A light-stimulated increase of cyclic GMP in squid photoreceptors

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Photoreceptor outer segments isolated from squid retina are known to contain a light-activated GTP-binding protein. Here it is shown that these photoreceptors contain around 0.01 mol cyclic GMP per mol rhodopsin. Adding GTP in the dark stimulates the production of 0.0003–0.001 mol cyclic GMP/mol rhodopsin per min. GTP and light cause a 2-fold faster increase in cyclic GMP. These results show that either (1) squid rhodopsin activates a guanylate cyclase, or (2) there is a constant guanylate cyclase activity and photoexcited rhodopsin inhibits a cyclic GMP phosphodiesterase.

Invertebrate photoreceptor *GTP-binding protein* *Cyclic nucleotide* *Visual transduction*

1. INTRODUCTION

Vertebrate rods contain a well described enzyme cascade in which photoexcited rhodopsin activates a GTP-binding protein [1,2] which in turn activates a cyclic GMP phosphodiesterase (PDE) [3–5]. The action of this PDE plays an important role in regulating the light-sensitive conductance decrease of the rod outer segment leading to the hyperpolarizing response [6,7]. Although cephalopod photoreceptors differ structurally from those in vertebrates [8] and depolarize in response to light [9], they contain a related GTP-binding protein [10–13]. Cephalopod rhodopsin is capable of activating bovine PDE [14], and rhodopsin and GTP-binding protein from the two systems cross-react [13]. Neither PDE nor cyclase activity has been previously reported however, nor has any clear physiological effect of cyclic nucleotides been demonstrated in an invertebrate photoreceptor. Anomalous results on light activation of apparent GTPase activity in the squid photoreceptor preparation [13] suggested the presence of other enzyme activities. Measurements of cyclic nucleotide concentrations are presented here.

2. MATERIALS AND METHODS

The photoreceptor outer segment layer was isolated from dark-adapted, frozen squid retinas (*Alloteuthis subulata*) as in [8,13]. Reagents were obtained from Sigma, except for LiGTP which was from Boehringer. Manipulations of photoreceptors up to the acid denaturation step were done in darkness or deep-red light (Kodak Safelight Filter no.2 with a 25 W bulb at 0.5 m). The outer segments were shaken off into buffer [400 mM KCl, 20 mM $MgCl_2$, 10 mM EGTA, 1 mM dithiothreitol, 20 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.4)] and homogenized to give total outer segment protein (OS). The microvillar membranes (M_s) were collected by centrifugation at 10000 × *g* for 4 min in the cold. They were purified by flotation on 40% sucrose, washed with buffer and suspended in a volume equal to the starting homogenate. The supernatant of this homogenate (E_s) was collected and respun to remove any membrane contaminants. The polypeptide compositions of M_s and E_s are shown in [13], where it is demonstrated that both M_s and E_s contain GTP-binding protein on the basis of light-dependent changes in turbidity and GTPase

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activity, and by peptide mapping. Rhodopsin concentration was measured by the absorbance change at 494 nm on illumination at pH 10.

Cyclic nucleotide concentrations were measured by radioimmunoassay using kits from New England Nuclear based on the method in [15]. Timed reactions were initiated by addition of 0.1–0.2 mM NTP, 15 mM creatine phosphate and 0.2 mg/ml creatine phosphokinase to samples containing 1 μM rhodopsin and/or equivalent amounts of E_s at 20°C. Samples were divided into two sets for light/dark comparisons; the order of measurement did not affect the results. Illumination was provided by switching on a dim white light simultaneously with addition of nucleotide [3 $\times$ 10^{12} $h\nu/cm^2$ per s (λ_{max} equivalent) incident from a lamp which did not increase the sample temperature]. One min was considered to be the shortest interval from manual mixing of nucleotide which could be accurately timed. Trichloroacetic acid (6%) was used to stop the reactions and precipitate the protein, which was removed by centrifugation. The supernatants were then extracted 4 times with ether to remove the acid, freeze-dried and redissolved in 50 mM acetate buffer. Pairs of samples were assayed without acetylation at two different concentrations according to the procedure described in the radioimmunoassay kit instructions, and results were proportional to sample concentration. Background levels of antibody binding by cross-reaction with other nucleotides were measured by assaying samples with acid added before NTP. These were always less than 50% of total values and were subtracted from sample values. The cyclic GMP antibody has a working range of 0.02–5.0 pmol/sample and was used in the most sensitive range of 0.05–0.5 pmol.

A direct test of guanylate cyclase activity, suitable for comparison of many samples in parallel, is available [16]. However this method is about 50-times less sensitive than the radioimmunoassay and was found to be impracticable for measurement of low activity in light-sensitive samples.

3. RESULTS

3.1. *Cyclic nucleotide content of squid photoreceptors*

The cyclic nucleotide contents of crude outer segments are shown in table 1. Variable amounts were isolated with the OS, probably reflecting variable degrees of contamination with inner segment material, but cyclic AMP levels were generally about 5-times those of cyclic GMP. Cyclic GMP compares closely to that observed in dark-adapted frog rod outer segments (~0.015 mol cGMP/mol rhodopsin) [17]. A 15 min incubation with 1 mM $CaCl_2$ reduced the cyclic AMP to 50% of its initial level but did not affect the cyclic GMP level. In contrast, incubation with 0.1 mM ATP + 0.1 mM GTP had little effect on the cyclic AMP content but caused a 2–3-fold increase in cyclic GMP, demonstrating the presence of a guanylate cyclase. Without added GTP the cyclic GMP level was stable for $\geqslant$30 min.

3.2. *Light-sensitive variations in cyclic GMP content of M_s and E_s*

The membrane and soluble extract fractions M_s and E_s were assayed as a function of time after adding 0.2 mM GTP in darkness or in light. Fig.1a shows the light-insensitive linear increase of cyclic GMP with time in M_s, giving a net guanylate cyclase rate of 0.0003 mol cGMP/mol rhodopsin per min. Total OS gave rates of 0.001 mol/mol per min. Frog rod outer segment guanylate cyclase, measured in the presence of cyclase activators, is about 0.05 mol/mol per min [18,19] and has been characterized in detail [20]. Fig.1b shows the effect of adding an equivalent amount of E_s. At 1 min cyclic GMP has risen twice as fast in the light. A light activation of 1.99 ± 0.47 has been observed in 5 independent sets of experiments, either on crude OS or on M_s + E_s. Cyclic GMP continues to rise at the slower, dark rate after the first minute. It also increases slowly in E_s alone (fig.1c).

Table 1

Cyclic nucleotide content of squid photoreceptor outer segments

	OS (mol/mol rhodopsin)	(OS + Ca^{2+})/ OS	(OS + ATP + GTP)/OS
cAMP	0.015–0.06	0.38–0.53	1.1
cGMP	0.005–0.015	0.85–1.0	2.0–3.0

Values are ranges of two or more measurements. Incubations with Ca^{2+} or NTP were for 15 min at 20°C

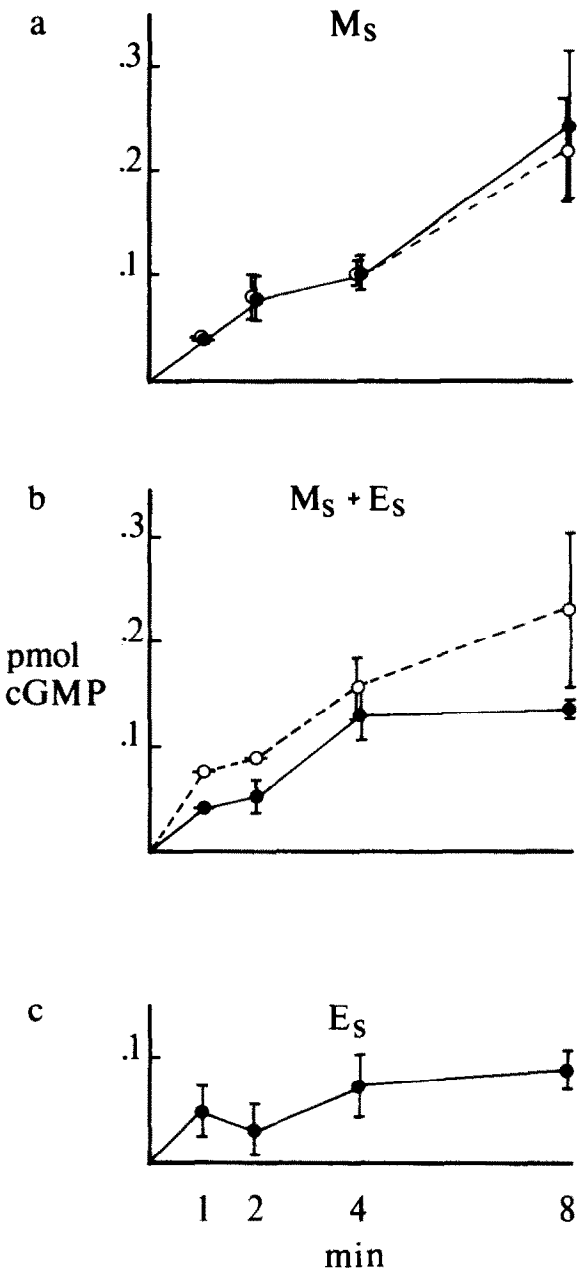

Fig.1. Cyclic GMP increase with time after adding 0.2 mM GTP in dark (●) and illuminated (○) samples. Error bars give ranges of duplicate measurements. (a) M_s contained equal volumes of photoreceptor membranes and buffer; (b) $M_s + E_s$ contained equal volumes of membranes and soluble extract; (c) E_s contained equal volumes of extract and buffer and was measured in the light, since it contained no visual pigment.

4. DISCUSSION

The measurements described here were done on preparations identical to those in which rhodopsin activates GTP-binding protein present in both M_s and E_s [13]. It is therefore likely that the light-stimulated cyclic GMP changes are mediated through the GTP-binding protein. They can be interpreted in one of two ways:

(i) Squid photoreceptors contain a light-activated guanylate cyclase. This would be unusual in requiring GTP both as a co-factor for the GTP-binding protein and as substrate for the cyclase.

(ii) There is a constant guanylate cyclase activity combined with a slower cyclic GMP phosphodiesterase which is inhibited by light. This could be done through an inhibitory GTP-binding protein similar to that described in the hormone receptor system [21].

It has previously been reported that cyclic AMP levels in *Limulus* photoreceptors are reduced after 1 h of illumination in seawater [22]. The present results suggest that this decrease is caused by increased intracellular calcium: Calcium reduces cyclic AMP content in squid photoreceptors within 15 min (table 1), and intracellular calcium is known to rise in invertebrate photoreceptors during light adaptation [9].

This is the first demonstration of guanylate cyclase activity and of light-dependent changes in cyclic GMP levels in an invertebrate photoreceptor. Although the net cyclase rates are low in purified preparations, the cyclic GMP levels in whole outer segments are similar to those in vertebrate rod outer segments. It is striking that the cyclic GMP increase by light in squid mirrors a decrease in bleached rods [17]. Cephalopod photoreceptors depolarize in response to light by opening sodium conductances [9] with a similar intracellular voltage waveform, apart from the sign reversal, to vertebrate photoreceptors [23]. The present findings are consistent with a scheme in which cyclic GMP or a product of its metabolism [24,25] plays a similar role in opening light-sensitive channels in both cephalopod and vertebrate photoreceptors. Although there is physiological evidence for the action of a GTP-binding protein in the *Limulus* ventral eye [26], little effect of introducing cyclic nucleotides has been

found in this system [27]. However, it may not be absolute concentrations, but rates of cyclic GMP metabolism which are important [24], and the site of cyclic GMP action in photoreception is still unknown.

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