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Interaction of GTP-binding protein Gq with photoactivated rhodopsin in the photoreceptor membranes of crayfish

Akihisa Terakita[a,*], Takahiko Hariyama[b], Yasuo Tsukahara[b,c], Yuki Katsukura[c], Hideo Tashiro[c]

[a]*Institute of Biology, Faculty of Education, Oita University, Oita 870–11, Japan*
[b]*Graduate School of Information Sciences, Tohoku University, SKK Building, Sendai 980, Japan*
[c]*Photodynamics Research Center, The Institute of Chemical and Physical Research (RIKEN), Aoba-ku Koeji, Sendai 980, Japan*

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Interaction of G-protein with photoactivated rhodopsin (Rh*) in crayfish photoreceptor membranes was investigated by immunoprecipitation using an antibody against rhodopsin. Two kinds of protein were co-precipitated with rhodopsin. One is an α subunit of class-q G-protein (42 kDa, CGqα) which showed light-induced, dose-dependent binding to rhodopsin, and the other is an actin-like protein (44 kDa) with light-independent binding. Most of the CGqα was available for binding to Rh* but was dissociated from Rh* in the presence of GTPγS. These findings demonstrate that, in the crayfish photoreceptor, a Gq class of G-protein is activated by Rh*.

Photoreceptor membrane; Rhodopsin; G-protein; Gq; Signal transduction; Phospholipase C; Crayfish; *Procambarus clarkii*

1. INTRODUCTION

Heterotrimeric G-proteins play an important role in many signal transduction pathways. Different subclasses of G-protein are known to be involved in coupling specific receptors to effector enzymes (see [1,2] for reviews). Transducin (Gt) is a major G-protein in the vertebrate rod photoreceptor cell and is readily isolated without detergents [3]. The rhodopsin–Gt-PDE cascade is therefore well studied as a model for receptor–G-protein-effector enzyme systems (see [4,5] for reviews).

The molecular interactions in invertebrate phototransduction are still unclear, even at the level of rhodopsin–G-protein interactions, but electrophysiological, biochemical and molecular biological studies suggest that PLC is important in the invertebrate phototransduction cascade [6,7 for reviews]. In mammalian systems, subclass q G-protein (Gq) activates PLC-β [2,8,9] and the occurrence of a Gq-type of G-protein in the photoreceptor cells of several invertebrates has been reported (*Drosophila* [10,11], crayfish, shrimp and octopus [12], and squid [12,13]). There is evidence for the activation of guanine-nucleotide-binding and GTPase activities of Gq by irradiation of squid photoreceptor membranes [14] and the relative amount of squid Gq has been calculated to be about 10% of the rhodopsin [13,14], indicating that Gq is probably an important component of the photoreceptor cell membranes.

In the present study, we provide further evidence supporting the presence of a receptor-Gq-PLC cascade in the phototransduction pathway of invertebrate photoreceptors: immunoprecipitation experiments were performed using a monoclonal antibody against rhodopsin to investigate the interaction of Gq with photoactivated rhodopsin in crayfish photoreceptor membranes.

*Corresponding author. Fax: (81) (975) 688319.

Abbreviations: ATPγS, Adenosine 5′-*O*-[3-thiotriphosphate]; G-protein, GTP-binding protein; GDPβS, guanosine 5′-*O*-[2-thiodiphosphate]; GTPγS, guanosine 5′-*O*-[3-thiotriphosphate]; Gt, rod transducin; HPLC, high-performance liquid chromatography; PDE, phosphodiesterase; PLC, phospholipase C; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

Crayfish (*Procambarus clarkii*) were maintained in the dark at 20°C at least 3 days before use. All procedures prior to the addition of SDS-PAGE sample buffer were carried out under infrared ($\lambda > 800$ nm), using an image converter (Noctvision, NEC).

2.1. *Isolation of photoreceptor membranes and protein extraction*

Crayfish rhabdomeric photoreceptor membranes were isolated from the dark-adapted retinas by passing through meshes of 94 μm and 19 μm, using buffer A (10 mM PIPES, pH 7.2, 200 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF) [15]. Isolated membranes from one retina contained about 7 μg protein including about 3 μg of opsin. The isolated membranes were solubilized with detergent, 0.4% sucrose-monolaurate (SML) in buffer A (10 μl per 10 μg protein), kept one hour at 4°C and centrifuged at 16,000 × *g* for 30 min at 4°C to obtain a protein extract in the supernatant. For SDS-PAGE analysis, an aliquot of the membranes was solubilized with SDS-PAGE sample buffer (20 μl per 10 μg protein) and an aliquot of the protein extract was mixed with an equal volume of 2 × SDS-PAGE sample buffer [15,16].

2.2. *Irradiation*

Isolated photoreceptor membranes or protein extracts were irradi-

ated for 15 s at 4°C with red light (λ > 590 nm, 200-W slide projector with VR-60 cutoff filter, Toshiba) at a distance of 30 cm.

2.3. *Immunoprecipitation*

For monoclonal antibody generation against crayfish rhodopsin, BALB/c female mice were immunized 3 times with 50 μg of SDS-PAGE-purified opsin at intervals of 2 weeks. Standard techniques were employed for hybridoma fusion, screening and cloning [17]. The monoclonal antibody against rhodopsin (Rh3A5) was purified from ascitic fluid and covalently coupled with protein A-agarose (Bio-Rad), using 0.2 M dimethylpimeliminate (AbRh-agarose) [18]. 30 μl of protein extract from the membranes was applied to a 1 ml column containing 20 μl of AbRh-agarose, equilibrated with buffer A. It was shaken for 1.5 h at 15°C, washed with 2 ml of buffer A containing 0.2% SML, and bound (immunoprecipitated) proteins were eluted with SDS-PAGE sample buffer containing 2.5% 2-mercaptoethanol. IgG light-chain was eluted but little heavy-chain, due to the covalent attachment to protein A [18]. The eluate was analyzed by SDS-PAGE (12% acrylamide gel) and immunoblotting [15,16,19]. For immunological visualization, the blots were treated with a primary antibody, a biotinylated secondary antibody, an avidin:biotinylated horseradish peroxidase complex, and diaminobenzidine (ABC kit, Vectastain). Antibodies against rhodopsin (culture fluid of Rh3A5), GTP/GDP-binding site of Gα (GA/1, Daiichi Chemicals Co.), and actin (clone C4, Boehringer Mannheim Biochemica) were used at titers of 1:10, 1:500 and 1:500, respectively.

2.4. *Quantitative analyses of eluted proteins and metarhodopsin*

The relative amounts (percentage of total rhodopsin) of the 42 kDa and the 44 kDa proteins were estimated on the basis of the density of the proteins per the density of opsin (monomer + dimer) by densitometry of SDS-PAGE gel lanes. The relative amount of metarhodopsin to total rhodopsin (i.e. rhodopsin + metarhodopsin) was calculated on the basis of chromophore analysis by HPLC [15].

2.5. *Amino acid sequences*

15 μg of the 42 kDa immunoprecipitated polypeptide cut from SDS-PAGE gels were digested with *Staphylococcus aureus* V8 protease and separated by SDS-PAGE [20]. The digested peptides were transfered to immobilization membranes (ProBlott, Applied Biosystems) [19] and sequenced (Applied Biosystems sequence analyzer Model 473A).

3 RESULTS AND DISCUSSION

Fig. 1 shows the SDS-PAGE profiles of the immunoprecipitated proteins. Isolated photoreceptor membranes contain several major proteins as well as opsin (lane 1, 'membranes'). The protein pattern of the extract with detergent SML (lane 2) is similar to that of the membranes. The AbRh-agarose step (immunoprecipitation) with an anti-rhodopsin antibody Rh3A5 removed most of the proteins, leaving 5 major bands (lanes 3 and 4). Three of these were identified as opsin monomer and dimer, based on staining with Rh3A5 (lane 9), and IgG light-chain. The two remaining bands were labelled P42 and P44 according to their respective apparent molecular masses. The profiles of immunoprecipitation show P42 and P44 were co-precipitated with rhodopsin and their association with rhodopsin. Irradiation of the protein extract converted more than 90% of rhodopsin to metarhodopsin but produced little change in the amount of P44 (lanes 3 and 4): the ratio of irradiated/non-irradiated was 0.9 ± 0.2 (n = 6). However, the amount of P42 increased markedly: the ratio of irradiated/non-irradiated was 13.4 ± 4.5 (n = 6). P44 reacted with anti-actin antibody (lane 8), so we conclude that it is an actin-like protein. P42 reacted with anti-Gα antibody (lane 5) in the irradiated sample, but not in the

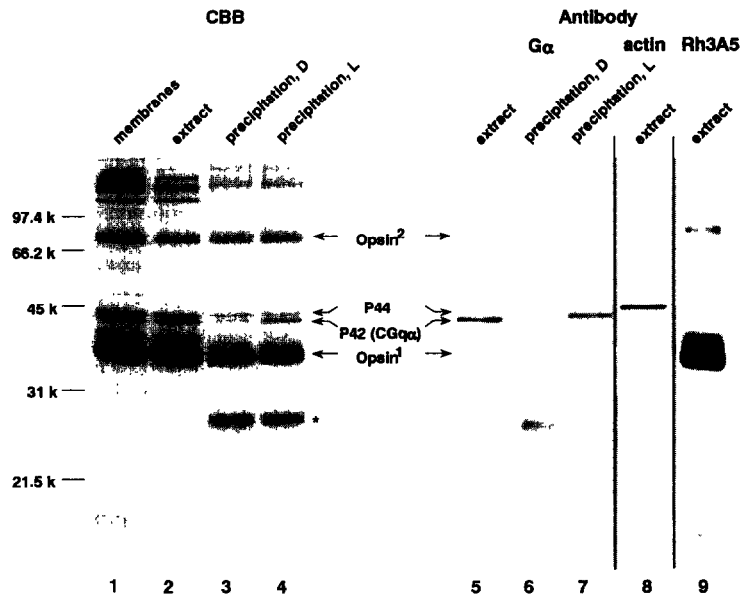

Fig. 1. SDS-PAGE profiles of immunoprecipitated proteins. The precipitation was performed using non-irradiated (D) and irradiated (L) protein extracts. In the 'L' samples, more than 90% of rhodopsin was converted to metarhodopsin. Lanes 1–4 show the SDS-PAGE gel stained with Coomassie brilliant blue (CBB). Lanes 5–7, 8 and 9 show the immunoblots. Lanes 1, 2, 5, 8 and 9, each contain 10 μg of protein; the rest, 7 μg of protein. Asterisk indicates IgG light-chain eluted from the AbRh-agarose. The light-chain was non-specifically stained with anti-Gα antibody (lanes 6 and 7). Opsin^1 and Opsin^2 indicate monomer and dimer, respectively. High molecular weight proteins in lanes 3 and 4 are opsin polymers. Apparent molecular weights (Bio-Rad) are indicated to the left.

Gα	Sequence	Identity (%)
CGqα	QKRIN QEIER QLRKD KRDAR REVKL LLLGT GE	--
DGqα	***** ****K ***R* ***** **L** ***** **	91
Gqα	AR*** D**** HV*R* ***** **L** ***** **	78
Gα11	S**** A***K ***R* ***** **L** ***** **	84
DGα1	AIERS KN*D* A**AE GER*A S*V** ****A **	44
DGo	AIQRS KQ**K N*KE* GIQ*A KDI** ****A **	41
Gsα	*REA* KK**K **Q** *QVV* ATHR* ****A **	53
Giα2	AAERS KM*DK N**E* GEK*A **V** ****A **	47
Gtα	SRELE K---K -*KE* AEKDA *TV** ****A **	34

Fig. 2. Partial amino acid sequence of P42 (CGqα) and comparison with known Gα. Peptide sequence data of CGqα were aligned with those for similar regions of other Gαs: DGqα from *Drosophila* photoreceptor [10,11], *Drosophila* DGα1 [21] and DGo [22–25], mouse Gqα and Gα11 [10], human Gsα [26] and Giα2 [27], and human rod transducin (Gt) [28]. Asterisks denote amino acids identical to CGqα.

non-irradiated sample (lanes 6 and 7, respectively). This suggests the binding of Gα to photoactivated rhodopsin.

The partial amino acid sequence of co-precipitated P42 is shown in Fig. 2. It has highest identity with the a subunits of Gq from *Drosophila* (DGqα, [10,11]) and rat (Gqα and Gα11, [10]). The apparent molecular mass of P42 on SDS-PAGE (Fig. 1) is the same as Gqα in squid photoreceptors [13]. These similarities suggest that the P42 bound to the photoactivated rhodopsin is a kind of Gqα (CGqα). The ratios of CGqα to total rhodopsin in the membranes and in the protein extract (Fig. 1, lanes 1 and 2) were 11 ± 2% (n = 7) and 10 ± 1% (n = 7), respectively. This value (i.e. 10–11%) is in agreement with the amount of Gqα in squid photoreceptors [13,14].

The relationship between the amount of CGqα-binding and the amount of metarhodopsin (irradiated rhodopsin, Rh*) is shown in Fig. 3. The maximum binding of CGqα to Rh* was determined to be 9 ± 1% of total rhodopsin (n = 7), comparable with the above estimates of the CGqα/opsin ratio in photoreceptor membranes and detergent extract, and demonstrating that most of the available CGqα can bind to Rh*. The binding rate reached maximum (i.e. 9%) when about 10% of the rhodopsin was converted to Rh*. In short, this demonstrates that one CGqα molecule binds to one photoactivated rhodopsin molecule, even in the detergent-extracted condition.

It is known that the activated GTP-binding a subunit of G-protein is released from receptors, for example GTP-Gtα [29,30], so we tested the effects of non-hydrolysable analogs (GTPγS, GDPβS and ATPγS) on the binding of CGqα to Rh* (Fig. 4). Irradiated membranes were incubated with and without the analogs for 30 min at 4°C. The proteins were then extracted and analyzed by immunoprecipitation using AbRh-agarose. The SDS-PAGE profiles of the extracts did not change on incubation of the membranes with the analogs (Fig. 4, upper lanes). The lower lanes in Fig. 4 show that CGqα was not co-precipitated with Rh* when incubated with 20 μM GTPγS (lane 2), but 20 μM GDPβS and 20 μM ATPγS did not affect the precipitation of CGqα (lanes 3 and 4); cf. incubation without the analogs, lane 1. These results suggest that GDP-CGqα was bound to activated rhodopsin, exchanged GDP for GTP, and then GTP-CGqα dissociated from Rh*, as with Gt [29,30]. The ATP analog, ATPγS, showed no effect on CGqα binding but there was an effect on the co-precipitation of P44, which is an actin-like protein (lanes 1 and 4).

It has been reported for Gt that α, β and γ subunits bind to Rh* [3] and that the association of α and βγ subunits is necessary for GTP-GDP exchange [31]. In Fig. 1, CGqα was co-precipitated with Rh* but βγ subunits were not precipitated (a possible β subunit is a 38 kDa protein present in Figs. 1 and 4, on the basis of the similarity of the molecular mass to that in cephalopods and *Drosophila* photoreceptors [13,32,33].). The effect of GTPγS on the co-precipitation of CGqα with Rh* is greater in the membrane preparation than in the detergent SML-extract (data not shown). This is probably explained by the weaker interaction between α and βγ subunits of the crayfish Gq in the detergent SML than in the membranes. The weak interaction of Gqα with Gβγ in SML has been suggested also for squid Gq [13]. It has been reported that the interaction of a with βγ subunits of Gt is affected by Mg^{2+} concentration [34]. Unlike Gt, the αβγ subunits of crayfish Gq were not co-precipitated together with irradiated rhodopsin in the presence or absence of Mg^{2+} and/or Ca^{2+} (0.1, 2, 5 and 10 mM; data not shown).

In summary, an immunoprecipitation system using anti-rhodopsin monoclonal antibody has facilitated the demonstration of light-dependent and GTP-modulated binding of Gqα to rhodopsin in crayfish photoreceptors: i.e. photoactivated rhodopsin activates Gq. The Gq class of G-protein possibly plays a central role in invertebrate phototransduction, which is therefore a

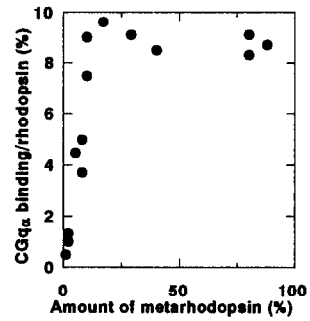

Fig. 3. Relationship between binding rate of CGqα and the amount of *meta*-rhodopsin. The protein extracts were irradiated for 15 s. using several kinds of neutral density filters (Toshiba) to produce various amounts of metarhodopsin on the extracts. Aliquots of non-irradiated extracts were analyzed by HPLC to estimate the relative amount of metarhodopsin. The rest extracts were used for immunoprecipitation to estimate relative amount of CGq binding to rhodopsin.

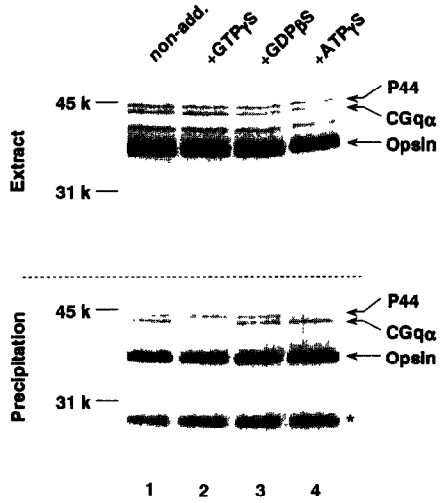

Fig. 4. Effect of nucleotide analogs on the binding of CGqα to irradiated rhodopsin. SDS-PAGE profiles showing proteins extracted from the irradiated photoreceptor membranes after incubation with nucleotide analogs (upper) and immunoprecipitated proteins using AbRh-agarose (lower). 50–20 kDa regions of CBB stained gels are shown. Lane 1, after incubation without nucleotide analogues; lane 2, with 20 μM GTPγS; lane 3, with 20 μM GDPβS; lane 4, with 20 μM ATPγS. Each sample contained 7 μg protein. Asterisk indicates IgG light-chain.

model for studies of the receptor-Gq-PLC cascade. Our precipitation system also provides evidence for direct or indirect association of rhodopsin with an actin-like protein. At present, the relevance of this association to the visual process is unknown.

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