Activation of the GTP-binding protein Gq by rhodopsin in squid photoreceptors

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Photoaffinity labelling by a GTP analogue has been used to identify a 42 kDa band as the major G α subunit in squid photoreceptor membranes, recently identified by partial sequence analysis to be a member of the Gq sub-group of GTP-binding proteins [Pottinger, Ryba, Keen & Findlay (1991) *Biochem. J.* 279, 323–326]. Guanine-nucleotide-binding displacement analysis gave a stoichiometry of 1 G-protein per 12.5 rhodopsin molecules, the same as in vertebrate rod photoreceptors. Binding was not detected above background in the dark, but was rapidly activated by light. Unlike vertebrate transducin, this G-protein is very temperature-sensitive. GTP binding is maximal at temperatures less than 10 °C and is much decreased after several minutes above 18 °C. The light-stimulated GTPase rate is maximal around 10 °C, above which the loss of binding sites counteracts the increase in hydrolytic rate per site. Earlier studies described light-sensitive G α components of 40 and 45 kDa, by ADP-ribosylation in the presence of cholera and pertussis toxins. These are now shown to be very minor components, as the prolonged treatment at elevated temperature required for ADP-ribosylation is sufficient to inactivate the major G α totally. Unlike the minor G α components, the 42 kDa G α is not inhibited by Ca²⁺.

INTRODUCTION

Visual transduction in photoreceptors provides a specialized example of the signalling pathway in which the visual pigment rhodopsin activates a GTP-binding protein (G-protein) that in turn activates an effector enzyme (for reviews see [1,2]). In vertebrate rods the effector is a phosphodiesterase that hydrolyses cyclic GMP, the intracellular messenger of light excitation. Because rod outer segments contain large amounts of these proteins, the transduction system in vertebrates has been an important source of information on molecular mechanisms in the cyclic nucleotide cascade. The photoreceptors of invertebrates, on the other hand, use the phosphoinositide signalling system, a more recently discovered pathway which is equally widespread but less well understood. An abundant source of invertebrate photoreceptor membranes is the squid retina, from which a preparation of signalling components approaching the purity of that from rod outer segments can be obtained. The phosphoinositide signalling components in squid are arranged in a structurally ordered honeycomb-like network of microvilli [3].

In the photoreceptors of cephalopods (e.g. squid and octopus), a rhodopsin–G-protein cascade [4–6] stimulates phospholipase C (PLC) [7–10]. The G-protein and PLC involved in invertebrate phototransduction have not yet been functionally characterized. But molecular-genetics studies of *Drosophila* have shown that PLC is essential for the light response [11], and a *Drosophila* clone [12] and partial sequencing of a squid protein [13] have recently shown that invertebrate photoreceptors contain a Gq-like G-protein. This class of G-proteins is responsible for the activation of PLC- β in mammalian systems [14–16]. Here we present results on the activation and biochemical properties of the major G-protein in squid photoreceptors, a Gq protein accounting for the activation of light- and GTP-stimulated PLC [9,10].

EXPERIMENTAL

Membrane preparation

Squid photoreceptor membranes were prepared as described in [17]. Briefly, photoreceptor outer segments were detached from *Loligo forbesi* or *Alloteuthis subulata* retinas which had been rapidly frozen in low-Ca²⁺ artificial sea water. Photoreceptor membranes were detached by shaking and purified by flotation on a 0–36% (w/v) sucrose step. The assay buffer contained 200 mM-KCl, 2.5 mM-MgCl₂, 50 mM-Mops and 10 mM-EGTA at pH 7.5. All manipulations were done in darkness or with i.r. illumination, by using an i.r. viewer (FindRScope; FJW Industries, Mt. Prospect, IL, U.S.A.) mounted on a helmet, unless otherwise stated. Rhodopsin concentration was measured by the change in *A*₄₉₄ after bleaching at pH 10 [18].

U.v. cross-linking

Samples (200 μ l) of membrane suspension containing 7.5 μ M-rhodopsin, mixed with 1.5 μ M- or 7 μ M-[³H]p[NH]ppG (14.3 Ci/mmol; Amersham International, Amersham, Bucks., U.K.) in assay buffer under room light, were irradiated with u.v. light (0.7 mW/cm² at 254 nm) for 30 min on ice [19]. Protein was precipitated with cold acetone, and the pellet was washed twice with assay buffer. One-tenth of each sample was electrophoresed on an SDS/10%-acrylamide gel. The gel was soaked in Amplify fluorographic reagent (Amersham) and dried. Kodak X-Omat X-ray film was exposed for 3 weeks with an intensifying screen.

p[NH]ppG-binding assays

Membranes containing 1.5 μ M- or 3.5 μ M-rhodopsin were mixed with 1.4 μ M- or 0.145 μ M-[³H]p[NH]ppG (14.3 Ci/mmol) at 4 °C. At timed intervals after adding nucleotide, 10 μ l samples were removed and washed on Millipore filters (HA; 0.45 μ m pore) with 2 $\times$ 3 ml of assay buffer to remove unbound nucleotide.

Abbreviations used: PLC, phospholipase C; p[NH]ppG, guanosine 5'-[β -imidazo]triphosphate.

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Fig. 1. U.v.-induced cross-linking of $[^3\text{H}]\text{p}[\text{NH}]\text{ppG}$ to squid photoreceptor membrane proteins

(a) Coomassie Blue staining; (b) autoradiograph. Lanes: M, unirradiated photoreceptor membranes; 1, membranes + $7\ \mu\text{M}$ - $[^3\text{H}]\text{p}[\text{NH}]\text{ppG}$ and 10 mM unlabelled $\text{p}[\text{NH}]\text{ppG}$; 2, membranes, $7\ \mu\text{M}$ - $[^3\text{H}]\text{p}[\text{NH}]\text{ppG}$; 3, membranes + $1.5\ \mu\text{M}$ - $[^3\text{H}]\text{p}[\text{NH}]\text{ppG}$ + 10 mM unlabelled $\text{p}[\text{NH}]\text{ppG}$; 4, membranes, $1.5\ \mu\text{M}$ - $[^3\text{H}]\text{p}[\text{NH}]\text{ppG}$. Abbreviations: R, rhodopsin; $\text{G}\beta$, β -subunit of the G-protein. The $\text{G}\gamma$ subunit is not resolved from the dye front. Rhodopsin is the broad band at 45 kDa.

Fig. 2. Light-activated $\text{p}[\text{NH}]\text{ppG}$ binding

Binding of $1.4\ \mu\text{M}$ - $[^3\text{H}]\text{p}[\text{NH}]\text{ppG}$ to membranes containing $1.5\ \mu\text{M}$ -rhodopsin, in darkness (●) and in light (○). Points on the plot are averages of three separate experiments, each done in triplicate.

The filters were dissolved in scintillant and counted for ^3H radioactivity by liquid-scintillation spectroscopy. This experiment was done in light and in darkness under i.r. illumination. Because of the difficulty of sampling at short intervals in the dark, the 1 min time point was omitted from the dark series. Data values plotted were averages of three separate experiments (except where indicated otherwise), each done in triplicate. Background counts were determined by processing filters in the absence of membranes; these values were subtracted from the measured data points. Temperature- and Ca^{2+} -dependence were measured after a 30 min incubation at the higher temperature and/or Ca^{2+} concentration. Ca^{2+} buffering was done with EGTA and Ca^{2+} concentrations given by the program of Perrin & Sayce [20].

Binding displacement analysis

Binding experiments were done in the light, at 4°C , with $1.5\ \mu\text{M}$ -rhodopsin and $1.4\ \mu\text{M}$ - $[^3\text{H}]\text{p}[\text{NH}]\text{ppG}$ ($14.3\ \text{Ci}/\text{mmol}$), and with the addition of increasing amounts of unlabelled $\text{p}[\text{NH}]\text{ppG}$ from 0 to 1 mM. The curve was fitted to the data by using the relation $L^*R = B_{\text{max}} \cdot L^*/(L^* + K + I)$, where L^*R is the amount of bound radiolabelled ligand, B_{max} , the total number of binding sites, L^* the concentration of labelled ligand in μM , K the dissociation constant in μM , and I the amount of unlabelled ligand added in μM . This relation incorporates the assumption that the ligand binds to a homogeneous population of reversible sites. Values of L^*R are measured in d.p.m. and converted into concentration for a $10\ \mu\text{l}$ sample. The data points were fitted by a curve with this equation, treating B_{max} and K as parameters. Label bound in the presence of 10 mM added unlabelled nucleotide was taken as the non-specific background.

GTPase assays

GTPase activity was measured by release of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (New England Nuclear), used at a specific radioactivity of $10\ \text{Ci}/\text{mmol}$ and a concentration of $0.59\ \mu\text{M}$ in the assay. The rhodopsin concentration was $1.5\ \mu\text{M}$, and 1 mM-ATP was also included in the assay buffer. The reactions were stopped by mixing samples with 0.2 M- HClO_4 . Released phosphate in the acid supernatants was separated from unhydrolysed nucleotide by binding to activated charcoal. The supernatant of the charcoal assay was counted for radioactivity by Čerenkov counting to give the amount of free phosphate. Temperature- and Ca^{2+} -dependence measurements were done after a 30 min incubation under the new conditions as above.

RESULTS

U.v.-induced cross-linking of $[^3\text{H}]\text{p}[\text{NH}]\text{ppG}$ to squid photoreceptor membrane proteins

Fig. 1 shows that photoaffinity labelling revealed only a 42 kDa band (tracks 2 and 4). The labelled band coincides with the $\text{Gq}\ \alpha$ -subunit isolated by Pottinger *et al.* [13]. Since this component migrates very close to the position of actin, previously identified in these photoreceptors [21], it was not detected by Coomassie Blue staining. Simultaneous addition of unlabelled $\text{p}[\text{NH}]\text{ppG}$ abolished the labelling (tracks 1 and 3), showing that the cross-linking is specific. No difference was seen between samples incubated with $1.5\ \mu\text{M}$ or $7\ \mu\text{M}$ radiolabelled $\text{p}[\text{NH}]\text{ppG}$. The u.v. irradiation usually caused a diffuse background in the gel tracks, probably due to some protein cross-linking.

Light-activated $\text{p}[\text{NH}]\text{ppG}$ binding

If the membranes were prepared from retinas dissected in dim light, no binding was seen in the dark, and substantial light activation was observed (Fig. 2). Under the conditions of this experiment, the binding saturated in less than 5 min.

This allowed us to estimate the number of guanine-nucleotide binding sites, so that we could determine the molar ratio of activatable G-protein to rhodopsin in these membranes. Binding-displacement analysis (Fig. 3) gave curve-fit results for the parameters $B_{\text{max}} = 0.117 \pm 0.007\ \mu\text{M}$ and $K = 0.29 \pm 0.11\ \mu\text{M}$. The B_{max} value shows that there is one guanine-nucleotide binding site for every 12.5 rhodopsin molecules. This molar ratio is consistent with the density of Coomassie Blue staining of the $\text{G}\beta$ band [3,22] relative to rhodopsin. The value of the affinity constant K is less reliably determined by this data.

Temperature-dependence of $\text{p}[\text{NH}]\text{ppG}$ binding

A surprising finding was the strong temperature-dependence of $\text{p}[\text{NH}]\text{ppG}$ binding (Table 1). Taking the level of binding at

Fig. 3. Displacement of radiolabelled p[NH]ppG binding by unlabelled p[NH]ppG

Plot of binding as a function of unlabelled nucleotide added. The curve fitted to the data points is given by $L^*R = B_{max} \cdot L^* / (L^* + K + I)$, as defined in the Experimental section.

Fig. 4. Light-activated GTPase as function of temperature

GTP hydrolysed as a function of time, in light (○) and dark (●), at different temperatures. Hydrolysis was measured by release of [³²P]P_i from [γ-³²P]GTP as described in [23].

4 °C as 100 %, the binding decreased significantly at 10 °C and was below 50 % at 18 °C. At 24 °C, the binding decreased to 5 % of the starting value within 5 min of incubation, although the loss of binding was slower at 10 °C (time-course results not shown). The loss of binding was irreversible.

GTPase activity

Increasing temperature has the opposing effects of decreasing the number of binding sites while increasing the hydrolytic rate

Table 1. Temperature-dependence of p[NH]ppG binding

Binding was measured in the light, after 30 min incubation at 4, 10, 18 and 24 °C, and normalized to 100 % binding at 4 °C. The rhodopsin concentration was 1.5 μM and the p[NH]ppG concentration was 1.4 μM. Binding at 4 °C was 0.057 ± 0.01 mol of p[NH]ppG/mol of rhodopsin. Values were determined in triplicate in two independent experiments.

Temp. (°C)	p[NH]ppG bound (% of that at 4 °C)
4	100
10	85 ± 3.3
18	42.7 ± 10.4
24	5.6 ± 1.3

Fig. 5. Ca²⁺ effect on GTPase activity at different temperatures

Light-stimulated GTPase activity was measured after incubation with 0.01 μM (○) and 1 μM (●) free Ca²⁺ at the three temperatures used in Fig. 4.

(Fig. 4). At 10 °C, most binding sites are still present, and the light-activated GTPase activity is maximal. At 4 °C, the dark background is virtually zero, and the rate in the light is 0.03 mol of GTP/min per mol of G-protein (0.06 in the set of experiments shown in Fig. 5). At 10 °C the dark rate is still close to zero and the light rate is 0.12 mol of GTP/min per mol of G-protein. At 24 °C, the background activity in the dark is increased.

Ca²⁺ effects

In a previous study, membranes subjected to prolonged treatment at room temperature lost GTPase activity in the presence of micromolar concentrations of Ca²⁺ [23]. In order to test for the presence of minor heat-resistant G-proteins which might be Ca²⁺-sensitive, the GTPase assay was repeated after 30 min incubation with 0.01 μM and 1 μM free Ca²⁺ at various

Fig. 6. Effect of Ca^{2+} on p[NH]ppG binding

Binding was measured in the light, at 4 °C and 0.01 μM - Ca^{2+} (○), at 4 °C and 1 μM - Ca^{2+} (●), at 23 °C and 0.01 μM - Ca^{2+} (▽), and at 23 °C and 1 μM - Ca^{2+} (▼). The rhodopsin concentration was 3.5 μM , and p[NH]ppG was 0.145 μM in this experiment. Results were obtained in triplicate from a single experiment.

temperatures (Fig. 5). A slight inhibition of activity by Ca^{2+} was seen at 24 °C, but was not statistically significant. The p[NH]ppG-binding assay was also repeated as a function of temperature and Ca^{2+} concentration (Fig. 6). This showed no effect of Ca^{2+} on the major binding at 4 °C, but a significant inhibition of the residual heat-resistant binding by 1 μM - Ca^{2+} at 23 °C. The presence of 1 mM-ATP did not affect the results in similar experiments using guanosine 5'-[γ -thio]triphosphate (results not shown). These results suggest that the previous experiments, in which the membranes were incubated at elevated temperature for longer times in order to carry out the covalent modifications by cholera and pertussis toxins, were measuring a very small residual activity with different properties, and that the bacterial toxin substrates are very minor components of the photoreceptor preparation.

DISCUSSION

The G-protein activity studies presented here establish that the Gq identified previously by purification and sequencing [22,13] is the major G-protein activated by squid rhodopsin. Our binding studies now show that this Gq protein is present at a molar ratio of about 1:12.5 rhodopsins, very similar to that found in bovine photoreceptors [24]. Its activation by photoexcited rhodopsin is highly specific, with virtually no G-protein activity in the dark. It is surprisingly temperature-sensitive, a property that previously obscured the detection of its activity in this preparation. Loss of binding occurs above normal physiological temperatures for the North Atlantic species investigated here. Since there are other squid species in warm waters, this temperature-sensitivity is unlikely to occur *in vivo*, and is probably related to the conditions of membrane isolation. Very minor components, whose α -subunits are substrates for cholera and pertussis toxins respectively, are also capable of light activation [23,25], but these account for less than 2% of the total G-protein in our preparation. The toxin substrates are inhibited by micromolar Ca^{2+} concentrations [23], but the Gq activity is unaffected by Ca^{2+} in

this concentration range. In squid, the effector enzyme PLC is activated by light and GTP [9,10], and this effector is essential for the light response in *Drosophila* photoreceptors [11]. Electrophysiological results also point to InsP_3 and Ca^{2+} fluxes as major messengers in visual excitation and adaptation in the retinas of invertebrates [26,27]. An unresolved issue is the site of Ca^{2+} negative feedback. On the basis of the Ca^{2+} effect on our activity measurements (Figs. 5 and 6), a feedback pathway with Ca^{2+} as an inhibitor of rhodopsin-Gq activation is unlikely. Regarding receptor activation of the Gq, squid rhodopsin is homologous to other rhodopsins and to G-protein-coupled receptors, but has a proline-rich C-terminal extension [28,29]. This C-terminal tail is not present in *Drosophila* rhodopsin, and does not appear to play a role in G-protein activation or specificity.

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