A novel photointermediate of octopus rhodopsin activates its G-protein

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Abstract The photointermediate of octopus rhodopsin responsible for G-protein activation was examined by a GTPγS-binding assay in a reconstituted system with purified rhodopsin and photoreceptor G-protein. When octopus rhodopsin alone was incubated in the dark after illumination, its ability to stimulate GTPγS-binding by the G-protein decreased in a time-dependent manner. We associate this decay with the decay of a novel photointermediate, transient acid metarhodopsin, which lies between mesorhodopsin and acid metarhodopsin. Spectroscopic evidence for its existence was suggested by its effects on the turbidity of the vesicles. These results suggest that the transient acid metarhodopsin, not the stable final photoproduct, acid metarhodopsin, activates a G-protein in octopus photoreceptors.

Key words: Octopus rhodopsin; G-protein; Metarhodopsin; Photointermediate; Laser photolysis

1. Introduction

The central theme in seven-helical membrane receptors is their activation of G-proteins. Rhodopsin is a system par excellence to study the molecular mechanisms of the activation of a G-protein by its receptor [1–3]. Upon absorbing a photon, the retinal chromophore isomerizes from 11-*cis* to all-*trans* which leads to protein conformational changes of the pigment [4]. Although vertebrate rhodopsin has several photointermediates, most evidence has suggested that a particular intermediate, metarhodopsin II, is the one that can activate transducin in vertebrate photoreceptors. Unlike vertebrate visual pigments, the final photoproduct of octopus and probably all invertebrate rhodopsins, acid metarhodopsin, is thermally stable. It has been reported that the stable final photoproduct of squid rhodopsin activates phospholipase C (PLC) by activating G_q [5]. We wished to investigate this issue.

Because of the reversible interconversion between acid metarhodopsin and rhodopsin by light, octopus rhodopsin is a particularly attractive system to study the molecular mechanisms involved in G-protein activation [6–8]. The photochemical sequence of octopus rhodopsin has been extensively studied by UV and visible absorption spectroscopy over a wide time range from fs to s [4,9–11]. These results suggested that the final transformation in this sequence was from mesorhodopsin to acid metarhodopsin, with a lifetime of 31 μs at 15°C [4]. Fourier-transform infrared (FTIR) spectroscopy provides a powerful tool for studying the side-chain structure and main-chain conformation of the apoprotein as well as structural changes in the retinal moiety [12–15]. Recently, using FTIR spectroscopy we have found evidence for a new intermediate, a precursor of acid metarhodopsin, decaying within a minute [12]. During this transition, a band at 1743 cm^{-1} increased concomitantly with the decrease of a band at 1721 cm^{-1}. These bands were assignable to changes in the environment of a protonated carboxyl group of the pigment, however no absorption changes in visible wavelength were observed after the decay of mesorhodopsin. Thus, it appears that this transient species has the same absorption spectrum as acid metarhodopsin. These results prompted us to reexamine which photointermediate of octopus rhodopsin can activate its G-protein.

2. Materials and methods

2.1. Purification of photoreceptor microvillar membranes and rhodopsin

Microvillar membranes of octopus photoreceptors were prepared from eyes of *Octopus dofleini* described previously [16]. After isolation by sucrose floatation, the microvillar membranes were repeatedly washed with buffer A (400 mM KCl, 10 mM $MgCl_2$, 10 mM MES (pH 6.5), 1 mM DTT, 1 mM benzamidine-HCl, 20 μM APMSF) and then with buffer B (10 mM MES (pH 6.5), 1 mM DTT, 1 mM benzamidine-HCl, 20 μM APMSF).

After the microvillar membranes were solubilized in 1% (w/v) sucrose monolaurate, 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM DTT, 1 mM benzamidine-HCl, and 20 μM APMSF as described previously [11], the octopus rhodopsin was affinity purified by Con-A (concanavalin-A) Sepharose (Amersham Pharmacia Biotech).

2.2. Reconstitution of octopus rhodopsin into phospholipid vesicles

Reconstitution of octopus rhodopsin into phospholipid vesicles was carried out as described by Cerione et al. [17]. The purified rhodopsin (approximately 8 μM) in a buffer containing 2.5 mg/ml of azolectin (Sigma), 1% (w/v) octyl glucoside, and 1 mg/ml bovine serum albumin was passed through a 1-ml column of Extractigel-D (Pierce) which had been equilibrated in a buffer containing 20 mM Tris-HCl (pH 7.4), 25 mM $MgCl_2$, and 100 mM NaCl (referred henceforth to as TMN). Turbid fractions after the void volume were collected.

2.3. Purification of octopus photoreceptor G_q

G_q, one of the G-proteins in octopus photoreceptors, was isolated as described previously [16]. Briefly, G_q, in the supernatant of the solubilized microvillar membranes was successively purified by a DEAE cellulose column (2.5×2 cm, bps), Sephacryl S-300 HR (2.2×90 cm, Amersham Pharmacia Biotech), and a high resolution Mono Q PC 1.6/5 column using the SMART System (Amersham Pharmacia Biotech).

2.4. GTPγS-binding assay

All procedures before the filtration were done under dim red light. The rhodopsin-reconstituted vesicles (3.4 μM rhodopsin) were excited by a flash (pulse width; 10 ms) of panchromatic light. Immediately after the irradiation of the vesicles, the two halves of the sample were preincubated either in the presence (case A) and absence (case B) of G_q (about 380 nM) at 20°C in the dark. After the prescribed period of dark preincubation, the reaction mixture (25 mM Tris-HCl (pH 7.4), 25 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.5 μM [^{35}S]GTPγS

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Abbreviations: FTIR, Fourier-transform infrared spectroscopy; G-protein, guanine nucleotide-binding protein; PLC, phospholipase C; APMSF, 4-(amidinophenyl)methanesulfonyl fluoride; DTT, dithiothreitol; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; MES, 2-(*N*-morpholino) ethane sulfonic acid

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(4×10^3 cpm/pmol)) was added to the vesicle suspension to initiate GTPγS-binding. In case B, an amount of G_q equal to that used in case A was added to the vesicle suspension along with the mixture. After the prescribed time of incubation, a 20-μl aliquot was withdrawn and diluted with 250 μl of ice-cold TMN further supplemented with 1 μM cold GTPγS. The diluted mixture was filtered through nitrocellulose filters, and the filters, after being washed 3 times with ice-cold TMN, were counted for ^{35}S by a liquid scintillation counter.

2.5. Spectroscopic measurements

Transient transmittance changes were measured using a modified version of a laser photolysis apparatus that had been described previously [4]. When the chromophore absorption changes were measured at 530 nm over a long time range, the probe light was reduced by a neutral density filter to minimize the photolysis by the probe beam. The turbidity changes of the microvillar membranes were measured at 700 nm where there was no absorption by the pigment's chromophore. To avoid excitation of the sample by the second order diffraction light from the monochromator, a cut-off filter ($\lambda > 590$ nm O-590, Toshiba) was placed in front of the cuvette. The actinic light for rhodopsin at 460 nm was a dye laser (UNISOKU, TSP-611) with Coumarin 460 in ethanol pumped by the third harmonic (355 nm) of the Nd-YAG laser (a 8-ns pulse width (fwhm), Continuum NY 60 model). The actinic light for acid metarhodopsin was the second harmonic (532 nm) of the Nd-YAG laser.

The excitation pulse and the probe beam were focused onto the sample cuvette (2×2 mm) at right angle to each other. The photolyzed sample was photoregenerated back to the original pigment by irradiation with light of the appropriate wavelength, orange light (O-58, Toshiba) for rhodopsin and blue light (V-42, Toshiba) for acid metarhodopsin. The sample temperature was maintained at 15°C with a thermal bath (Lauda RMS6).

3. Results and discussion

3.1. Kinetics of GTPγS-binding

If there is a transient species that activates G_q, then the ability of this species to activate G_q so it can bind GTPγS will decay with time. On the other hand, GTPγS-binding to G_q will be constant or increase with time if acid metarhodopsin is the species which can activate G_q. To test this assumption, we performed the following experiments using a reconstituted system of the purified rhodopsin and G_q. Immediately after a Xenon flash irradiates the rhodopsin-reconstituted vesicles, the two halves of the sample were preincubated in the absence (case A) or presence (case B) of G_q in the dark. After the period of dark preincubation indicated, the initial rate of GTPγS-binding was measured in the dark for each case. The inset of Fig. 1 shows a typical time course of GTPγS-binding to the irradiated vesicles.

In the case where the irradiated rhodopsin vesicles were preincubated in the absence of G_q (case A), GTPγS-binding was initiated right after G_q was added to the irradiated rhodopsin vesicles. As shown by curve 1 in Fig. 1, the initial rate of GTPγS-binding drastically decreased as the time, when the G_q was added, increased after a flash, suggesting that the active form of irradiated rhodopsin was gradually decaying. The initial binding rate decreased to almost zero level in an hour. The time course of the decrease in the initial rate was not a single exponential. The time constant of the slow component of the decrease was estimated to be 20 min and that of the fast component was estimated to be less than one minute, because this is the limitation of our assay.

In this experiment, we performed another set of assays in parallel (case B). Right after excitation of the vesicles by a flash, a half of the irradiated vesicles was mixed with G_q in the dark. After the mixture was incubated for the indicated periods in the dark, the initial rate of GTPγS-binding was measured. In contrast to case A, the initial rate of GTPγS-binding did not significantly change during preincubation as shown by curve 2 in Fig. 1. These results suggest that G_q in the irradiated rhodopsin/G_q complex stays in an active state, which is able to bind GTPγS, for over an hour.

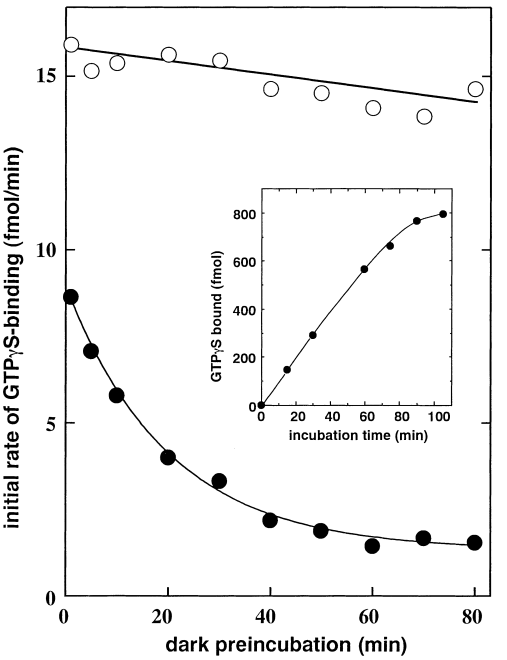

Fig. 1. Initial rates of GTPγS-binding to the purified G_q and octopus rhodopsin-reconstituted vesicles in case A and B were plotted against time after the vesicles were excited by a flash. Curve 1: After the irradiated rhodopsin vesicles were incubated for the indicated periods in the dark, G_q and GTPγS were added to the vesicles and the initial rate of GTPγS-binding was measured (case A). Curve 2: Immediately after rhodopsin-reconstituted vesicles were excited by a flash, G_q was added to the vesicles and the mixture incubated for the indicated periods in the dark. The initial rate of GTPγS-binding was measured by addition of GTPγS (case B). Inset: Typical time course of GTPγS-binding to the irradiated vesicles with G_q.

Previously it has been reported that the stable final photoproduct (corresponding to acid metarhodopsin) of squid rhodopsin activates phospholipase C (PLC) through the activation of G_q [5]. These experiments were performed with rhabdomeric membranes which contain rhodopsin, G_q, and phospholipase C. As shown in Fig. 1, GTPγS-binding in the mixture of irradiated rhodopsin and G_q did not change over an hour of dark incubation (curve 2), while GTPγS-binding to G_q when incubated separately with the irradiated rhodopsin in the dark decreased with time (curve 1). Thus, the duration of PLC activation in rhabdomeric membranes can not be simply attributed to the lifetime of the active form of rhodopsin. The active form of rhodopsin intermediates can best be identified by reconstituted system as shown in the present study.

3.2. Light-initiated turbidity changes

Are there any spectral changes that correspond to what appears to be the decay of the activating intermediate? Upon illumination of the octopus rhodopsin vesicles with a 460 nm flash, an abrupt decrease in transmittance (increase in absorbance) at 530 nm was observed, as shown by curve 1 in

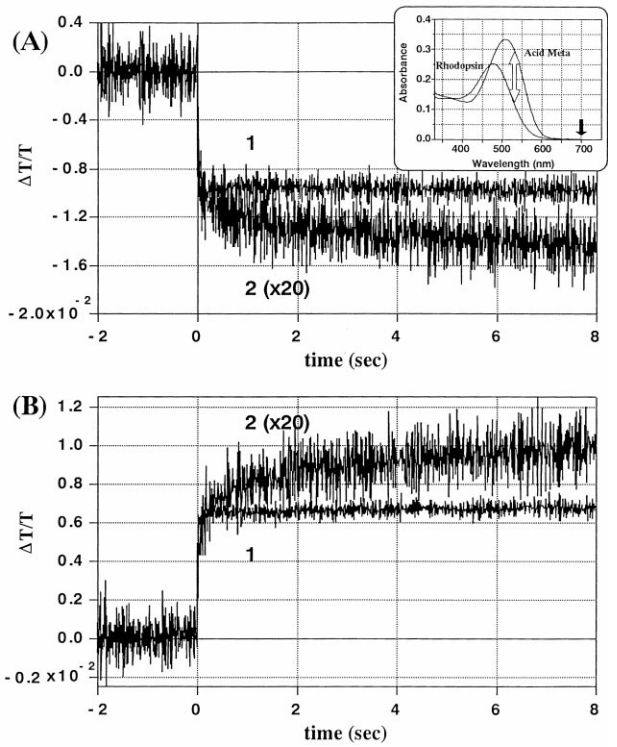

Fig. 2. Time course of the light-induced transmittance changes of azolectin vesicles containing octopus rhodopsin. Panels A and B showed the transformation of rhodopsin to acid metarhodopsin upon a 460 nm flash and that of acid metarhodopsin to rhodopsin upon a 532 nm flash, respectively. Curve 1 in each panel is the transmittance change at 530 nm. Curve 2 in each panel shows the light-induced transmittance changes at 700 nm, which was multiplied by a factor of 20 to be displayed with other curves in the corresponding scale. Inset: Reversal photoconversion between rhodopsin and acid metarhodopsin by blue and orange light illumination.

Fig. 2. Previously, we assigned this change to the final photochemical change in the photolysis of octopus rhodopsin, the transformation from mesorhodopsin to acid metarhodopsin [9]. Both the increase at 530 nm and the decrease at 460 nm have lifetimes of 31 ms. The present results (curve 1 in Fig. 2A) also seem to support this conclusion, because no additional transmittance changes at 530 nm were observed even up to 8 s. Since there are no absorbance changes over this time range, the activating intermediate must have the same absorption spectrum as acid metarhodopsin and so we will call it transient acid metarhodopsin.

Interestingly, a light-induced decrease in transmittance was observed at 700 nm, where there is no chromophore absorbance (curve 2 in Fig. 2A). The transient transmittance changes at 700 nm showed multi-component kinetics with three lifetimes of 97 ms, 2.5 s and 46 s. When the sample was solubilized with detergent, the abrupt decrease in transmittance at 530 nm was retained, but the slow transmittance changes at 700 nm completely disappeared. These results suggested that the flash-induced transmittance changes of the vesicles at 700 nm were not due to chromophore absorption changes but due to the light-induced turbidity changes of the rhodopsin vesicles.

As shown in the inset in Fig. 2, rhodopsin is converted to acid metarhodopsin upon illumination with blue light, with a maximum increase in absorbance at 530 nm. On the other hand, acid metarhodopsin photoregenerated to rhodopsin upon illumination of orange light, with a maximum decrease in absorbance at 530 nm. However, there are no absorption changes above 600 nm.

Curve 1 and curve 2 in Fig. 2B shows the time course of the transmittance changes at 530 nm and at 700 nm for acid metarhodopsin vesicles upon a 532 nm flash, respectively. An abrupt increase in transmittance (decrease in absorbance) at 530 nm was observed, due to the transformation of acid metarhodopsin to rhodopsin. The light-induced transmittance changes at 700 nm were in just the opposite direction to those observed for rhodopsin vesicles upon a 460 nm flash (curve 2 in Fig. 2A). These results strongly suggest that the turbidity changes were related to changes of the membranes induced by protein conformational changes in the transformation of transient acid metarhodopsin to acid metarhodopsin.

Light-induced turbidity changes of the photoreceptor membranes were first observed in bovine rod outer segment preparations [18]. Kuhn et al. [19] demonstrated a stoichiometric relation between the association and dissociation of the rhodopsin/G-protein complex and the light-scattering signal in the bovine system. Since we used the vesicles containing purified rhodopsin, the light-induced turbidity changes observed for these vesicles should be induced solely by conformational changes of octopus rhodopsin. Moreover, the flash-induced turbidity changes of the rhodopsin vesicles were not affected by the presence or absence of GTPγS (data not shown). This result excluded the possibility that the flash-induced transmittance changes of the octopus membranes were related to the binding and dissociation of rhodopsin with the G-protein.

In summary, our results showed that a novel photointermediate (transient acid metarhodopsin), whose absorption spectrum is the same as that of the final photoproduct, acid metarhodopsin, is the active photointermediate of octopus rhodopsin which activates its G-proteins.

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