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Light-dependent in vivo phosphorylation of an inhibitory subunit of cGMP-phosphodiesterase in frog rod photoreceptor outer segments

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Abstract

In vivo phosphorylation of P_γ, an inhibitory subunit of cGMP-phosphodiesterase of frog (Rana catesbeiana) photoreceptor rod outer segments, was investigated using a quick-freezing technique and a newly developed method for the preparation of rod outer segments. Light-dependent phosphorylation of P_γ was observed. Okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A, enhanced the apparent incorporation of ^{32}P into P_γ, suggesting that P_γ is in equilibrium between phosphorylation and dephosphorylation. Neither phorbol ester, a potent activator of protein kinase C, nor changes in the extracellular Ca^{2+} concentration affected the in vivo phosphorylation of P_γ.

Key words: Photoreceptor; Rod outer segments; Phosphorylation; cGMP-phosphodiesterase; Inhibitory subunit; Protein phosphatase

1. Introduction

In vertebrate photoreceptor rod outer segments (ROS), light evokes a rapid decrease in the cytoplasmic cGMP concentration via the activation of cGMP-phosphodiesterase (PDE). Highly complex and sophisticated molecular mechanisms underly this PDE activation and subsequent deactivation processes. PDE is a heterotetrameric protein consisting of P_α, P_β and two P_γ subunits located in the disc membrane of photoreceptor ROS [1,2]. Both P_α (88 kDa) and P_β (84 kDa) subunits show cGMP hydrolytic activities [3], while the two P_γ subunits (13 kDa) inhibit the hydrolytic activities of P_α and P_β [4]. Light activates the photoreceptor, rhodopsin, resulting in the activation of the photoreceptor-specific GTP-binding protein, transducin, which is a heterotrimeric protein consisting of T_α, T_β and T_γ. Activated transducin exchanges bound GDP with GTP, and the GTP–$T_{\alpha\beta\gamma}$ complex dissociates into GTP–T_α and membrane-bound $T_{\beta\gamma}$ [2,5,6]. GTP–T_α binds to P_γ to form the GTP–T_α–P_γ complex. This complex formation leads to the activation of PDE by releasing P_γ from the catalytic region of P_α and/or P_β [7–9]. After a certain period of time, GTP on the complex is hydrolyzed by the GTPase activity of T_α itself. GDP–T_α releases P_γ when it rebinds to $T_{\beta\gamma}$ on the membrane [10]. Furthermore, P_γ can affect the GTPase activity of GTP-T_α and determine the life time of activated transducin [11–13] . Released P_γ rebinds to the catalytic subunits of PDE and inactivates them. In this context, the interaction of P_γ with GTP–T_α or $P_{\alpha\beta}$ is very important in signal transduction in photoreceptors.

We have already reported that P_γ is the preferred substrate for an unidentified protein kinase in the soluble fraction of frog photoreceptor ROS [14]. It is noteworthy that the phosphorylation of P_γ by the protein kinase is activated by phosphatidylinositol but not by cyclic nucleotides. In this paper, I have tried to observe in vivo phosphorylation of P_γ in frog photoreceptor ROS. By using a quick freezing method and a newly developed technique for ROS preparation, I have observed light-stimulated phosphorylation of P_γ in an in vivo system.

2. Materials and methods

[^{32}P]Orthophosphate was purchased from Amersham (PBS13). Okadaic acid was purchased from Wako Chemical Co., Ltd. Other chemical reagents were the purest grade commercially available.

2.1. Phosphate incorporation in frog retina photoreceptor cells

Bull frogs (*Rana catesbeiana*) were dark-adapted overnight, and decapitated in the dark. All procedures were performed under complete darkness using an infrared image converter until the reactions in ROS were quenched by quick freezing. The eyeballs were excised immediately, and the frontal hemispheres were removed with a razor blade to make 'eye cups'. Each eye cup was cut into two pieces longitudinally to make 'half eye cups'. Each half eye cup was placed with its vitreous

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Abbreviations: PDE, phosphodiesterase; P_α, P_β and P_γ, α, β and γ subunits of phosphodiesterase; ROS, rod outer segments; T_α, T_β and T_γ, α, β and γ subunits of transducin; GTP, guanosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; NEPHGE, non-equilibrium pH gel electrophoresis; SDS, sodium dodedcyl sulfate.

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body downward on two layers of filter paper (Toyo No. 5, 3 × 5 cm) which had been bent so that the curved surface faced up. After 3–4 min, the filter papers were extended flat, and the sclera and pigment epithelium were removed. By this procedure we can obtain a retina which is flat on a piece of filter paper and has its photoreceptor side facing up. Retinas on the filter papers were immediately and gently wetted with Ringer's solution (115 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl pH 7.5), and the margins of the filter papers around the retinas were removed with scissors. Each half retina was placed in a moist chamber made of Teflon (2.5 cm in diameter, and 5 mm in depth with a plastic lid) and refluxed with a moisturized O_2/CO_2 mixture (95:5). Ringer's solution (200 μl) containing 0.1 mCi [^{32}P]orthophosphate was poured over each half retina, and the retinas were incubated in the solution for 30 min in the dark or exposed to dim white light (a 20 W tungsten lamp from 1 m distance) for 1 min after the dark treatment. At the end of the incubation, the retinas were gently rinsed with Ringer's solution and then placed on the flat end of a plastic plunger. Excess Ringer's solution was removed, and the ROS layer was quickly frozen by attaching its surface to the mirrored surface of a copper block at liquid nitrogen temperatures.

2.2. Isolation of ROS from frozen retina

ROS were prepared from frozen retinas by a procedure similar to that established for the preparation of retinas from frozen fly heads [15,16]. Frozen retinas were transferred into cold acetone (−80°C, 5 ml/half retina) in a glass bottle and kept overnight in a freezer at −80°C. The bottle was sequentially transferred to −40°C and −25°C freezers (overnight incubation for each step) with several changes of acetone at the same temperatures. Acetone was removed by decantation and the dehydrated retinas were lyophilized immediately. ROS were harvested from the lyophilized retinas using adhesive tapes commercially available (Scotch 810). A piece of adhesive tape was placed on a yellow rod outer segment layer of the lyophilized retina and the tape was pressed with the round tip of a glass rod to produce an adheasive contact. The filter paper under the retina was removed with forceps. Another piece of tape was placed under the white neural retina layer and pressed to make close contact. Opening the sandwiches of the retina in two tapes, a yellow carpet-like ROS layer can be harvested on a tape, while white neural retina is on the other tape. The use of a binocular microscope is recommended to perform this procedure. On the basis of optical microscopic observation, the ROS layer was fairly separated from the white layer consisting of the inner segment and the neural retina layer. Protein analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis showed that the protein profile of the ROS preparation was totally different from that of the inner segment and the neural retina preparation. Rhodopsin acounted for over 80% of the total protein in the ROS preparation, although it was very minor (less than 2%) in the inner segment and the neural retina preparation.

Proteins in the ROS were separated by two-dimensional gel electrophoresis. P_γ is a typical basic protein; therefore non-equilibrium pH gel electrophoresis (NEPHGE) [17] was used for the first dimension and SDS-polyacrylamide gel electrophoresis (gradient: 8–20%) was used for the second. The ROS layers from 3 retinas were dissolved in 100 μl of a sample buffer containing 1% Nonidet P-40, 0.2% SDS, 0.2% Biolyte (pH 3–10) and 100 mM dithiothreitol. To the solution of ROS, was added 80 mg of solid urea. The protein concentration in the sample was determined by combining the sodium deoxycholate–trichloroacetic acid precipitation method [18] and the bicinchoninic acid protein assay method [19]. Solubilized ROS (400 μg protein) was poured on a first-dimensional tube gel (3 mm diameter), and NEPHGE and SDS-polyacrylamide gel electrophoresis were performed as described [17]. After the gel was dried, phosphorylated proteins were detected on an image analyzer (BAS 2000, Fuji Film).

P_γ was purified from frog ROS as described by Yamazaki et al. [10]. Purified P_γ was ^{32}P-labeled using [γ-^{32}P]ATP and a cytosolic fraction of intact frog ROS containing an endogenous protein kinase as described previously [14].

3. Results and discussion

The effect of light on the ^{32}P-incorporation into basic phosphoproteins including P_γ in ROS on frog retinas was analyzed by using NEPHGE/SDS two-dimensional gel electrophoresis. Fig. 1 shows the autoradiograms of the phosphorylated proteins in ROS after 30 min incubation in Ringer's solution containing [^{32}P]orthophosaphte in the dark (Fig. 1A) and those after 30 min in the dark follwed by 1 min exposure to dim white light (Fig. 1B). A radioactive spot was observed in each autoradiogram at the lowest end (11 kDa) and the most alkaline region of the two-dimensional gel (indicated with an arrow head). In a separate experiment, this radioactive spot comigrated exactly with [^{32}P]P_γ which had been produced from purified frog P_γ and [γ-^{32}P]ATP in the presence of the cytosolic fraction of intact frog ROS as described [14] (data not shown). Thus, the ^{32}P-labeled 11 kDa protein observed in the in vivo system was identified as phosphorylated-P_γ. Phosphorylated-P_γ was observed both in the dark and after white light irradiation, and 1 min exposure to dim white light considerably stimulated the phosphorylation of P_γ (compare Fig. 1A and B). The relative enhancement of the incorporation of ^{32}P into P_γ brought about by light exposure was approximately 5-fold. No proteins, except for P_γ and rhodopsin (high molecular weight diffuse radioactive bands in Fig. 1B; rhodopsins tend to form polymers and are hard to penetrate into the second-dimension gel under the conditions employed) were phosphorylated in a light-dependent manner.

[^{32}P]Orthophosphate was used as a radiotracer in this experiment. ^{32}P emits high-energy β-rays which irradiate dim blue-white light (Cerenkov radiation) in Ringer's solution surrounding the retina. Therefore the darkness was not complete in this experiment. It can be thought that a larger difference in the extent of P_γ phosphorylation could be observed between the light and completely dark conditions if we used [^{33}P]orthophosphate which does not emit the Cerenkov radiation.

In the in vitro system, we observed that the phosphorylation of P_γ can be catalyzed by an unidentified protein kianse in the cytosolic fraction of frog ROS in a phosphatidylinositol-dependent manner [14]. Therefore, it can be expected that this protein kinase also phosphorylates P_γ in vivo, though the contribution of another protein kinase cannot be excluded. It has recently been reported that P_γ can also be phosphorylated by protein kinase C [20]. Thus, the effect of an activator of protein kinase C was examined. However, upon addition of phorbol 12-myristate-13-acetate (300 nM) in Ringer's solution, no stimulation of P_γ-phosphorylation was observed (not shown). On addition, changes in the concentration of extracellurar Ca^{2+} (10^{-8}–10^{-6} M) caused no significant effect on the phosphorylation of P_γ under either dark or light conditions (not shown). The unknown protein kinase, which phosphorylates P_γ in the presence of phosphatidylinositol, or rhodopsin kinase may contribute to this light-dependent P_γ phosphorylation.

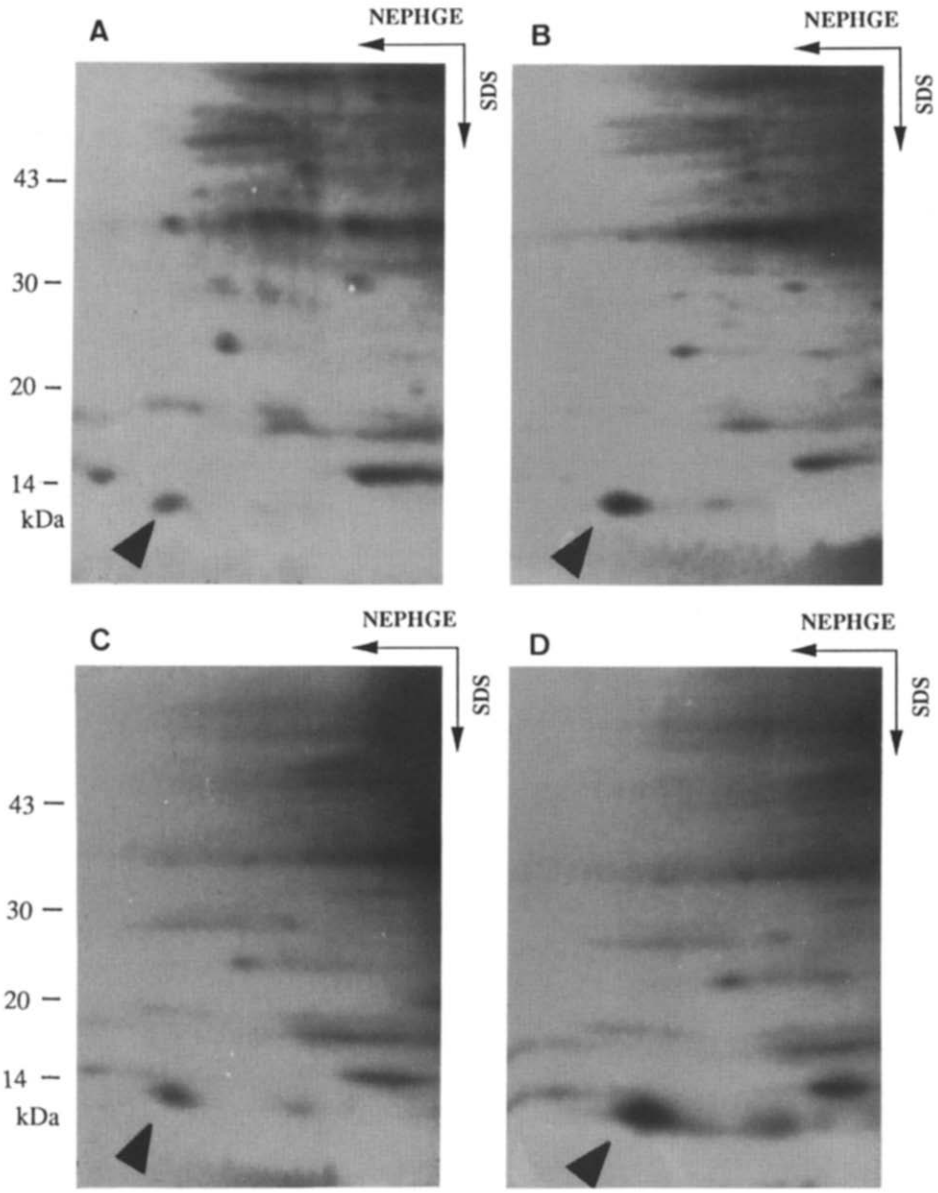

Fig. 1. Autoradiograms indicating phosphoryated basic proteins in intact frog ROS separated on two-dimensional (NEPHGE and SDS) gel electrophoresis. (A) 30 min incubation in the dark. (B) 30 min incubation in the dark followed by 1 min light exposure. (C and D) The same conditions as in A and B, respectively, except for the presence of 100 nM okadaic acid. Arrowheads indicate phosphorylated P_γ. Frog retinas were incubated in Ringer's solution containing 0.1 mCi [^{32}P]orthophosphate in the dark for 30 min. Following 1 min irradiation of the retina with dim white light or in the dark, retinas were quickly frozen by a copper block at liquid nitrogen temperatures. Rod outer segments were harvested from the dehydrated and freeze-dried retinas, and phosphorylated proteins in the rod outer segments were analyzed as described in section 2. 400 μg of protein of ROS was used for each experiment.

If the phosphorylation of P_γ is involved in the regulation of its function, it is expected that a dephosphorylation mechanism is required to return the system to its original state. In the presence of 100 nM of okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A, significant enhancement of P_γ phosphorylation was observed (Fig. 1C and D). These data indicate that P_γ is phosphorylated in a light-dependent manner and also dephosphorylated by an endogenous protein phosphatase. Therefore, it can be concluded that a flash of light causes transient phosphorylation of P_γ in the in vivo system.

It is very likely that the phosphorylation of P_γ affects its interactions with $P_{\alpha b}$, and/or T_α-GTP. If this is the case, the phosphorlation of P_γ may contribute to the adaptation mechanism of this signal transduction system. The physiological role of P_γ phosphorylation is to be studied extensively in order to understand the regulatory mechanism of phototransduction in vertebrate photoreceptors.

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