Effects of Cyclic GMP on the Kinetics of the Photocurrent in Rods and in Detached Rod Outer Segments

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ABSTRACT We investigated the effects of high concentrations of cytoplasmic cyclic GMP on the photocurrent kinetics and light sensitivity of the tiger salamander rod both in intact cells and in detached outer segments. Photoreceptors were internally perfused with cGMP by applying patch pipettes containing cGMP to the inner or outer segment. Large increases in the concentration of cGMP in the outer segment cytoplasm were achieved only when the patch pipette was applied directly to the outer segment. The dark-current amplitude increased with increasing cGMP concentrations up to ~1,400 pA. Internal perfusion with 5.0 mM cGMP introduced a delay of 1–3 s in the photocurrent. The magnitude of the delay was inversely proportional to the light intensity. In addition, the photocurrent time course was slowed down and the light sensitivity, measured 1 s after the flash, was decreased ~100-fold when compared with that of the intact cell. The observed effects of cGMP were compared with those predicted by a model that assumes that the initial photocurrent time course is determined by the kinetics of the light-activated phosphodiesterase (PDE) and the cGMP dependence of the light-sensitive channels. At high concentrations of cGMP, the experimental data were similar to those predicted by the model and based on the known biochemical properties of the light-activated PDE and cGMP-activated channels.

INTRODUCTION

Illumination of rod photoreceptors in the vertebrate retina leads to the closure of ionic channels in the plasma membrane of the outer segment (Hagins et al., 1970; Bodia and Detwiler, 1985). The time course and amplitude of the resulting photocurrent are determined by the intensity of the stimulus (Baylor et al., 1979*a*). Cyclic GMP molecules are thought to act as an internal messenger coupling the photoexcitation of rhodopsin in the rods to the closure of the ion channels (reviewed by Pugh and Cobbs, 1986). This hypothesis is supported by the findings that photoexcited rhodopsin activates a cGMP-specific phosphodiesterase (PDE) (reviewed by Stryer, 1986) and that cGMP opens channels in

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excised patches of outer segment membrane (Fesenko et al., 1985; Haynes et al., 1986; Zimmerman and Baylor, 1986), in intact rods (Miller, 1982; Matthews et al., 1985; Cobbs and Pugh, 1985), and in truncated rod outer segments (Yau and Nakatani, 1985). The cGMP-activated channels appear to be identical to the light-sensitive channels (Matthews, 1986).

To understand the role of cGMP in phototransduction, it is necessary to determine the relationship between the hydrolytic and synthetic processes that control the concentration of cGMP in the outer segment cytoplasm and the amplitude and kinetics of the photocurrent. Investigation of the effects of increased cytoplasmic concentrations of cGMP on the photoresponse provides an experimental tool to correlate the biochemical and electrophysiological processes. General agreement exists on the experimental finding that, in the dark, an increase in the cytoplasmic concentration of cGMP depolarizes the rod membrane (Miller and Nicol, 1979; Brown and Waloga, 1981; Lipton, 1983) and increases the membrane conductance (MacLeish et al., 1984; Cobbs and Pugh, 1985; Matthews et al., 1985). However, less agreement exists on the experimental effects of increased cGMP on the kinetics of the photoresponse and the light sensitivity of the cell.

Nicol and Miller (1978) first reported that injection of cGMP into rod outer segments delays the response to a flash of light. They proposed that the increased latency occurred because hydrolysis of the added cGMP was necessary before the ionic channels could close. These observations are central to the acceptance of the role of cGMP as an internal messenger in phototransduction. However, similar experimental results were not obtained by others (Waloga, 1983; Lipton, 1983). Cobbs and Pugh (1985) perfused isolated rods with cGMP using patch pipettes and found that cGMP can increase the photocurrent amplitude without changes in its latency. Cobbs and Pugh (1985) recognized that their observations might have resulted from ineffective perfusion of cGMP into the outer segment. Brown and Waloga (1981) reported that injections of cGMP did not effect the rod light sensitivity. However, Lipton (1983) found that cGMP injection reduced the sensitivity, while Cobbs et al. (1985) reported that internal perfusion of cGMP increased the sensitivity.

We investigated the effects of cytoplasmic cGMP on the photocurrent with an improved method for the internal perfusion of rod outer segments with cGMP. Our results resolve some of the experimental discrepancies described above and allow us to quantitatively relate the characteristics of the photocurrent to the known biochemical controls of cGMP.

METHODS

Materials

Larval-stage tiger salamanders (*Ambystoma tigrinum*) were maintained at 4°C with continuously recirculating water on a 12-h dark-light cycle. They were fed goldfish once a week. Minimum essential medium (MEM) amino acids and MEM vitamins were obtained from the tissue culture facility at the University of California at San Francisco. The calcium chelator BAPTA [1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid) was purchased from Molecular Probes, Inc. (Eugene, OR). The lectin concanavalin A (Con A),

type IV, cGMP, ATP, GTP, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, MO).

Mechanical Dissociation

The procedure for mechanical isolation of single rods was similar to that described by Baylor et al. (1984) and by Hestrin (1987). A dark-adapted animal was decapitated and pithed. The retina was isolated from the eyecup under infrared (IR) illumination and placed in a supplemented salt solution containing (millimolar): 95 NaCl, 2 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 $NaHCO_3$, 10 glucose, 10 HEPES, MEM amino acids, MEM vitamins, and 0.1 mg/ml bovine serum albumin, pH 7.4. The osmolality of this solution was 224 mosM as measured in a freezing point depression osmometer (Advanced Instruments, Inc., Needham Heights, MA). To dissociate cells, an isolated retina in supplemented salt solution without glucose was positioned with its photoreceptor side up on the Sylgard-coated (Dow Corning Corp., Midland, MI) bottom of a petri dish. The retina was finely chopped with a razor blade. The resulting suspension of retina fragments and isolated cells was transferred onto a Con A–coated coverslip (Bader et al., 1982). Cells were allowed to adhere to the coverslip for 20 min, after which the incubation medium was replaced with a supplemented salt solution containing 10 mM glucose.

Electrical Recording

The coverslip with the attached cells was placed on the stage of an upright microscope and observed with differential interference contrast optics under IR illumination (830 ± 10 nm) with the aid of a TV camera and monitor. Cells were continuously observed during electrode manipulations, but the IR source was turned off while photoresponses were recorded. Photoreceptors were illuminated on the microscope stage with light flashes produced by a tungsten source. The flash duration was 10 ms and was controlled by a mechanical shutter. The stimulating light was filtered with a narrow-band interference filter (500 ± 10 nm half-bandwidth; Ealing Corp., South Natwick, MA) and its intensity was adjusted with neutral density filters. The intensity of the stimuli was measured with a calibrated photodiode (model UV100, United Detector Technology, Hawthorne, CA) placed on the microscope stage. The effective collecting area of unpolarized light (A) of the outer segment was calculated by the following expression (Baylor et al., 1979*b*; Miller and Korenbrot, 1987):

$$A = \frac{\pi d^2 l}{4} Q_{isom}\, 2.303 f\alpha, \tag{1}$$

where l is the outer segment length, d is its diameter, Q_{isom} is the quantum efficiency of isomerization (0.67; Dartnall, 1972), α is the transverse specific density (0.012; Hárosi, 1975), and f is 0.63 for unpolarized light and a dichroic ratio of 3.78 (Hárosi, 1975). Assuming an outer segment length of 22 μm and a diameter of 11 μm, A = 23.2 μm^2. The collecting area is corrected for the difference between the stimulus used and the λ_{max} (516 nm) of the salamander rod photopigment (Hárosi, 1975).

Patch-clamp recordings were conducted in either the cell-attached or whole-cell mode (Hamill et al., 1981). The patch pipettes had a tip diameter of 0.5–1.5 μm and were coated with Sylgard. The pipettes were filled with a solution of the following composition (millimolar): 74 KOH, 4 $MgCl_2$, 5 Na_2ATP, 2 Na_4GTP, 0.05 EGTA, and 15 KCl. HEPES was added in its acidic form until a pH of 7.4 was reached. The final concentration of HEPES was ~90 mM. When cGMP was included in the pipette, the concentration of KOH was reduced. The osmolality of the solutions was ~224 mosM. All recordings were made with cells bathed in supplemented salt solution. The reference electrode was

connected to the experimental chamber via a 120 mM NaCl/agar bridge. The junction potential at the tip of the pipette was measured as the voltage difference between the pipette filled with the internal solution and the salt solution in the experimental chamber. The membrane potential was corrected for the measured junction potential.

Membrane currents were recorded with a patch-clamp amplifier (EPC-7, List-Medical, Darmstadt, Federal Republic of Germany). After establishing a tight seal with the cell's membrane, the capacitance of the pipette and headstage were canceled. To obtain a whole-cell recording, continuous suction was applied until the membrane within the pipette was disrupted and there was a sudden increase in capacitance. Pulses of 5 mV were applied to the pipette to monitor the capacitance of the cell. In all the experiments, unless specified otherwise, the cell membrane potential was held at −30 mV. The series resistance that we measured in the whole-cell recordings was 4–14 MΩ. Typically, the series resistance was at the low end of this range. Under these conditions, an inward current of 1,500 pA would make the actual membrane potential more positive than its nominal value by ~10 mV. This error was neglected in the analysis of the photocurrent because the photocurrent is nearly voltage independent in the voltage range of the holding potential (Baylor and Nunn, 1986). On the other hand, in experiments in which the effects of the membrane potential were studied, a correction for the series resistance error was made.

A microcomputer (PDP 11/23, Digital Equipment Corp., Maynard, MA) was used to acquire data and apply command voltages to the patch-clamp amplifier, as well as to control light stimuli. Analog data were filtered with an eight-pole Bessel filter (model 902LPF, Frequency Devices, Haverhill, MA), digitized at 12-bit accuracy (model DT2782, Data Translation, Marlboro, MA), and stored on computer disks. The data were analyzed on a Micro VAXII computer (Digital Equipment Corp.).

RESULTS

Whole-Cell Recording of the Photoresponse of Isolated Rods

To interpret the effects of internally perfusing cGMP on phototransduction, it was important to first determine the characteristics of photocurrents measured in intact, unperfused rods. Fig. 1*A* illustrates photocurrents recorded from a cell-attached membrane patch with the pipette applied to the outer segment. Dim flashes produced a current that reached a peak in ~0.75 s and then declined. As the intensity of the light increased, the peak amplitude of the photocurrent increased until it saturated; further increases in light intensity prolonged the time spent in the saturated state. Fig. 1*B* illustrates whole-cell photocurrents recorded from an intact rod with the pipette applied to the inner segment. The holding potential was −30 mV. The amplitude of the maximum photocurrent in this experiment was 94 pA. In some recordings, the kinetics of the photoresponse changed during the course of the experiment. These data were not used for further analysis. The time course of the photocurrent of most of the cells we recorded from did not change for up to 30 min. Typically (but not always), however, the amplitude of the dark current decreased by ~30% within 2–3 min after the membrane was ruptured. The average amplitude of the initial dark current from five different cells was 75.2 ± 12.9 pA (mean ± SD). The whole-cell photocurrents are similar in time course and photosensitivity to those recorded from a cell-attached membrane patch. Whole-cell recordings with patch pipettes applied to the inner segment were, in general, not different from recordings obtained with the pipette applied to the outer segment.

Recording of whole-cell currents with patch pipettes results in diffusional exchange of the cytoplasm with the pipette-filling solution (Marty and Neher, 1983). Therefore, whole-cell recordings from isolated rods may interfere with phototransduction by removing diffusable cytoplasmic substances or by introducing foreign substances. To compare the phototransduction process in internally perfused rods with that in intact cells, we measured the photosensitivity and kinetics of the photoresponse of perfused rods. Fig. 2 illustrates the depend-

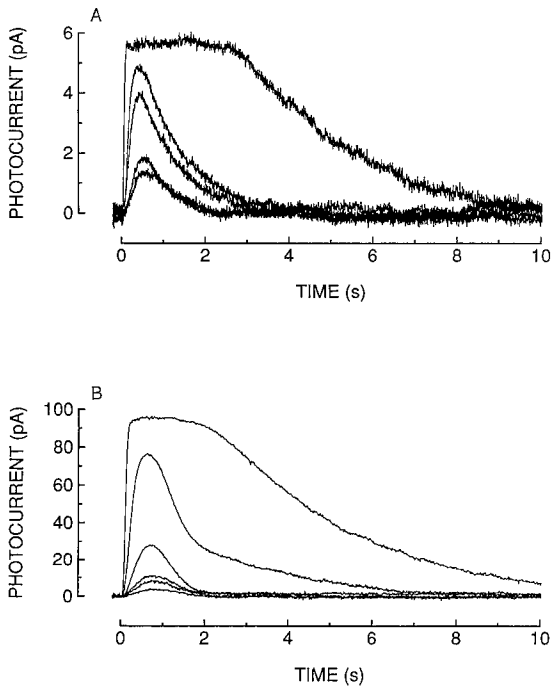

FIGURE 1. (*A*) Photocurrents recorded from a cell-attached patch of outer segment membrane. Flashes of diffused, unpolarized 500-nm light were presented at time zero. The light flashes, 10 ms in duration, delivered 1.7, 3.6, 10.1, 28.6, and 340.0 photons·μm^{-2}. The current amplitude is given in reference to the dark current, which is taken to be zero. Outward current is plotted upward. (*B*) Whole-cell photocurrents recorded from an intact rod. The patch pipette was sealed onto the inner segment. The pipette contained the standard filling solution (see Methods) without cGMP. The holding potential was set at −30 mV. The responses to flashes that delivered 0.39, 0.5, 1.1, 3.0, 16.9, and 99.7 photons·μm^{-2} are shown.

ence of photocurrent peak amplitude on light intensity measured in five different rods. This dependence can be described by the Michaelis function:

$$\frac{R}{R_{max}} = \frac{I}{I + I_0}, \tag{2}$$

where R and R_{max} represent the amplitude (picoamperes) of the response at the peak and at saturation, respectively, I is the flash intensity (photons per square micron), and I_0 is the half-saturating intensity (the light intensity at which the peak amplitude reaches 50% of the saturating flash response). The average value of I_0 was 7.9 photons·μm^{-2}, which corresponds to ~183.3 photoexcited rhodopsin molecules, assuming an effective collecting area of the rod of 23.2 μm^2 (see

Methods). The waveform of the response to a dim flash (inset) can be described by the independence equation of Baylor et al. (1974):

$$r(t) = kIe^{-t/\tau}\,(1 - e^{-t/\tau})^3, \tag{3}$$

where $r(t)$ is the response in picoamperes as a function of time t, k is a sensitivity factor, I is the flash intensity, and τ is the characteristic time constant. The average value of τ that we found in five cells was 0.534 ± 0.065 s (mean ± SD), which corresponds to t_{peak} = 0.74 s.

The whole-cell photocurrents we measured are similar in their photosensitivity and kinetics to photocurrents measured from unperfused salamander rods (Bader

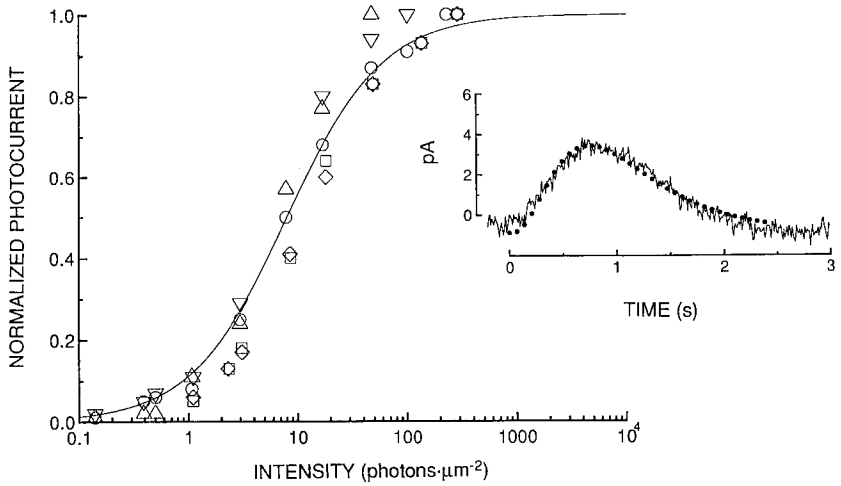

FIGURE 2. Dependence of the photocurrent peak amplitude on the intensity of the light stimulus. Data were collected from whole-cell photocurrents measured from five intact rods using a patch pipette without cGMP. The peak photocurrents measured from each cell were normalized by the maximal response of that cell. Flash intensities, given in photons per square micron, can be converted into rhodopsin isomerizations using an effective collecting area of 23.2 μm^2. The line superimposed on the data represents the Michaelis function (Eq. 2), with I_0 = 7.9 photons·μm^{-2}. The inset shows the kinetics of the photocurrent, measured from a single rod, in response to a flash intensity of 0.39 photons·μm^{-2}. The filled circles represent the independence function (Eq. 3) with τ = 620 ms, which corresponds to t_{peak} = 860 ms.

et al., 1979; Baylor and Nunn, 1986). The amplitude of the dark current we measured was ~40% larger than the dark current measured with the suction pipette method. However, this difference is expected since suction pipettes collect only a fraction of the total outer segment current. Thus, internal perfusion with our medium apparently does not interfere with the physiology of phototransduction in the rod.

Photoresponse of Intact Rods Internally Perfused with cGMP

Fig. 3 illustrates whole-cell photocurrents recorded from isolated rods that were internally perfused with 5.0 mM cGMP with the pipette applied either to the

inner segment (*A*) or the outer segment (*B*) of different rods. The membrane potential was held at −30 mV in both cases. Typically, the amplitude of the maximum photocurrent was much larger when the pipette was applied to the outer segment. In the experiments illustrated in Fig. 3, the maximal photocurrent amplitude was 280 pA with the pipette on the inner segment and 1,500 pA with the pipette on the outer segment. The photoresponses illustrated in Fig. 3 also differed in the duration of the delay between flash presentation and the onset of the photocurrent. When the pipette was applied to the outer segment this delay was ~1–3 s, whereas it was at most 300 ms when the pipette was applied to the inner segment.

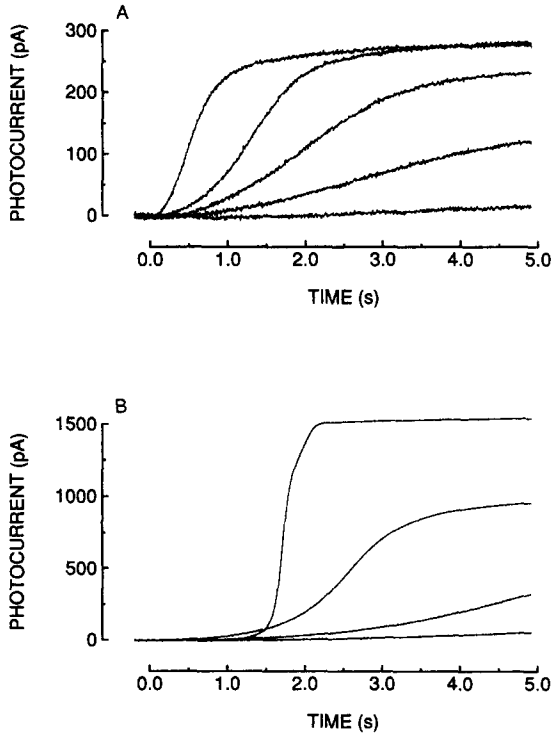

FIGURE 3. Whole-cell photocurrents measured with a patch pipette containing 5 mM cGMP added to the standard filling solution. (*A*) Photocurrents recorded with the patch pipette sealed onto the inner segment of an intact rod. Illustrated are responses to 10-ms flashes that delivered 3.02, 8.4, 15.4, 48, and 133 photons·μm^{-2}. The responses are slower in the time course than those recorded in the absence of added cGMP, but they show relatively little delay. (*B*) Photocurrents recorded with the patch pipette sealed onto the outer segment of an intact rod. Responses to flashes that delivered 5.1, 11.3, 29.8 and 306.0 photons·μm^{-2}. The photocurrents exhibit a pronounced delay between the flash presentation and the rising phase of the response.

Zimmerman et al. (1985) have found that the diffusion of dyes from a patch pipette applied to the inner segment to the cytoplasm of the outer segment is a slow process requiring ~1 min. Therefore, the difference in the effects of cGMP perfusion when the pipette was applied to the inner or the outer segment may have been the result of insufficient time for diffusion of cGMP from the patch pipette on the inner segment to the outer segment. We attempted to test this by allowing several minutes for cGMP to diffuse into the cells in the dark from a pipette applied on the inner segment before stimulating with light. In virtually all the cells studied (n = 26), we observed a rapid rundown of the dark current

during this waiting period. The dark current rundown was irreversible and we were unable to maintain the photoreceptors in the dark, in the presence of cGMP, for more than 1 min. In contrast, when the pipette was applied to the outer segment, we obtained stable recordings of dark currents >1,000 pA in amplitude for prolonged periods of time, up to 30 min.

We have not investigated the mechanism that produced the rundown of the dark current when the pipette was sealed onto the inner segment. It is possible that the rundown was initiated by the large dark current that resulted from the increase in the cytoplasmic cGMP. Under these conditions, it can be expected that both Ca and Na ions will enter the outer segment. The increase in the cytoplasmic concentration of these ions might lead to reduction in the dark current (Yau and Nakatani, 1984; Hodgkin et al., 1985). When a pipette is sealed onto the outer segment, the concentrations of cytoplasmic Ca^{2+} and Na^{+} are likely to be maintained at a value close to that of the pipette-filling solution. In support of that notion, we found that detached outer segments, in the whole-cell mode, can sustain cGMP-induced dark currents of >1 nA for up to 30 min (see below). These results suggest that patch pipettes, when applied to the outer segment, are effective in maintaining low internal Ca and Na ions.

The kinetics and amplitude of the photocurrents that we recorded from rods perfused with cGMP with patch pipettes applied onto the inner segment are, in general, similar to those first reported by Cobbs and Pugh (1985) and Matthews et al. (1985). However, as detailed in the Appendix, given the dissociation constant of the cGMP-activated channels, it would be expected that if cGMP is an internal messenger, then an increase in its concentration should introduce a delay in the response to light. The lack of a delay in the photoresponses, when perfusing cGMP from the inner segment, is inconsistent with this expectation. On the other hand, the photocurrents obtained with the pipette applied to the outer segment exhibited the predicted delay. The difference between the records from the inner and outer segments suggests that a patch pipette, when applied to the inner segment, is not very effective in increasing the concentration of cGMP in the outer segment. This conclusion is also supported by the observation that the amplitude of the dark current when the pipette is applied to the outer segment is larger than when the pipette is applied to the inner segment. We think that the failure to effectively increase the outer segment cGMP with pipettes placed on the inner segment resulted from the long pathway for diffusion between the pipette and the outer segment. Since we were not able to maintain the dark current with a pipette applied to the inner segment without severe rundown, we were prevented from allowing sufficient time for loading of the outer segment with cGMP. Under these conditions, a large increase in the concentration of internal cGMP can be obtained only with patch pipettes placed directly on the outer segment. Therefore, the studies of the effects of internal perfusion with cGMP described below were carried out with pipettes applied to the outer segment.

Properties of the cGMP-activated Conductance in Detached Outer Segments

If it is experimentally more reliable to perfuse rods internally with patch pipettes placed on the outer segment, it may be of further advantage to study detached

outer segments to avoid uncertainties introduced by ionic channels and enzymatic processing in the inner segment. Therefore, we examined the possibility of obtaining photoresponses from detached outer segments. Previously, Yau and Nakatani (1985), using suction electrodes, have shown that truncated rod outer segments can respond to light when perfused with cGMP, GTP, and ATP. Indeed, we were able to record photocurrents from detached outer segments patch-clamped in the whole-cell mode, with pipettes containing 2 mM GTP, 5.0 mM ATP, and 0.2–5.0 mM cGMP. At a holding potential of −30 mV and in the presence of 5.0 mM cGMP, steady dark currents of 1,000–1,500 pA could be recorded for periods of up to 30 min.

To establish that the channels opened by cGMP in the detached outer segment are the same as the light-sensitive channels in intact cells, we investigated the current-voltage characteristics and the reversal potential of the cGMP-dependent current. Fig. 4*A* illustrates the effects of a saturating flash of light on the conductance of the outer segment membrane. A test voltage from −30 to +20 mV was applied before and after a saturating flash. The resistance of the outer segment was increased 55-fold 5 s after the flash, which indicates that ~98% of the conductance in the dark is light sensitive. Under continuous bright light, the current-voltage relation was linear, with an equivalent input resistance of ~1,000 MΩ (data not shown). Fig. 4*B* illustrates the current-voltage relation in the dark. The current-voltage relation shows a pronounced outward rectification and a reversal potential close to 0 mV. Similar results were obtained in four detached outer segments. Thus, the cGMP-activated conductance in detached rod outer segments is light sensitive and exhibits a current-voltage relation and reversal potential that are similar to the corresponding characteristics of the light-sensitive conductance measured from intact salamander rods by Bader et al. (1979) and by Baylor and Nunn (1986). The presence or absence of an inner segment did not make a significant difference in the records of the light response or the dark current obtained by internally perfusing outer segments with cGMP.

Effects of cGMP on the Dark Current

The average dark current that we measured with a pipette that was applied to the outer segment and contained 5.0 mM cGMP was 1,374 pA (see Table I). When the pipette contained 0.2 mM cGMP, the dark current was 294 pA. These results are surprising since, if the K_d of the cGMP-sensitive channels is ~10 μM (Zimmerman and Baylor, 1986), it would be expected that 0.2 mM cGMP would be sufficient to saturate the outer segment cGMP-dependent conductance. One possible interpretation of these results is that the K_d of excised patches is different from the K_d of intact cells. Alternatively, these experimental results might simply reveal a difference between the concentration of cGMP in the pipette and the cytoplasm of the rod outer segment. Such a difference might be generated by the activity of a cGMP PDE in the dark and by restricted diffusion of cGMP into the outer segment. To examine these possibilities, we recorded from a detached outer segment with a pipette containing 0.2 mM cGMP and tested the effects of the PDE inhibitor IBMX on the dark current and the photoresponse. We found that superfusion of supplemented salt solution containing 100 μM IBMX resulted in an average increase of the dark current from 206 to 696 pA (n = 6). The

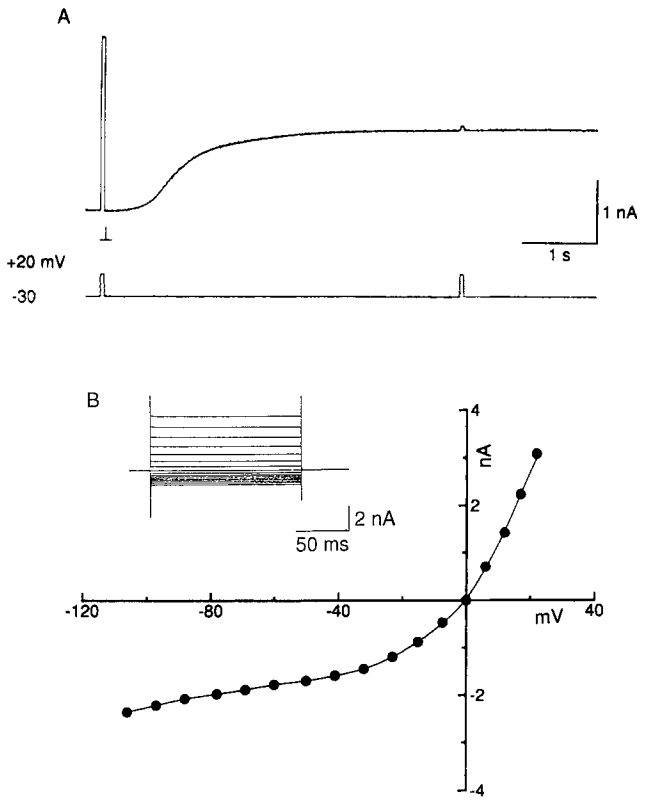

FIGURE 4. cGMP-activated currents from a detached rod outer segment. (*A*) Whole-cell recording from a detached outer segment with 5 mM cGMP in the pipette. The outer segment dark current was 1,200 pA at a holding potential of −30 mV. A bright flash of light was presented at the time indicated by the mark below the current trace. The conductance of the outer segment membrane was tested in the dark, 7.8 s after the light stimulus. The conductance was estimated by recording the current produced by a 50-ms step of voltage from −30 to +20 mV. The lower trace in *A* shows the time course of the command voltage. The light stimulus decreased the conductance 55-fold. (*B*) The current-voltage relation of the cGMP-activated conductance. The inset shows a series of current responses to voltage steps ranging from −100 to +40 mV. The amplitude of the voltage steps used to obtain the current-voltage relation were corrected for series resistance error. The series resistance was 5.8 MΩ. The reversal potential of the current was ~0 mV. The line connecting the data points was drawn by hand.

TABLE I

Dark Current

cGMP	I_d	n
	pA	
Intact rod	75.2±13	5
0.2 mM	294.4±91	9
1.0 mM	787.5±345	10
5.0 mM	1,374.1±175	8

Averaged data (mean ± SD) from whole-cell recordings. Intact rods were perfused with a solution that did not contain cGMP. cGMP was internally perfused with the pipette applied to the outer segment of both intact rods and detached outer segments.

effects of 100 μM IBMX on the normalized time course of the photocurrent are illustrated in Fig. 5. The response to a dim flash of light resulted in a much slower response after the introduction of IBMX. The effects of IBMX were reversed upon superfusion with supplemented salt solution without IBMX. The effects of IBMX are those expected if the outer segment contains a PDE that is active in the dark and reduces the concentration of cGMP in the cytoplasm. Application of IBMX can increase the level of cGMP in the dark by slowing down the hydrolytic activity of this PDE. The photoresponse under these conditions would be slowed because of the slower velocity of the light-activated PDE in the presence of IBMX (Capovilla et al., 1983). Our results therefore suggest that the cytoplasmic concentration of cGMP in the outer segment, studied

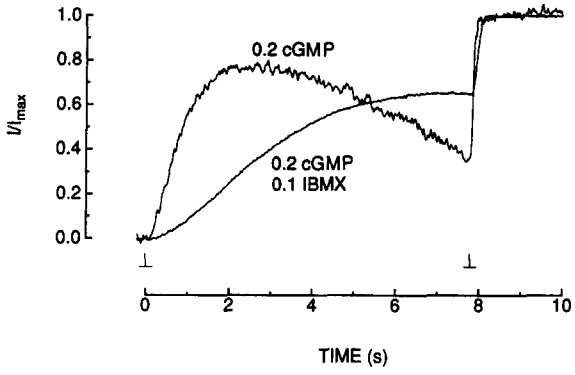

FIGURE 5. The effects of IBMX on whole-cell photocurrents measured from a detached outer segment internally perfused with 0.2 mM cGMP. A dim flash delivered 11.3 photons·μm^{-2} at t = 0, followed by a bright flash delivering 1,492.8 photons·μm^{-2} at t = 7.8 s. The photoresponses recorded before (labeled: 0.2 cGMP) and after superfusion with salt containing 100 μM IBMX (labeled: 0.2 cGMP, 0.1 IBMX) are shown. The dark current increased from 189 to 585 pA after IBMX was added. In order to compare the photocurrents, they were normalized by the maximum current amplitude measured in response to the bright flash.

with pipettes containing 0.2 mM cGMP, was less than or about equal to the channel's K_d. When the concentration of cGMP in the pipette was 5 mM, the dark current was ~20-fold larger than its value in the intact cell. We present below evidence suggesting that with 5.0 mM cGMP in the pipette, the concentration of cGMP in the outer segment is manyfold larger than the channel's K_d.

Effects of cGMP on the Delay in the Photoresponse

Fig. 6 illustrates the rising phase of photocurrents measured from detached outer segments with two different concentrations of cGMP in the pipette. The data shown in *A* were recorded with a pipette containing 5.0 mM cGMP, while those in *B* were recorded with a pipette containing 0.2 mM cGMP. In the presence of 5.0 mM cGMP, the photocurrents exhibited a pronounced delay between the stimulating flash and the onset of the response. Photocurrents

measured with 0.2 mM cGMP showed no such delay. The asterisks in *A* and *B* indicate photocurrents generated by flashes of nearly equal intensity.

Fig. 7 illustrates photocurrents generated by saturating flashes of light in three different detached outer segments perfused with 0.2, 1.0, and 5.0 mM cGMP. The dark current under these condition differed, as discussed above. Therefore, to compare the photocurrents, we normalized the photocurrents in relation to the maximum amplitude. Only the response in the presence of 5.0 mM cGMP showed a pronounced delay. The photoresponse in the presence of 1 mM cGMP had a slower rate of rise than that observed in the presence of 0.2 mM cGMP at all light intensities (data for dim flashes not shown).

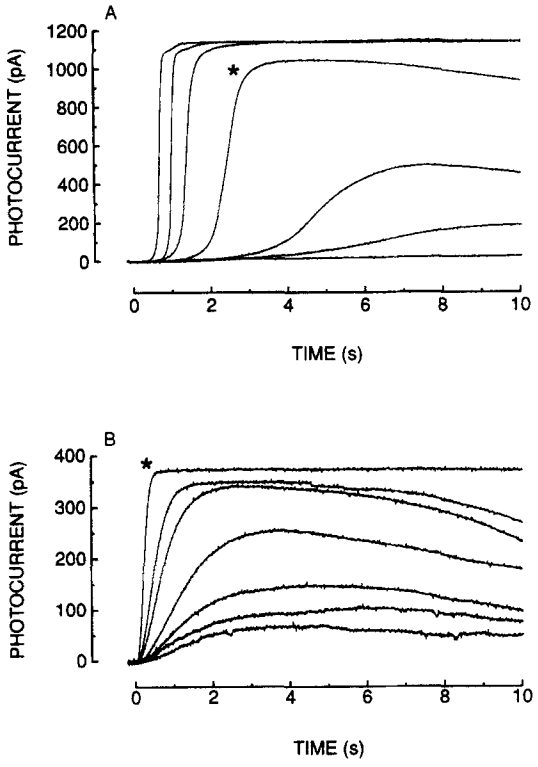

FIGURE 6. Photocurrents measured from a detached outer segment internally perfused with cGMP. (*A*) Responses measured with a pipette containing 5.0 mM cGMP. The dark current was 1,140 pA. The light stimuli delivered 28.5, 61.0, 133.0, 398.0, 654.0, 1,429.0, and 2,665.0 photons·μm^{-2}. Flashes that delivered <30.0 photons·μm^{-2} did not produce a detectable response. (*B*) Responses recorded with a pipette containing 0.2 mM cGMP. The dark current was 370 pA. The light stimuli delivered 1.3, 2.4, 5.1, 11.3, 29.8, 63.7, and 320.0 photons·μm^{-2}. Note the lack of delay in these photoresponses.

Effect of a Prior Dim Flash on the cGMP-induced Delay

When a saturating test flash was presented in the dark to an outer segment containing a high concentration of cGMP, the photocurrent exhibited a delay. If this delay arises directly from the elevated cGMP, then the response to the same flash should be without delay when it is preceded by a dim flash that decreases the concentration of the cytoplasmic cGMP. Fig. 8 illustrates currents recorded from a detached outer segment perfused with 5.0 mM cGMP, in response to two bright test flashes of equal intensity. Trace *a* shows the response

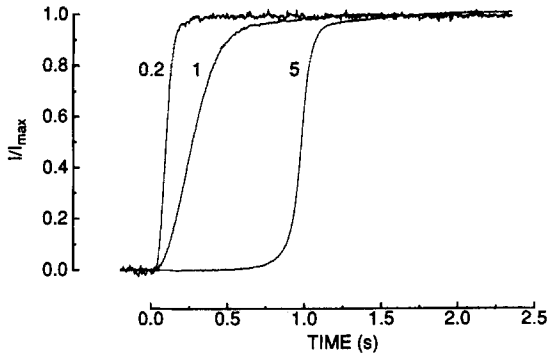

FIGURE 7. The effects of cGMP on the rising phase of the photocurrent. Initial responses of three different detached outer segments to the same bright flash (1,492.8 photons·μm^{-2}). Photocurrents were measured with pipettes containing 0.2, 1.0, and 5.0 mM cGMP. The photocurrents are normalized by the maximal current measured in each experiment. The dark currents were 218.7, 603.0, and 1,131.0 pA for 0.2, 1.0, and 5.0 mM cGMP, respectively.

to a test flash presented after a dim flash. Trace *b* shows the response to the test flash in the absence of prior stimulation. The response without prior stimulus was delayed by >1 s. In contrast, the response that followed a dim flash did not exhibit a significant delay. We have repeated these observations in seven detached outer segments. These experiments suggest that the delay of the photoresponse depends on the cytoplasmic concentration of cGMP and is not a consequence of the large dark current measured in the presence of high concentrations of cGMP. Moreover, these results indicate that in the course of experiments, successive

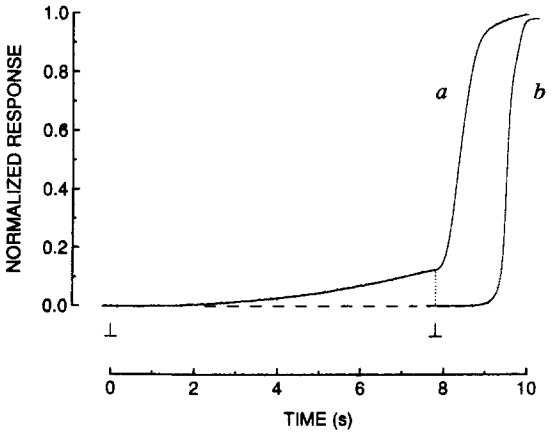

FIGURE 8. Comparison of the kinetics of the response to a saturating flash, with or without a prior dim flash. Photocurrents were measured from the same detached outer segment with a pipette containing 5.0 mM cGMP. Trace *a* shows a dim flash (5.1 photons·μm^{-2}) presented at the first time mark. The dim flash was followed by a bright flash (320.0 photons·μm^{-2}). Trace *b* shows the same bright flash presented without prior stimulation. The dark current was 1,374 pA in trace *a* and 1,541 pA in trace *b*. The responses were normalized by their maximum photoresponse amplitude.

light flashes must be delivered with sufficient intervals between them to permit complete recovery. In the experiments described below, we allowed the cells to be in the dark for 2–3 min between stimuli. Under these conditions, the kinetics and the delay of the photoresponses were reproducible. A further increase of the time interval between flashes to 5–10 min had no effect on the photoresponses.

Effects of Light Intensity on the Delay of the Photoresponse

In the following sections, we present a quantitative analysis of the effects of cytoplasmic cGMP on the photoresponse. In the intact cell, the cytoplasmic concentration of cGMP is determined by the rates of its synthesis and hydrolysis. However, in the presence of high concentrations of cGMP in the outer segment sustained by a patch electrode, the hydrolytic activity of the light-activated PDE defines the rate of change of the cGMP concentration (see Appendix). Under these conditions, the concentration change of the cGMP can be derived from the K_m and V_{max} of the PDE and the characteristics of the photocurrent can then be calculated from the cGMP dependence of the light-sensitive conductance.

We have defined the delay t_d as the time interval between the light flash and the time when the photocurrent reaches 10% of its maximum value. Fig. 9*A* illustrates photocurrents measured from a detached outer segment with a pipette containing 5.0 mM cGMP. In panel *B*, the delay measured in this experiment is plotted as a function of the inverse of the light intensity. The data confirms the expectation, detailed in the Appendix, that the delay is inversely proportional to the light intensity. Furthermore, the slope of the linear relationship can be used to estimate the concentration of cGMP before the flash of light (Eq. 8). In the experiment shown in Fig. 9, we estimated that the initial cytoplasmic cGMP concentration was 3.3 mM. The average initial concentration of cGMP that we calculated in five experiments was 1.3 mM. Although the precise value of the internal concentration of cGMP as calculated depends on the parameters used in the model, the linear relationship between the delay and the inverse of the light intensity is predicted by the model independently of the particular values selected. For the data illustrated in Fig. 9, the fitted straight line intersects the ordinate at 0.6 s. Similar nonzero intersects were found in all the experiments that were analyzed.

Intensity-Response Relation of Rod Outer Segments Perfused with 5 mM cGMP

We determined the dependence of the photocurrent amplitude on light intensity in the presence of high concentration of cGMP by measuring the fraction of the current that was suppressed at 1 s after the flash. Fig. 10 illustrates the averaged result from seven experiments on detached outer segments that were perfused with 5.0 mM cGMP (filled circles). For intensities of <200 photons·μm^{-2}, there was no detectable current suppression at 1 s after the flash. At an intensity of ~800 photons·μm^{-2}, half of the dark current was suppressed. For comparison, the peak photocurrent amplitude of intact rods not perfused with cGMP as a function of light intensity is also illustrated in Fig. 10. The photocurrents of intact cells peaked at ~0.75 s after the flash. Intact rods (squares, $n = 5$) were ~100-fold more sensitive than rods perfused with 5.0 mM cGMP.

The theoretical intensity-response functions, calculated as described in the Appendix, are illustrated in Fig. 10 as continuous lines. The form of the theoretical functions depends on the initial concentration of the cytoplasmic cGMP. One function shown was calculated assuming that the initial concentration of cGMP was 5.0 mM cGMP and that the half-saturating intensity was 500 photons·μm^{-2}, values close to the experimental ones. The slope of the theoretical relation, however, is considerably steeper than the experimentally obtained averaged data. This discrepancy is addressed in the Discussion.

We estimated the concentration of cGMP in the intact rod to be 3.75 μM through the use of Eq. 9, taking I_{max} = 1,347 pA, the average dark current in the presence of 5.0 mM cGMP, and I = 75 pA, the average dark current in

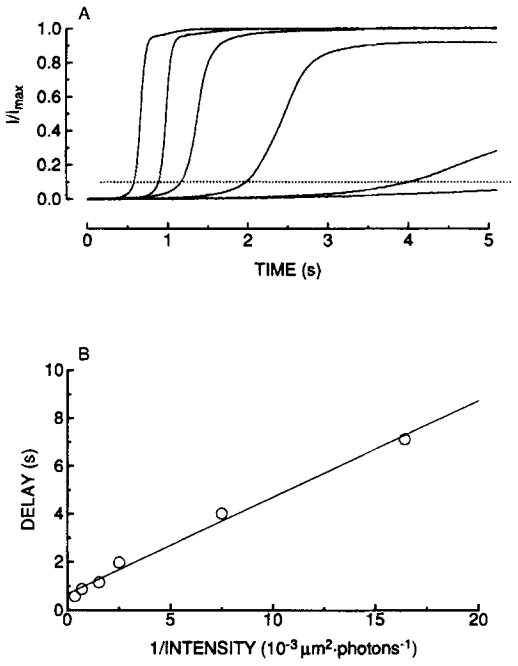

FIGURE 9. Dependence of the photocurrent delay on light intensity. (*A*) Photocurrents measured from a detached outer segment in response to flashes that delivered 61.0, 133.0, 398.0, 654.0, 1,429.0, and 2,665.0 photons·μm^{-2}. The pipette contained 5.0 mM cGMP. The dotted line indicates 10% change in the normalized photocurrent. t_d is the time interval between the flash presentation and the 10% change in the photocurrent. (*B*) Dependence of t_d on the inverse of the light intensity. The straight line was obtained by a least-squares fit to the data. The slope of this line indicates a concentration of 3.3 mM cGMP in the outer segment (see Appendix).

intact cells. The intensity-response relationship, assuming an initial concentration of cGMP of 3.75 μM, was calculated as the fraction of the current that was suppressed at t = 0.75 s and is plotted in Fig. 10. As is apparent from this figure, the position and the steepness of the theoretical function are similar to the experimentally obtained data. It is important to emphasize that the theoretical functions illustrated in Fig. 10 were calculated based on known parameters of the light-activated PDE and the cGMP-activated channels and not by fitting the experimental data.

Effects of cGMP on the Falling Phase of the Photoresponse

In intact cells, injection of cGMP produces a pronounced prolongation of the falling phase of the photocurrent (Miller and Nicol, 1981; Cobbs and Pugh,

1985). We found that, in the detached outer segment, the effects of cGMP on the falling phase are similar to those that have been reported in intact cells. Fig. 11 illustrates a series of photocurrents measured with a patch pipette containing 5.0 mM cGMP, in response to flashes of increasing intensity. The photocurrent peaked at ~8 s after the flash for relatively dim stimuli and the peak occurred at progressively earlier times for brighter flashes. The falling phase of the responses had a time constant of ~15 s. This behavior was observed in all cells perfused with high concentrations of cGMP. We also found that the prolongation of the falling phase also occurred in cells perfused with 0.2 mM cGMP (see Fig. 6*B*).

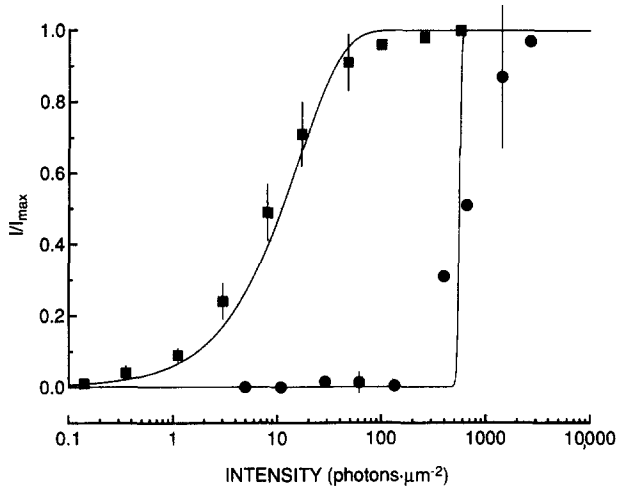

FIGURE 10. The effects of cGMP on the intensity-response relation. The figure shows the average of normalized peak photocurrent amplitudes measured from intact rods without added cGMP (squares) and the average of normalized photocurrent amplitudes measured 1 s after the flash from detached outer segments perfused with 5.0 mM cGMP (circles). The lines are drawn according to Eqs. 8 and 9 (see Appendix); I_{max} in intact rods was 5% of the I_{max} of rods outer segments perfused with 5.0 mM cGMP. For the cells without added cGMP, it was assumed that C_0 = 3.75 μM. Under both conditions, it was assumed that the PDE's K_m was 500 μM and that the channel's K_d was 10 μM. The data points represent the average of five experiments with pipettes without cGMP and seven experiments with pipettes containing 5.0 mM cGMP. Error bars indicate the standard deviation.

We also observed that, as previously reported by Cobbs and Pugh (1985), the prolongation of the falling phase can occur even when there is little or no increase in its delay.

Fig. 12*A* illustrates the photoresponse of an intact cell, compared with that of a detached outer segment perfused with 5.0 mM cGMP. The noisy trace represents the response of the intact cell, which rose with little delay and then rapidly declined. In contrast, the response of the detached outer segment to a light stimulus of similar intensity rose with a delay and the photocurrent remained at saturation for a prolonged period of time. About 15 s after the flash, the current began to decline with a much slower time course than the unperfused

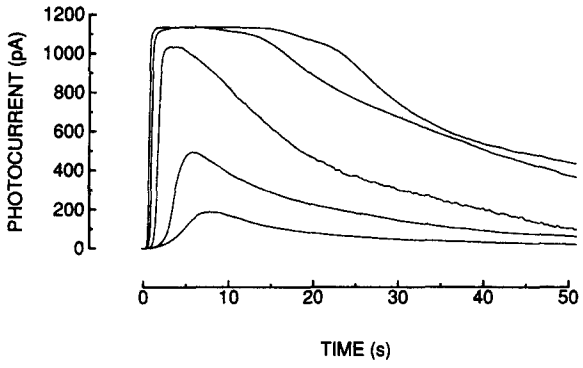

FIGURE 11. Whole-cell photocurrents measured from a detached outer segment with a patch pipette containing 5.0 mM cGMP. Responses to flashes that delivered 61.0, 133.0, 398.0, 654.0, and 1,429.0 photons·μm^{-2} are shown. The peak of the response occurred at earlier times as the intensity of the flashes increased. The falling phase of the photocurrent was ~10-fold slower than the response of rods without added cGMP.

rod. To compare quantitatively the effects of cGMP on the falling phase, we integrated the normalized photoresponses at different light intensities. In Fig. 12*B*, a log-log plot of the average time integrals of intact cell without added cGMP (diamonds) is compared with the time integral of responses from detached outer segments perfused with 5.0 mM cGMP (squares).

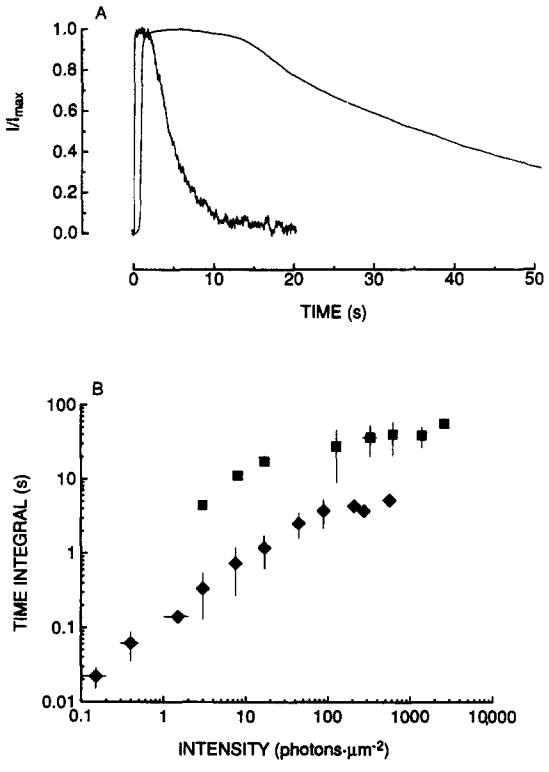

FIGURE 12. Comparison of the photocurrent time course recorded from intact rods and from detached outer segments perfused with 5.0 mM cGMP. (*A*) Responses to a flash delivering 650 photons·μm^{-2}. The photocurrents are normalized to the peak response. cGMP produced a significant delay in the photocurrent, prolonged its duration, and slowed its rate of recovery, when compared with the response of the intact rod. (*B*) Effects of flash intensity on the normalized time integral of the photocurrents. The data were measured from intact cells (diamonds) and from rod outer segments perfused with 5.0 mM cGMP (squares). The time integral increases with light intensity, but at all intensities it is lower in the presence of 5.0 mM cGMP than in its absence.

DISCUSSION

The role of cGMP as an internal messenger in retinal rods has been supported by both biochemical and electrophysiological observations (recently reviewed by Schwartz, 1985; Korenbort, 1985; Stryer, 1986; Pugh and Cobbs, 1986). However, it has, so far, not been possible to combine these different data into a unique quantitative description. We report here that detached outer segments provide a preparation in which the components of the phototransduction machinery are functional and in which the composition of the intracellular milieu can be under reliable experimental control, thus permitting critical examination of the role of cGMP and other metabolites in phototransduction.

Whole-cell recording with patch pipettes does not appear to dilute important cytoplasmic components or to introduce substances that interfere with the normal transduction process since the kinetics of the photoresponse and its light sensitivity, in the absence of cGMP in the patch pipette, are similar to those obtained from intact rods.

In detached outer segments perfused with 5.0 mM cGMP, ~98% of the membrane conductance could be suppressed by light. The current-voltage relationship and the reversal potential of the cGMP-dependent current in the dark are similar to those of the light-sensitive (Bader et al., 1979; Baylor and Nunn, 1986) and cGMP-activated (MacLeish et al., 1984) conductance in intact rods. These data suggest, as has previously been reported by MacLeish et al. (1984) and Matthews (1986), that cGMP specifically activates the light-sensitive channels, which are likely to be the only type of ionic channel functioning in the outer segment plasma membrane (Baylor and Nunn, 1986; Hestrin and Korenbrot, 1987).

The maximum cGMP-activated current we measured in the salamander rod corresponds to a current density of 1.4 $pA \cdot \mu m^{-2}$, if we assume a homogeneous spatial distribution of outer segment channels. Assuming a single channel current of 5 fA (Gray and Attwell, 1985; Bodia and Detwiler, 1985), our data indicate that the maximum density of open cGMP-activated channels is 280 μm^{-2}. This estimate is close to the estimates obtained from excised patches of membrane (Haynes et al., 1986; Zimmerman and Baylor, 1986). The density of channel molecules in the plasma membrane is expected to be higher than the density of open channels because the open probability of the cGMP-activated channels, under a saturating concentration of cGMP, is <0.5 (Matthews, 1986).

Effects of cGMP on the Latency and the Light Sensitivity

We have shown that a large increase in the concentration of cytoplasmic cGMP in the outer segment delays the photoresponse by several seconds. The effects of internal perfusion with cGMP on the delay of the photocurrent are in general agreement with the effects of cGMP injection on the photovoltage reported by Miller and Nicol (1981) in the intact retina. The failure of others to observe this delay (Waloga, 1983; Cobbs and Pugh, 1985; Cobbs et al., 1985) is probably the result of the experimental difficulty in introducing a sufficiently high concentration of cGMP into the outer segment cytoplasm. The kinetic effects of cGMP require a very high increase in its cytoplasmic concentration. Consider, for

example, an increase in the cytoplasmic concentration of cGMP to three times the binding constant of the cGMP-activated channels, i.e., 30 μM. Under these conditions, the dark current would be 96% of its maximum: 1,000–1,500 pA. However, the predicted delay would be only 75 ms in response to a flash of 200 photons·μm^{-2}. Thus, even when the effect of cGMP on the dark current is nearly saturated, its predicted effect on the photocurrent latency is relatively small.

Yau and Nakatani (1984) and Hodgkin et al. (1985) have shown that Ca^{2+} can permeate through the light-sensitive conductance. Since perfusion with cGMP increases the light-sensitive conductance, it is possible that the increased influx of Ca^{2+} in the dark increases the internal concentration of Ca^{2+} and this is the cause of the delay of the photocurrent. This explanation implies that the amplitude of the dark current would be closely related to changes in the delay of the photocurrent. However, as shown in Fig. 8, a prior dim flash that reduces the dark current by only 12% can eliminate the delay of the photoresponse. That is, there appears to be no causal relation between the amplitude of the dark current and the delay. In addition, we found in two experiments (data not shown) that the delay of the photoresponse was not eliminated by the addition of 10 mM of the Ca^{2+} chelator BAPTA to a cGMP-containing perfusate. These results indicate that the effects of cGMP on the delay of the photoresponse are not mediated by an increase of cytoplasmic Ca^{2+}. The role of cytoplasmic Ca^{2+} in phototransduction is not presently understood. However, the lack of effect of cytoplasmic Ca^{2+} buffers on the rising phase of the photocurrent (Matthews et al., 1985; Torre et al., 1986; Korenbrot and Miller, 1986) is in agreement with our findings and suggests that Ca ions do not play a role in the initial phase of the photoresponse.

We have found that internal perfusion with 5.0 mM cGMP decreased the light sensitivity ~100-fold when measured 1 s after the flash. We have chosen to measure the sensitivity at a fixed time after the flash because it is easier, using our model, to interpret these measurements. The photocurrent of rods perfused internally with cGMP peak at 5–8 s after the flash, in contrast to the photocurrent of intact rods, which peaks at 0.75 s. When the sensitivity is measured at the peak of the response, the effects of cGMP are much less dramatic.

A Model of the Initial Phase of the Photoresponse

We used the model that is described in the Appendix to predict the effects of high concentrations of cGMP on the photocurrent delay and the light sensitivity. The model assumes that light-activated PDE alone regulates cytoplasmic cGMP and that cGMP controls the light-sensitive channels. We carried out experiments under high concentrations of cGMP, where, we reasoned, the time course of the concentration changes of cGMP indeed reflects the hydrolytic activity of the PDE. The observed delay and intensity-response relation under perfusion of 5.0 mM cGMP are reasonably close to those predicted. However, the predicted intensity-response relation is much steeper than the experimentally observed one.

In deriving the predicted intensity-response relation, we presumed that, for 1

s after the flash, the contributions of diffusion from the patch pipette and of guanylate cyclase activity to the concentration of cytoplasmic cGMP are negligible. The flux of cGMP from the pipette into the outer segment can be estimated from the time course of the current increase after membrane rupture. In the fastest case that we recorded, the dark current reached half its maximum amplitude ~2 s after the membrane rupture. Thus, the most rapid cGMP flux from the pipette increased the cytoplasmic concentration of cGMP to ~10 μM (the K_d value of the membrane channels) in 2 s or a rate of 5.0 μM/s. The synthesis of cGMP by guanylate cyclase has been estimated to be ~10 μM/s (Pannbacker, 1973; Goldberg et al., 1983). The combined contribution of cGMP from the pipette and from the cyclase is then estimated to be 15 μM/s. To assess the importance of these sources, we must compare them with the velocity of the light-activated PDE. Solving Eq. 6, using the estimates of K_m and V_{max} given in the Appendix and assuming an initial concentration of cGMP of 5.0 mM, shows that the initial velocity of the PDE is in the range of several millimolar per second (for a saturating flash of light), and indeed, cGMP synthesis and replenishment can be neglected.

When the concentration of cGMP is not high, the replenishment of cGMP cannot be neglected, and the model is likely to be in error. This error is apparent in the intensity-response relation shown in Fig. 10: the theoretical function in the presence of 5.0 mM cGMP is much steeper than the observed data. This difference probably arises because, at the lower cGMP concentrations present once the photocurrent has developed, the guanylate cyclase activity and the cGMP flux from the pipette are not negligible. Under these conditions, current suppression would require PDE activity that is higher than predicted to hydrolyze the additional cGMP.

In deriving the expressions of the model, we assumed that, after a flash of light, the PDE activity increases in a stepwise manner. In fact, the PDE activates and deactivates with complex kinetics (Wheeler and Bitensky, 1977; Yee and Liebman, 1978; Robinson et al., 1980; Liebman and Pugh, 1980; Sitaramayya and Liebman, 1983). The activation appears to be a first-order kinetic process and is likely to have a time constant of ~0.5 s (Yee and Liebman, 1978; Vuong et al., 1984). We have not investigated in detail the turn-off process. However, in vitro, under conditions that produce a maximum turn-off rate, the deactivation time constant of PDE has been found to be ~4 s (Sitaramayya and Liebman, 1983). If we assume that the PDE in our preparation turned off at a similar rate, then ignoring the turning-off process would produce an error in our calculation on the order of 12% at 1 s and 21% at 2 s.

The model we have used is restricted to high concentrations of cGMP and bright flashes of light. A more general scheme would include the kinetics of the PDE turn-on and turn-off and the contribution of the guanylate cyclase. It is convenient to artificially separate the processes that control cGMP into two pathways:

$$\mathrm{Rh}^* \rightarrow \mathrm{PDE} \uparrow \rightarrow \mathrm{cGMP} \downarrow \rightarrow g_{h\nu} \downarrow \quad (4)$$

$$(\mathrm{PDE} \downarrow, \text{ cyclase} \uparrow) \rightarrow \mathrm{cGMP} \uparrow \rightarrow g_{h\nu} \uparrow \quad (5)$$

Eq. 4 describes the initial phase during which the concentration of cGMP decreases because of the light-activated PDE. Eq. 5 describes the later phase of the response, in which PDE is turned off and guanylate cyclase restores the concentration of cGMP. The experiments presented in this article examined only the initial phase of the response. The premise of the model was that, at high concentrations of cGMP and with bright flashes, the contribution of Eq. 5 to the initial response is small. However, a complete description of the photoresponse of the intact rod to dim flashes of light would have to consider both of these processes.

We found that the known properties of the light-activated PDE can be used to predict the initial phase of the photocurrent when the cytoplasmic concentration of cGMP is high. Since cGMP does not modulate the activity of the PDE (Yee and Liebman, 1978), it is likely that its light sensitivity in the intact cell, at low concentrations of cGMP, is similar to that found at high concentrations of cGMP. It is not known whether the initial phase of the light response in intact rods depends on the activation of the PDE alone. However, we found (Fig. 10) that the light sensitivity of intact rods can be estimated from the properties of the PDE used to estimate the light sensitivity at high concentrations of cGMP. These results suggest that the turn-off of the PDE and the activation of the guanylate cyclase might play a role only in the later phase of the photoresponse.

APPENDIX

The model we develop is adopted from one originally proposed by Cobbs and Pugh (1985). The model proposes that a brief flash of light activates the PDE in a stepwise fashion. The model further assumes that the activity of the PDE is the rate-limiting reaction regulating the cytoplasmic concentration of cGMP. Given the known properties of PDE, it is possible to compute the concentration of cGMP as a function of time after the flash. The model further proposes that the photocurrent can be calculated from the concentration of cGMP and the known cGMP sensitivity of the rod's channels.

The kinetics of the light-activated PDE are assumed to follow a Michaelis-Menten expression (Miki et al., 1975; Baehr et al., 1979; Yee and Liebman, 1978):

$$\frac{dC}{dt} = -\frac{V_{max}C}{C + K_m}, \tag{6}$$

where C is the free concentration of cGMP at time t after bleaching rhodopsin, K_m is the Michaelis constant, and V_{max} is the maximum enzyme velocity. Eq. 6 can be rearranged in an integral form as follows:

$$\int_{C_0}^{C} (1 + K_m/C)dC = -\int_0^t V_{max}dt, \tag{7}$$

where C_0 is the concentration of cGMP before the flash. Integration leads to the following expression:

$$C - C_0 + K_m(\log_e C - \log_e C_0) = -tV_{max}. \tag{8}$$

Eq. 8 cannot be rearranged to give C as an explicit expression of time. However, the concentration C can be computed, at any given time, by conventional numerical methods.

Fesenko et al. (1985) have shown that, in excised patches of outer segment plasma

membrane, the cGMP-activated conductance depends on the cGMP concentration according to:

$$\frac{g}{g_{max}} = \frac{C^h}{C^h + K_d^h}, \tag{9}$$

where g is the membrane conductance, g_{max} is the maximum conductance obtained at a saturating concentration of cGMP, C is the free concentration of cGMP, h is the Hill coefficient, and K_d is the dissociation constant of the binding reaction between cGMP and the channels. The value of K_d in the rod of tiger salamander was estimated by Zimmerman and Baylor (1986) to be 10 μM and the value of h was estimated to be 3.

Combining Eqs. 8 and 9 gives the fraction of open channels at any given time t. Since the light-sensitive channels control the current in the outer segment, we can calculate the membrane current of the rod outer segment as a function of time. If large concentrations of cGMP are introduced into the outer segment ($C_0 \gg K_d$), then the dark current will be approximately equal to I_{max}. After a flash of light, the concentration of cGMP decreases, and if $h = 3$, then, from Eq. 9, when its concentration reaches 2.08 K_d, the current would be 90% of I_{max}. By combining Eqs. 8 and 9, we obtain the following approximation:

$$t_d = [C_0 - 2.08\ K_d + K_m \log_e(C_0/2.08\ K_d)]\ 1/V_{max}, \tag{10}$$

where t_d is the delay between the flash and the moment when the dark current is reduced by 10%.

The K_m of the light-activated PDE of bovine rods has been estimated by Sitaramayya et al. (1986) to be 0.87–1.42 mM. The K_m in toad rods has been estimated to be 360 μM by Barkdoll et al. (1986). We will assume that $K_m = 500$ μM. The maximal activity of the PDE has been estimated to be 4×10^5 molecules of cGMP hydrolyzed per second for each bleached rhodopsin (Rh*) (Yee and Liebman, 1978). The enzymatic activity would translate in the rod's outer segment internal volume (excluding disk space) to $V_{max} = 0.63$ μM/s per Rh*. The activation of the PDE was shown to be a linear function of light up to 10^4 Rh*. Our experiments were conducted in the linear range of the PDE activation. Under these conditions, V_{max} is proportional to the intensity of the stimulating flash, and thus Eq. 10 predicts that the relation between the inverse of the flash intensity and the delay of the photoresponse will be linear.

The delay that is predicted for $C_0 = 5{,}000$ μM and for a saturating flash intensity of 200 photons·μm^{-2} is 2.6 s. In addition to the photocurrent time course and the t_d, the model can predict the intensity-response relationship. The response is defined as I/I_{max}, where I is the amplitude of the outer segment current at 1 s after the flash, and I_{max} is the amplitude of the dark current. The current I can be calculated by first calculating the concentration of cGMP from Eq. 8 at 1 s and then using Eq. 9 to compute the current. For example, for an initial concentration of cGMP of 5,000 μM, half-saturation (at 1 s) will require a flash intensity of 554.0 photons·μm^{-2} or 12,852.8 Rh*.

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