for guanylyl cyclase activity as described. (●) Incubation in the presence of 0.1 mM ATP; (○) control without ATP. Data points represent triplicates with standard deviation.

varies between 1:100 and 1:250 [8,9] phosphate incorporation may be as high as 0.5 mol P<sub>i</sub> per mol recoverin.

Analysis of the membrane fraction (see Materials and Methods) revealed that rod GC [25,26], a 112 kDa protein, was not phosphorylated under our assay conditions (data not shown). Incubation of ROS with 0.1 mM ATP enhanced the calcium-dependent activation of GC (Fig. 3). The effect was most pronounced below 200 nM free calcium, i.e. the range of free calcium where phosphorylation becomes significant. GC activities were about 2-times higher in the presence of ATP than in control incubations without ATP (Fig. 3). We suppose that this effect of ATP is due to the phosphorylation of recoverin. Maximal activities of GC measured at low calcium concentrations (<200 nM) in the presence of ATP showed a variation between 6 and 18 nmol/min/mg rhodopsin. Modulation of GC activity by phosphorylation of recoverin may explain the differences of GC activities found in the literature: basal GC activities range from 0.6–2.4 nmol/min/mg rhodopsin and increased GC activities range from 6–20 nmol/min/mg rhodopsin [7–9]. Thus, these values would reflect different amounts of pre-phosphorylated recoverin in purified ROS. GC activities shown in Fig. 3 were corrected for hydrolysis of cGMP by measuring the recovery of [<sup>3</sup>H]cGMP [7,8]. Thus, the effect of ATP shown in Fig. 3 does not reflect the quenching of the PDE [1].

We tested whether phospholipid-dependent protein kinase C catalyzed this phosphorylation reaction. Preparations of enriched photoreceptor kinase C, kindly provided by I. Weyand (Forschungszentrum Jülich), were incubated with purified recoverin (Fig. 1A, lane a) in the presence of phosphatidyl serine (10 µg/assay). No phosphorylation above or below 200 nM free calcium was observed. Pre-incubation of whole ROS with the rhodopsin kinase inhibitor 5'-[p-(fluoro-sulfonyl)benzoyl]adenosine [27] did not prevent phosphorylation of recoverin. These experiments indicate that protein kinase C and rhodopsin kinase are probably not involved. The kinase activity is extractable from ROS in a low ionic strength buffer and can be separated from recoverin by gel filtration on a Superdex 75 column (Materials and Methods). Its identity and how its calcium dependence can be interpreted, however, remains unclear. The effect of calcium could be due to a conformational change of recoverin. It would only become a substrate in its calcium free form and/or the kinase only operates at a low calcium concentration.

We propose that the modulation of GC activity by phosphorylation of recoverin is an additional mechanism in the recovery of the excitation signal and in light adaptation. Significant phosphorylation *in vitro* was observed after several minutes of incubation. The light-induced decrease of calcium therefore would trigger at least two subsequent events. First, after a delay of a few hundred milliseconds GC is activated by recoverin. Second, constant illumination over a longer period of time

would keep the cytoplasmic calcium concentration low, but it would also increase the number of activated PDE molecules. Thus, phosphorylation of recoverin leads to a further increase of GC activity in order to balance higher hydrolysis of cGMP. After re-opening of the cGMP-gated channels, re-entry of calcium leads to binding of calcium to recoverin and consequently to a decrease of GC activity. According to the current data we cannot discriminate between two alternative routes of inactivation, i.e. binding of calcium to the phosphorylated or the dephosphorylated form of recoverin. Further experiments, as well as investigations concerning the activity of (a) possibly involved phosphatase(s), are needed to shed light on this point.

**Acknowledgements:** We thank Mrs. D. Höppner-Heitmann for technical assistance and Drs. E. Eismann, U.B. Kaupp and I. Weyand for critical reading of the manuscript.

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