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Photosensitivity of 8BrcGMP-induced conductance in ROS-excised patches

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Inside-out patches from ROS plasma membranes contain the basic enzymes of the phototransduction cascade. Similar to a native photoreceptor cell, such patches are capable of responding to light, the effect of which suppresses the cGMP-activated current. Photoresponses are observed only in the presence of GTP, whereas ATP essentially accelerates the current recovery to a dark level. Photoresponses are also observed in the presence of 8BrcGMP. Phosphodiesterase (PDE) hydrolyzes 8BrcGMP two orders of magnitude slower than cGMP, so the light inhibition of the 8BrcGMP-induced current cannot be accounted for by PDE activation. It seems that activity of cGMP-gated channels depends not only on cGMP concentration, but is additionally controlled by some other regulatory mechanisms.

Retinal rod; Phototransduction; cGMP-gated channel

1. INTRODUCTION

Excised cell membrane patches are usually considered as simple systems composed of ionic channels only. At the same time it is evident that being excised from the plasma membrane the patches may contain some membrane-bound enzymes. Electrophysiological [1] and morphological [2] data suggest that ROS inside-out patches do contain fragments of photoreceptor disk membranes. Thus, the phototransduction enzymatic cascade must operate, at least, in some patches. This has been shown directly in experiments with dark-adapted rods the inside-out patches of which respond to light [3]. Hence, excised patches of ROS may appear to be a convenient model system to study phototransduction despite a partial loss of water-soluble components from the cytoplasm of photoreceptor cells. In the present studies this model seems to reveal some unexpected details of the phototransduction mechanism.

2. MATERIALS AND METHODS

The experiments were performed on ROS, mechanically isolated from retina of the dark-adapted frog, *Xenopus laevis*. Dark adaptation lasted for 3–5 h just before the experiments.

A conventional patch-clamp technique was used to obtain gigaseal inside-out patches of ROS and to measure their electrical characteristics. The patch current was low-pass filtered at 1 kHz and stored on an FM tape-recorder, then refiltered at 200 Hz and digitized at 25 Hz for computer analysis. The patch pipettes were filled with solution (mM): 100 NaCl, 0.1 EGTA, 0.1 EDTA, 10 HEPES, pH 7.4. The cytoplasmic side of the inside-out patches were bathed by solution (mM): 100 NaCl, 0.8 $MgCl_2$, 0.1 $CaCl_2$, 0.12 EGTA; pCa 7, 10 HEPES, pH 7.4. All experiments were carried out under infra-red light and room temperature (20–22°C). In the present study we used cGMP, 8BrcGMP, HEPES from Boehringer (Austria), and ATP, GTP, EGTA, EDTA from Serva (Germany).

Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; ROS, rod outer segment.

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3. RESULTS AND DISCUSSION

The experiments were carried out on 52 gigaseal inside-out patches from dark-adapted frog ROS; 39 patches responded to cGMP or 8BrcGMP application by a reversed increase in conductance. In 11 out of 18 cases in the presence of GTP (50 μM), illumination led to inhibition of the cGMP-induced current, whereas in the absence of GTP no photoresponses were observed. ATP (100 μM) insignificantly altered the rising phase of the photocurrent and accelerated its recovery by more than one order of magnitude (data not shown).

The GTP- and ATP-dependent light sensitivity of the cGMP-induced current can be explained by assuming that ROS-excised patches contain the main phototransduction enzymes, rhodopsin, transducin and phosphodiesterase (PDE), as well as rhodopsin kinase. Some portion of the cGMP molecules from the bathing solution diffuses into the patch, where it is hydrolyzed by PDE. The concentration of cGMP near the membrane is mediated by a diffusion-hydrolysis equilibrium and decreases with light activation of PDE. Transducin activates PDE in the precence of GTP, whereas PDE recovery to a dark-adapted state is accelerated by ATP [4].

In our experiments performed with light-adapted

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ROS, the hydrolysis-resistant cGMP analog, 8BrcGMP, also activated cGMP-gated channels. Its efficiency is one order of magnitude higher than that of cGMP (EC_{50}'s are approximately 1 and 20 μM, respectively). In 12 out of 21 cases light suppressed the 8BrcGMP-induced current in an intensity-dependent manner (Fig. 1). Just as in the case of cGMP, inhibition of the 8BrcGMP-induced current was observed only in the presence of GTP (data not shown). ATP essentially accelerated the recovery of the 8BrcGMP-induced current and decreased the amplitude of photoresponses (Fig. 2).

It should be noted, that 8BrcGMP is not completely resistant to PDE hydrolysis [5]. In principal, the photoresponsiveness of the ROS patches in the presence of 8BrcGMP can be explained just as in the case of cGMP.

However, in some experiments in the dark, 40 μM cGMP evoked a current the value of which was equal to that induced by 2 μM 8BrcGMP. The rates of agonists hydrolysis (at a given concentration) differ by two orders of magnitude [5], so it could be expected that the extents of patch photoresponses in the presence of cGMP or 8BrcGMP will be essentially different. As a matter of fact, in the presence of cGMP, photosensitivity of the patch current is actually higher than that in the presence of 8BrcGMP but not by two orders of magnitudes (Fig. 3A).

On the other hand, a competitive PDE inhibitor, isobutylmethylxanthin (IBMX) [6], could decrease the rate of cGMP hydrolysis and make it equal to that by 8BrcGMP (in the absence of IBMX). If three processes, namely (1) diffusion and (2) hydrolysis of cyclic nucleotides, as well as (3) their interaction with cGMP-gated channels, underlie all peculiarities of the responses of ROS-excised patches to agonist application and their photoresponsiveness, then 2 μM 8BrcGMP must be equivalent to 40 μM cGMP + 1 mM IBMX.

In fact, the responses of ROS inside-out patches to application of 2 μM 8BrcGMP or 40 μM + 1 mM IBMX are drastically different. In the presence of IBMX, 40 μM cGMP induce the currents (Fig. 3B), the value of which essentially exceeds that of the current induced by 2 μM 8BrcGMP (Fig. 3A). It should be noted that in the presence of IBMX cGMP-dependent current photosensitivity remains higher than that of the 8BrcGMP-dependent one (see Fig. 3).

As expected, the photoresponses of ROS-excised patches are due to variation of the cGMP (8BrcGMP) concentration near the membrane caused by both PDE light activation and a change in the equilibrium between diffusion and hydrolysis. The diffusion–hydrolysis equilibrium can also be shifted by alteration of the diffusive flow of agonist. For instance, light may evoke structural rearrangements in the patch leading to an increase in the diffusion mean path of the agonist molecules. Even at a constant rate of hydrolysis, this may lead to alteration of the agonist concentration change inside the patch.

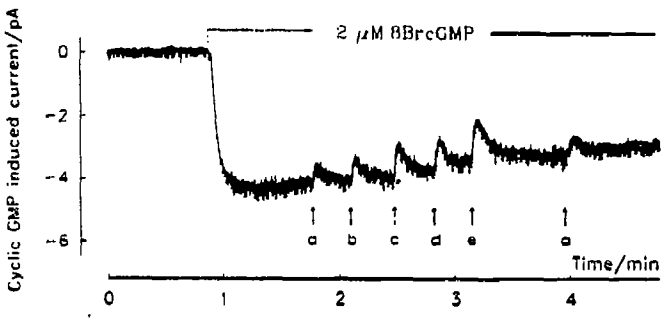

Fig. 1. Photoresponses of ROS inside-out patches in the presence of 2 μM 8BrcGMP. Arrows indicate 50 ms light flashes applied with intensities (10^3 photons·μm^{-2}): a, 0.750; b, 12.7; c, 42.1; d, 55.1; e, 74.5. The bath solution contained 50 μM GTP and 100 μM ATP. The membrane voltage was −10 mV.

Whatever the true mechanism of cyclic nucleotide concentration change near the patch membranes, in the presence of 2 μM 8BrcGMP the dark and light effects must be approximately similar to that in the presence of 40 μM cGMP + 1 mM IBMX. However, the experiments described point in an opposite direction. That is why the processes: diffusion, hydrolysis, and cGMP-gated channel activation do not account for all the peculiarities of patch photoresponses in the presence of 8BrcGMP.

Previously, cGMP sorption–desorption was supposed to participate in the phototransduction of vertebrates [7]. Probably, light-evoked sorption of cyclic nucleotides leads to the decrease of its concentration in ROS-excised patches and thereby may play an essential role in patch photoresponsiveness. It was easy to estimate, that no less than 10^5 8BrcGMP molecules per second are supplied by diffusion into the patch (the dimension of which is approx. 1 μm), when a double concentrational gradient exists and micromolar amounts of agonist are contained in the bathing solution. In the absence of ATP the 8BrcGMP-induced current was light-suppressed for at least several minutes after a flash (Fig. 2). No less than 10^7 agonist molecules must be bound during this period of time to compensate for an agonist diffusive flow. The patch volume is ap-

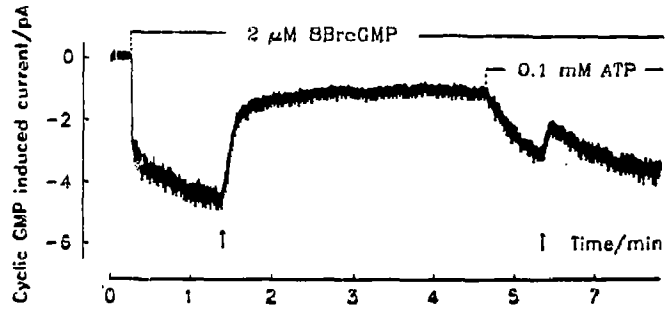

Fig. 2. ATP-dependent recovery of 8BrcGMP-induced patch current after exposure to light flashes (50 ms, 12,700 photons·μm^{-2}). The bath solution contained 50 μM GTP; the membrane voltage was −20 mV.

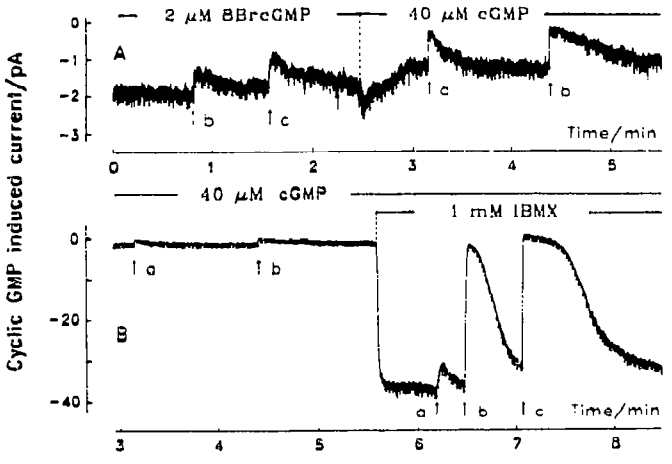

Fig. 3. Photoresponses of patches in the presence of 2 μM 8BrcGMP, 40 μM cGMP (A), or 40 μM cGMP + 1 mM IBMX (B). Light flash intensities as in Fig. 1. The bath solution contained 50 μM GTP and 100 μM ATP; the membrane voltage was −20 mV.

proximately 0.1% of ROS one, therefore a patch may contain no more than 10^6 molecules of rhodopsin. Thus, if sorption of 8BrcGMP leads to patch photoresponses, the amount of cGMP-binding sites in a patch must be one order of magnitude higher than that of rhodopsin; this seems to be improbable.

According to the data [3], guanylate cyclase operates in ROS inside-out patches. In the presence of GTP in the bathing solution, the patch could contain some amount of de novo synthesized cGMP. The content of cGMP in the patch depends on the balance between its synthesis and removal at the expense of diffusion and hydrolysis. A number of experiments in the dark showed neither an appreciable increase in patch conductance nor, correspondingly, photoresponses (data not shown) when the bathing solution contained GTP in the absence of cyclic nucleotides. Thus, cGMP removal from the patches sufficiently exceeds its synthesis in the absence of 8BrcGMP. In the presence of 2 μM 8BrcGMP it takes place as well, since the constant of PDE inhibition by 8BrcGMP is equal to about 1 mM [5]. This evidence indicates that in the experiments a possible increase of the patch conductance in the dark (above the 8BrcGMP-induced level) due to cGMP synthesis is negligible. At the same time, light inhibits an essential portion of the patch current in the presence of 8BrcGMP (Figs. 1–3), so the light suppression of 8BrcGMP-induced conductance is just valid.

Thus, we have considered some obvious mechanisms which could account for the photoresponses of ROS-excised patches in the presence of 8BrcGMP. Every mechanism suggests that light evokes changes in the agonist concentration near the patch membranes. However, neither of them, either alone or in combination, could explain all the peculiarities of the patch photoresponses in the presence of 8BrcGMP. In our opinion, an adequate interpreation of the data obtained requires the assumption of the existence of some other light-dependent mechanism, which controls the activity of cGMP-gated channels.

The question arises, how the activity of cGMP-gated channels could be controlled at unchangeable cGMP concentration. Moreover, it is also not evident whether this mechanism plays physiological role or whether it is only peculiar to excised-ROS patches. To clear up these problems, further experiments are required.

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