Volume 261, number 2, 419-422 FEBS 08182 February 1990

Binding of GTP to transducin is not inhibited by arrestin and phosphorylated rhodopsin

Yoshitaka Fukada, Tôru Yoshizawa, Tetsuya Saito*, Hiroshi Ohguro* and Toyoaki Akino*

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606 and **Department of Biochemistry, Sapporo Medical College, Sapporo 060, Japan*

Received 25 December 1989

In the presence of a photobleaching intermediate of unphosphorylated or phosphorylated rhodopsin (Rh*), the binding of GppNHp to transducin was measured with or without arrestin for elucidation of the shut-off mechanism of the visual transduction process in bovine rod outer segments. The ability of Rh* to catalyze the formation of the transducin-GppNHp complex in the absence of arrestin was independent of the degree of phosphorylation of Rh*. Furthermore, the catalyzing ability of the phosphorylated Rh* was not reduced by the addition of arrestin. These observations indicate that the interaction between phosphorylated Rh* and transducin was not inhibited by arrestin. Thus, the hypothesis was not supported that the PDE shut-off process is a simple competition between transducin and arrestin for binding to phosphorylated Rh*.

Rod outer segment; Guanosine triphosphate-binding protein; Transducin; Arrestin; Rhodopsin; Phosphorylation; (Bovine retina)

1. INTRODUCTION

A photobleaching intermediate of rhodopsin (Rh*) catalyzes the GDP-GTP exchange reaction of a GTP-binding protein, transducin ($T\alpha/T\beta\gamma$), in rod outer segments (ROS). The transducin in its GTP-bound form ($T\alpha$-GTP complex), in turn, activates cGMP-phosphodiesterase (PDE), which decreases intracellular cGMP concentration (visual transduction process in rod cells). On the other hand, phosphorylation of Rh* by rhodopsin kinase inhibits the activity of light-activated PDE in the presence of the 48 kDa protein, arrestin (PDE shut-off process), though the mechanism is not fully understood. Wilden et al. [1] suggested that arrestin, which has a high affinity for phosphorylated Rh*, may compete with $T\alpha/T\beta\gamma$ for binding to phosphorylated Rh*, inhibiting the formation of active $T\alpha$-GTP complex. Sitaramayya [2] demonstrated that the light-induced activation of the PDE was terminated by phosphorylation of Rh* even in the absence of arrestin. The inhibition of the light-activated PDE could be accelerated by arrestin [3]. Alternatively, Zuckerman and Cheasty [4] proposed that arrestin would be activated by Rh* in the presence of ATP, inhibiting the PDE activity through a direct interaction. Most of these workers have assessed the PDE activities in ROS or reconstituted system.

Since the activation and subsequent deactivation processes of the PDE involve several protein-protein interaction steps, it has been difficult to make clear which step is affected by arrestin from the measurements of the PDE activities. In order to clarify it, we measured the amount of $T\alpha$-GppNHp (GTP analogue) complex, which is a stoichiometric activator of the PDE, formed in a liposome system consisting of purified $T\alpha$, $T\beta\gamma$, arrestin and Rh*, the latter of which had been phosphorylated to varying degrees. The present experiments display that the light-induced formation of $T\alpha$-GppNHp complex is independent of the phosphorylation of Rh* in the presence or absence of arrestin. This finding is in disagreement with not only the competitive inhibition model for the role of arrestin [1] but also the model that the phosphorylation of Rh* may bring the activation of transducin to a termination [2,3]. Thus, our result is not contradictory to the proposition by Zuckerman and Cheasty [4] that arrestin directly interacts with the PDE to suppress its activity.

2. EXPERIMENTAL

All procedures were performed at 4°C unless otherwise stated. ROS were isolated [5] from freshly dissected bovine retinas. Rh in the membranes was phosphorylated by the method of Wilden and Kühn [5] in the presence of 3 mM [γ-^{32}P]ATP (1.2 Ci/mol; Amersham). The membranes thus prepared contained unphosphorylated and diversely phosphorylated Rh, each of which was isolated from the mixture as described previously [6]. Briefly, after regeneration of Rh in the membranes by adding molar excess of HPLC-purified 11-*cis*-retinal, it was

Correspondence address: T. Yoshizawa, Department of Biophysics, Faculty of Science, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo-Ku, Kyoto 606, Japan

Abbreviations: ROS, rod outer segments; Rh, rhodopsin; Rh*, photobleaching intermediate of rhodopsin; DTT, dithiothreitol; GppNHp, guanosine-5'-(β,γ-imido)triphosphate

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

solubilized with 16 mM CHAPS and purified on a Con A-Sepharose (Pharmacia) column. Incorporated phosphates were estimated to be an average of 3.1 mol/mol Rh (P/R ratio) in this eluate. Then, unphosphorylated and phosphorylated Rh were separated from each other through a DEAE-Toyopearl 650S (Toyo Soda) column with a linear gradient of NaCl. The early fraction contained unphosphorylated Rh, while the late ones had phosphorylated Rh with high P/R ratios. Three fractions containing unphosphorylated (P/R = 0) or phosphorylated (P/R = 3.0 or 4.6) Rh were collected and then separately incorporated into egg yolk L-α-phosphatidylcholine (Sigma; XI-E) liposomes at a molar ratio of 1:150 by dialysis against buffer A (10 mM Mops-NaOH, 0.14 M CaCl, 2 mM $MgCl_2$, 1 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5). The Rh in liposomes was irradiated at 4°C for 1 min with orange light (1 kW tungsten lamp; wavelengths longer than 500 nm) just before mixing with transducing for measurements of binding of [^{3}H]GppNHp to transducin.

Tα or T$\beta\gamma$ was purified as described previously [7]. Arrestin was isolated by the method of Wilden et al. [8] and then purified by a Mono-Q column equipped with a Fast Protein Liquid Chromatography system (Pharmacia). According to the method of Zuckerman and Cheasty [4], we confirmed that arrestin thus purified exhibited an ability to suppress the light-activated PDE activity of bovine ROS in the presence of both ATP (110 μM) and GTP (85 μM).

Rh in liposomes and the purified arrestin were divided into several aliquots and stored at −80°C until use. Purified Tα and T$\beta\gamma$ were kept at 4°C and used within a week after purification without freezing.

3. RESULTS AND DISCUSSION

The purified proteins were subjected to an SDS-polyacrylamide gel electrophoresis (fig.1). After confirming high purities of the proteins, constant amounts of Tα and T$\beta\gamma$ were mixed with various amounts of Rh* in liposomes which had been phosphorylated to varying degrees. As shown in fig.2, the addition of Rh* with different P/R ratios at a concentration (<0.1 μM) far lower than that of transducin subunits (1.5 μM each) remarkably enhanced the binding of GppNHp to Tα. The binding was nearly saturated by the addition of unphosphorylated (fig.2A: P/R = 0) or phosphorylated (fig.2B and C: P/R = 3.0 and 4.6, respectively) Rh* at a concentration of 0.2 μM. This fact indicates that both unphosphorylated and phosphorylated Rh* act like a catalyst in the formation of T_α-GppNHp complex. A noteworthy feature is that these curves (fig.2A, B and C; open circles) can be superimposed on each other. Even if the phosphorylated Rh might be contaminated with a small amount of unphosphorylated Rh, the similarity in profile among the curves enabled us to conclude that the phosphorylation of Rh* itself did not induce any change in an interaction site of Rh* with transducin. This result is in good agreement with the observation of Kühn et al. [9] in which both Tα and T$\beta\gamma$ bind with phosphorylated Rh* as well as unphosphorylated Rh* in the absence of GTP, and subsequently dissociate from the Rh* by the addition of GTP. None of unbleached Rh with different P/R ratios enhanced the binding of GppNHp to Tα (fig.2A, B and C, filled circles). Thus, the enhancement of the binding

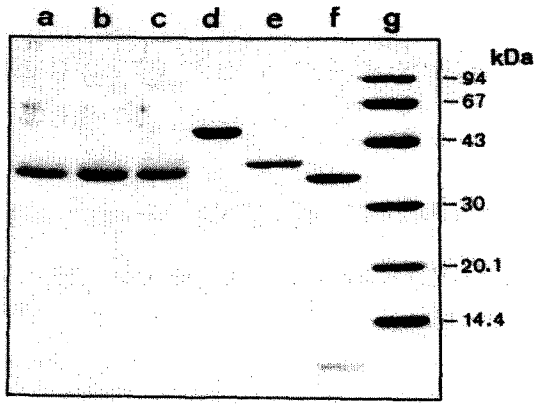

Fig.1. SDS-polyacrylamide gel (13%) electrophoresis of proteins used in these experiments. Reconstituted Rh (lane a: P/R = 0; lane b: P/R = 3.0; lane c: P/R = 4.6; 2.0 μg each), arrestin (lane d: 1.6 μg), Tα (lane e; 1.5 μg), T$\beta\gamma$ (lane f, 2.4 μg) and molecular weight standard proteins obtained from Pharmacia (lane g) were electrophoresed [11] and stained with Coomassie brilliant blue.

by Rh* cannot be ascribed to non-specific adsorption of GppNHp or transducin to liposomes with charges on the surface altered by the phosphorylation.

Then, we examined the effect of arrestin on the ability of phosphorylated Rh* to catalyze the formation of Tα-GppNHp complex. Two-fold molar excess of arrestin (3.0 μM) was added to a mixture of Tα and T$\beta\gamma$ (1.5 μM each), with which highly phosphorylated Rh* was mixed. Although arrestin has so high an affinity for phosphorylated Rh* as to form an arrestin-Rh* complex [9], a molar excess amount of arrestin over transducin did not induce any decrease in the ability of phosphorylated Rh* to enhance the binding of GppNHp to Tα (fig.3, open and filled circles). As shown in the inset of fig.3, the time course of the formation of Tα-GppNHp complex catalyzed by a small amount (0.1 μM) of highly phosphorylated Rh* indicated that the interaction of the phosphorylated Rh* with transducin was not interrupted at any time of the incubation by 30-fold molar excess amount (3.0 μM) of arrestin over phosphorylated Rh*. However, there exists a possibility that the bound GppNHp measured might be associated not with Tα but with arrestin, which is supposed to have a purine nucleotide-binding site [10]. In order to exclude such a possibility, Tα or T$\beta\gamma$ (or both) was omitted from the reaction mixture of the GppNHp binding experiment. The deletion of Tα (fig.3, open triangles), T$\beta\gamma$ (filled triangles) or both (filled squares) almost completely suppressed the GppNHp binding even in the presence of arrestin. Thus it was confirmed that the GppNHp bound in the presence of both arrestin and transducin (fig.3, filled circles) was substantially associated with Tα. However, we could not elucidate the origin of the slight increase in the formation of Tα-GppNHp complex by the addition of arrestin (compare open with filled circles in

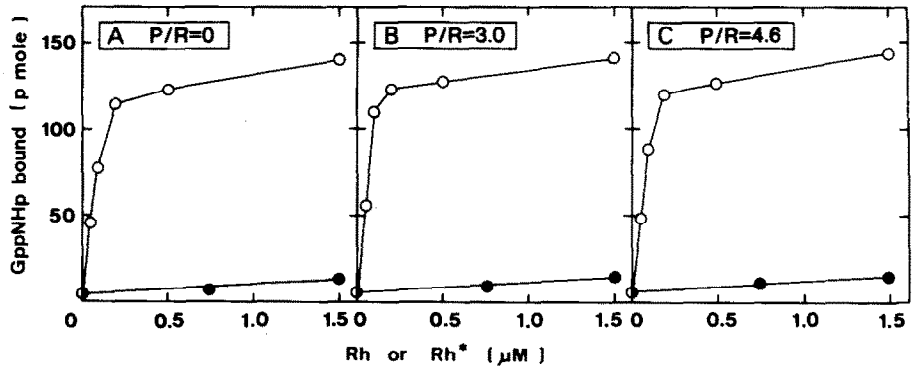

Fig.2. Formation of Tα-GppNHp complex catalyzed by Rh* with different P/R ratios. Each of the Rh in liposomes with different P/R ratios (A: P/R = 0; B: P/R = 3.0; C: P/R = 4.6) was kept in the dark (●) or irradiated at 4°C for 1 min with the orange light (>500 nm) just before the following mixing (○); various amounts of the Rh or Rh* were mixed with 1.5 μM Tα, 1.5 μM Tβγ and 10 μM [^{3}H]GppNHp (0.27 Ci/mmol, Amersham) in 0.15 ml of buffer A, followed by incubation at 4°C for 30 min in the dark. After the incubation, [^{3}H]GppNHp bound to Tα was isolated from the free [^{3}H]GppNHp according to a conventional filtration method [12] by use of a nitrocellulose filter (0.45 μM, Toyo Roshi), and the radioactivity of the filter was measured.

fig.3). The increase is not derived from a non-specific adsorption of GppNHp to arrestin because no increase of the binding was observed in the presence of arrestin when Tα was motted (open triangles or filled squares).

In conclusion, no inhibition of the formation of Tα-GppNHp was observed by phosphorylation of Rh* in the absence or even presence of arrestin. Such observations are not expected from the models where arrestin may inhibit the binding of transducin to phosphorylated Rh* in competitive fashion [1] and where the ability of Rh* to activate transducin may be diminished by the phosphorylation [2,3]. The affinity of arrestin for phosphorylated Rh* might be lower than that of transducin as suggested [9]. A noticeable difference in the experimental conditions between what appears in the literature and ours lies in the bleached amount of Rh. In the measurements of PDE activities, very low bleaching conditions (10^{-5}-10^{-4} portion of total Rh) have been employed. When the bleached amount of Rh was increased, relatively high concentrations of arrestin were required to fully suppress the PDE activity [1]. On the other hand, we could not observe any inhibition of the formation of Tα-GppNHp complex in the presence of a small amount of phosphorylated Rh* (0.025 μM) by the addition of a large excess amount of arrestin (3.0 μM) (fig.3). Since the molar ratio of Rh:transducin is about 10 in the ROS, 0.025 μM of Rh* in the reaction mixture, which contains 1.5 μM of each subunit of transducin, may correspond to about 10^{-3} bleached condition in vivo. Such a bleaching amount should not be far from the physiological condition. Although the reason why the ability of arrestin to suppress the PDE activity depends on the bleached amount of Rh is not clear yet, some shut-off mechanism of the PDE might function only at very low bleaching condition. Alternatively, a large amount of unbleached Rh which is absent in our reaction mixture might be cooperatively involved in the PDE shut-off process. In any case, our results do not contradict the proposition by Zuckerman and Cheasty [4] that arrestin may directly deactivate the PDE.

Recently, we have found that Tβγ characterized so far is composed of two components, Tβγ-1 and Tβγ-2, with distinctive γ-subunits [6]. Although the mixture of Tβγ-1 and Tβγ-2 was used in this study, addition of either of them instead of the mixture displayed no significant difference in the effect of arrestin on the formation of Tα-GppNHp complex catalyzed by phosphorylated Rh* (data not shown). Further studies are required for complete elucidation of the shut-off process of the visual transduction process.

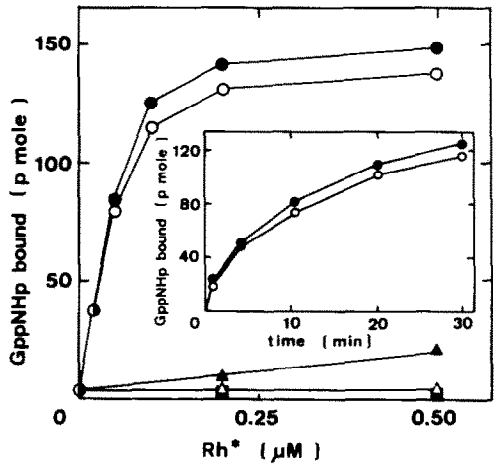

Fig.3. Effect of arrestin on the formation of Tα-[^{3}H]GppNHp complex catalyzed by highly phosphorylated Rh*. Highly phosphorylated Rh (P/R = 4.6) in liposomes was irradiated at 4°C for 1 min with the orange light (>500 nm) just before the following mixing; various amounts of Rh* were mixed with 3.0 μM arrestin, 1.5 μM Tα, 1.5 μM Tβγ and 10 μM [^{3}G]GppNHp (●). Other symbols represent similar mixtures except that arrestin (○), Tα (△), Tβγ (▲) or Tαβγ (■) was omitted from the mixtures. Other experimental conditions are the same as in fig.2. (Inset) Time course of the formation of Tα-[^{3}]GppNHp with or without arrestin. In the presence (●) or absence (○) of arrestin (3.0 μM), a mixture of Tα (1.5 μM), Tβγ (1.5 μM), [^{3}H]GppNHp (10 μM) and highly phosphorylated Rh* (P/R = 4.6, 0.1 μM) was incubated at 4°C and filtreated at the indicated time for the quantitation of Tα-[^{3}H]GppNHp.

Acknowledgements: We wish to express thanks to Mr K. Kokame for his assistance of the experiments. This work was supported in part by Grants-in-Aid for Specially Promoted Research and for Scientific Research on Priority Areas on Molecular Physiology of Photoreception from the Japanese Ministry of Education, Culture and Science and by a Special Coordination Fund of the Science and Technology Agency of the Japanese Government.

REFERENCES

[1] Wilden, U., Hall, S.W. and Kühn, H. (1986) Proc. Natl. Acad. Sci. USA 83, 1174–1178.
[2] Sitaramayya, A. (1986) Biochemistry 25, 5460–5468.
[3] Bennett, N. and Sitaramayya, A. (1988) Biochemistry 27, 1710–1715.
[4] Zuckerman, R. and Cheasty, J.E. (1986) FEBS Lett. 207, 35–41.
[5] Wilden, U. and Kühn, H. (1982) Biochemistry 21, 3014–3022.
[6] Fukada, Y., Ohguro, H., Saito, T., Yoshizawa, T. and Toyoaki, A. (1989) J. Biol. Chem. 264, 5937–5943.
[7] Fukada, Y. and Akino, T. (1986) Photobiochem. Photobiophys. 11, 269–279.
[8] Wilden, U., Wüst, E., Weyand, I. and Kühn, H. (1986) FEBS Lett. 207, 292–295.
[9] Kühn, H., Hall, S.W. and Wilden, U. (1984) FEBS Lett. 176, 473–478.
[10] Wistow, G.J., Katial, A., Craft, C. and Shinohara, T. (1986) FEBS Lett. 196, 23–28.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Fung, B.K.-K. and Stryer, L. (1980) Proc. Natl. Acad. Sci. USA 77, 2500–2504.