Guanylate cyclase in rod outer segments of the toad retina

Effect of light and Ca^{2+}

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Guanylate cyclase activity was measured in disrupted rod outer segments of the toad retina. The experiments showed that cGMP is synthesized from GTP at a rate of 3 ± 1 nmol/min per mg protein. In darkness this value is largely independent of the Ca^{2+} concentration, while it is enhanced by flashes of light of increasing intensity upon lowering Ca from 10^{-5} to 10^{-8} M. In view of recent observations that shortly after a flash of light calcium activity inside the photoreceptor cell decreases, it seems likely that calcium plays a regulatory role in cGMP metabolism in visual excitation.

Visual transduction *Guanylate cyclase* *Rhodopsin* *cyclic GMP metabolism* *Photoreceptor*

1. INTRODUCTION

Phototransduction in vertebrate rods requires an internal transmitter [1] linking photon absorption by rhodopsin with the conductance change of the plasma membrane. While calcium does not appear to play such a role [2–4], the more recent candidate cGMP is receiving growing support [5,6]. In this view, cGMP acts directly on light-sensitive channels, holding them open in darkness to allow the Na current to flow in. By stimulating PDE activity, light causes a drop of cGMP concentration, with consequent closure of light-sensitive channels and hyperpolarization of the photoreceptor membrane. However, calcium could play the role of regulating cGMP metabolism as suggested by the recent findings that its concentration inside the cell decreases during illumination [7,8]. We have examined this possibility, studying guanylate cyclase activity as a function of different concentrations of Ca^{2+} in disrupted ROS of the toad. We have found that in darkness cyclase is not affected by changing calcium concentration while, following a flash of light, cyclase activity shows a marked increase when the calcium level falls from 10^{-5} to 10^{-8} M.

Abbreviations: ROS, rod outer segments; PDE, phosphodiesterase; IBMX, isobutylmethylxanthine; Mops, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol

2. MATERIALS AND METHODS

2.1. *Preparations*

ROS were obtained from dark-adapted eyes of toads (*Bufo bufo*) enucleated under dim red light. The eyes were hemisected under infrared light and retinae were removed and gently shaken in 35% sucrose (w/w) in Ringer solution (115 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, buffered at pH 7.5 with 10 mM Hepes and tetramethylammonium hydroxide). The ROS suspension was centrifuged for 10 min at 6000 rpm ($3000 \times g$). The pellet was discarded and the supernatant diluted with Ringer solution and centrifuged at 4000 rpm for 10 min. The pellet containing ROS from 4 retinae was suspended in 70 μl of 4 mM Mops (pH 7.1) containing 5 mM DTT

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and homogenized with a 100 μl pipette with a plastic tip (0.5 mm diameter orifice).

2.2. *Guanylate cyclase assay*

Guanylate cyclase was assayed following the method of Pannbacker [9] with minor modifications. The reaction mixture contained 100 mM Mops (pH 7.1), 140 mM KCl, 20 mM NaCl, 10 mM $MgCl_2$, 5 mM GTP, 5 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase, 5 mM IBMX, 4 mM cGMP, 2 μCi [^{14}C]GTP (46 mCi/mmol) and 12 μCi [^{3}H]cGMP /15 Ci/mmol). The reaction was initiated by adding 10 μl ROS homogenate to 10 μl reaction mixture (final concentration about 1–2 mg/ml protein) at room temperature and stopped by adding 5 μl of 100 mM EDTA and boiling for 2 min. After centrifuging, 3 μl of the supernatants were analysed by TLC on polyethyleneimine-cellulose developed in one dimension with 1 M LiCl. 5′-GMP, GDP and guanosine were added as internal standards. The separated spots, visualized under UV light, were shaved into scintillation vials, eluted in 0.3 ml of 25 mM $MgSO_4$ and counted in an LKB liquid scintillation counter.

2.3. *Experiments with different Ca concentrations*

ROS suspensions were prepared as described above except for Ringer solution which did not contain $CaCl_2$. Different concentrations of Ca^{2+} were obtained adding 2 mM EGTA to samples containing 1.8 mM $CaCl_2$ (1.7×10^{-6} M [Ca^{2+}]), 1 mM $CaCl_2$ (1.9×10^{-7} M [Ca^{2+}]) and 0.2 mM $CaCl_2$ (2.1×10^{-8} M [Ca^{2+}]). Samples without $CaCl_2$ and without EGTA were assumed to contain roughly 10^{-5} M [Ca^{2+}]. Samples with the smallest amount of calcium ($<10^{-10}$ M [Ca^{2+}]) were obtained with 2 mM EGTA without the addition of $CaCl_2$. Protein concentration was determined with a method based on Coomassie blue staining [10].

3. RESULTS

3.1. *Cyclase activity in darkness*

A typical experiment with an ROS homogenate from 4 retinae gave the results shown in fig.1. After 25 min darkness tritium-labelled cGMP decreased by about 20% of its initial level, indicating that PDE was still effective even in the presence of 5 mM IBMX. The formation of

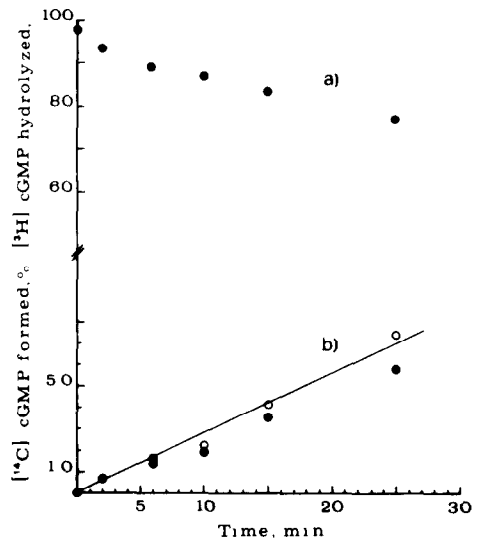

Fig.1. cGMP hydrolysis (a) and formation (b) during the same experiment, measured as percentage of ^{3}H and ^{14}C radioactivity contained in cGMP spots after TLC on polyethyleneimine-cellulose. In (b) full points represent the observed formation of [^{14}C]cGMP, open points the values corrected for cGMP hydrolysis by PDE. In this experiment 70 μl ROS homogenate were added to 50 μl reaction mixture (see section 2) and aliquots of 20 μl were collected at different times.

[^{14}C]cGMP from [^{14}C]GTP was linear with time at least for the duration of the experiment and reached about 6% of the total ^{14}C radioactivity in 25 min. In fig.1 the straight line joins the values z of [^{14}C]cGMP corrected for the hydrolysis by PDE according to: $z = v/(1 - y)$ where v is the observed value and y the percentage of hydrolysis by PDE.

Guanylate cyclase activity was assumed to be equal to the rate of formation of [^{14}C]cGMP/mg protein. In the experiment shown in fig.1 the percentage of cGMP formed/min (slope of the straight line) was 0.27 and the initial amount of GTP was about 290 nmol in a sample containing 0.2 mg protein. The cyclase activity therefore amounts to $0.27 \times 10^{-2} \times 290/0.2 = 3.9$ nmol cGMP formed/min per mg protein.

3.2. *Effect of light in low calcium*

To verify the hypothesis that calcium may play a role as a modulator of cGMP metabolism, the cyclase activity was measured at different Ca concentrations ranging from 10^{-4} to 10^{-9} M. In

darkness the cyclase activity calculated from about 10 experiments was 3 ± 1 nmol cGMP formed/min per mg protein, and showed no significant variation upon changing the Ca^{2+} concentration. By contrast, when a flash of light was given to the samples, the cyclase activity increased as the Ca level decreased. Fig.2 shows the results of typical experiments where a flash of the same intensity was given to samples with two different Ca concentrations. The corrections for cGMP hydrolysis by PDE were made till 1.5 min after the flash of light. After this time these corrections were no longer reliable as the cGMP hydrolyzed was more than 90%. In fig.2 the points at 6 min were not corrected for PDE and therefore the behaviour of the cyclase 2.5 min after the flash was lost.

The cyclase activity stimulated by light, upon decreasing Ca concentration, is compared to that in the dark in fig.3. The stimulating effect became apparent for flash intensities bleaching 7×10^{-4}% of rhodopsin and increased up to intensities bleaching 7×10^{-2}% of rhodopsin. The range of Ca concentrations affecting the cyclase was expanded by increasing the intensity of light. For lights bleaching about 10^{-5}% of rhodopsin or less

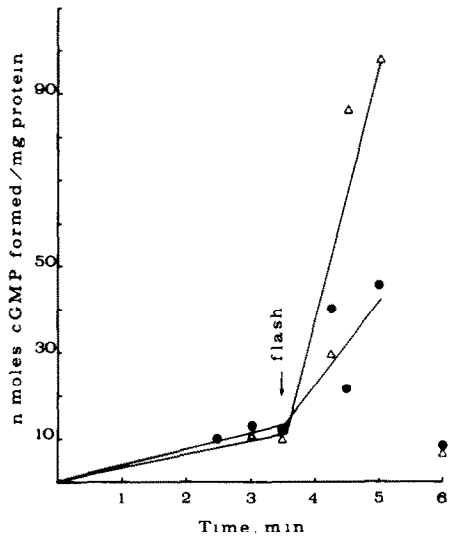

Fig.2. Effect of light on cyclase activity at two different concentrations of Ca^{2+}: (●) 1.9×10^{-7} and (△) 2.1×10^{-8} M. Samples were flashed with the same light bleaching about 10^{-2}% of the total rhodopsin present (about 0.6 nmol rhodopsin per retina; 4 retinae). Each point resulted from a different experiment.

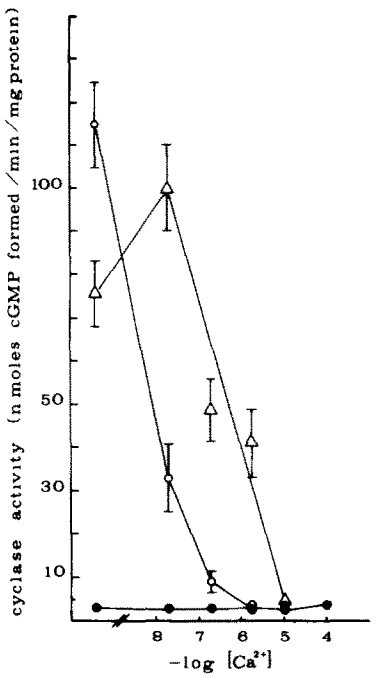

Fig.3. Effect of light and calcium on cyclase activity: (●) in the dark; each value represents the mean ± SD of 5–10 experiments. (○) After a flash bleaching about 7×10^{-4}% of rhodopsin. (△) After a flash bleaching about 7×10^{-2}% of rhodopsin. Each value in the light represents the mean ± SD of 2–4 experiments. Samples were incubated for 4 min in darkness for cyclase assay and flashed for 1 min before the reaction was stopped.

the action of Ca on cyclase was negligible. Flashes of light of intensity bleaching more than 10^{-1}% of rhodopsin were not used as the degradation of cGMP by the PDE was so fast that the cyclase activity values corrected for PDE were no longer reliable.

4. DISCUSSION

The values of cyclase activity obtained in darkness are in the range of those found by other investigators [9,11–13]. In the presence of light (0.07% of rhodopsin bleached) these values increase by a factor of 30, when $[Ca^{2+}]$ is 10^{-8} M. The light activation of cyclase is depressed to dark value by increasing calcium to 10^{-5} M. At 10^{-4} M $[Ca^{2+}]$ light inhibited cyclase activity of about 80% of the dark value (not shown) in agreement with

the results of experiments carried out in relatively high calcium concentration [9,12,13,15].

Our results indicate that the mere lowering of calcium level in darkness does not affect the cyclase. In rat visual cells Lolley and Racz [11] found that cyclase activity in dark-adapted animals increased by more than 50% when Ca concentration was lowered from about 10^{-6} to 10^{-9} M. This apparently different result can be explained by the fact that Lolley and Racz carried out their measurements under red light, which is known to bleach a small percentage of rhodopsin, sufficient to activate PDE [15]. We obtained similar results from experiments where complete darkness was not achieved.

The mechanism by which light stimulates guanylate cyclase in low Ca remains to be investigated. The simplest way to enhance the activity of the enzyme would be the rapid clearing away of its product (cGMP) caused by PDE activated by light. However, control experiments carried out in the dark at about 10^{-8} M [Ca^{2+}] with 0.2 mM cGMP gave no differences from the values of cyclase activity obtained in the presence of 2 mM cGMP.

In conclusion, our observations suggest that calcium may regulate the activity of guanylate cyclase during visual excitation. In darkness, with the cytoplasmic calcium levels presumably between 10^{-7} and 10^{-6} M, the cyclase activity would be minimal. After a flash of light, the activation of PDE causes a drop in cGMP level and consequent hyperpolarization of the plasma membrane. The reopening of the light-sensitive channels restoring the normal membrane potential could be due to the cyclase activation caused by the decrease in internal Ca concentration. Eventually, the initial levels of calcium and cGMP would be restored by the increase of Ca influx through the opened light-sensitive channels [3] which would drive back the cyclase activity to minimal values [16,17].

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