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Light-Induced Changes of Cyclic GMP Content in Frog Retinal Rod Outer Segments Measured with Rapid Freezing and Microdissection

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Abstract. Cyclic GMP concentration was measured in the rod outer segments (ROS) of the isolated frog retinas. Retinas were quickly frozen in 0.5 s after the short light flash producing 90%-saturated late receptor potential (2,000 rhodopsins bleached per rod). ROS were obtained by microdissection, and cGMP levels were determined by radioimmunoassay method. No detectable changes in cGMP concentration was found in this stimulus condition. Dark-adapted ROS contained 46.3 ± 1.5 pmole cGMP per mg dry weight, flash-illuminated ones −45 ± 2 pmole/mg. 3-s bright illumination (ca. 10^7 rhodopsins bleached per rod per second) led to approximately 30% drop in cGMP content. It is supposed that the main part of cGMP within the ROS is in the bound state and therefore fast light-induced changes in its minor free fraction may escape the detection.

Key words: Retinal rods – Intracellular messenger – cGMP

There is an accumulated evidence that 3′, 5′-cyclic guanosinemonophosphate can be the intracellular messenger in vertebrate retinal rods. The concentration of cGMP in rod outer segments (ROS) drastically decreases under illumination, presumably due to a light-activated, cGMP-specific phosphodiesterase (PDE) (Miki et al. 1973; Goridis and Virmaux 1974; Goridis et al. 1976; Farber and Lolley 1977; Cohen et al. 1978). The gain of PDE reaction (up to $4 \cdot 10^5$ cGMPs hydrolysed per second per rhodopsin bleached) is sufficient to transmit the signal from the disks to the plasma membrane (Yee and Liebman 1978; Liebman and Pugh 1979). cGMP, injected intracellularly, can control the membrane potential in rods (Miller and Nicol 1979). PDE inhibitors strongly influence the amplitude and time course of the aspartate-isolated rod receptor potential (Govardovskiĭ and Berman 1977, 1978). The effects can be explained on the assumption that the light-activated hydrolysis of cGMP is the initial stage of the transduction process.

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It is not clear, however, whether the light-induced drop in cGMP concentration is fast enough to be responsible for the generation of the electrical response. The published data seems to suggest that when the intensity of light is low the activation of PDE in frog ROS occurs with a significant delay, so that it takes several seconds before the reaction rate can achieve its maximum (Yee and Liebman 1978). Using frog and cattle isolated retinas, Goridis et al. (1976) found the first detectable changes in cGMP level to occur not earlier than 3 s after the light flash. On the other hand, Woodruff et al. (1977) and Woodruff and Bownds (1979) reported a noticeable decrease in cGMP concentration in isolated ROS within 100–200 ms after onset of illumination. Both groups used chemical fixation by perchloric acid to stop light reactions. This procedure does not seem quick enough to prevent possible artifacts. The data obtained by Goridis et al. (1976) was supported recently by Kilbride and Ebrey (1979) whose results of the analysis of quickly frozen samples of whole isolated frog retina were published at the time of writing the present paper. We tried to detect the light-induced changes in cGMP concentration in ROS of isolated frog retina by rapid freezing and microdissection of ROS layer. No measurable decrease was found within 0.5 s after the short flash producing 90%-saturated receptor potential.

Methods

Frogs (*Rana ridibunda*) were dark-adapted for at least 24 h before use. All the dissections were performed under very dim red light ($\lambda > 650$ nm). After pithing, eyes were removed and hemisected. The posterior part of the eye was cut in two and the halves were immersed into freshly oxygenated Ringer's solution (100 mM NaCl, 2 mM KCl, 0.1 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5 mM glucose and 5 mM Na-phosphate buffer pH 7.35–7.4). The retinas were detached from the pigment epithelium and spread receptor side up on pieces of filter paper. The pieces were then placed on the plastic plates and transferred for dark adaptation into the dark moist chamber filled with oxygen. Recordings of the aspartate-isolated late receptor potential made inside the chamber showed that the dark adaptation was complete within 3 min. The plates with the retinas were subsequently attached to four levers driven by a motor inside light-tight box (Fig. 1) and some more 3 min were allowed for dark adaptation. Then the motor was started and the levers pressed the retinas to the polished copper blocks cooled by liquid oxygen. A pair of samples, each taken from the different eye of the same frog, was frozen in the dark, and another pair after the onset of the stimulating light. Two stimulus conditions were used: 50 ms flash and continuous light. The flash bleaching ~2,000 rhodopsins per ROS produced approx. 90%-saturated receptor potential which nearly reached its maximum 0.5 s after the flash (Fig. 2A). Most of light samples were frozen at that moment. Measurements by means of a copper-constantan thermocouple inserted between the retina and the filter paper showed that the retina was frozen throughout within approximately 100 ms (Fig. 2B). ROS layer froze much faster. When the thermocouple was placed on the outer retinal surface, its temperature fell below zero in less than 20 ms. Some of the "light" samples were frozen after 3 s of continuous illumination bleaching ca. 10^7 rhodopsins/s per ROS.

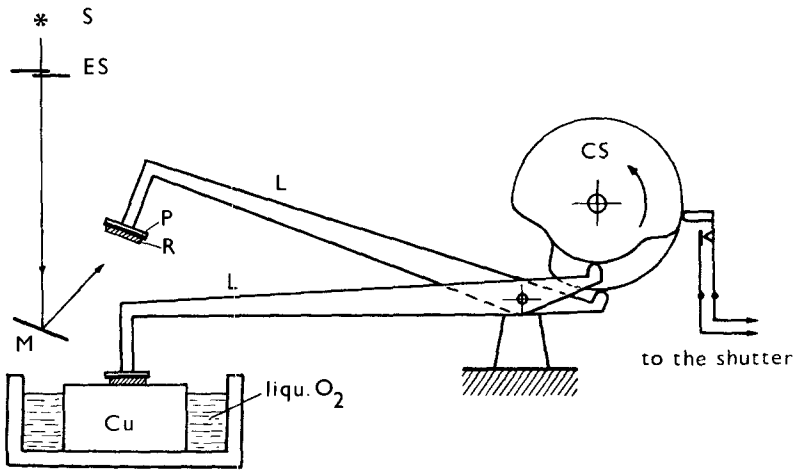

Fig. 1. The outline of the experimental set-up. Two of four levers *L* with the plastic plates *P*, filter papers and retinas *R* on them are shown. *CS* = motor-driven camshaft; *S* = light source; *M* = mirror; *ES* = electromagnetic shutter

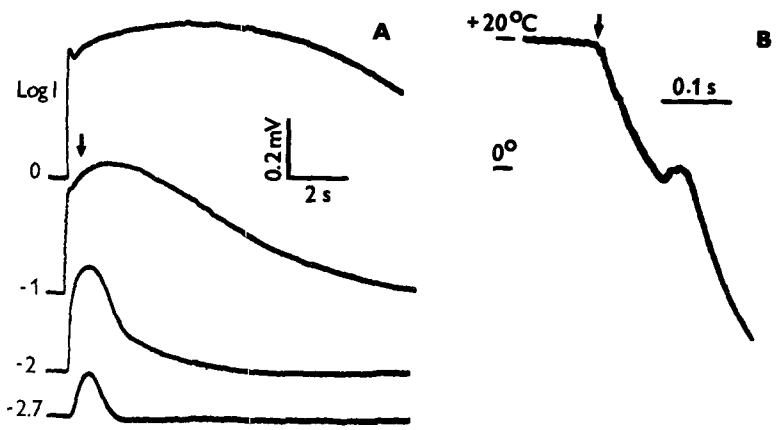

Fig. 2. A. Aspartate-isolated mass receptor potential of the frog retina recorded within the freezing chamber. 20 mM of NaCl in Ringer's solution serving for retinal isolation were substituted by equal amount of the sodium aspartate. Electrical response was recorded by means of two cotton wick electrodes, one placed under the retina and another touching outer retinal surface. 50-ms, 494-nm flash. Log I = 0 corresponds to 20,000 rhodopsins bleached per rod per flash. **B** Time course of the temperature under the retina during freezing. Arrows indicate the moment at which freezing was initiated

Since the retinas were frozen down to −183° C, all the subsequent operations were performed under room light. Some of the samples were lyophylized at −50° C and used for determining the cGMP content in the whole retina. Rod outer segments were obtained in two ways. Usually a plastic plate with the frozen retina on it was placed receptor side up on the Peltier microtome stage at −30° C and 5−10 μm sections of the ROS layer were cut by dry-ice cooled knife. Under

binocular microscope rod outer segments can be easily distinguished from the remaining retina owing to their color. The colored sections without white patches were solely used for the analysis. The sections were lyophylized and weighted. Sometimes frozen retinas were lyophylized and ROS were scraped off under binocular microscope by means of small knife made of a razor blade. In this case the remaining part of the retina was analyzed for cGMP as well. The samples of ROS thus obtained could be contaminated by the photoreceptor inner segments.

Lyophylized and weighted samples (0.2–1 mg of dry weight) were placed into tubes, cooled on dry ice, poured over with the cooled absolute ethanol, 0.5 ml per sample, and incubated at −78° C for 1 h. Then the tubes were heated up to +60° C for 2 min, 0.5 ml of hot distilled water was added and the incubation proceeded for 2 min more. This procedure led to denaturation of proteins and prevented an enzymatic degradation of cGMP. Samples were kept for 1 h at room temperature for cGMP extraction and centrifuged at 10,000 *g* for 30 min. The supernatant was sampled, lyophylized, dissolved in Tris-EDTA buffer (50 mM Tris- and 4 mM EDTA) and used for determining the cGMP content by radioimmunoassay method (cGMP RIA kit, Amersham Co., England). cGMP concentrations were expressed as picomoles of cGMP per milligram of dry weight. In control experiments lyophylized samples were treated with 5% trichloracetic acid (TCA) or with 0.05 M EDTA to prevent cGMP hydrolysis. Incubation with TCA (0.2 ml per sample) lasted 1 h at 0° C. Then 0.1 ml of 0.3 M HCl were added to convert TCA in an undissociated form, and TCA was extracted by 1 ml of the ethyl ether. Extraction was repeated six times. After extraction of TCA samples were lyophylized, dissolved in Tris-EDTA buffer and sedimented by centrifugation. Supernatant was used for determining cGMP content.

When EDTA was used to stop phosphodiesterase reaction, samples were poured over with 0.5 ml of 0.05 M EDTA at 0° C, heated up to 100° C for 3 min, cooled and centrifuged. Supernatant was diluted 1 : 5 by Tris-buffer and used for the analysis.

TCA and absolute ethanol produced similar results (22.6 ± 0.7 and 23.5 ± 0.7 picomoles of cGMP per mg of dry weight of the dark-adapted retina, respectively). Treatment with EDTA resulted in approximate 80% loss of cGMP.

Results and Discussion

Our levels of cGMP in the dark-adapted ROS (46.3 ± 1.5 pmole/mg dry weight) are in fair agreement with the measurements by de Azeredo and Lust (1978) in *Rana pipiens* (43.5 pmole/mg dry weight). Similarly, the cGMP content of the whole retina (23.5 ± 0.7 pmole/mg dry weight) corresponds to the value reported by Kilbride and Ebrey (1979) (44 pmole/mg protein). ROS contained approximately 60% of the total retinal cGMP (see legend to Table 1).

Short flash capable of exciting nearly saturated receptor potential did not produce any significant decrease either in ROS or in retinal cGMP at the peak of

Table 1. Effect of illumination on the cGMP concentration in the frog retina. Light/dark ratio in percents. Mean ± SEM. Number of samples is given in parentheses

	0.5 s after the short flash	3 s step of light
Dissected ROS	97 ± 3.5 (18)	71 ± 8 (10)
Whole retina	97 ± 5 (6)	–

cGMP content (in picomoles per mg of dry weight):

Dark-adapted ROS	46.3 ± 1.5 (21)
Whole retina	23.5 ± 0.7 (8)
Retina without ROS layer	13.1 ± 0.5 (18)

Dry weight of the ROS layer comprises 27.9 ± 1% (18) of that of the whole retina

electrical response. A marked drop in cGMP concentration was only observed after 3 s of continuous bright illumination. This is in line with the data of Goridis et al. (1976) and Kilbride and Ebrey (1979) obtained on the whole retina.

Our results are in apparent disagreement with the measurements of Woodruff et al. (1977) and Woodruff and Bownds (1979) who found a marked decrease in cGMP level in isolated frog ROS within 100–200 ms after onset of illumination. Beside chemical fixation, this group used low-calcium Ringer's solution as an incubation medium which can increase cGMP concentration and enhances light effects (Cohen et al. 1978). Another cause of the discrepancy in the results might be related to the difference between the isolated ROS and our retinal preparation which is a more close simulation of the in vivo conditions.

Thus, we can conclude that the generation of nearly saturated late receptor potential is not accompanied by an noticeable concomitant changes in the cGMP content of ROS. However, we cannot rule out a possibility that the main part of ROS cGMP might be in an "inactive" (bound) state. Comparison of the maximum activity of ROS guanylate cyclase (2.6 nmole/min mg protein, Goridis et al. 1976) with the V_m and K_m of phosphodiesterase (150 nmole/min mg protein and 100 μM, respectively, Goridis et al. 1976) shows that the steady-state free cytoplasmic concentration of cGMP should be approximately 2 μM. It makes up not more than 5% of the total cGMP content (ca. 50 μM). If this estimation is correct, significant changes in minor free cGMP fraction could be left undetected. This point can be the subject of a further study. We believe however, that cyclic GMP still remains the most probable candidate among others (Ca^{2+}, H^+), for the role of an intracellular messenger in vertebrate rods on account of an extremely high gain of light-activated phosphodiesterase reaction.

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