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Selective activation of G-protein subtypes by vertebrate and invertebrate rhodopsins

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Abstract We have quantitatively investigated specificities in activating G-protein subtype by bovine and squid rhodopsins to examine whether or not the phototransduction cascade in each of the photoreceptor cells is determined by the colocalization of a large amount of G-protein subtype (Gt or Gq). In contrast to the efficient activation of respective Gt and Gq, bovine and squid rhodopsins scarcely activated G-protein counterparts. Exchange of α- and βγ-subunits of Gt and Gq indicated the critical role of the α-subunit in specific binding to respective rhodopsins. Thus the specific recognition of G-protein subtype by each rhodopsin is a major mechanism in determining the phototransduction cascade.

Key words: Rhodopsin; Transducin; Gq-type G-protein; Phototransduction cascade; Visual cell

1. Introduction

In the photoreceptor cells of animal's eyes, there is a G-protein-mediated signal transduction cascade which generates an electrical response of the cells. So far, at least three kinds of signal cascades mediated by different subtypes of G-protein have been reported, that is, Gt(transducin)-mediated system in vertebrate photoreceptor cells [1] and Gq- and Go-mediated ones in invertebrate rhabdomeric [2–4] and ciliary photoreceptor cells [5], respectively. Since these types of G-protein respectively activate the different types of effector enzymes (i.e. cGMP phosphodiesterase, phospholipase C (PLC)β, and so on) [6], animals have utilized different types of phototransduction cascades to capture vision signal from the outer environments.

Several lines of evidence have now suggested that the subtype specificity of the G-protein to activate an effector enzyme (or channel) is relatively strict and a cascade system elicited by signal is dependent on what subtype of G-protein is activated by the receptor [7]. In other words, coupling specificity between receptor and G-protein subtype is one of the major factors to determine the cascade system utilized. In this context, it is important to note the report that the coupling specificities of vertebrate and invertebrate (cephalopods) rhodopsins to G-protein subtypes are relatively low so that they can activate the different types of G-protein [8]. Also the light absorption of invertebrate rhodopsin was reported to cause an activation of cGMP phosphodiesterase present in the vertebrate photoreceptor cells [9]. Recent investigations revealed that G11, a kind of Gq-type G-protein, is colocalized with PLC-β4 in the mammalian rod outer segment [10] and PLC is activated in a light-dependent manner [11–14]. In addition, octopus photoreceptor cells contain Gi types of G-protein other than the Gq type as evidenced by the light-dependent toxin-labeled experiments [15]. All these results suggest that the main pathway of the phototransduction in each of the photoreceptor cells might not be determined by the coupling specificities of rhodopsins to G-protein subtypes, but the presence of a large amount of specific G-protein subtype colocalized with rhodopsin.

However, we recently indicated from the phylogenetic analysis that visual pigments cluster into three groups and suggested that visual pigments in each of the groups would couple with a specific subtype of G-protein [5]. This speculation stands on the accumulated evidence from the other G-protein coupled receptors such as muscarinic and adrenergic receptors [16–19]. Therefore, to get a clue to the mechanism in determining the cascade, we have quantitatively investigated the coupling specificity between rhodopsins and G-proteins which were extracted and purified from bovine and squid photoreceptor cells. Our current findings clearly showed that the coupling specificity is considerably high to conclude that the specific molecular recognition by rhodopsin of G-protein subtype is a major mechanism in determining the cascade. In this paper the possibility that the rhodopsin system possesses a molecular mechanism in selective activation of G-protein subtype different from other G-protein coupled receptors is also discussed.

2. Materials and methods

2.1. Animals

Fresh bovine eyes were purchased from a local slaughterhouse. Squid, *Todarodes pacificus*, which were collected in the Japan Sea at midnight, were kindly supplied by the TYK Institute for Photobiology in Toyama Prefecture.

2.2. Purification of G-proteins

Purification of transducin from the bovine rod outer segments (ROS) was carried out according to the methods previously described [20].

Gq-type G-protein was purified from the squid photoreceptor membranes according to [21] with slight modifications. Squid photoreceptor membranes were solubilized with 1% sucrose monolaurate (SML) in 30 mM Tris-Cl (pH 7.2), centrifuged at 30 000 × *g* to prepare clear supernatant. The supernatant was applied to DEAE-Sepharose (DE52, Wattman) column (φ1.5 × 4 cm) which had been equilibrated with 0.1% SML in 30 mM Tris-Cl (pH 7.2), and βγ- and α-subunits of

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Abbreviations: Gt, transducin; Gq, Gq-type G-protein; PLC, phospholipase C; G11, G11 subclass of G-protein; ROS, rod outer segments; SML, sucrose monolaurate; DM, dodecylmaltoside; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; PC, L-α-phosphatidylcholine from egg yolk; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); mACh, muscarinic acetylcholine

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Gq were eluted with the same buffer containing 100 mM and 250 mM NaCl, respectively.

2.3. Preparation of rhodopsins

Bovine and squid rhodopsin were extracted from the respective photoreceptor membranes with 2% dodecylmaltoside (DM) and purified with concanavalin A-Sepharose column according to [22,23] with 0.02% DM as a detergent.

2.4. GTPγS-binding assay

G-protein activation assays were carried out by measuring the amount of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) bound to G-protein. Rhodopsin solutions were irradiated with orange light for 30 s at 15°C or kept in the dark, and they were immediately mixed with G-protein solution. The buffer composition of the mixture is 50 mM HEPES (pH 7.0), 5 mM $MgCl_2$, 200 mM NaCl, 1 mM dithiothreitol (DTT), 0.01% DM, 0.025% SML, 0.8 mg/ml of L-α-phosphatidylcholine from egg yolk (PC), 1 μM [^{35}S]GTPγS (1000–2000 cpm/pmol) and 2 μM GDP. After the mixture was incubated at a selected time in the dark, an aliquot (20 μl) was mixed with 200 μl of stop solution (20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 25 mM $MgCl_2$, 2 μM GTPγS and 2 μM GDP) and it was immediately filtrated with nitrocellulose membrane to trap [^{35}S]GTPγS bound to G-proteins. The membrane was then washed 4 times with 200 μl of washing buffer (20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 25 mM $MgCl_2$) to diminish free [^{35}S]GTPγS and put into 2 ml of scintillator (ACS II, Amersham) to assay by a liquid scintillation counter (LS 6000IC, Beckman).

2.5. Spectroscopic analysis of the formation of extra meta II

Irradiation of bovine rhodopsin in the presence of C-terminal peptide of Gt results in formation of a large amount of meta II (extra meta II), which is one of the assay systems to monitor the interaction between rhodopsin and Gt [24]. C-terminal peptides of Gt (Ac-IKENLKDCGLF) and Gq (Ac-LQLNLKEYNLV) were synthesized, purified and characterized by Kurabo (Osaka). Urea-washed ROS containing 1 μM rhodopsin, which were prepared according to [25], were mixed with or without each G-protein peptide at 4°C in 140 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, and 50 mM Tris-Cl (pH 7.8) and the spectrum before irradiation was recorded by Shimazu MPS 2000 spectrophotometer. The sample was then irradiated with orange light (Toshiba O55 filter) for 10 s at 4°C and the spectrum was recorded. The amount of meta II formed in the presence and absence of the peptide was then compared by calculating the difference spectra before and after irradiation.

3. Results and discussion

3.1. Activation of G-proteins by rhodopsins

Rhodopsins and G-proteins were purified from the bovine and squid visual cells and time-courses of GTPγS binding to G-proteins in the presence of rhodopsins were measured (Fig. 1). GTPγS binding to Gt in the presence of bovine rhodopsin (A) and the binding to Gq in the presence of squid rhodopsin (B) were greatly stimulated by irradiation of rhodopsins. We then investigated G-protein activation by photo-stimulated rhodopsin in a dose-dependent manner. Gq was mixed with various concentrations of bovine or squid rhodopsins and light-activated GTPγS binding to Gq for 3 min was measured (Fig. 2B). Squid rhodopsin dose-dependently elevated GTPγS binding to Gq, while bovine rhodopsin scarcely accelerated the binding. Light-activated GTPγS binding to Gt for one minute was measured to investigate activation of Gt by bovine and squid rhodopsins (Fig. 2A). Bovine rhodopsin stimulated GTPγS binding to Gt in a dose-dependent manner but squid rhodopsin scarcely elevated the binding rate compared with bovine rhodopsin. The results demonstrate that bovine rhodopsin activates Gt much more efficiently than squid rhodopsin does. In contrast, the activation of Gq was much more effective by squid rhodopsin than by bovine rhodopsin. Thus we can safely conclude that the specificity of each rhodopsin to couple with the corresponding G-protein subtype is reasonably high and the specific recognition of G-protein subtype is a major mechanism in determining the cascade.

It has been reported that the C-terminal regions of both α- and γ-subunits of Gt are involved in interaction of Gt with rhodopsin [24,26–28]. The bovine Gt and squid Gq are different from each other in not only the C-terminal structure of the α-subunit but also that of the γ-subunit, although their β-subunits are more than 80% identical. Thus, we then prepared Gt and Gq chimeras, namely $T_\alpha Q_{\beta\gamma}$ (trimer of the α-subunit of Gt and βγ-subunits of Gq) and $Q_\alpha T_{\beta\gamma}$ (trimer of the α-subunit of Gq and βγ-subunits of Gt), and their activation by the rhodopsins was measured (Fig. 3). Activation of the G-proteins by bovine rhodopsin is shown in Fig. 3A. As described above, bovine rhodopsin activated transducin ($T_\alpha T_{\beta\gamma}$) but scarcely Gq ($Q_\alpha Q_{\beta\gamma}$). The activation profiles of $T_\alpha Q_{\beta\gamma}$ and $Q_\alpha T_{\beta\gamma}$ were almost identical to those of $T_\alpha T_{\beta\gamma}$ and $Q_\alpha Q_{\beta\gamma}$, respectively, showing that the difference in activation efficiency of the G-proteins by bovine rhodopsin is dependent on the α-subunit rather than βγ-subunits. The result of the activation of G-proteins by squid rhodopsin is shown in Fig. 3B. The time-courses in activation of $T_\alpha Q_{\beta\gamma}$ and $Q_\alpha T_{\beta\gamma}$ were

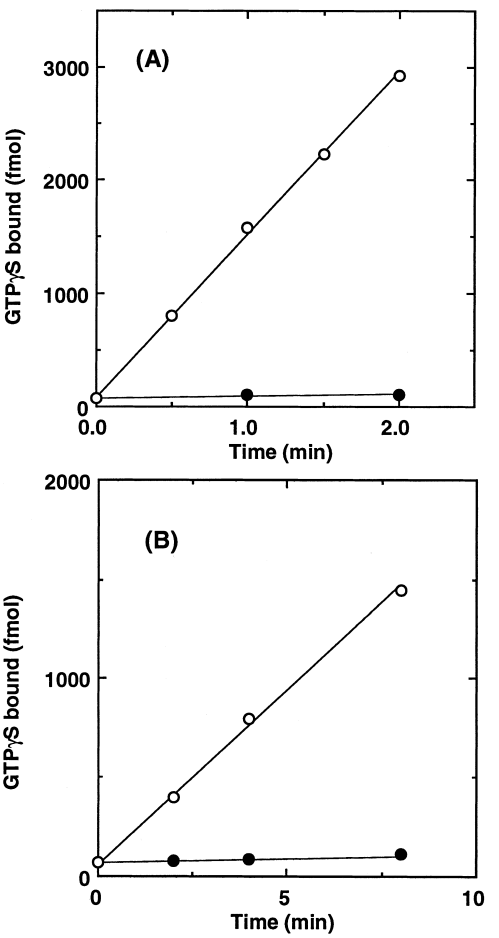

Fig. 1. Time-course of G-protein activation by rhodopsins. A: Gt activation by 2.5 nM of non-irradiated (closed circles) and irradiated bovine rhodopsin (open circles). The concentration of Gt is 300 nM. B: Gq activation by 70 nM of non-irradiated (closed circles) and irradiated squid rhodopsin (open circles). The concentration of Gq is 200 nM. It should be noted that Gt and Gq activation show a different time profile which is due to difference of G-protein subtypes.

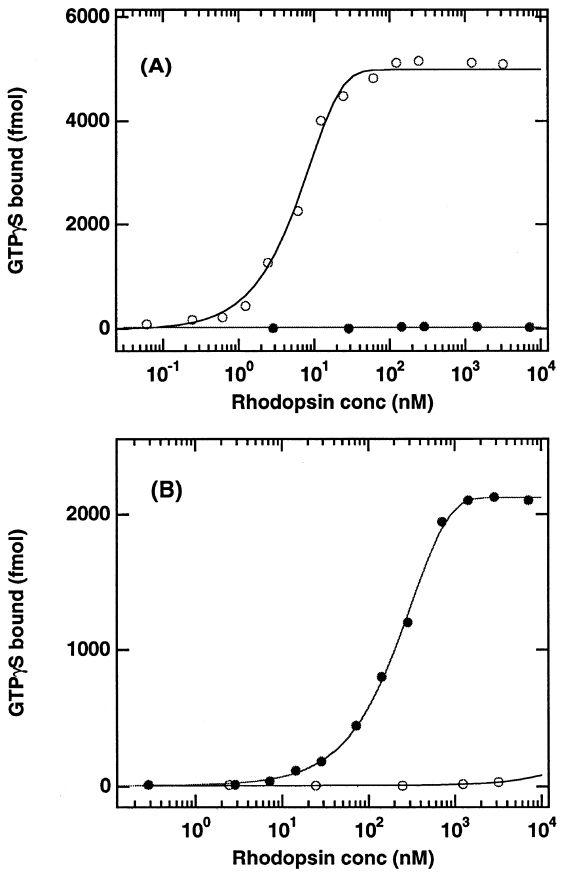

Fig. 2. Light-dependent activation of heterotrimeric G-proteins by rhodopsins. Irradiated and non-irradiated bovine (open circles) and squid (closed circles) rhodopsins in various concentrations were mixed with Gt (A, 300 nM) and Gq (B, 200 nM). GTPγS binding to Gt for 1 min and Gq for 3 min at 15°C was measured. Note that the dose-dependent manners of Gq and Gt activation are different.

clearly different from each other and almost identical to those of $T_{\alpha}T_{\beta\gamma}$ and $Q_{\alpha}Q_{\beta\gamma}$, respectively. These results demonstrate that the α-subunit plays a critical role in selective activation of G-protein by rhodopsin. Squid rhodopsin activated Gq much more efficiently than Gt, whereas squid rhodopsin also activated Gt very slightly (Fig. 3B). This observation does not contradict the previous reports that cephalopod rhodopsin activates vertebrate phototransduction enzyme [9].

3.2. Binding of Gα peptide to rhodopsin

The result of GTPγS-binding assay as shown in Figs. 1–3 gave information about the activation of G-protein by rhodopsin (GDP/GTP exchange in the α-subunit) but less about the binding of G-protein to rhodopsin, since the interaction of rhodopsin with G-protein is divided into two steps in native [20,29] and mutant [30] rhodopsins, namely binding of G-protein to rhodopsin and exchange of GDP to GTP in G-protein. In bovine rhodopsin, binding of Gt to photoactivated rhodopsin results in the shift of the meta I and meta II equilibrium; formation (stabilization) of larger amounts of meta II (extra meta II) [31,32]. The formation of extra meta II is also observed by using a C-terminal 11 amino acid peptide of the Gt α-subunit instead of heterotrimeric Gt [18]. As the C-terminal region of the G-protein α-subunit is important for selective coupling of G-proteins with receptor proteins [33], we then investigated the binding of Gq to bovine rhodopsin by the measurement of the formation of extra meta II using the C-terminal peptide of Gq (Fig. 4). Each curve of Fig. 4 is shown as the difference spectrum before and after irradiation. In the presence of the Gt peptide, the MI peak at 470 nm decreased, and the meta II peak at 380 nm and >500 nm increased compared with that in the absence of the peptides, showing the formation of extra meta II. The spectrum in the presence of the Gq peptide, however, was almost identical to that without any peptide, showing that the Gq peptide did not bind to bovine rhodopsin in a similar way as the Gt peptide. This result suggests that Gq does not bind to bovine rhodopsin with the same efficiency as Gt.

3.3. Comparison with the other G-protein coupled receptor systems

Our results presented here strongly suggest that, in both bovine and squid systems, the activation of G-protein by rhodopsin is based on the molecular recognition of α-subunit of G-protein by rhodopsin. The similar selective coupling to G-protein was reported in many G-protein coupled receptor systems [16–19]. Molecular phylogenetic analyses on G-protein coupled receptors suggest that receptors specific to different

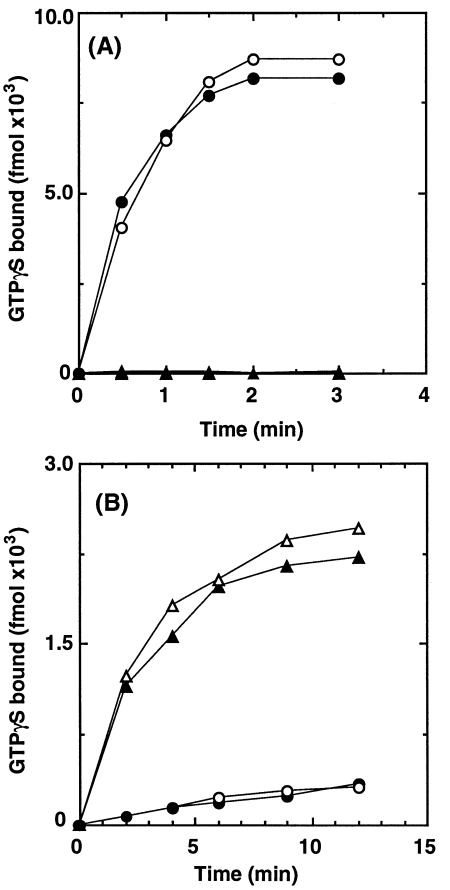

Fig. 3. Activation of α- and βγ-chimerical G-proteins by rhodopsins. Time-course of light-induced GTPγS binding to G-proteins by using 10 nM bovine (A) and 300 nM squid (B) rhodopsins. 300 nM of 4 kinds of G-proteins were used here, namely α-subunit of Gt and βγ-subunits of Gt (TT, closed circles), α-subunit of Gt and βγ-subunits of Gq (TQ, open circles), α-subunit of Gq and βγ-subunits of Gq (QQ, closed triangles) and α-subunit of Gq and βγ-subunits of Gt (QT, open triangles).

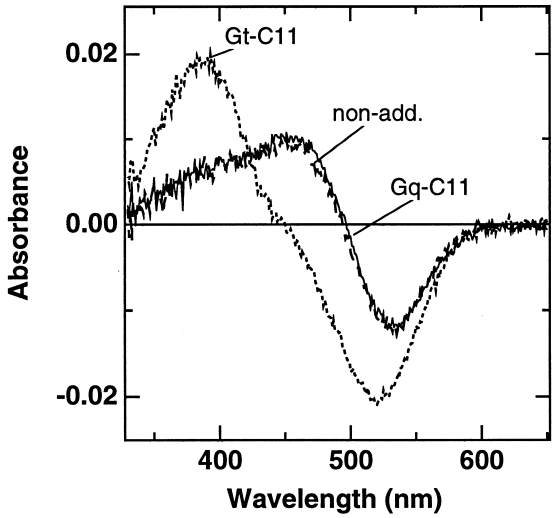

Fig. 4. Recognition of G-protein C-terminal peptides by bovine rhodopsin. Urea washed ROS were mixed with the C-terminal peptide of Gt (dotted line) or Gq (solid line) which are composed of 11 amino acids, irradiated with an orange light for 10 s, and difference spectrum before and after irradiation was obtained. As a control, urea washed ROS without peptide were examined (broken line).

ligands first diverged from an ancestral type and then each receptor was individually diversified into several subclasses which showed different specificity to G-protein subclasses [34]. Thus there is a possibility that molecular mechanisms of the selective activation of G-protein are different among different ligand receptors, and it is evolutionarily important to compare molecular mechanisms of selective activation of G-protein between rhodopsin and other G-protein coupled receptors. In the muscarinic acetylcholine (mACh) receptor systems, it has been suggested that the exchange of the 3rd loop between Gi/Go and Gq-coupled subtypes results in changing of the coupling of G-protein subtypes [35]. To test the effect of the loop replacement of rhodopsin on the coupling to G-protein subtypes, we prepared chimerical mutants of bovine rhodopsin in which the 2nd and/or the 3rd loops were replaced by the corresponding loops of squid rhodopsin. Although the mutants lessened the ability of activation of Gt, they scarcely activated Gq compared with squid rhodopsin (data not shown). The result suggests that the mechanism of selective activation of G-protein by rhodopsin is similar but not identical to the well-studied mACh receptor system. To resolve the difference, a study on the molecular mechanism of selective activation of Gq by rhodopsin is now in progress by using loop replaced mutants.

As described, G11 and PLCβ4 are localized in mammalian ROS [10]. In addition, light-elevated PLC activity in vertebrate ROS has been reported [11–14]. Although in this study we used Gq which was prepared from an invertebrate system but not G11 from mammalian ROS, their C-terminal sequences which were important for receptor-G-protein coupling [16,24,26] are the same. Thus we regarded squid Gq as a model for G11 of mammalian ROS in view of the interaction between rhodopsin and G-protein. Although our experimental results presented here suggest that bovine rhodopsin scarcely activates Gq, previous reports [10–13] show the possibility that vertebrate rhodopsin stimulates the G11/PLC cascade. If in ROS the efficient activation of G11 by rhodopsin occurs, unknown molecular systems would make their coupling more efficiently. Several mechanisms can be speculated based on other G-protein coupled receptor systems. As in the β2-adrenergic receptor system, phosphorylation in the 3rd loop of the receptor by protein kinase A switches the coupling of the receptor to G-protein subtype from Gs to Gi [36], one could speculate the switching of rhodopsin specificity to G-protein subtypes from Gt to Gq/G11 by phosphorylation. As a recent report pointed out the possibility that a rhodopsin similar to Gq-coupled scallop and cephalopod rhodopsins is present in the vertebrate [37], another kind of rhodopsin, which is similar to Gq-coupled invertebrate rhodopsin, would be present in the ROS. Our attention will next focus on what makes efficient activation of G11 in vertebrate ROS.

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