Light-dependent GTP-binding proteins in squid photoreceptors

Phyllis R. ROBINSON,*†† Susan F. WOOD,†‡** Ete Z. SZUTS,†§ Alan FEIN,|| Heidi E. HAMM¶ and John E. LISMAN*

*Biology Department, Brandeis University, Waltham, MA 02254, †Laboratory of Sensory Physiology, Marine Biological Laboratory, Woods Hole, MA 02543, ‡Boston University Marine Program, Marine Biological Laboratory, Woods Hole, MA 02543, §Department of Physiology, Boston University School of Medicine, Boston, MA 02118, ||Department of Physiology, University of Connecticut Health Center, Farmington, CT 06032, and ¶Department of Physiology and Biophysics, University of Illinois Medical College, 835 South Wolcott, Chicago, IL 60612, U.S.A.

Previous biochemical and electrophysiological evidence suggests that in invertebrate photoreceptors, a GTP-binding protein (G-protein) mediates the actions of photoactivated rhodopsin in the initial stages of transduction. We find that squid photoreceptors contain more than one protein (molecular masses 38, 42 and 46 kDa) whose ADP-ribosylation by bacterial exotoxins is light-sensitive. Several lines of evidence suggest that these proteins represent distinct α subunits of G-proteins. (1) Pertussis toxin and cholera toxin react with distinct subsets of these polypeptides. (2) Only the 42 kDa protein immunoreacts with the monoclonal antibody 4A, raised against the α subunit of the G-protein of vertebrate rods [Hamm & Bownds (1984) *J. Gen. Physiol.* **84**, 265–280]. (3) In terms of ADP-ribosylation, the 42 kDa protein is the least labile to freezing. (4) Of the 38 kDa and 42 kDa proteins, the former is preferentially extracted with hypo-osmotic solutions, as demonstrated by the solubility of its ADP-ribosylated state and by the solubility of the light-dependent binding of guanosine 5'-[γ -thio]triphosphate. The specific target enzymes for the observed G-proteins have not been established.

INTRODUCTION

In most types of invertebrate photoreceptors, light evokes a transient depolarization of the cell due to an increase in permeability to cations. A second messenger system links the absorption of light by rhodopsin to the opening of the light-dependent channels. In contrast with vertebrate photoreceptors, in which cyclic GMP has been firmly established as the second messenger of photoexcitation, the details of the biochemical cascade underlying phototransduction in invertebrates have yet to be firmly established. Biochemical and electrophysiological evidence strongly suggests that the two photoreceptor systems have the same initial stage of transduction, i.e. activation of GTP-binding protein(s) (G-proteins) by photoactivated rhodopsin. Exposure of invertebrate photoreceptors to a variety of pharmacological probes that activate G-proteins produces discrete depolarizations similar to those caused by the absorption of a single photon, although smaller in size (Fein & Corson, 1979, 1981; Bolsover & Brown, 1982; Minke & Stephenson, 1985; Kirkwood *et al.*, 1989). Injection of guanosine 5'-[β -thio]diphosphate, a compound known to block receptor activation of G-proteins, prevents photoexcitation and adaptation in *Limulus* ventral photoreceptors (Fein, 1986; Kirkwood *et al.*, 1989).

The biochemical evidence for the involvement of G-proteins in phototransduction comes from a wide variety of invertebrates. Photoreceptor membranes from flies, *Limulus* and cephalopods have been probed using a variety of biochemical assays that are diagnostic for the presence of a G-protein. Light-activated GTPase activity has been observed in photoreceptor membranes of *Musca* (Blumenfeld *et al.*, 1985), *Calliphora* (Paulsen & Bontrop, 1986), octopus (Calhoun *et al.*, 1980) and squid (Vandenberg & Montal, 1984; Saibil & Michel-Villaz, 1984). Proteins cross-reacting with antibodies specific for G-proteins

have been observed in blowfly (Paulsen *et al.*, 1988), octopus (Tsuda *et al.*, 1986; Tsuda, 1987) and *Sepia* (Stieve & Lumme, 1989). Finally, light-dependent ADP-ribosylation catalysed by bacterial toxins has been described in squid (Vandenberg & Montal, 1984; Brown *et al.*, 1987), blowfly (Bontrop & Paulsen, 1986) and octopus (Tsuda, 1987).

In the experiments reported here, we have found evidence for the existence of three light-activated G-proteins that are clearly distinct from each other. These multiple G-proteins may play different roles in phototransduction.

EXPERIMENTAL

Materials

Guanosine 5'-[γ -³⁵S]thio]triphosphate ([³⁵S]GTP[S]) was purchased from New England Nuclear (Boston, MA, U.S.A.), and [³²P]NAD⁺ was obtained from ICN (Irvine, CA, U.S.A.). Boehringer–Mannheim Biochemicals (Indianapolis, IN, U.S.A.) was the source for non-radioactive GTP[S]. All other reagents (unless otherwise specified) were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Photoreceptor membrane preparation

Live squid (*Loligo pealei*), obtained from the Marine Biological Laboratory (Woods Hole, MA, U.S.A.), were dark-adapted for 1–2 h before decapitation. All subsequent handling was performed under infra-red illumination. Eyes were removed and hemisected and their posterior portion, largely consisting of retinal tissue, was rinsed in cold oxygenated artificial sea water (393 mM-NaCl, 12 mM-KCl, 20 mM-MgCl₂, 10 mM-CaCl₂, 25.5 mM-MgSO₄ and 10 mM-Hepes, pH 7.8).

Abbreviations used: G-protein, GTP-binding protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; AC, artificial cytosol.

** Present address: Department of Neuroscience, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205, U.S.A.

†† To whom correspondence should be addressed.

All analyses were performed using photoreceptor distal segments (photoreceptor membranes) that were preferentially removed from the retinas by mechanical agitation (Wood *et al.*, 1989). Between two and four retinas were gently shaken for 1 min in 1–2 ml of a medium resembling artificial cytosol (AC) and then, to increase yield, AC was forcefully directed at the vitreal side of the retinas from a syringe with a 14-gauge needle. AC contained 80 mM-NaCl, 335 mM-potassium isethionate, 200 mM-sucrose, 4 mM-MgCl₂, 10 mM-Hepes, 10 kallikrein inhibitory units of aprotinin/ml, 100 μ M-phenylmethanesulphonyl fluoride, 5 mM-dithiothreitol and 1 mM-EDTA, pH 7.4 (DiPollo, 1973). The resulting suspension of distal segments was then filtered through a 37 μ m-pore-size nylon mesh to remove large pieces of tissue. Subsequently the suspension was homogenized (10–15 strokes) using a glass/glass homogenizer.

In some experiments, photoreceptors were obtained from frozen dark-adapted retinas. In these cases, freshly dissected retinas were frozen in liquid N₂ (Kito *et al.*, 1982) and then either thawed for immediate use or placed in a vial and wrapped in aluminium foil and stored at –80 °C for use at a later time. Isolation of distal segments from thawed retinas used procedures identical to those described for the fresh tissue.

Toxin-catalysed ADP-ribosylation

Squid photoreceptor membranes were obtained as described above. The photoreceptors were centrifuged at 13 000 *g* for 1 min, and the pellet was resuspended in 500 μ l of a hypo-osmotic buffer containing 50 mM-Tris/HCl, 5 mM-MgCl₂ and 1 mM-EGTA, pH 8.0. A 200 μ l portion of the membrane suspension was then added to a tube containing 50 μ l of toxin (either pertussis or cholera) and 50 μ l of 'nucleotide mixture'. In terms of final concentrations, the contribution of the nucleotide mixture to the pertussis toxin reaction was 100 mM-Tris/HCl, 2.5 mM-ATP, 10 μ M-NAD⁺, 26 μ Ci of [³²P]NAD⁺/ml (4 Ci/mmol), 5 μ M-thymidine, 4 mM-MgCl₂, 0.5 mM-ADP-ribose and 2 mM-EDTA. The final concentrations for the cholera toxin reactions were 7.5 mM-Tris/HCl, 20 μ M-NAD⁺, 26 μ Ci of [³²P]NAD⁺/ml (4 Ci/mmol), 16 μ M-arginine, 5 mM-ATP and 5 mM-thymidine. Before use, toxins (50 μ g/ml) were activated by incubation at 30 °C for 30 min in the presence of 10 mM-dithiothreitol. In experiments in which the effect of illumination on toxin-catalysed ADP-ribosylation was measured, half of the membranes remained in the dark and the remainder were exposed to white illumination that photoactivated over 50 % of the rhodopsin. Membranes were incubated for 1–2 h and quenched by the addition of sample solubilization buffer (Laemmli, 1970).

Electrophoresis

Solubilized samples were sonicated at room temperature and their proteins were separated on SDS/polyacrylamide gels, 10 % total acrylamide/2.6 % bisacrylamide (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue (Weber & Osborne, 1969) and then dried under a vacuum using a slab gel dryer. For autoradiography, preflashed X-ray films (Kodak X-Omat AR; Eastman Kodak Co., Rochester, NY, U.S.A.) were exposed at –80 °C with the use of intensifying screens. Films were analysed with scanning densitometers (Hoefer GS 300 and LKB UltroScan XL). Molecular masses of the G-proteins were determined by comparing their relative mobilities with those of protein standards.

Western blots

The protein bands of unstained gels were electroblotted overnight on to nitrocellulose paper (Towbin *et al.*, 1979). Nitrocellulose strips were then treated with either control serum or monoclonal antibody 4A, which was raised in the mouse

against the α subunit of bovine transducin (Hamm & Bownds, 1984). Immunoreactivity was revealed using an immunoperoxidase procedure (Vectastain ABC kit; Vector Labs, Burlingame, CA, U.S.A.) based on biotinylated secondary antibodies and a horseradish-peroxidase-labelled avidin-biotin complex. Diaminobenzidine was the enzyme substrate.

Illumination conditions

A flash of varying intensity was used as the light stimulus and was delivered by an electronic flash unit (Sunpak; flash duration ~ 1 ms). The following filters were interposed between the flash and the samples: Schott no. KG3 to absorb infra-red rays that may heat the sample; and Wratten no. 25 filter (Kodak) and calibrated Wratten neutral density filters (Kodak) to vary light intensity. With 1 absorbance neutral density filter in place, approx. 10 % of the rhodopsin was photoactivated per flash.

Guanine-nucleotide-binding assay

The protocol of Robinson *et al.* (1986) was used for these assays. In experiments using freshly dissociated squid photoreceptor membranes, 0.05 % saponin was added to the solutions to permeabilize the distal segments. Dark-adapted photoreceptor membranes in AC were added to a reaction mixture which contained final concentrations of 10 μ M-GTP[S] and 1–10 μ Ci of [³⁵S]GTP[S] (1 Ci/mol). Portions of 25 μ l were filtered at defined times through 0.46 μ m-pore-size nitrocellulose filters (Millipore, type HA25), and were rinsed with 15 ml of 400 mM-KCl/10 mM-Hepes, pH 7.8. The filters were then dissolved in 1 ml of 2-ethoxyethanol and 5 ml of scintillation fluid. Radioactivity retained by the filter was determined using a liquid scintillation counter. Frog rod outer segments were assayed as described in Robinson *et al.* (1986).

Assays for rhodopsin and protein

The concentration of squid rhodopsin was measured spectroscopically (Hara & Hara, 1967; Hagins, 1973). Total protein was assayed using a bicinchoninic acid procedure (Smith *et al.*, 1985), with BSA as standard. In our samples of distal segments, rhodopsin accounts for approx. 15 % of the total protein.

RESULTS

Identification of several light-stimulated G-proteins

The α subunits of putative G-proteins from fresh squid photoreceptor membranes were identified by ADP-ribosylation with either cholera toxin or pertussis toxin, as shown in Figs. 1 and 2. These toxins normally catalyse the transfer of ADP from NAD⁺ to the α subunits of stimulatory or inhibitory G-proteins respectively. Transducin, the G-protein of the vertebrate visual transduction, is unique in its ability to react with both toxins (Stryer, 1986). In squid, the bacterial exotoxins specifically reacted with as many as three polypeptides, with molecular masses of 38, 42 and 46 kDa. However, not all were labelled by a single toxin. The 42 and 46 kDa proteins were specific substrates for pertussis toxin and cholera toxin respectively. By contrast, the 38 kDa polypeptide, like transducin, can be ADP-ribosylated by both toxins; it reacted with cholera toxin in all five experiments and with pertussis toxin in six out of ten experiments. (The identification of these three polypeptides as α subunits of G-proteins is putative, since the effector enzymes remain unidentified.)

ADP-ribosylation appears to depend on the receptor membrane preparation and on incubation conditions. Freezing the membranes at any time prior to the toxin reactions abolishes the labelling of the 38 kDa and 46 kDa polypeptides (see below). Incubation conditions also appear to affect the results. For

Fig. 1. Pertussis toxin and cholera toxin labelling of squid photoreceptor proteins

Freshly dissociated squid photoreceptor distal segments were labelled in the dark as described in the Experimental section. Lane a contains Coomassie Blue-stained protein standards, and lane b contains squid photoreceptor proteins. The remaining lanes are autoradiograms, showing pertussis toxin labelling of the 38 and 42 kDa proteins with [32 P]NAD $^{+}$ (lane c), cholera toxin labelling of the 38 kDa protein with [32 P]NAD $^{+}$ (lane d), and a control, in which toxin was omitted from the reaction mixture (lane e).

Fig. 2. Cholera toxin labelling of squid photoreceptors using alternative conditions

Freshly dissociated squid photoreceptors were labelled in the dark by cholera toxin as described in the Experimental section, but with the addition of 1 mM-GTP and 0.5 mM-ADP-ribose at pH 7.1. Lane a contains Coomassie Blue-stained protein standards and lane b contains squid photoreceptor membranes. Lane c is an autoradiogram of cholera toxin labelling of both the 38 kDa and 46 kDa proteins.

example, the 46 kDa protein was not labelled under the conditions cited in the Experimental section, but could be routinely ribosylated by cholera toxin with the addition of 1 mM-GTP and 0.5 mM-ADP-ribose at pH 7.1. GTP has been shown to specifically enhance cholera toxin labelling of the stimulatory G-protein G_s in some hormone-stimulated adenylate cyclase systems (Doberska *et al.*, 1980). Therefore it is likely that the presence of GTP during cholera toxin labelling allowed us to observe the additional labelling of the 46 kDa protein. Most of the experi-

Table 1. Effect of light on toxin labelling

Freshly detached distal segments were labelled by either cholera toxin or pertussis toxin in the dark or in the presence of bright ambient illumination. All experiments were performed as described in the Experimental section, except for the experiments in which cholera toxin catalysed labelling of the 46 kDa band. In these experiments, 1 mM-GTP and 0.5 mM-ADP-ribose (pH 7.1) were also added. ADP-ribosylation was observed by autoradiography. Autoradiograms were analysed by densitometry, as described in the text. Values shown are means $\pm$ s.d., with numbers of experiments given in parentheses.

Molecular mass (kDa)	ADP ribosylation ratio (light/dark)	
	Pertussis toxin	Cholera toxin
46	0	1.40 $\pm$ 0.09 (4)
42	0.77 $\pm$ 0.06 (15)	0
38	0.59 $\pm$ 0.14 (7)	1.30 $\pm$ 0.20 (5)

Fig. 3. Lability of 38 kDa protein to freeze-thawing

Lane a contains Coomassie Blue-stained protein standards. Autoradiograms illustrate pertussis toxin labelling of fresh or freeze-thawed photoreceptors (lanes b and c respectively), cholera toxin labelling of fresh or freeze-thawed photoreceptors (lanes d and e respectively) and a control sample in which toxin was omitted from the reaction mixture (lane f). Arrows indicate the positions of the 38 kDa and 42 kDa proteins.

ments presented in this paper were performed under conditions where the labelling of this protein was not observed. Therefore the experiments presented here focus on the 38 and 42 kDa proteins. The smaller protein does not appear to be the proteolytic product of the other, because its radioactivity relative to the other remains constant with incubation time. These two proteins can also be distinguished by differences in solubility, lability to freeze-thawing and antigenicity (see below).

The light-dependence of toxin labelling is quantified in Table 1. In these experiments, identical portions of freshly detached distal segment membranes were incubated with toxin either in the dark or in the presence of bright ambient illumination. Light-stimulation is expected to decrease the ribosylation by pertussis toxin because the inactive state of the G-protein is the substrate for pertussis toxin (Van Dop *et al.*, 1984; Watkins *et al.*, 1985; Stryer & Bourne, 1986). In contrast, receptor activation is predicted to increase the amount of cholera toxin labelling, because the portion of the protein that is ribosylated is made accessible by activation (Abood *et al.*, 1982; Navon & Fung, 1984; Stryer & Bourne, 1986). This was indeed observed. Light

Fig. 4. Preferential extraction of the 38 kDa G-protein with a hypo-osmotic solution

Lanes a-c illustrate the protein patterns observed on SDS/PAGE when initial squid photoreceptor proteins (total, lane a) were washed with a hypo-osmotic medium and were fractionated by centrifugation into pellet (lane b) and hypo-osmotic supernatant (lane c) fractions. Autoradiograms of pertussis-toxin-labelled proteins of total proteins (lane d), pellet (lane e) and hypo-osmotic supernatant (lane f) are also shown.

Fig. 5. Immunoreactivity of monoclonal antibody 4A with squid photoreceptor proteins

Lane c is an autoradiogram illustrating the labelling of both the 38 kDa and 42 kDa proteins by pertussis toxin in the dark. The appearance of a doublet at 42 kDa is caused by a breakdown of [32 P]NAD $^{+}$ and the subsequent formation of radiolabelled ATP, which results in the phosphorylation of rhodopsin. This band is not observed when fresh [32 P]NAD $^{+}$ is used (see Figs. 1, 3 and 4). Lanes a and b are from corresponding immunoblots of squid photoreceptor proteins showing the cross-reactivity of the 42 kDa protein with monoclonal antibody 4A (lane a) and with a control non-specific antibody (lane b). Note that anti-transducin cross-reacts with the 42 kDa protein only.

decreased the amount of labelling of both the 42 kDa and the 38 kDa proteins by pertussis toxin, while increasing the ADP-ribosylation of both the 38 kDa and the 46 kDa proteins by cholera toxin.

Effects of freezing

The experiment depicted in Fig. 3 demonstrates that ribosylation of the 38 kDa protein by either cholera toxin or pertussis toxin is labile to freezing. Photoreceptors were obtained either from freshly dissected retinas or from similar retinas that were frozen and thawed prior to the isolation of the photoreceptors. This latter method of preparing squid photoreceptors is a method used by many investigators to yield a large amount of material per retina. As seen in Fig. 3, however, the freezing and subsequent thawing of the retinas abolished our ability to observe ADP-

Fig. 6. Time course of light-activated GTP[S] binding

A suspension of distal segments from previously frozen retinas was added at time zero to radiolabelled GTP[S] in the dark. At $t = 2$ min (arrowed), the membranes were stimulated with a flash of light that photoactivated 30% of the rhodopsin. Portions containing 40 μ g of protein were removed and filtered at the times indicated. O, Light-activated membranes; ●, membranes remaining in the dark.

ribosylation of the 38 kDa protein. This was the case in all experiments with retinas or isolated distal segments that were frozen and subsequently thawed. The 42 kDa protein is less labile to freeze-thawing, as it can still be ribosylated after freezing. Under our conditions, freeze-thawing also abolished ribosylation of the 46 kDa protein by cholera toxin (results not shown).

Preferential extraction with hypo-osmotic solution

The 38 kDa and 42 kDa proteins can also be distinguished on the basis of their solubility properties. Freshly detached squid photoreceptors were labelled in the dark with pertussis toxin in a hypo-osmotic buffer, as described in the Experimental section. After 1 h of incubation, 0.1% saponin was added to permeabilize the photoreceptors without solubilization. Saponin itself had no effect on the labelling (results not shown). The membranes were then centrifuged and the supernatant and pellet were analysed by SDS/PAGE. As shown in Fig. 4, the 38 kDa protein was present in the supernatant of the hypo-osmotic solution, whereas the 42 kDa protein remains in the membrane fraction, demonstrating that the 38 kDa protein can be preferentially extracted under hypo-osmotic conditions. As shown in later experiments (see Fig. 7), the extraction correlates with hypo-osmoticity and not with the presence of the detergent.

Immunoreactivity with anti-transducin antibodies

Squid photoreceptors were probed with monoclonal antibody 4A which specifically cross-reacts with the α subunit of the vertebrate photoreceptor G-protein transducin. As seen in the Western blots depicted in Figs. 5a and 5b, this monoclonal antibody cross-reacts with only the 42 kDa band. Therefore, of the several putative G-proteins, only the 42 kDa polypeptide can be recognized by this monoclonal antibody under these conditions.

Binding of GTP[S]

To complement these antibody and toxin-labelling experiments, we characterized the light-dependent binding of GTP to squid photoreceptor membranes with its hydrolysis-resistant analogue, GTP[S]. This light-dependent binding activity is shown in Fig. 6. Dark-adapted squid photoreceptor membranes in the presence of GTP[S] were exposed to a brief (1 ms) flash of light. The light-stimulated binding reached its maximum approx. 3 min after the flash.

Fig. 7. Distribution of bound GTP[S] on washing of squid photoreceptors with hypo-osmotic (or iso-osmotic) solutions

Light-stimulated binding occurred in iso-osmotic medium when initial photoreceptor material (A) was stimulated by a flash of light. The suspension was then pelleted and washed with hypo-osmotic medium (see the Experimental section) and fractionated by centrifugation into pellet (D) and hypo-osmotic supernatant (C). Pellet and supernatant were diluted with fresh iso-osmotic medium to the same volume as the initial suspension, so that equivalent portions could be filtered for the binding assays. To test for iso-osmotic extraction, the supernatant from the first centrifugation (B) was assayed in parallel with the hypo-osmotic wash. As an additional control, some of the starting material was washed with hypo-osmotic medium before the incubation with GTP[S]. These receptor membranes were not fractionated any further and were assayed directly for bound nucleotide (E). □, Light-stimulated membranes; ■, membranes kept in the dark.

Binding of GTP[S] is dependent on the permeability of photoreceptors. Squid distal segments are highly resistant to permeabilization (Wood *et al.*, 1989). Both freeze-thawing and solubilization with saponin increase the access for small molecules. Maximal nucleotide binding can be achieved using 0.1 % saponin, regardless of freeze-thawing. In contrast, $53 \pm 15\%$ of maximal binding was seen in four experiments after freeze-thawing alone. Therefore 0.1 % saponin was used to permeabilize cells in subsequent experiments on fresh tissue.

Since our toxin labelling study had indicated that hypo-osmotic media could solubilize the 38 kDa protein, we tested whether nucleotide binding occurred for both soluble and membrane fractions. Fig. 7 illustrates that in freshly dissociated saponin-treated squid membranes there is light-dependent binding of GTP[S] that is associated with both the membrane and supernatant fractions. Approx. 40 % of the binding is removed into the supernatant with the hypo-osmotic wash, which was performed after the incubation with GTP[S]. If the hypo-osmotic wash was performed before the incubation, approximately half as much nucleotide was bound by the membranes, consistent with the hypo-osmotic medium removing some of the binding proteins. The extraction is clearly due to the hypo-osmoticity of the solution and not to solubilization by saponin. This is because 0.1 % saponin in iso-osmotic medium did not significantly decrease binding capacity, as seen in Fig. 7. These results are consistent with the toxin labelling studies described above, in which hypo-osmotic conditions solubilized the 38 kDa protein.

Toxin and antibody effects on nucleotide binding

To further relate the binding studies with the labelling observations, the effect of pertussis toxin and antibody 4A on GTP[S] binding were tested. Incubation of squid photoreceptor membranes with the monoclonal antibody 4A before incubation had no effect on light-induced binding activity (results not shown). This is in contrast with the frog photoreceptor where antibody 4A inhibits 90 % of the light-induced GTP[S] binding (Hamm & Bownds, 1984). However, incubation of squid photoreceptors with pertussis toxin in the dark for 1 h eliminates 50 % of the

Table 2. Inhibition of light-activated GTP[S] binding by pertussis toxin

Squid and frog photoreceptors were incubated with pertussis toxin for 1 h at 30 °C and then assayed for GTP[S] binding as described in the Experimental section. 'Squid' refers to experiments performed on squid distal segments; 'frog' refers to those on frog rod outer segments. Binding is expressed as a percentage of maximum light-induced binding, as assayed in a control sample that lacked the toxin. Values are means $\pm$ S.D.

Preparation	GTP[S] binding (% of max.)
Squid	44 $\pm$ 26
Frog	16.2 $\pm$ 0.4

Fig. 8. Intensity-response curve for GTP[S] binding

Binding (as a percentage of maximal light-activated binding) is plotted as a function of relative light intensity. Light-stimulated binding was assayed 3 min after the light stimulus. Values are means $\pm$ S.D. ($n = 4$). Where not shown, error bars are smaller than symbols. Half-maximal stimulation occurs at 1.5 log units of attenuation of the flash or when 0.5 % rhodopsin is photoactivated.

light-induced binding (Table 2). With a similar incubation protocol, pertussis toxin eliminated approx. 85 % of the light-activated binding activity in frog photoreceptor membranes. Increasing the pertussis toxin preincubation time in squid photoreceptor membranes did not enhance its effects on light-induced binding activity, since incubation alone (in the absence of toxin) for longer than 1 h substantially diminished the amount of light-induced binding that could be measured subsequently. The GTP[S] binding that is pertussis-toxin-insensitive may in fact involve binding to the 46 kDa protein, which is not a substrate of this exotoxin.

Light-sensitivity of GTP[S] binding

In the next GTP[S]-binding experiments, frozen squid retinas were used to prepare photoreceptors. Fig. 8 illustrates the relationship between light intensity and binding. Light modulates the binding of GTP[S] over an intensity range of approx. 3 orders of magnitude. The average maximal binding in the three experiments depicted in Fig. 8 was 498 pmol/mg of protein. This gives a ratio of 0.13 mol of GTP per mol of rhodopsin, based on our determination that rhodopsin constitutes about 15 % of the protein present. Assuming that one GTP molecule is bound per G-protein, we can use the maximal amount bound as an approximate measure of the number of membrane-bound G-proteins in the squid photoreceptor membranes. Such a calculation yields a rhodopsin/G-protein ratio of 8:1. This is a conservative estimate, as freeze-thawing may decrease binding capacity.

DISCUSSION

Using a variety of techniques, we have identified three light-dependent G-proteins in the Atlantic squid *Loligo pealei*. We base our identification on several criteria. First, the polypeptides with apparent molecular masses of 38 kDa, 42 kDa and 46 kDa are ADP-ribosylated by bacterial exotoxins in a light-dependent manner (Table 1). Secondly, the G-proteins can be distinguished by differences in lability to freeze-thawing and cross-reactivity with an anti-transducin antibody. These different properties suggest that we are dealing with separate and distinct proteins that are unlikely to be related by proteolysis. Thirdly, the 38 and 42 kDa proteins can be differentiated by differences in solubility under hypo-osmotic conditions; the solubility of the ADP-ribosylated 38 kDa protein is mirrored by the solubility of GTP[S] binding, (Figs. 4 and 7). The three G-proteins appear to be localized to the distal segment of the receptor cell, where phototransduction takes place. This conclusion is based on the light-sensitivity of the toxin labelling (Table 1), and indicates that rhodopsin interacts with all three of the subunits, suggesting that all three are components of the microvillar membranes where phototransduction occurs.

The G-proteins that we have identified in *Loligo pealei* resemble G-proteins previously identified in other cephalopods. Vandenberg & Montal (1984) also observed a light-dependent cholera toxin substrate (44 kDa) in the photoreceptors of *Loligo opalescens* that was also labile to freezing. However, they did not report on the presence of any other putative G-proteins. The G-proteins identified by Saibil & Michel-Villaz (1984) in the squids *Alloteuthis subulata* and *Loligo vulgaris*, and in the cuttlefish *Sepia officinalis*, had molecular masses of 46 kDa and were mostly soluble under all conditions. Recently, Baverstock *et al.* (1989) observed light-dependent pertussis toxin labelling of a 40 kDa protein in *Alloteuthis subulata*, as well as light-dependent cholera toxin labelling of a 46 kDa protein. Tsuda (1987) reported light-dependence for the labelling of one of the pertussis toxin substrates in octopus photoreceptors (41 kDa) and a transient light effect for the labelling of another (34 kDa). The single pertussis toxin substrate identified by Brown *et al.* (1987) in *Loligo pealei* had a molecular mass of 39 kDa. Only one G-protein was ADP-ribosylated by pertussis toxin, which is consistent with our results, since Brown *et al.* (1987) used frozen retinas for their labelling.

On the basis of size, correspondence may exist among all the reported G-proteins in cephalopods. For example, our 46 kDa protein is very likely to be identical with the 44 kDa subunit described by Vandenberg & Montal (1984), given the uncertainty in mass measurements by PAGE. Similarly, our 42 kDa subunit appears to correspond to those described by Tsuda (1987) (41 kDa), Baverstock *et al.* (1989) (40 kDa) and Brown *et al.* (1987) (39 kDa). Surprisingly, previous workers observed only one or two of the three putative G-proteins that we have found in *Loligo pealei*. This may be partly due to differences in experimental conditions that can greatly alter the efficiency of ADP-ribosylation. As indicated in the Results section, one crucial factor is freezing, which we found to consistently abolish the labelling of the 38 kDa and 46 kDa polypeptides. However, freezing may not be the only explanation, because Baverstock *et al.* (1989) have obtained conditions in which the reactivity of the 46 kDa protein in frozen retinas of *Alloteuthis* was recovered. Although the optimal conditions for ADP-ribosylation have not been identified, our results clearly indicate the presence of as many as three G_x subunits in the photoreceptors of the Atlantic squid.

We have also characterized the guanine-nucleotide binding of squid photoreceptors. Using GTP[S] binding, we have meas-

ured the light-dependence of binding (half-maximal saturation with the photoactivation of 0.5% rhodopsin and a ratio of rhodopsin/G-protein of 8:1). Using this ratio of rhodopsin/G-protein, we calculate a gain of 12 activated G-proteins per photoactivated rhodopsin. Over the range of light intensities tested, the relationship between GTP[S] binding and the number of rhodopsin molecules photoactivated is non-linear. At low light intensities, however, this relationship is linear, and the number of G-proteins activated per photoactivated rhodopsin can be recalculated, yielding a gain of 30 G-proteins activated per photoactivated pigment molecule. This calculation represents a lower limit for gain, as one G-protein may be activated twice by a rhodopsin molecule. This amplification measured *in vitro* is lower than that seen in vertebrate rods, where the gain has been estimated to be 100–500 (Fung & Stryer, 1980), and is consistent with estimates in *Limulus in vivo* of a gain of 8 (Kirkwood *et al.*, 1989). GTP[S] binding is decreased by pertussis-toxin-catalysed ADP-ribosylation. This decrease is not complete and may be due either to the relatively short incubation times that have to be used, or to the presence of a pertussis-toxin-insensitive G-protein.

Why should there be several distinct light-stimulated G-proteins within squid photoreceptors? Could they be involved in separate pathways? Both InsP_3 and cyclic GMP have been proposed as second messengers for invertebrate phototransduction (Fein *et al.*, 1984; Brown *et al.*, 1984; Johnson *et al.*, 1986), and Fein (1986) has suggested that a G-protein mediates production of InsP_3 . The light-stimulated production of InsP_3 has been linked to a G-protein (Devary *et al.*, 1987; Baer & Saibil, 1988; Wood *et al.*, 1989). The G-proteins that we have observed in squid may act on these different effectors or may modulate the same effector in different ways. A precedent for the idea of a receptor activating two different G-proteins comes from experiments with M2 muscarinic receptors of pig brain (Haga *et al.*, 1986; Ashkenazi *et al.*, 1987). The M2 receptor, which is evolutionarily related to rhodopsin, has been shown to activate both G_i and G_o . Light activates both excitation and adaptation within photoreceptors, and these processes can be interconnected. Although we have not yet identified the effectors modulated by the light-activated G-proteins, the possibility of multiple pathways involving separate G-proteins activated by a single receptor may stimulate new ideas and approaches to the mechanisms involved in invertebrate phototransduction.

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