Squid photoreceptor phospholipase C is stimulated by membrane Gqα but not by soluble Gqα

Tatsuo Suzuki[a,*], Akihisa Terakita[b], Kinya Narita[c], Kazuo Nagai[a], Yasuo Tsukahara[d], Yuji Kito[c]

[a]*Department of Pharmacology, Hyogo College of Medicine, Nishinomiya, Hyogo 663, Japan*
[b]*Department of Biophysics, Kyoto University, Kyoto, Kyoto 606, Japan*
[c]*Department of Biology, Osaka University, Toyonaka, Osaka 560, Japan*
[d]*Graduate School of Information Sciences, Tohoku University and Photodynamics Research Center, The Institute of Chemical and Physical Research (RIKEN), Sendai, Miyagi 980, Japan*

Received 2 October 1995; revised version received 3 November 1995

Abstract Phospholipase C (PLC) was purified from squid retina. Soluble Gqα, membrane Gqα and Gβγ were isolated from GTPγS-treated and light-illuminated photoreceptor membranes. The membrane Gqα stimulated phosphatidyl inositol-phospholipase C (PI-PLC) activity in a dose-dependent manner. Soluble Gqα and membrane Gβγ showed no stimulating effects on PLC. GTPγS-binding was found exclusively in membrane fraction, with very little present in the KCl-soluble fraction which contained soluble Gqα. These results indicate that light-activated rhodopsin activates PLC through membrane-bound Gqα and suggest that the rhodopsin/Gq/PLC cascade might be the pathway of phototransduction in squid photoreceptors.

Key words: Phospholipase C; GTP-binding protein; Signal transduction; Photoreceptor; Vision; Squid

1. Introduction

Invertebrate rhabdomeric photoreceptor cells depolarize in response to light, in contrast to the hyperpolarizing response in vertebrate photoreceptor cells. The molecular mechanism of phototransduction is well understood in vertebrate photoreceptors [1], but it is still unclear in invertebrate photoreceptors [2]. Molecular genetics of the fruitfly *Drosophila* have shown that phospholipase C is an essential enzyme for phototransduction [3]. Photoreceptor-specific GTP-binding proteins have been identified in fly [4,5], crayfish [6] and squid [7,8], and they belong to the Gq class, which is believed to couple to β-type phospholipase C (PLCβ) [9]. A recent study of the fly mutant of *dgq* gene has suggested an essential role for Gq in invertebrate phototransduction [10].

In previous papers [11,12], we demonstrated the presence of Gq-type G-protein and PIP_2-specific PLC in squid photoreceptor membranes. Light and GTP stimulated the squid PI-PLC and the effects were synergistic, suggesting that, following light absorption, rhodopsin activates PLC via Gq. Some PLCβs are reported to be activated not only by the α-subunit of Gq but also by βγ-subunits (reviews [9,13]). In the squid photoreceptor, however, there have been no studies to confirm which subunits activate the PLC. About half of Gqα was found in the soluble fraction of squid photoreceptors [12]. The role of soluble Gqα is another interesting problem. Although a recent paper reported the activation of PI-PLC by soluble Gqα [14], the functional difference between soluble and membrane-bound Gqαs remains to be clarified.

In the present study, we isolated soluble Gqα, membrane Gqα and Gβγ from squid photoreceptors and studied the effects of these G-protein subunits on purified PLC. GTPγS-binding to soluble Gqα was also investigated to clarify the function of soluble Gqα.

2. Materials and methods

Living squids, *Watasenia scintillans*, were collected in the Japan Sea at night, and the eyes were enucleated under dim red light in a dark room. Eye cups were immediately frozen in liquid nitrogen and kept at −80°C until use.

2.1. Purification of PLC

Eye cups were quickly thawed in 20 mM HEPES buffer (pH 7.2) containing 1 μM APMSF, 0.1 mM EGTA and 0.1 mM DTT (buffer A) and agitated in fresh buffer to suspend the outer segments of photoreceptor cells. The outer segment suspension was homogenized in buffer A and centrifuged at 15,000 rpm for 45 min at 4°C. The precipitate was homogenized with 0.4 KCl in buffer A, centrifuged at 15,000 rpm for 45 min, and the supernatant was used as a crude extract. PLC was purified by monitoring PIP_2 hydrolysis activity and reactivity to antibody against PLC, as reported previously [12]. The 0.4 M KCl extract was diluted to 0.1 M and applied to a DEAE-column equilibrated with 0.1 M NaCl in 20 mM Tris-HCl (pH 7.2)/1 μM APMSF/0.1 mM EGTA/0.1 mM DTT (buffer B). Fractions passed through the DEAE-column were applied to a Heparin column (HiTrap Heparin, Pharmacia) and eluted with a 0.1–1.0 M NaCl gradient in buffer B. Active fractions were pooled and buffer was exchanged by gel-filtration (Econo-Pac 10DG, Biorad) to 20 mM phosphate buffer (pH 7.2) containing 1 μM APMSF, 0.1 mM EGTA and 0.1 mM DTT (buffer C). This was applied to cation-exchange column (Resource S, Pharmacia) and eluted with a 0.1–0.5 M NaCl gradient in buffer C. The peak fraction eluted at about 0.2 M NaCl was applied to a gel-filtration column (Superose 12H, Pharmacia) and eluted with buffer B. PLC was obtained as a single peak near 130 kDa.

2.2. Activation and purification of soluble Gqα, membrane Gqα and Gβγ

Rhabdomal membranes were isolated by sucrose floatation under hypotonic buffer conditions (20 mM HEPES) as described previously [12]. The membranes were suspended in 20 mM HEPES/10 μM GTPγS/2 mM $MgCl_2$ and incubated at 4°C for 30 min in the light. 4.0 M KCl was added to the membrane suspension to a final concentration of 0.4 M, and the membranes were homogenized and centrifuged at 15,000 rpm for 45 min. The supernatant was diluted to 0.1 M KCl, applied to

*Corresponding author. Fax: (81) 7984 56332.

Abbreviations: GTPγS, guanosine-γ-thio-triphosphate; G-protein, heterotrimeric GTP-binding protein; PIP_2, phosphatidyl inositol bisphosphate; PLC, phospholipase C; PI-PLC, phosphatidyl inositol-phospholipase C; APMSF, (*p*-amidinophenyl) methansulfonyl fluoride; DTT, (±)-dithiothreitol (*threo*-1,4-dimercapto-2,3-butanediol); CBB, Coomassie brilliant blue.

SSDI 0014-5793(95)01364-4

an anion-exchange column (HiTrap Q, Pharmacia) and eluted with a 0.1–0.5 M NaCl gradient in buffer B. A peak fraction eluted at about 0.4 M, which was reactive to Ab GqC (antibody against C-terminus of Gqα [12]), was collected, concentrated in the presence of 1% *n*-octyl-β-D-glucoside (O.G.), applied to Superose 12H and eluted with 1% O.G. in buffer B. Soluble Gqα was obtained as a single peak near 40 kDa.

The residual 0.4 M KCl-washed rhabdomal membranes were solubilized with 2% sucrose monolaurate in buffer A (EGTA-omitted) and applied to a ConA-Sepharose column (Pharmacia) to trap rhodopsin. The fraction passed through the ConA-column was applied to HiTrap Q and eluted with a 0.1–0.5 M NaCl gradient in buffer B containing 1% O.G. A peak fraction reactive to antibody against common Gβ (SW/1, DuPont) was eluted at about 0.2 M and a peak fraction reactive to Ab GqC was eluted at about 0.4 M. Each fraction was concentrated, applied to Superose 12H and eluted with 0.1 M NaCl in buffer B containing 1% O.G. A single peak at about 40–50 kDa was obtained in both fractions.

2.3. Reconstitution and PI-PLC assay

The purified soluble Gqα, membrane Gqα and Gβγ in 1% O.G. were mixed with an equal volume of phosphatidyl ethanolamine (PE)/phosphatidyl serine (PS) mixed vesicles (each 150 μg/100 μl) and passed through a small Sephadex G50 (Pharmacia) column equilibrated with 20 mM HEPES, to remove detergent. The lipid vesicles containing G-proteins were mixed with purified PLC and PI-PLC activity was determined by the same method as in the previous paper [12]. [^{3}H]PIP_2/PE mixed vesicles (5 nmol PIP_2) were added to enzyme/G-protein mixture at 0°C and incubated at 20°C for 20 min. Free calcium concentration was kept at 0.1 μM with Ca^{2+}/EGTA buffer as described [12].

2.4. GTPγS-binding assay

Rhabdomal membranes prepared under hypotonic buffer conditions were suspended in 20 mM HEPES (pH 7.2) containing 2.5 mM $MgCl_2$. A hundred μl of the membrane suspension (12.5 μg protein) was mixed with 25 μl of 5 μM [^{35}S]GTPγS (NEM, 24,000 cpm/μl) in 20 mM HEPES/1 mM DTT. After incubation at 4°C for 5 min, 500 μl of ice-cold 0.5 M KCl in buffer D (20 mM HEPES/2 mM $MgCl_2$) was added to the sample, mixed vigorously and centrifuged at 15,000 rpm for 20 min at 4°C. Supernatant was collected and precipitate was re-suspended into 500 μl of buffer D containing 0.4 M KCl. Aliquots (one fifth portion) of supernatant and membrane suspension were blotted to a nitrocellulose (NC) membrane (Schleicher and Schuell) of 4 mm diameter, using a 96-well dot-blotter, and the NC membrane was washed 5 times with 300 μl of buffer D. After drying, the NC membrane was punched at 6 mm diameter and the radioactivity of each was determined in 2 ml scintillator fluid (Aquasol 2, DuPont) with a liquid scintillation counter (Packard 2500TR). Control experiments were carried out with reaction medium containing 2 mM EDTA instead of $MgCl_2$, Mg^{2+}-free KCl solution and Mg^{2+}-free washing buffer with 2 mM EDTA. The binding capacity of the NC membrane was checked by applying 0.4 M KCl extracts and residual membrane suspension, and fractions passed through NC membrane were blotted to a different NC membrane. No immunoreactivity to Ab GqC was found in the passed-through fractions, indicating that all Gqαs were trapped by the NC membrane.

2.5. SDS-PAGE and immunoblotting

SDS-PAGE was performed on slab gels following Laemmli [15] at an acrylamide concentration of 10%. Part of the gel was stained with Coomassie brilliant blue (CBB) and the rest was used for immunoblotting. Proteins were electrically transferred to PVDF membrane (PVDF-plus, Micron Inc.) and treated with anti-peptide antibodies: Ab GqC [12] and Ab DGqN [11] for Gqα; Ab PLCβ4/norp A(Y) [12] for PLC; and antibody against common Gβ (GW/1, DuPont). Proteins reactive to antibodies were visualized by the peroxidase/diaminobenzidine (DAB) method.

2.6. Rhodopsin and protein concentrations

Rhodopsin concentration was determined by spectroscopy. Rhabdomal membranes were solubilized with 2% sucrose monolaurate, adjusted to pH 11.0 and irradiated with $\lambda > 520$ nm light. Rhodopsin concentration was calculated from the absorbance difference at 482 nm and a molar extinction coefficient of 35,000 (this squid has three kinds of visual pigment [16], the extinction coefficient has not been determined, so the value of another squid rhodopsin was used [17]).

Protein content was determined by BCA reagent (Pierce) using bovine serum albumin as standard.

3. Results

3.1. PLC and Gqα released from rhabdomal membranes by 0.4 M KCl

When rhabdomal membranes prepared under hypotonic conditions were treated with 0.4 M KCl, some proteins were

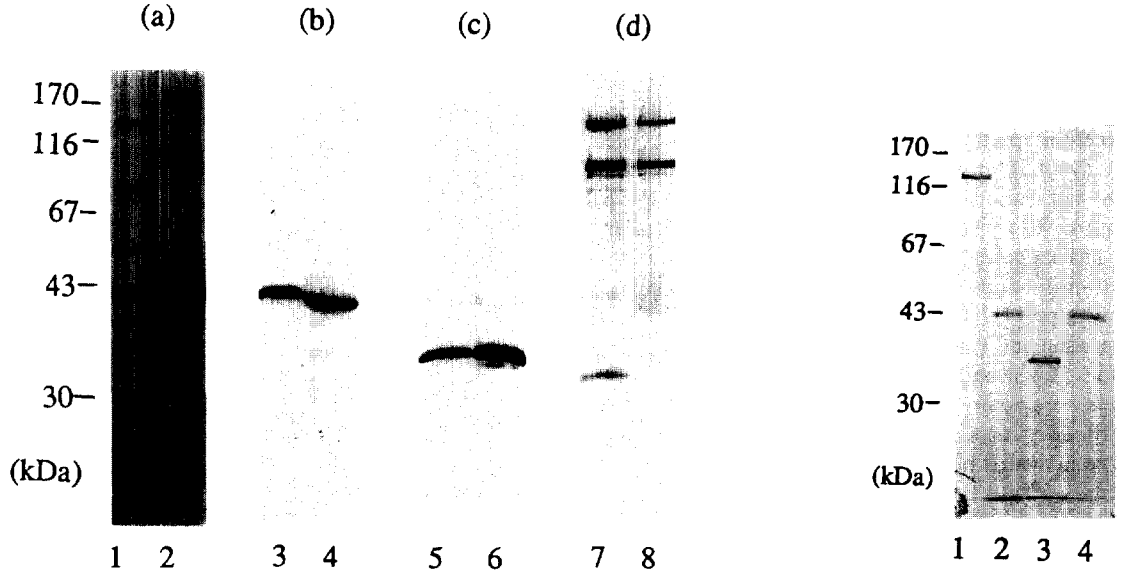

Fig. 1. SDS-PAGE of the rhabdomal membrane extracts and purified PLC and Gq subunits. (Left) Membrane extracts. Lanes 1, 3, 5 and 7 are 0.4 M KCl extracts; lanes 2, 4, 6 and 8 are detergent extracts. The protein amount in each lane corresponds to 1/100 retina. (a) CBB stain of gel. (b)–(d) immunostain with anti-peptide antibodies Ab GqC (b), GW/1 (c) and Ab PLCβ4/norp A(Y) (d). Molecular weights are indicated to the left. (Right) Purified PLC, soluble Gqα, membrane Gqα and Gβγ. G-proteins were purified from GTPγS-treated rhabdomal membranes and 0.4 μg of each were subjected to SDS-PAGE and stained with CBB. Lane 1, PLC; lane 2, soluble Gqα; lane 3, Gβγ; lane 4, membrane Gqα (see section 2 for details).

liberated from the membrane. The proteins extracted with 0.4 M KCl and the membrane solubilized with 2% sucrose monolaurate were subjected to SDS-PAGE and immunoblotting, and the results are shown in Fig. 1. About a half of the protein reactive to Ab GqC was extracted with 0.4 M KCl (Fig. 1b). This protein was also reactive to antibody against the N-terminus peptide of Gqα, Ab DGqN (data not shown). From the antibody reactivity and apparent molecular weight of 42 kDa, the protein is considered to be the buffer-soluble form of Gqα. Most of the Gβγ remained in the membrane (Fig. 1c). A 130 kDa protein reactive to Ab PLCβ4/norp A(Y) was identified as PLC and a large portion of it was extracted with 0.4 M KCl (Fig. 1d). The immunoreactive bands of around 95 kDa in Fig. 1d are thought to be degradation products of PLC, because the increase in these bands accompanies a decrease in the 130 kDa band when the sample was left without protease inhibitor and EGTA.

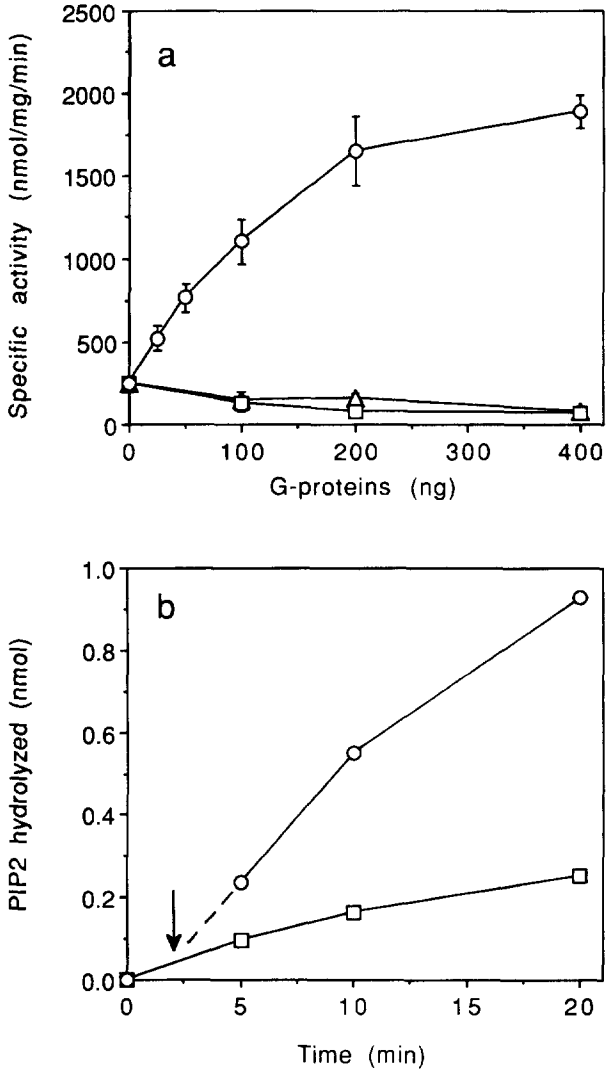

Fig. 2. Effects of G-proteins on PI-PLC activity. (a) Effects of soluble Gqα (△), Gβγ (□) and membrane Gqα (○) on purified PLC (40 ng). Means ± S.D. of three determinations. (b) Time course of PLC activation by membrane Gqα. Two minutes after starting the PIP_2 hydrolysis by PLC (40 ng), activated membrane Gqα (GTPγS-bound form, 200 ng) was added to the reaction mixture (arrow). The values at 0 min were determined at 0°C immediately after mixing PLC and PIP_2. ○, with Gqα; □, without Gqα. Means of duplicate determinations.

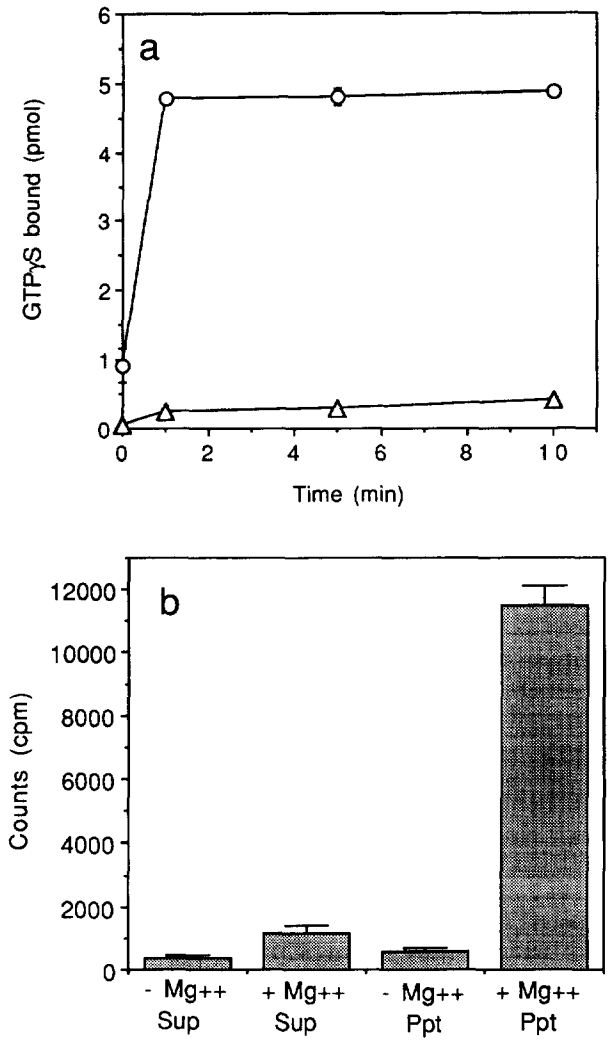

Fig. 3. GTPγS-binding to rhabdomal membrane and KCl-soluble fractions. (a) Time course of GTPγS-binding with (○) and without (△) Mg^{2+}. Rhabdomal membranes were mixed with [^{35}S]GTPγS and incubated at 4°C in the presence or absence of 2 mM $MgCl_2$. The values at 0 min were determined on nitrocellulose membrane in a dot-blotter under suction. Means ± S.D. of three determinations. (b) Rhabdomal membranes were incubated with [^{35}S]GTPγS at 4°C in the reaction mixture with ($+Mg^{2+}$) or without ($-Mg^{2+}$)$MgCl_2$. After 5 min incubation, samples were mixed with 0.4 M KCl, and soluble (Sup) and insoluble (Ppt) fractions were obtained by centrifugation. Means ± S.D. of five determinations.

3.2. *Purified PLC, soluble Gqα, membrane Gqα and Gβγ*

We purified PLC from 0.4 M KCl extracts with four steps of chromatography; after the final step of purification, PLC was the single component with apparent molecular weight of 130 kDa (lane 1 in Fig. 1, right).

The soluble Gqα , membrane Gqα and Gβγ were each purified with two chromatography steps (lanes 2–4 in Fig. 1, right). The molecular weight of soluble Gqα was exactly the same as that of membrane Gqα. In the Gβγ fraction (lane 3), the γ-subunit was invisible due to location at the dye-front, but we

confirmed the γ-subunit with a 5–25% gradient gel (data not shown).

3.3. Effects of three G-proteins on PI-PLC activity

The three form of purified G-protein subunits, soluble Gqα, membrane Gqα and Gβγ, were obtained (Fig. 1, right) and each of them were incorporated into lipid vesicles (Soluble Gqα is considered to be bound to lipid vesicle under hypotonic buffer conditions). The effects of these proteins on PI-PLC activity were then investigated and the results are shown in Fig. 2. Membrane Gqα stimulated PI-PLC activity in a dose-dependent manner, maximally about 8-fold: soluble Gqα and Gβγ, however, had no stimulating effects on PI-PLC (Fig. 2a). The stimulating effects quickly appeared and showed no apparent time-lag (Fig. 2b). Without pre-treatment of rhabdomal membranes with GTPγS, no stimulating effect of membrane Gqα on PI-PLC was observed (data not shown).

3.4. [^{35}S]GTPγS-binding to KCl-soluble and insoluble fractions

GTPγS-binding to KCl-soluble and insoluble fractions was performed to investigate a cause of the inability of soluble Gqα to stimulate PI-PLC. For control experiments, the Mg^{2+}-free system was employed, because Mg^{2+} is essential for the GDP-GTP exchange reaction on the α-subunit of G-protein [18].

The [^{35}S]GTPγS-binding reaction in the presence of Mg^{2+} was rapid: complete within 1 min at 4°C; without Mg^{2+}, however, binding was very slow and at a low level (Fig. 3a). In the presence of 100 μM GTP, [^{35}S]GTPγS-binding was reduced to the level of the Mg^{2+}-free control (data not shown). When the GTPγS-binding in the absence of Mg^{2+} is subtracted from the value in the presence of Mg^{2+}, 4.5 pmol GTPγS binds to the rhabdomal membranes which contains 100 pmol rhodopsin. If Gqα is assumed to be present at one tenth the concentration of rhodopsin, as estimated in squid [7] and crayfish [6], nearly half the Gqα binds GTPγS under the experimental conditions.

After incubating with [^{35}S]GTPγS for 5 min, high radioactivity was found in the insoluble membrane fraction but very low in the KCl-soluble fraction (Fig. 3b). Again, when the binding without Mg^{2+} is assumed to be non-specific, the soluble fraction binds less than 7% of total bound GTPγS. These results show that soluble Gqα fails to bind GTPγS.

4. Discussion

Squid Gqα exists in two different forms, soluble and membrane Gqαs. We extracted soluble Gqα, membrane Gqα and Gβγ from GTPγS-treated rhabdomal membranes and purified each by the same procedures to the grade of single band in SDS-PAGE (see Fig. 1, right). Of these subunits, only membrane Gqα stimulated PIP_2 hydrolytic activity of the purified PLC. Soluble Gqα and Gβγ had no ability to stimulate PLC (see Fig. 2a). It has been reported that PLCβs are stimulated not only by α-subunits of Gq-class G-proteins but also by βγ-subunits (reviews [9,13]). However, PLCβ4, recently found in the vertebrate retina [19], was stimulated only by α-subunits of Gq-class [20]. The stimulation of squid photoreceptor PLC is therefore similar to that of vertebrate PLCβ4.

The results for soluble Gqα in the present study are inconsistent with those of Mitchell et al. [14], who reported that soluble Gqα stimulated their purified PLC. They extracted soluble Gqα with 2 M KCl from rhabdomal membranes and used it for PLC stimulation without further purification. Since, according to our data, squid PLC dissociates from membrane under high ionic conditions, one explanation of the discrepancy is that PLC was present in their soluble Gqα preparation. A second explanation is that AlF_4^- was used in their experiments as the activator of Gq: the GDP-bound form of Gαs can be activated by AlF_4^- without interaction with ligand-activated receptors [21].

Our results of the GTPγS-binding experiment in soluble and membrane fractions demonstrated that soluble Gqα does not bind GTPγS. The reason why soluble Gqα does not stimulate PLC in our experiments is therefore explained by its failure to bind the nucleotide. Since interactions with Gβγ and with ligand-activated receptors are required for the GDP-GTP exchange on α-subunit of G-protein (review [18]), one may suggest that soluble Gqα is not able to interact with active metarhodopsin or Gβγ-subunits. In the soluble fraction, however, about 7% of total bound GTPγS was detected (see Fig. 3b). If small amounts of the active form of Gqα (GTPγS-bound form) are produced, some activation of PLC would be observed at higher concentrations of soluble Gqα, but this did not occur in our results (see Fig. 2a). There is also the possibility that the GTPγS-bound form of soluble Gqα, if produced, does not interact with PLC.

Our recent immunocytochemical and biochemical studies on crayfish photoreceptors have demonstrated that Gqα translocates from the rhabdomal membrane to the cytosol during light-adaptation and from cytosol to membrane during dark-adaptation [22]. Chemical treatment to cleave thioester linkage results in an increase in the soluble form of Gqα [22]. These facts suggest that palmitoylation and depalmitoylation (reviews [23,24]) occur on photoreceptor Gqα in response to light stimulation. In our present study, soluble Gqα did not bind GTPγS and did not stimulate PLC. The soluble Gqα might therefore be a depalmitoylated form which does not interact with activated rhodopsin or Gβγ.

The GDP-GTP exchange on the α-subunit of Gq, which is catalyzed by the active metarhodopsin and Gβγ-subunits, was mimicked by GTPγS-binding, which was very rapid and completed within 1 min at 4°C (see Fig. 3a and also [25]). The next reaction, the stimulation of PLC by GTP-Gqα, also was quite rapid, with little apparent time-lag (see Fig. 2b). Therefore, although the conditions of our present in vitro experiments are far from those in vivo, the rhodopsin/Gq/PLC pathway is rapid enough to offer a candidate mechanism for cephalopod phototransduction.

Acknowledgements: We thank Ms. E. Takai for her help, Dr. I. Gleadall (Ohu University) for comments on the manuscript, and Messrs. T. Nakajima, Y. Matsubara and K. Hamada for their technical suggestions. This work was supported by the Grant-in-Aids for Scientific Research (07839018) from Japanese Ministry of Education, Science and Culture.

References

[1] Stryer, L. (1986) Annu. Rev. Neurosci. 9, 87–119.
[2] Rayer, B., Naynert, M. and Stieve, H. (1990) J. Photochem. Photobiol. 7, 107–148.
[3] Bloomquist, B.T., Shortridge, R.D., Schneuwly, S., Perdew, M., Montell, C., Steller, H., Rubin, G. and Pak, W.L. (1988) Cell 54, 723–733.

[4] Strathmann, M. and Simon, M.I. (1990) Proc. Natl. Acad. Sci. USA 87, 9113–9117.
[5] Lee, Y.-J., Dobbs, M.B., Verardi, M.L. and Hyde, D.R. (1990) Neuron 5, 889–898.
[6] Terakita, A., Hariyama, T., Tsukahara, Y., Katsukura, Y. and Tashiro, H. (1993) FEBS Lett. 330, 197–200.
[7] Pottinger, J.D.D., Ryba, N.J.P., Keen, J.N. and Findlay, J.B.C. (1991) Biochem. J. 279, 323–326.
[8] Ryba, N.J.P., Findlay, J.B.C. and Reid, J. (1993) Biochem. J. 292, 333–341.
[9] Exton, J.H. (1994) Annu. Rev. Physiol. 56, 349–369.
[10] Lee, Y.-J., Shah, S., Suzuki, E., Zars, T., O'Day, P.M. and Hyde, D.R. (1994) Neuron 13, 1143–1157.
[11] Suzuki, T., Narita, K., Yoshihara, K., Nagai, K. and Kito, Y. (1993) Zool. Sci. 10, 425–430.
[12] Suzuki, T., Narita, K., Yoshihara, K., Nagai, K. and Kito, Y. (1995) Vision Res. 35, 1011–1017.
[13] Clapham, D.E. and Neer, E.J. (1993) Nature 365, 403–406.
[14] Mitchell, J., Gutierrez, J. and Northup, J.K. (1995) J. Biol. Chem. 270, 854–859.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Seidou, M., Sugahara, M., Uchiyama, H., Hiraki, K., Hamanaka, T., Michinomae, M., Yoshihara, K. and Kito, Y. (1990) J. Comp. Physiol. A 166, 769–773.
[17] Suzuki, T., Uji, K. and Kito, Y. (1976) Biochim. Biophys. Acta 428, 321–338.
[18] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[19] Lee, C.-W., Park, D.J., Lee, K.-H., Kimm, C.G. and Rhee, S.G. (1993) J. Biol. Chem. 268, 21318–21327.
[20] Jiang, H., Wu, D. and Simon, M.I. (1994) J. Biol. Chem. 269, 7593–7596.
[21] Smrcka, A.V., Hepler, J.R., Brown, K.O. and Sternweis, P.C. (1991) Science 251, 804–807.
[22] Terakita, A., Takahama, H., Tamotsu, S., Suzuki, T., Hariyama, T. and Tsukahara, Y. (1994) Zool. Sci. 11 (Suppl.), 99.
[23] Casey, P.J. (1994) Curr. Opin. Cell Biol. 6, 219–225.
[24] Wedegaertner, P.B., Wilson, P.T. and Bourne, H.R. (1995) J. Biol. Chem. 270, 503–506.
[25] Nobes, C., Baverstock, J. and Saibil, H. (1992) Biochem. J. 287, 545–548.