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Immunochemical Detection of GTP-binding Protein in Cephalopod Photoreceptors by Anti-peptide Antibodies

TATSUO SUZUKI[1], KINYA NARITA[2], KAZUO YOSHIHARA[3], KAZUO NAGAI[1] and YUJI KITO[2]

[1]*Department of Pharmacology, Hyogo College of Medicine, Nishinomiya, Hyogo 663,* [2]*Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560,* [3]*Suntory Institute for Bioorganic Research, Shimamoto, Osaka 618, Japan*

ABSTRACT—We prepared polyclonal antibodies (Pab) against the following peptides: partial sequences of bovine transducin α subunit including the ADP-ribosylation sites sensitive to cholera toxin (CTX) and pertussis toxin (PTX) and the N-terminus of *Drosophila* GTP-binding protein qα (DGqN) ; Pab CTX, Pab PTX and Pab DGqN, respectively. These antibodies were specific to the peptides used as antigen and no crossreactivity was observed. Pab CTX and Pab PTX reacted with bovine transducin α subunit and the reactivity was lost by preincubation with the specific antigen peptide. Proteins of 41–42 kDa in octopus and squid photoreceptor membranes were recognized by Pab DGqN but not by Pab CTX or Pab PTX. Anti-α antibody (GA/1) reacted with the same bands as Pab DGqN recognized. These results suggest that the major GTP-binding protein in cephalopod photoreceptors is a Gq-type, similar to *Drosophila* Gq.

INTRODUCTION

Signal transduction of vertebrate photoreceptors has been well studied and the main sequence of events at the molecular level is well established [16, 20]. Light triggers rhodopsin to activate GTP-binding protein (G-protein), transducin, which in turn activates an effector enzyme, phosphodiesterase, resulting in a decrease of cyclic guanosine monophosphate (cGMP) and the closure of cGMP-dependent cation channels.

In invertebrate photoreceptors, electrophysiological studies have suggested that, besides cGMP, inositol trisphosphate (IP_3) and/or calcium are candidates for intracellular second messengers [2, 6, 16]. Biochemical studies have shown light-dependent binding of non-hydrolysable GTP analogues to the photoreceptor membrane [17, 23] and light-dependent GTPase activity [3, 4, 13, 23], which indicate involvement of G-protein in phototransduction in invertebrate photoreceptors.

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Transducin, the key molecule in vertebrate phototransduction, is susceptible to modification (ADP-ribosylation) both by cholera and pertussis toxins [1, 24]. ADP-ribosylation by bacterial toxins had been applied to identify G-proteins of invertebrate photoreceptors (mainly in cephalopods) but the reported results were contradictory [7, 17, 22, 23], so the type of G-protein, as identified by the ADP-ribosylation, has remained uncertain.

Recently, G-protein was partially purified from squid photoreceptor membrane and its partial sequence was suggesting a Gq-type G-protein [15]. Genes encoding the α subunit of G-protein coupling to phosphoinositide-specific phospholipase C have been cloned and amino acid sequences were deduced from cDNAs in both vertebrates and invertebrates (Gqα, $G_{11}\alpha$, DGqα) [12, 18]. These three Gq-type proteins have some unique sequences which are not found in other G-proteins so far reported. In the present study, we prepared anti-peptide antibodies which react specifically with the N-terminus of *Drosophila* Gqα subunit (DGqα) and with peptides including ADP-

ribosylation sites of transducin α subunit. The G-protein of cephalopod photoreceptors was investigated using these anti-peptide antibodies.

MATERIALS AND METHODS

Preparation of anti-peptide antibodies

We synthesized three peptides: EQDVLRSRVKTTGI (residues 167–180) and DIIIKENLKDCGLF (337–350), corresponding to amino acid sequences around the ADP-ribosylation sites of bovine transducin [8]; and CLSEEAKEQKRINQE (4–19) of the N-terminal region of *Drosophila* Gqa [18]. The synthetic peptides were purified by reversed phase HPLC (C4 column), and amino acid composition was confirmed by amino acid analysis of each purified peptide. Each peptide of 5 mg was conjugated to 10 mg of bovine serum albumin (BSA, Sigma) using 1 mg of *m*-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS, Sigma) as a cross-linker.

Japanese white rabbits were immunized with 1 ml emulsion of 1 mg BSA-peptide conjugates mixed with Freund's complete adjuvant. Three weeks later, the rabbits were boosted with the same amount of conjugates and Freund's incomplete adjuvant, and after two weeks antisera were obtained. The immunoglobulin fraction was purified by ammonium sulfate fractionation and DEAE-column chromatography. BSA-Sepharose complex was made by coupling BSA to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instruction, and anti-BSA antibodies were removed by the BSA-Sepharose column. The antibodies against peptides around the ADP-ribosylation sites of transducin, sensitive to cholera and pertussis toxins, and against the N-terminus of *Drosophila* Gqα were named Pab CTX, Pab PTX and Pab DGqN, respectively.

Activities and specificities of the anti-peptide antibodies were determined by the method of enzyme-linked immunosorbent assay (ELISA), using the above three peptides as adsorbed antigens (each 0.5 μg/well). Antibodies diluted serially were reacted with adsorbed peptides. The bound antibodies were determined by anti-rabbit IgG (goat)-peroxidase (Wako Chemicals) by the standard method with *o*-phenylenediamine/hydrogen peroxide as substrates; absorbance at 492 nm was determined with a microplate reader.

Preparation of photoreceptor membranes and transducin

Outer segment membranes of octopus (*Octopus vulgaris*), squid (*Todarodes pacificus*) and bovine photoreceptors were prepared by the method of sucrose-density-gradient centrifugation [10]. Membranes floated on 32% sucrose were diluted with 50 mM Tris-HCl buffer (pH 7.4) and precipitated by centrifugation. The membrane preparations were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without further treatment.

Bovine transducin was prepared by the method of Kuhn [11]. Outer segment membranes were illuminated and washed three times with hypotonic buffer (5 mM Tris-HCl, pH 7.4), and once with 100 mM Tris-HCl buffer (pH 7.4). The membranes were then washed three times with the hypotonic buffer containing 200 μM GTP, and soluble fractions, containing transducin, were combined and concentrated.

SDS-PAGE and immunoblotting

The photoreceptor membranes were solubilized with sample buffer, subjected to SDS-PAGE and transferred to nitrocellulose sheet. The sheet was blocked with 1% gelatin/casein solution. Anti-peptide antibodies were diluted with 1% gelatin/casein in phosphate buffered saline (PBS) containing 1% BSA and preincubated at room temperature for 1 hr (1/500 dilution for Pab CTX and Pab PTX, 1/1000 for Pab DGqN), and then incubated with the nitrocellulose sheet at 4°C overnight. The sheet was then treated with anti-rabbit IgG (goat)-peroxidase, and immunoreactive proteins were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB)/hydrogen peroxide solution.

Anti-α antibody (GA/1; specific to the GTP/GDP-binding site, GAGESGKSTIVK; common to Gt, Gs and Go) and anti-β antibody (SW/1, specific to the C-terminus of common β) were purchased from Daiichi Chemicals Co., and immunoblot analyses were performed as above (1/300 dilution for GA/1 and 1/500 for SW/1).

RESULTS

The specificity and reactivity of the anti-peptide antibodies are shown in Fig. 1. Each antibody preparation reacted specifically with the peptide used as antigen, with no crossreactivity. The reactivities to BSA were 1/500–1/1000 of those to the specific peptides.

The transducin fraction extracted from bovine outer segment membranes was subjected to SDS-PAGE, transferred to nitrocellulose sheet and treated with the anti-peptide antibodies. The main components of this fraction were transducin α and β subunits (Fig. 2a). Pab CTX and Pab PTX recognized the transducin α subunit: apparent molecular mass 39 kDa (Fig. 2b). The reactions were completely inhibited by preincubation of Pab CTX and Pab PTX with respective peptide antigen.

Immunoblot analyses of octopus, squid and bovine photoreceptor membranes were performed using the three anti-peptide antibodies, as shown in Fig. 3b. Pab DGqN recognized a 41 kDa protein of octopus and a 42 kDa protein of squid membranes but no bovine protein. The reactivity of Pab DGqN against the 41–42 kDa proteins was significantly reduced by preincubation with the N-terminal peptide of DGqα (data not shown). Pab CTX and Pab PTX did not recognize the 41–42 kDa proteins of octopus and squid membranes, though unknown 35 kDa bands were weakly stained by Pab CTX. The results of analyses with anti-α antibody (GA/1) and anti-β antibody (SW/1) are shown in Fig. 3c. GA/1 reacted with the same 41–42 kDa proteins in octopus and squid membranes as Pab DGqN recognized, although the reactivity was lower than with transducin α. The bands with apparent molecular mass of about 35 kDa were equally recognized by anti-β antibody (SW/1) in the membranes of three species. These results show that cephalopod photoreceptor membranes contain Gq-type G-protein and no transducin-like G-protein.

DISCUSSION

The antibodies, Pab CTX and Pab PTX, were proved to be good tools for detection of G-proteins

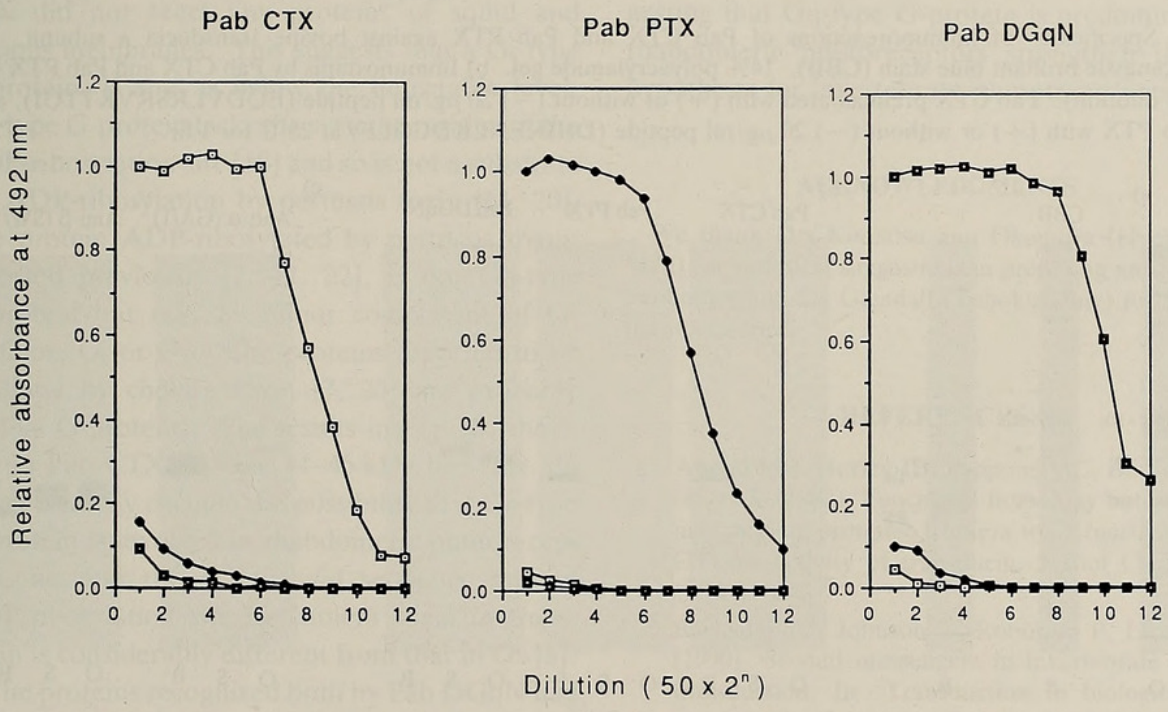

FIG. 1. Reactivities and specificities of anti-peptide antibodies determined by ELISA. Three peptides corresponding to: bovine transducin sequences around ADP-ribosylation sites sensitive to cholera toxin (EQDVLRSRVKTTGI, □), and pertussis toxin (DIIIKENLKDCGLF, ◆); N-terminus of Drosophila Gqα (CLSEEAKEQKRINQE, ■), were used as adsorbed antigens.

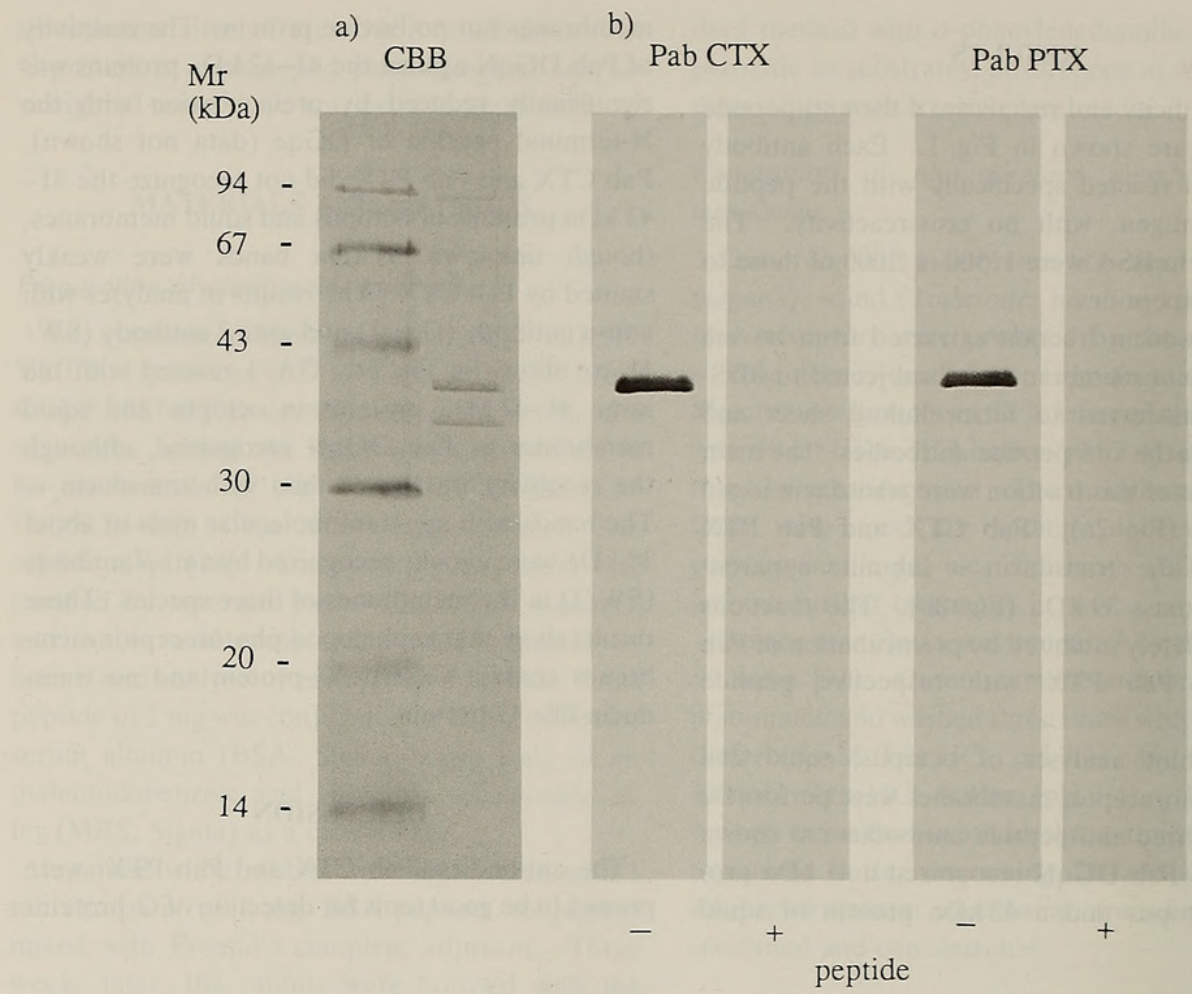

FIG. 2. Specificities of immunoreactions of Pab CTX and Pab PTX against bovine transducin α subunit. a) Coomassie brilliant blue stain (CBB). 14% polyacrylamide gel. b) Immunostains by Pab CTX and Pab PTX (1/500 dilution). Pab CTX preincubated with (+) or without (−) 20 μg/ml peptide (EQDVLRSRVKTTGI), and Pab PTX with (+) or without (−) 20 μg/ml peptide (DIIIKENLKDCGLF) at 25°C for 1 hr.

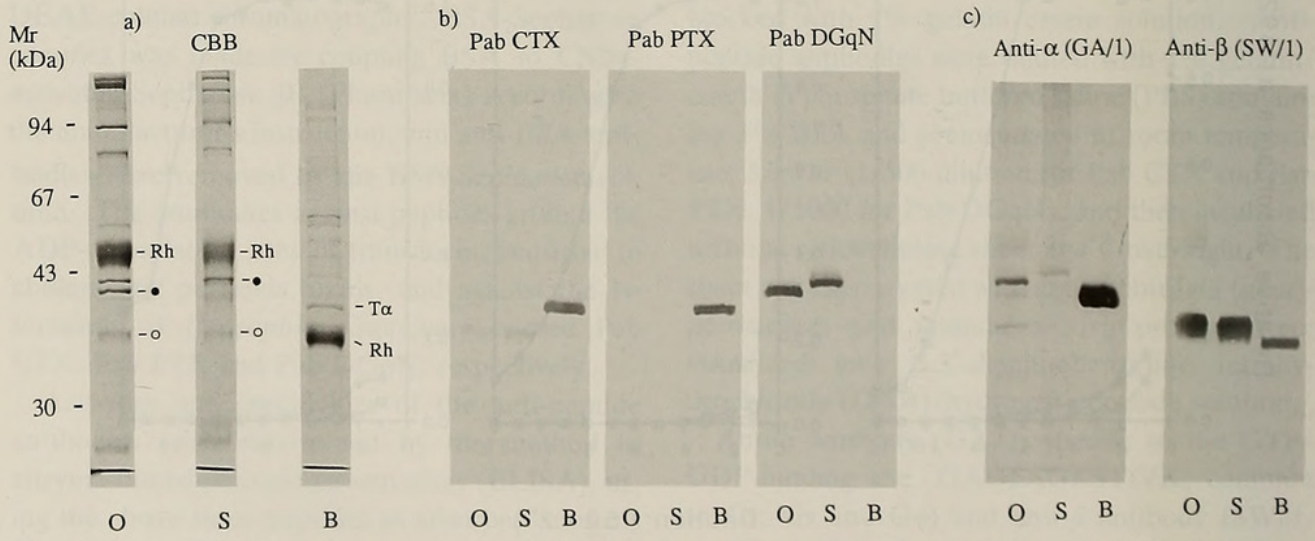

FIG. 3. Immunoblotting of octopus, squid and bovine photoreceptor membranes.a) Coomassie brilliant blue stain (CBB). 12.5% polyacrylamide gel. O, octopus; S, squid; B, bovine. The bands stained by Pab DGqN (●) and by anti-β antibody (○) are indicated. The broad bands around 45 kDa in octopus and squid and 35 kDa in bovine samples are the rhodopsins (Rh). b) Immunostains by Pab CTX, Pab PTX (1/500 dilution) and Pab DGqN (1/1000 dilution). c) Immunostains by anti-α antibody (GA/1, 1/300 dilution) and anti-β antibody (SW/1, 1/500 dilution).

having ADP-ribosylation sites similar to bovine transducin. The peptide sequence corresponding to the N-terminus of *Drosophila* Gqα is highly conserved among Gqα, $G_{11}\alpha$ and DGqα, and has low homology with other G-protein α subunits. The antibody Pab DGqN, therefore, is considered to be specific to these three members of Gq-class G-proteins. When these three antibodies were applied to immunoblot analyses, only Pab DGqN strongly reacted with the 41–42 kDa proteins in cephalopod rhabdomeric membranes.

Reports on ADP-ribosylation of cephalopod rhabdomeric photoreceptor membrane proteins by bacterial toxins have shown that proteins with apparent molecular weight around 40–42 kDa are ADP-ribosylated by pertussis toxin [7, 17, 22], 44–45 kDa proteins by cholera toxin [7, 23] and some others by both bacterial toxins [17, 22]. The results of our present work show that cephalopod photoreceptor membranes contain no transducin-like G-protein which is detectable by both Pab CTX and Pab PTX (Fig. 3b). The sequence around the ADP-ribosylation site by pertussis toxin in Gi-type G-proteins (Gi_1 and Gi_2) is very similar to that in transducin (12 of 14 residues are identical) [8]. Pab PTX did not react any proteins of squid and octopus membranes. This suggests that a Gi-type G-protein, if any, is below the detectable level. Gq-type G-protein lacks the cysteine residue of the ADP-ribosylation site [18] and so is not a substrate for ADP-ribosylation by pertussis toxin [14, 20]. The protein ADP-ribosylated by pertussis toxin, reported previously [7, 17, 22], is not Gq-type G-protein but may be minor component of G-proteins; Gi or Go. The proteins reported to be modified by cholera toxin [7, 23] are probably Gs-like G-proteins. The results in Fig. 3b, showing no Pab CTX-positive 44–45 kDa proteins, do not necessarily exclude the possibility that Gs-type G-protein is involved in rhabdomeric photoreceptors, because the amino acid sequence around ADP-ribosylation site by cholera toxin in transducin is considerably different from that in Gs [8].

The proteins recognized both by Pab DGqN had apparent molecular weight of 41–42 kDa, which is consistent with the reported molecular weight of α subunit of Gq, 42 kDa [14, 18, 21]. Anti-α antibody (GA/1) reacted with the same proteins as Pab DGqN recognized. The reactivity of GA/1 with 41–42 kDa proteins was lower than with transducin α (Fig.3c), suggesting that the sequence of GTP/GDP binding site of cephalopod Gα is slightly different from the common sequence recognized by GA/1. The Coomassie blue stain of the SDS-PAGE gel in Fig. 3a shows that the 41–42 kDa protein is a substantial component of cephalopod photoreceptor membranes, and that its ratio to rhodopsin is nearly the same as that of transducin α subunit to rhodopsin in bovine photoreceptor membrane. These results suggest that the major G-protein in cephalopod photoreceptors is a Gq-type, similar to *Drosophila* Gq. Our results are consistent with those of Pottinger *et al.* [15] suggesting that G-protein of squid photoreceptor is Gq-type.

The biochemical pathway of signal transduction is still obscure in invertebrate rhabdomeric photoreceptors. Both IP_3 and cGMP induce a depolarizing response mimicking light stimulation in *Limulus* ventral photoreceptor [2, 6, 9, 16]. Recent biochemical studies provide evidence suggesting that cGMP is not a second messenger in rhabdomeric photoreceptors [5, 19]. Our results suggesting that Gq-type G-protein is predominant in rhabdomeric photoreceptors strengthen the IP_3 hypothesis for invertebrate phototransduction.

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