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LIGHT-STIMULATED GTP BINDING TO A MEMBRANE PROTEIN IN ROD OUTER SEGMENTS

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SUMMARY

Bovine as well as frog rod outer segments contain a membrane-bound protein which binds the GTP analog GppNp in the light (Kd=0.3μM). The amount of GppNp bound is 2.5-3.5 mmole per mol rhodopsin. The binding protein (M.Wt.≃ 54,000) can be extracted from rod membranes with detergent and purified on an Agarose column. The chromatographic profile indicates that the binding protein is distinct from rhodopsin, GTPase or cyclic nucleotide phosphodiesterase.

INTRODUCTION

Various biochemical reactions which are stimulated by light have been found in rod outer segments. These reactions include rhodopsin phosphorylation (1-4), break-down of cyclic nucleotides (5,6) and hydrolysis of GTP (7-9). Marked decreases in cyclic GMP in light-illuminated rods (10-12) is attributed to activation of cyclic nucleotide phosphodiesterase which occurs in the presence of GTP (13). Light-activation of GTPase is therefore of particular interest because hydrolysis of the GTP associated with cyclic nucleotide phosphodiesterase may be a regulatory mechanism for the enzyme.

GTP is known to be an important requirement for hormone stimulation of adenyl cyclase in various tissues (14). A GTP binding protein has recently been isolated from hormone-responsive erythrocyte membranes (15). Since there are similarities between the light-stimulation of phosphodiesterase in the rod and hormone-activation of adenyl cyclase in non-photoreceptor cells, we investigated the possible presence of a GTP binding protein in rod membranes.

MATERIALS AND METHODS

Rod outer segments were prepared from fresh bovine or frog (Rana pipiens) eyes (16). For assay of binding activity, a reaction mixture (100μl, pH 7.0)

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Table I

Effect of light on GppNp binding to rod membranes

	GppNp bound (nmol/mol rhodopsin)	
	Dark	Light
Bovine ROS	3.2 (100)	5.5 (172)
Bovine ROS (+EDTA)	2.5 (63)	3.2 (100)
Bovine Disks (91% rhodopsin)	1.4 (44)	-
Bovine "PM fract." (9% rhodopsin)	8.4 (263)	-
Frog ROS	1.8 (100)	3.4 (189)

containing rod membranes (ca. 3 nmol rhodopsin), 1 μmol Hepes, 1 nmol [^{3}H] guanosine 5'-[β, γ-imido] triphosphate [GppNp, 20,000 DPM/pmol, Amersham, IL]or 1 nmol [^{3}H]adenosine 5' [β,γ-imido] triphosphate [AppNp, 20,000 DPM/pmol, Amersham, IL] was incubated for 30 minutes at 25°C in the dark. In some samples 100 nmol nonradioactive GppNp or AppNp was included in the mixture. The membranes were filtered through GFF Glass filters (Whatman, N.J.), washed with buffer, dissolved with tissue solubilizer and mixed with toluene-based mixture. Radioactivity of bound nucleotide was determined with a Beckman LS9000 scintillation counter. Cyclic nucleotide phosphodiesterase was determined by the method of Rangel-Aldao et al. (17). A reaction mixture containing 5 μl 10μM (^{3}H)c-GMP, 10 μl 15 mM $MgCl_2$, 25 μl bovine serum albumin (2mg/ml) and 10 μl enzyme was incubated for 5 minutes at 25°C. The reaction was stopped with 10 μl of a solution containing 1 μmol EDTA and 70 nmol each of guanosine, 5'GMP and c-GMP. The mixture was spotted on PEI cellulose plates and developed in 50 mM KCl. The spot containing 5'GMP was cut out and eluted for measurement of radioactivity. GTPase activity was assayed by a modification of the method of Bignetti et al. (9). A reaction mixture containing 15 μl 12 mM (α^{32}P)GTP, 10 μl 12 mM $MgCl_2$, 15 μl rod outer segment (ROS) extract (in 1 mM EDTA) and 20 μl eluate from the Agarose column was incubated for 15 minutes at 25°C. The reaction was stopped with 15 μl 20% trichloroacetic acid(TCA), centrifuged and the supernatant was spotted on PEI cellulose plates. After removing TCA with methanol, the plates were dried and developed in 4 M Na-formate)pH 3.4):1.5 M LiCl(1:1, v/v). Spots containing GDP were identified by autoradiography, eluted and measured for ^{32}P radioactivity. For agarose chromatography, ROS (44 nmol rhodopsin/ml) were incubated with 1 μM[^{3}H] GppNp in 50 mM Hepes buffer, pH 7.0, containing 2 mM Mg^{++} and 0.1 mM dithiothreitol for 1 hour in the dark. The labeled ROS were centrifuged and extracted with 5 ml 0.5% Ammonyx LO (Onyx Chemicals, N.J.) in 50 mM Tris buffer, pH 8.0. The supernatant from centrifugation at 100,000xg for 1 hour was loaded on an Agarose column(170x1.5 cm) and chromatographed as described by O'Brien et al. (18).

RESULTS

As shown in Table I, light irradiation of both bovine and frog ROS resulted in 70-90% increases in binding of the GTP analog GppNp to rod mem-

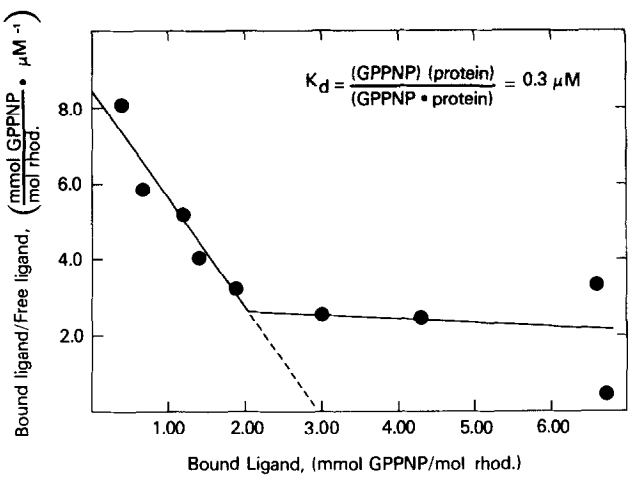

Figure 1. The binding of GppNp to bovine rod membranes in the light.

branes. The binding was inhibited by EDTA. When bovine rods were fractionated into disks and crude plasma membranes by the method of Smith, Stubbs and Litman (19), GppNp binding in the dark was predominantly found in the crude plasma membrane fraction. A Scatchard plot for binding of GppNp to membrane protein in the light is shown in Fig. 1. The dissociation constant for the GppNp protein complex was found to be 0.3μM. This value is of the same order of magnitude as the reported Km values for GTPase (8,20). The molar ratio of rhodopsin to binding protein in the rod was estimated to be 300-400.

Bovine rods contain GTP binding protein as well as ATP binding protein as shown by the competition studies in Table II. [^{3}H]GppNp binding was markedly reduced by incubation with excess unlabeled GppNp but not by AppNp. Conversely, binding of [^{3}H]AppNp was not affected by excess unlabeled GppNp. Two distinct binding proteins for GppNp and AppNp, respectively, could indeed be solubilized with non-ionic detergent Emulphogene BC720 from rod membranes (Fig. 2). Binding of radioactive GppNp or AppNp to these proteins was eliminated by addition of 100 fold excess unlabeled respective ligands prior to chromatography. To characterize the nature of the GppNp binding protein, rods were labeled with [^{3}H]

Table II

Binding specificity for GppNp and AppNp

	^{3}H-Lignad bound (pmol)	Relative binding (%)
10μM [^{3}H]GppNp	54±5	100
+ 1mM GppNp	7.6±1.5	14
+ 1mM AppNp	56±1	103
10μM [^{3}H]AppNp	28±7	100
+ 1mM AppNp	5.9±0.4	21
+ 1mM GppNp	22±0.2	78

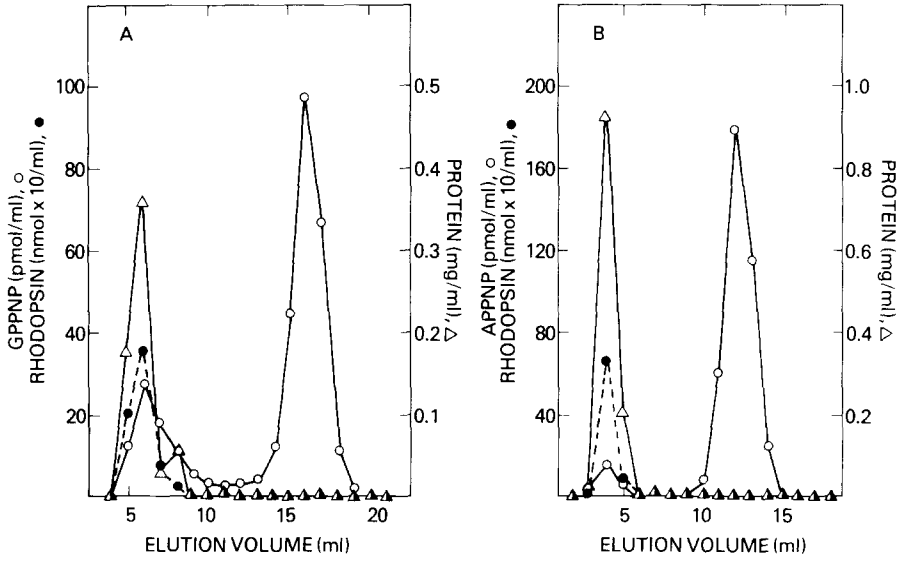

Figure 2. Identification of GppNp binding protein (A) and AppNp binding protein (B) in rod extracts.

GppNp, extracted with detergent and the extracted proteins were purified on an Agarose column (Fig. 3). The chromatographic profile indicates that GppNp binding protein is eluted in a fraction distinct from rhodopsin, GTPase or c-nucleotide phosphodiesterase. The GTPase activity of rhodopsin-containing fractions, as measured by release of GDP, was not due to rhodopsin phosphory-

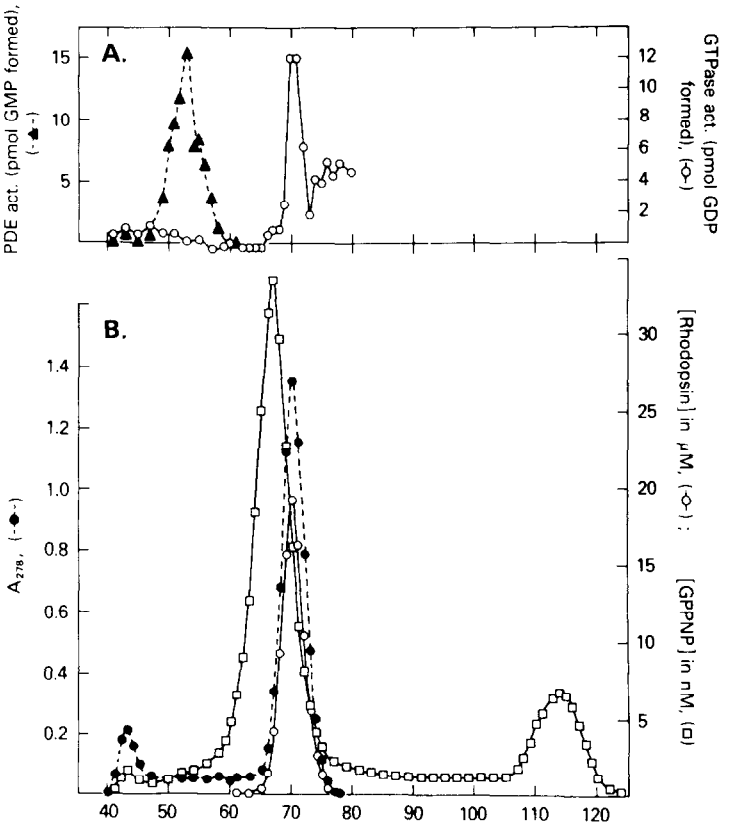

Figure 3. Separation of GppNp binding protein, rhodopsin, GTPase and cyclic nucleotide phosphodiesterase on an Agarose column.

lation because rhodopsin kinase is inactivated in detergent under the conditions (4). It should be noted that GTPase activity was restored by incubation of purified rhodopsin and an aqueous extract of rod membranes which itself had no activity. It awaits further studies whether GTPase was included in the extract or GTPase was associated with purified rhodopsin and the extract contained a cofactor required for activity. With reference to rhodopsin (Mol. Wt.=39,000) and c-nucleotide phosphodiesterase (Mol. Wt. = 240,000 (21)], a molecular weight of GTP binding protein estimated from the elution volume was approximately 54,000.

DISCUSSION

The GTP binding protein solubilized from pigeon erythrocyte membranes was suggested to be identical with GTPase (15). Separation of GTP binding protein from GTPase on an Agarose column indicates that the two proteins are not identical in the rod system. According to recent reports (9,22), a protein required for GTPase activity can be extracted from rod membranes without detergent. The enhanced binding of GTP to irradiated rod membranes resulted probably from depletion of bound GTP by light-activated GTPase and increased availability of unoccupied binding sites. The GTP binding protein isolated in this work may exist in vivo in close association with GTPase. The GTP concentration in dark-adapted frog rods were reported to be 1.0-1.6 mM (12,20). Assuming a rhodopsin concentration of 2.5 mM in the rod (23), the rhodopsin to GTP ratio is estimated to be about 2.5. The present result shows that rods contain one GTP binding protein per 300-400 rhodopsin molecules. Therefore, the bound GTP accounts for less than 1% of the total GTP.

In a study with γ-[^{32}P]GTP (24), GTP was reported to be a better substrate than ATP for rhodopsin phosphorylation, although ATP rather than GTP was the preferred substrate for highly purified rhodopsin kinase (4). The light-dependent GTP binding to rod membranes described in this work may provide an explanation for the discrepancy; ^{32}P incorporation into rod membranes from γ-[^{32}P]GTP was probably overestimated because of binding of the nucleotide to the membrane in the light.

GTP is known to be required in hormone-stimulated adenyl cyclase activation in various systems (14,25), in tubulin assembly (26) and peptide elongation (27). Light activation of nucleotide-metabolizing enzymes in vertebrate rods provides another example in which a GTP requirement and the presence of a GTP binding protein can be demonstrated. It remains to be seen how GTP, together with GTP binding proteins, exerts its effect on the diverse reactions.

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