

# Occupancy of the Chromophore Binding Site of Opsin Activates Visual Transduction in Rod Photoreceptors

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**ABSTRACT** The retinal analogue  $\beta$ -ionone was used to investigate possible physiological effects of the noncovalent interaction between rod opsin and its chromophore 11-cis retinal. Isolated salamander rod photoreceptors were exposed to bright light that bleached a significant fraction of their pigment, were allowed to recover to a steady state, and then were exposed to  $\beta$ -ionone. Our experiments show that in bleach-adapted rods  $\beta$ -ionone causes a decrease in light sensitivity and dark current and an acceleration of the dim flash photoresponse and the rate constants of guanylyl cyclase and cGMP phosphodiesterase. Together, these observations indicate that in bleach-adapted rods  $\beta$ -ionone activates phototransduction in the dark. Control experiments showed no effect of  $\beta$ -ionone in either fully dark-adapted or background light-adapted cells, indicating direct interaction of  $\beta$ -ionone with the free opsin produced by bleaching. We speculate that  $\beta$ -ionone binds specifically in the chromophore pocket of opsin to produce a complex that is more catalytically potent than free opsin alone. We hypothesize that a similar reaction may occur in the intact retina during pigment regeneration. We propose a model of rod pigment regeneration in which binding of 11-cis retinal to opsin leads to activation of the complex accompanied by a decrease in light sensitivity. The subsequent covalent attachment of retinal to opsin completely inactivates opsin and leads to the recovery of sensitivity. Our findings resolve the conflict between biochemical and physiological data concerning the effect of the occupancy of the chromophore binding site on the catalytic potency of opsin. We show that binding of  $\beta$ -ionone to rod opsin produces effects opposite to its previously described effects on cone opsin. We propose that this distinction is due to a fundamental difference in the interaction of rod and cone opsins with retinal, which may have implications for the different physiology of the two types of photoreceptors.

**KEY WORDS:** pigment regeneration • dark adaptation •  $\beta$ -ionone • visual pigments • bleaching adaptation

## INTRODUCTION

The visual cycle is the series of reactions occurring in the eye through which visual pigment proceeds from photoactivation to regeneration. Absorption of a photon by the pigment of a photoreceptor triggers within milliseconds a series of reactions leading to a cell response. This sequence of reactions, collectively termed the phototransduction cascade, has been extensively studied and is now well characterized (for reviews, see Lagnado and Baylor, 1992; Stryer, 1992; Pugh and Lamb, 1993; Yau, 1994). According to this scheme, photoisomerization of the chromophore from the 11-cis to the all-trans conformation (see Fig. 1, A and B) ultimately results in the closure of cation channels and the hyperpolarization of the cell. Another consequence of the photon absorption is the eventual dissociation of the chromophore from the protein moiety of the visual

pigment to form all-trans retinol and free opsin. The visual pigment is then regenerated through a series of reactions requiring less than a minute in cones and tens of minutes in rods (Wald et al., 1955). In the vertebrate retina, this is a complex process that involves both photoreceptors and the pigment epithelium (for reviews, see Rando, 1992; Crouch et al., 1996). Reduction of all-trans retinal to all-trans retinol must occur in the bleached outer segment of photoreceptors before the opsin binding site becomes accessible for regeneration. All-trans retinol is then removed from the outer segment and translocated to the pigment epithelium. There it is converted back to 11-cis retinal, and then returned to the receptor outer segment where it combines with opsin to form visual pigment.

Aside from the ultimate recovery of visual sensitivity (Pepperberg et al., 1978; Jones et al., 1989; Cornwall et al., 1990), very little is known about the physiological consequences of the reactions involved in the reconstitution of the visual pigment within photoreceptors. An important initial step in the recovery process is likely to be the binding of 11-cis retinal, supplied from the pigment epithelium, into the chromophore pocket of opsin (Matsumoto and Yoshizawa, 1975). The effects on phototransduction and regeneration of a whole host of

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retinal analogues have been studied with biochemical as well as with physiological methods (for reviews, see Corson and Crouch, 1996; Nakanishi and Crouch, 1996). Treating bleached pigment from rods with derivatives of retinal having shortened polyene chains produces a catalytically active complex that promotes phosphorylation of opsin by rhodopsin kinase (Buczylko et al., 1996). Competition studies with 11-cis retinal suggest that these compounds exert their effect by binding in the chromophore pocket of opsin (Matsumoto and Yoshizawa, 1975; Crouch et al., 1982). These retinoids, including  $\beta$ -ionone (Fig. 1 C), all-trans C17 aldehyde, and 9-cis C17 aldehyde do not form a covalent link with opsin via a Schiff base (Towner et al., 1981) because the shorter length of their side chain apparently does not allow them to span from the ring-binding site to the corresponding lysine of opsin (Daemen, 1978; Crouch et al., 1982). The fact that these compounds do not form a covalent bond with the protein suggests that occupancy of the chromophore binding site of rod opsin alone is sufficient for catalytic activation of the complex. Thus, these biochemical experiments indicate that, in rods, retinoids with shortened polyene chains act as agonists, activating the photoreceptor. On the other hand, physiological studies on intact isolated cone photoreceptor cells (Jin et al., 1993; Cornwall et al., 1995) show that in these cells short-chain retinal analogues produce an opposite effect: treatment of bleached cones with  $\beta$ -ionone or with 9-cis C17 aldehyde results in downregulation of transduction, characterized by slowed flash responses, increased sensitivity, and increased dark current. Thus, in cones, short-chain retinal analogues act as reverse agonists, inactivating the photoreceptor. These latter studies were interpreted as demonstrating that the noncovalent binding of retinal in the chromophore pocket of cone opsin might be an important initial step in dark adaptation.

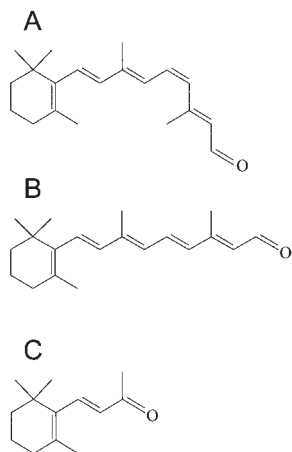


FIGURE 1. Structures of the retinoids. Structures of 11-cis retinal (A), all-trans retinal (B), and  $\beta$ -ionone (C). Both 11-cis retinal and  $\beta$ -ionone attach noncovalently in the chromophore pocket of opsin. Only 11-cis retinal is long enough to form a covalent bond with the protein.

The present study was undertaken to resolve the contradictory findings regarding the role retinoids play in regulating the activity of rod and cone photoreceptors. The experiments described here also address the question of whether the occupancy of the chromophore pocket of rod opsin by a retinoid produces physiological consequences that are separate from those due to the consequent formation of the Schiff base linkage. The retinal analogue chosen to address these questions was  $\beta$ -ionone because of its capability of binding in the chromophore pocket of opsin without forming a covalent bond. Furthermore, as we demonstrate here, its binding is rapidly and totally reversible. The use of  $\beta$ -ionone enabled us to study specifically the physiological effect of the noncovalent binding of retinal in the chromophore pocket of opsin without the interfering effects of the covalent bond between retinal and opsin. In addition, treating bleach-adapted rods with  $\beta$ -ionone allowed us to compare directly the effect of its binding in the chromophore pocket of rod opsin with its previously described effect in cones (Jin et al., 1993; Cornwall et al., 1995). We present evidence that, in bleach-adapted rods, occupancy of the chromophore binding site of opsin by  $\beta$ -ionone causes activation of the transduction cascade and desensitization of the cell. This observation is opposite to the expected downregulation of transduction during dark adaptation, when 11-cis retinal occupies the chromophore pocket. It is also opposite to the effect of  $\beta$ -ionone observed in bleach-adapted cone photoreceptors. This newly discovered distinction between rods and cones reconciles the conflict between biochemical and physiological results, and implies a fundamental difference in the interaction of rod and cone opsins with retinal.

#### MATERIALS AND METHODS

Single rod photoreceptors from the retina of the larval tiger salamander (*Ambystoma tigrinum*) were isolated in physiological solution as previously described (Cornwall et al., 1990). In brief, animals were dark-adapted overnight. They were decapitated and pithed in dim red light, following which the eyes were removed, hemisected, and placed in  $\sim 1.5$  ml saline solution. The retina was torn free of the pigment epithelium and chopped into small pieces with a razor blade. A fraction of the resulting suspension was transferred to a recording chamber located on the stage of an inverted microscope (Invertascope D; Carl Zeiss, Inc.). The remaining portion of the suspension was stored at 4°C in the dark and remained viable for several hours. The preparation was viewed via an infrared television camera (WV 5410; Panasonic) fitted to the inverted microscope and connected to a video monitor. Membrane currents were recorded from solitary rods with a suction microelectrode using methods similar to those reported previously (Baylor et al., 1979; Cornwall et al., 1990). The inner segment of the rod was drawn into the pipette so that the outer segment could be rapidly superfused with test solutions. The tip of the electrode was heat-polished to a diameter of  $\sim 10$   $\mu$ m. The current recorded from the cell was converted to voltage and amplified using a patch clamp amplifier (EPC-7; List Electronic),

and low-pass filtered with an active eight-pole filter at 20 Hz cut-off frequency (902LPF; Frequency Devices Inc.). The data were digitized at 250 Hz, stored on a computer, and subsequently analyzed using the pCLAMP 6 data acquisition and analysis software (Axon Instruments) and the Origin 4 graphics and data analysis software (Microcal Software, Inc.).

### Light Stimulation

A dual-beam optical stimulator provided test flashes as well as bleaching and background lights (Cornwall et al., 1990). The light source was calibrated at the beginning of each experiment with a photometer (80×; United Detector Technology). The absolute intensity of the test flash/bleaching beam was  $1.36 \times 10^7$  photons  $\mu\text{m}^{-2} \text{s}^{-1}$  (500 nm); the absolute intensity for the background beam was  $1.28 \times 10^7$  photons  $\mu\text{m}^{-2} \text{s}^{-1}$  (520 nm). Light intensity for each beam was attenuated with a series of calibrated neutral density filters. The wavelength was set with narrow band interference filters (10 nm bandwidth at 1/2 transmission; Corion Optics). A stimulus spot 1 mm in diameter was focused at the plane of the preparation by a 0.25 NA  $\times$  10 achromat objective located above the stage of the inverted microscope. Test flash duration was 20 ms. The fraction of bleached pigment was calculated according to the relation:

$$F = 1 - \exp(-Ipt), \quad (1)$$

where  $F$  is the fraction of bleached pigment,  $I$  is the light intensity in photons  $\mu\text{m}^{-2} \text{s}^{-1}$ , and  $t$  is the duration of light exposure in seconds. The value used for the photosensitivity of the cell  $P$  was  $6.2 \times 10^{-9} \mu\text{m}^2$  (Jones, 1995).

### Solutions

The cell was perfused with saline solution that contained 110 mM NaCl, 2.5 mM KCl, 1.6 mM  $\text{MgCl}_2$ , 1.0 mM  $\text{CaCl}_2$ , 10 mM dextrose, 10 mM HEPES, pH 7.8, and bovine serum albumin (100 mg/liter<sup>-1</sup>). The solution in which the guanylyl cyclase activity was measured was made by adding 3-isobutyl-1-methylxanthine (IBMX)<sup>1</sup> to normal saline solution to a final concentration of 500  $\mu\text{M}$  (IBMX solution). The solution in which phosphodiesterase activity was measured (Li<sup>+</sup> solution) was made by replacing NaCl in the saline solution with equimolar LiCl.  $\beta$ -Ionone was purchased from Sigma Chemical Co. and was repurified by double distillation. It was dissolved to 20 mM in EtOH and then to its final concentration with saline solution (final EtOH concentration <0.1%).  $\beta$ -Ionone concentration was measured with a spectrophotometer ( $\epsilon = 8,700 \text{ M}^{-1} \text{ cm}^{-1}$ ). Despite all attempts to minimize the binding of  $\beta$ -ionone to the plastic tubing by replacing most of it with glass, the concentration of  $\beta$ -ionone at the outflow of the microperfusion system in the recording bath was significantly reduced. For this reason, the concentration of  $\beta$ -ionone to which the cell was exposed was determined from samples collected from the outflow of the microperfusion tubing after each experiment.

The outer segment of the cell was exposed to  $\beta$ -ionone and test solutions using a double-barrel microperfusion system driven by a stepping motor (Cornwall et al., 1995). This system allowed for quick change of the solution perfusing the outer segment. The time required for a complete solution change, estimated from the junction current recorded while the cell was exposed to bright light, was  $\sim 25$  ms. Test solutions for estimation of rate constants of guanylyl cyclase and phosphodiesterase (to be referred to as rate of guanylyl cyclase and rate of phosphodi-

esterase) were delivered through one of the barrels of the microperfusion system using a multiple-way valve. The time for removing the dead volume in the system was  $\sim 45$  s. This allowed for effective estimation of both cyclase and phosphodiesterase rates within 1 min.  $\beta$ -Ionone was delivered to the cell through the other barrel of the microperfusion system. Initially,  $\beta$ -ionone flow was turned off so that jumps could be performed from saline solution into test solution (Li<sup>+</sup> and IBMX). Subsequently,  $\beta$ -ionone flow was turned on and its effect on cyclase and phosphodiesterase rates was estimated by jumping the cell from  $\beta$ -ionone into the test solution. The lack of  $\beta$ -ionone in the test solutions had only a negligible effect on the measurements of cyclase and phosphodiesterase rates.

The flow rates of the  $\beta$ -ionone and test solutions were regulated by injecting phenol red solution into the experimental chamber before each experiment. The sharp interface between the bath solution and the solutions flowing through the solution changer allowed us to observe and adjust the flow of both  $\beta$ -ionone and test solutions. The cells were not exposed to phenol red because in control experiments we observed that phenol red activates phototransduction in bleach-adapted rods.

### Data Analysis

To estimate the rates of cyclase and phosphodiesterase in the cell, we have used a modification of the method initially described by Hodgkin and Nunn (1988) and subsequently used by Cornwall and Fain (1994). The light-sensitive current in photoreceptors,  $i$ , is controlled by the concentration of cyclic GMP according to the relation (see Hodgkin and Nunn, 1988; Pugh and Lamb, 1990):

$$\frac{i}{i_m} = \frac{[\text{cGMP}]^N}{[\text{cGMP}]^N + K_{1/2}^N}, \quad (2)$$

where  $i_m$  is the maximum possible membrane current,  $K_{1/2}$  is the Michaelis constant for binding of cGMP to the light-sensitive channels, and  $N$  is the Hill coefficient, reflecting the cooperative binding of cGMP to the channels. The level of cGMP in the cell is regulated by the relative rates of guanylyl cyclase, responsible for cGMP synthesis, and cGMP phosphodiesterase, responsible for its hydrolysis. The change in free cGMP concentration in the cell is given by:

$$\eta \frac{d}{dt}[\text{cGMP}] = \alpha[\text{GTP}] - \beta[\text{cGMP}], \quad (3)$$

where  $\eta$  is the buffering capacity of the cell for cGMP,  $\alpha$  is the rate constant of cyclase, and  $\beta$  is the rate constant of phosphodiesterase. Following Hodgkin and Nunn (1988; see also Cornwall and Fain, 1994), we assumed that the cGMP binding sites are of high affinity and saturated ( $\eta = 1$ ). Combining Eqs. 2 and 3, one obtains a relation between the current and the rates of cyclase and phosphodiesterase:

$$\frac{d}{dt} \left[ K_{1/2} \left( \frac{i}{i_m - i} \right)^{1/N} \right] = \alpha[\text{GTP}] - \beta K_{1/2} \left( \frac{i}{i_m - i} \right)^{1/N}. \quad (4)$$

One can estimate the rate of cyclase or phosphodiesterase in the intact photoreceptor in different conditions of adaptation or in the presence of drugs by suddenly blocking one of these two enzymes. The rate at which the current changes immediately after the block provides an estimate of the velocity at which cGMP is being synthesized or hydrolyzed by the unaffected enzyme. Using this scheme, the rate of cyclase can be estimated by treating the cell with the phosphodiesterase inhibitor, IBMX. Sudden expo-

<sup>1</sup>Abbreviation used in this paper: IBMX, 3-isobutyl-1-methylxanthine.

sure of the cell to saline solution containing 500  $\mu\text{M}$  IBMX substantially inhibits phosphodiesterase and the continued production of cGMP by cyclase results in current increase. The rate of increase of the current provides an estimate of the rate of cGMP synthesis by cyclase. Alternatively, saline solution in which  $\text{Li}^+$  is substituted for  $\text{Na}^+$  can be used to estimate the rate of phosphodiesterase.  $\text{Li}^+$  permeates the light-sensitive channels but the presence of  $\text{Li}^+$  inside the cell blocks the  $\text{Na}^+/\text{Ca}^{2+}-\text{K}^+$  exchange mechanism (Yau and Nakatani, 1984; Hodgkin et al., 1985). As a result,  $[\text{Ca}^{2+}]_i$  increases rapidly and inhibits cyclase. The rate of the resulting decrease in current provides an estimate of the rate of hydrolysis of cGMP by phosphodiesterase.

Derivation of a useful expression of the current as a function of the enzymatic rates of cyclase or phosphodiesterase with the help of test solutions requires several important assumptions that were implicitly made in the original work of Hodgkin and Nunn (1988). However, new information makes necessary the explicit reexamination of these assumptions. The simpler case of the  $\text{Li}^+$  jump requires the assumption that the cGMP channel characteristics ( $K_{1/2}$  and  $N$ ), as well as  $i_m$ , do not change during or immediately after the solution change. Recent evidence has revealed that of these three parameters only  $K_{1/2}$  is not constant but rather varies as a function of the concentration of calcium (Hsu and Molday, 1993; Gordon et al., 1995; Nakatani et al., 1995; Bauer, 1996). As pointed out above, stepping into  $\text{Li}^+$  results in an increase of  $[\text{Ca}^{2+}]_i$ . A decrease in  $[\text{Ca}^{2+}]_i$  within the physiological range can cause a significant decrease of  $K_{1/2}$ . However, an increase in  $[\text{Ca}^{2+}]_i$  beyond the physiological range, as in the case of  $\text{Li}^+$  step, causes very little or no change of  $K_{1/2}$ . Thus, the proposition that  $K_{1/2}$ ,  $N$ , and  $i_m$  do not change during and immediately after the step in  $\text{Li}^+$  solution is valid. A second assumption is that the current  $i$  recorded with the suction electrode, is much smaller than  $i_m$  (respective typical values, 40 pA [Baylor et al., 1984] and  $>1,000$  pA [Cameron and Pugh, 1990]). Thus, the ratio  $i/(i_m - i)$  in Eq. 4 can be replaced by the more simple  $i/i_m$ . Using these assumptions for the case of blocked cyclase ( $\alpha = 0$ ), Eq. 4 simplifies to a form that relates the rate of phosphodiesterase to the normalized current:

$$\ln(J) = -N\beta t. \quad (5)$$

From Eq. 5, the relative change in the rate of phosphodiesterase compared with that of the dark-adapted cell will be given by:

$$\frac{\ln(J)}{\ln(J_D)} = \frac{\beta}{\beta_D}. \quad (6)$$

After subtracting the junction current resulting from the solution change, the normalized current was plotted on a semilogarithmic graph.  $\beta$  was estimated from the slope of the straight line fitted to the current trace, and then the ratio  $\beta/\beta_D$  was calculated.

In the more complicated case of the IBMX jump, one again has to assume that the cGMP channel characteristics ( $K_{1/2}$ ,  $N$ ) and  $i_m$  do not change during the solution jump. Since the step into IBMX causes an increase in the inward current, the level of  $[\text{Ca}^{2+}]_i$  should either stay the same or increase slightly. Thus, in addition to being valid for  $N$  and  $i_m$ , the above assumption still applies for  $K_{1/2}$ . One potential problem, however, derives from the current increase during the step into IBMX solution. The assumption  $i \ll i_m$ , which is again required, may no longer hold. To test the validity of this approximation, we calculated the rates of cyclase from a set of data with and without this assumption and compared the results. The relative velocities of cyclase in the two cases differed by  $<2\%$ , leading us to ignore this factor in further analysis. Using the same assumptions as made in the case of  $\text{Li}^+$ , Eq. 4 can be simplified for the case of blocked phosphodiesterase

( $\beta = 0$ ). Then, dividing by Eq. 2 written for the dark-adapted condition, one gets:

$$\frac{K_{1/2} \frac{d}{dt} [(i)^{1/N}]}{K_{1/2}^D [(i^D)^{1/N}]} = \frac{[\text{GTP}]}{[\text{cGMP}]_D} = \alpha', \quad (7)$$

where  $D$  indicates the parameters of the cell in its dark-adapted state. Following Hodgkin and Nunn (1988), we have used  $\alpha'$  as a measure of the relative changes in the rate of cyclase (see also Cornwall and Fain, 1994). The expression for the relative rate of cyclase derived from Eq. 7 is:

$$\frac{K_{1/2} \frac{d}{dt} [(i)^{1/3}]}{K_{1/2}^D [(i^D)^{1/3}]} = \frac{\alpha'}{\alpha'_D} = \frac{\alpha}{\alpha_D}, \quad (8)$$

where we assumed  $N = 3$  (Yau and Baylor, 1989). Another potential complication is that  $K_{1/2}$  is likely to be different in rods that are dark-adapted, bleached, or chemically treated. As can be seen from Eq. 8, in contrast to the  $\text{Li}^+$  method, calculation of the relative rate of cyclase requires direct comparison of  $K_{1/2}$  in the dark-adapted and in the bleached or otherwise manipulated cell. As pointed out above,  $K_{1/2}$  of the cGMP-gated channel is not constant, but rather varies as a function of the concentration of calcium. The decrease in  $[\text{Ca}^{2+}]_i$  produced by the bleach (Sampath et al., 1998) and probably by  $\beta$ -ionone can cause a significant decrease of  $K_{1/2}$  (Nakatani et al., 1995). To improve the accuracy of the calculation of the rate of cyclase, we have incorporated an estimate of the change of  $K_{1/2}$  caused by the bleaching of pigment in our analysis. This was done in the following way. The time derivative of the current  $di/dt$  was calculated using the Savitzki-Golay method (Press et al., 1992). Since the data were acquired at 250 Hz (4 ms/point) and filtered at 20 Hz (50 ms/point), the linear regression was performed over 13 points ( $13 \times 4$  ms = 52 ms, corresponding to 19 Hz). The change in  $K_{1/2}$  after the bleach was calculated from the corresponding drop in the dark current using the relation between current and  $[\text{Ca}^{2+}]_i$  in Nakatani et al. (1995, see Fig 6). Using Eq. 6,  $\alpha'$  was obtained in each experimental condition, and then the ratio  $\alpha/\alpha_D$  (equal to  $\alpha'/\alpha'_D$ ) was calculated.

The measurements of the rates of cyclase and phosphodiesterase in this study were done pairwise in the same cell, under the same conditions, and within 1 min of each other. To minimize possible side effects, the order of measurements was varied from cell to cell. Thus, direct comparison between the two enzymatic rates should be much more accurate than when two different cells from two different animals are considered. Since all of the measurements of the velocities of cyclase and phosphodiesterase were done in steady state, it would be expected that the two relative velocities (see Eqs. 6 and 8) will be equal. Accounting for the change in  $K_{1/2}$  and modifying the analysis of IBMX jumps originally devised by Hodgkin and Nunn (1988) and later used by Cornwall and Fain (1994) compensated for the mismatch between the rates of cyclase and phosphodiesterase observed in these studies. In our experience, the two rates were very similar for activation of the phototransduction cascade by bleaches of up to 40%. For even higher transduction levels (higher bleaches), the  $\text{Li}^+$  method saturates due to the overlap of the initial rapid influx of  $\text{Li}^+$  ions, caused by the higher permeability of the light-dependent channels to  $\text{Li}^+$  than to  $\text{Na}^+$  (Hodgkin et al., 1985), and the decrease in cGMP-activated current, caused by the block of cyclase. In addition, after higher bleaches, the dark current is significantly reduced, which makes the rate of its decrease in  $\text{Li}^+$  solution more difficult to measure.

## RESULTS

### Photocurrent and Light Sensitivity

The experiment illustrated in Fig. 2 shows the effect that  $\beta$ -ionone has on the dark current and sensitivity of a bleach-adapted rod. The cell was stimulated with a series of test flashes of increasing intensity to monitor changes in the amplitude of the current and in sensitivity. Fig. 2 A illustrates a series of superimposed flash responses recorded from a dark-adapted rod. After a brief exposure to bright light that bleached 20% of the visual pigment, the cell was allowed to recover for 35 min until its dark (light-suppressible) current reached a new steady state level from which no further recovery occurred. The bleach caused a steady reduction in the dark current, as evidenced by the decreased amplitude of the saturating flash response (Fig. 2 B). When the rod was then exposed to  $\beta$ -ionone, the dark current declined further within several seconds to a new, lower level (Fig. 2 C), which persisted for as long as the cell

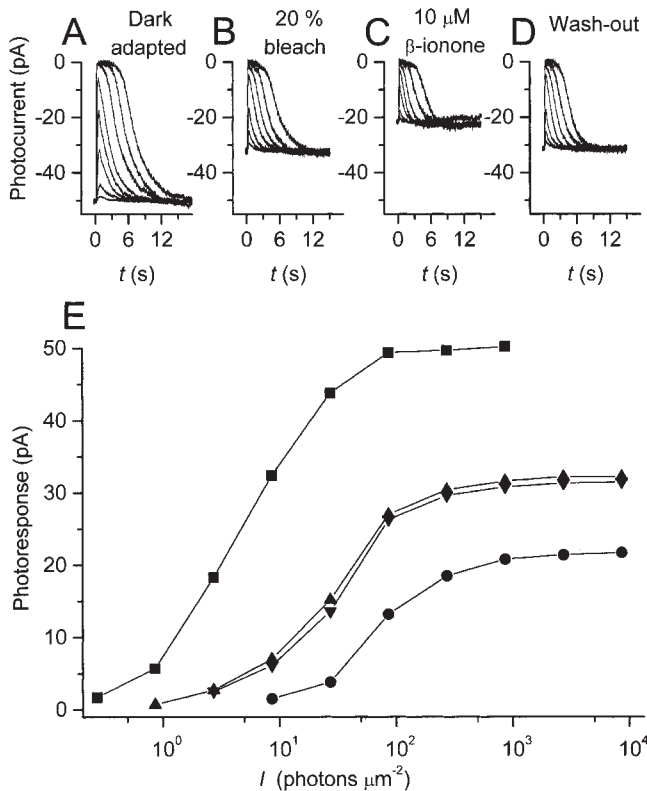


FIGURE 2. Effect of  $\beta$ -ionone on the dark current and on the sensitivity in a rod. Shown is a series of flash responses from one cell in its dark-adapted state (A), after a 20% bleach (B), in  $10 \mu\text{M}$   $\beta$ -ionone solution (C), and after washing out  $\beta$ -ionone (D). The corresponding intensity-response curves are shown in E: dark-adapted (■), bleach-adapted (▲), in  $\beta$ -ionone (●), and after washing out  $\beta$ -ionone (▼).  $\beta$ -Ionone caused a reversible decrease in the dark current and in the sensitivity of the cell.

remained in  $\beta$ -ionone solution. After returning the rod to normal saline solution, the dark current recovered to its bleach-adapted level within 5 min (Fig. 2 D). The amplitudes of the flash responses in each condition were plotted against flash intensity to create the corresponding intensity-response curves (Fig. 2 E). Bleaching increased the test flash intensity required to elicit a threshold response (Fig. 2 E, ▲) compared with the dark-adapted state (■). Thus, the sensitivity of the cell after the bleach was lower than that of its dark-adapted state. Treatment of the bleached cell with  $\beta$ -ionone reduced further its sensitivity (Fig. 2 E, ●), at the same time decreasing the maximal response amplitude. After returning the rod to normal saline solution, both sensitivity and maximal response amplitude recovered to their bleach-adapted levels (Fig. 2 E, ▼). Thus,  $\beta$ -ionone caused a reversible decrease in dark current and sensitivity in bleach-adapted rods. When cells were exposed to a sufficiently bright light to totally suppress the dark current and treated with  $\beta$ -ionone, only a small junction current was observed. Thus, the decreased amplitude of the responses elicited in  $\beta$ -ionone solution appeared to result from a decrease in the light-sensitive conductance.

The effect of  $\beta$ -ionone on the dark current and the sensitivity was tested in a total of 36 bleach-adapted cells. All of them exhibited a decrease in both dark current and sensitivity in the presence of  $\beta$ -ionone. The magnitude of pigment bleaching ranged from 3 to 80%.  $\beta$ -Ionone concentrations used varied from 1 to  $20 \mu\text{M}$ . For low concentrations of  $\beta$ -ionone (up to  $5 \mu\text{M}$ ), the decrease in the dark current was greater for larger bleaches until it saturated at  $\sim 20\%$  pigment bleach. For higher concentrations of  $\beta$ -ionone, the decrease in the dark current was also larger for larger bleaches, but the effect did not saturate in the range of bleaches that were tested. Owing to their variable nature, it was not possible to determine the exact relationship between the decrease in the dark current and the magnitude of the pigment bleach or the concentration of  $\beta$ -ionone. For this reason, we chose to restrict our analysis to the effect of  $10 \mu\text{M}$   $\beta$ -ionone in rods in which 20% of the pigment had been bleached. In a total of 21 cells tested under these conditions, the mean decrease in the dark current caused by  $\beta$ -ionone was  $51 \pm 8\%$  (SEM,  $n = 21$ ). The corresponding decrease in sensitivity was  $0.91 \pm 0.08$  log units (SEM,  $n = 10$ ).

### Dim Flash Kinetics

The response amplitude is a linear function of light intensity at the lower end of the intensity-response curve for both rods and cones (Baylor and Hodgkin, 1973; Kleinschmidt and Dowling, 1975). This implies that for dim flashes, the photoresponse is equal to the linear

sum of the responses to single photons. Thus, analysis of the kinetics of dim flash responses allows for investigation of the kinetics of the phototransduction cascade. Activation of transduction by a bleach or by background light accelerates the dim flash response (Cornwall et al., 1989, 1990; Matthews et al., 1990). To investigate whether the effect of  $\beta$ -ionone on the dark current and photosensitivity is a result of activation of phototransduction as opposed to a nonspecific effect, we looked at its impact on the kinetics of dim flash responses. Fig. 3 shows normalized dim flash responses elicited from a cell, first in the dark-adapted state, 40 min after 20% of the pigment had been bleached, and then during and after exposure to  $\beta$ -ionone. As expected, bleaching accelerated significantly the photoreponse. After bleaching, the time to peak of the dim flash response was reduced by >25%. Treatment of the bleached cell with  $\beta$ -ionone resulted in further acceleration of the kinetics of the response. The effect of  $\beta$ -ionone was completely reversible and several minutes after returning the cell to normal saline solution, the response slowed to its pretreatment, bleach-adapted level. Thus,  $\beta$ -ionone caused a reversible acceleration of the dim flash response in a bleach-adapted rod.

#### Rate of Guanylyl Cyclase

To investigate the effect of  $\beta$ -ionone on phototransduction in detail, we performed a series of experiments designed to measure directly the rates of cyclase and phosphodiesterase and to evaluate their change when the cell was exposed to  $\beta$ -ionone (see MATERIALS AND METHODS). Fig. 4 shows typical results from one experi-

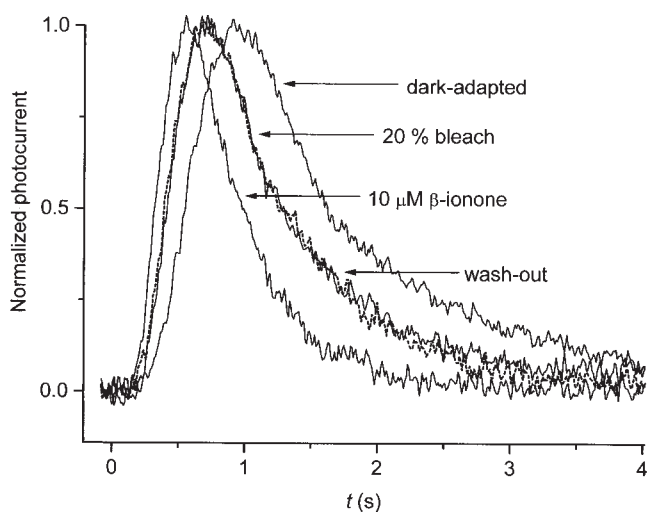


FIGURE 3. Effect of  $\beta$ -ionone on the kinetics of dim flash responses in a rod. Normalized dim flash responses from one rod in a dark-adapted state, after a 20% bleach, in the presence of 10  $\mu$ M  $\beta$ -ionone, and after washing out  $\beta$ -ionone.

ment in which cyclase was studied. Using the microperfusion system, the rod was quickly exposed to saline test solution containing 500  $\mu$ M IBMX in order to block phosphodiesterase. Fig. 4 (left) shows current recordings during the solution change. Fig. 4 (right) plots  $d(J^{1/3})/dt$  as a function of time, where  $J = i/i^D$ . The time course of the solution change in both cases is shown on the top of the figure. Since the derivative of  $J^{1/3}$  is proportional to the rate of change in cGMP concentration (see MATERIALS AND METHODS, Eq. 2), its maximum represents a measure of the rate of synthesis of cGMP by guanylyl cyclase. The rate of cyclase was first measured in the dark-adapted cell (Fig. 4 A). Here, cyclase had only a low basal activity. After a 20% bleach followed by a recovery period, the rate of cyclase was measured once again (Fig. 4 B). The rate of current increase upon jumping into IBMX solution (Fig. 4 B, left) was higher than in the dark-adapted state (Fig. 4 A, left). As shown previously (Cornwall and Fain, 1994), the rate of cyclase increases as a result of a bleach, as can be seen here from the greater than threefold increase in the peak of the derivative (Fig. 4 B, right) as compared with that measured before bleaching (Fig. 4 A, right). Exposure of the bleached cell to  $\beta$ -ionone produced a twofold further acceleration of cyclase (Fig. 4 C). The effect of  $\beta$ -ionone was completely reversed a few minutes after the cell was returned to normal saline solution (Fig. 4 D). Thus,  $\beta$ -ionone caused a reversible acceleration of guanylyl cyclase in a bleach-adapted rod.

The effect of  $\beta$ -ionone on the rate of cyclase was studied in a total of 30 bleach-adapted rods. In all of them,  $\beta$ -ionone caused acceleration of cyclase. The range of pigment bleaches used was from 3 to 80%, and the range of  $\beta$ -ionone concentrations was from 3 to 20  $\mu$ M. No clear quantitative correlation between the fraction of pigment bleached, or the concentration of  $\beta$ -ionone, and the corresponding acceleration of cyclase could be observed. However, in general, for a given bleached fraction of visual pigment, the relative rate of cyclase, compared with the bleach-adapted state, was higher for higher concentrations of  $\beta$ -ionone. On the other hand, for a given concentration of  $\beta$ -ionone, the relative acceleration of cyclase was higher for smaller bleaches. As in the experiments described in the previous section, we studied in detail the effect of 10  $\mu$ M  $\beta$ -ionone after a 20% pigment bleach. A total of 18 rods were studied under these conditions. The rate of cyclase in the presence of  $\beta$ -ionone was  $2.5 \pm 0.3$  (SEM,  $n = 18$ ) times higher than in the corresponding bleach-adapted state.

#### Rate of Phosphodiesterase

To substantiate further the conclusion that  $\beta$ -ionone activates phototransduction, the rate of phosphodi-

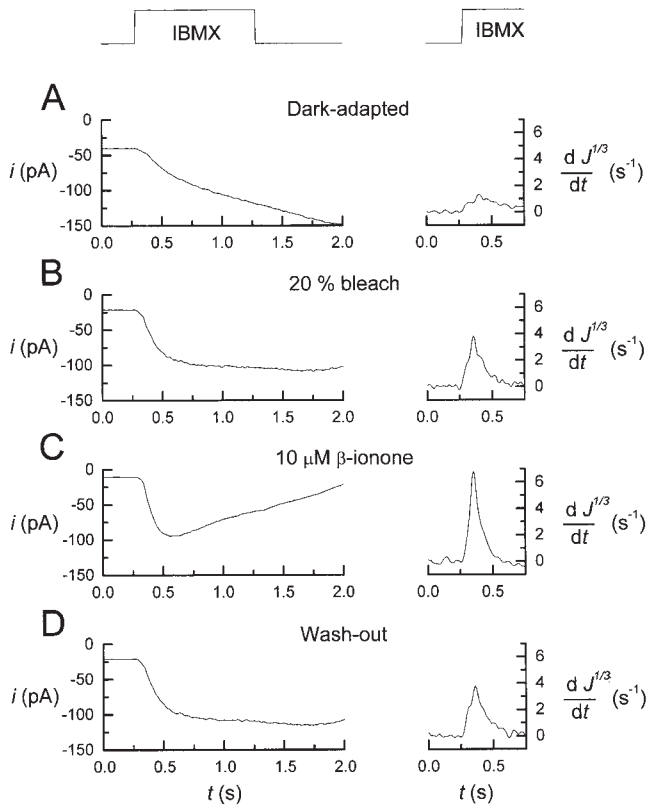


FIGURE 4. Effect of  $\beta$ -ionone on the rate of guanylyl cyclase in a rod. The left panels show current recordings from a rod during steps into 0.5 mM IBMX solution. The corresponding right panels show the derivative of the cube root of the normalized current,  $d(J^{1/3})/dt$ . The time course of the solution step for both panels is shown at the top. Recordings were made first in the dark-adapted state (A), after a 20% bleach (B), in 10  $\mu$ M  $\beta$ -ionone solution (C), and then after washing out  $\beta$ -ionone (D). Each current trace is the average of six measurements. All recordings were done in the same cell.

esterase was measured within 2 min of the cyclase rate measurement in these same rods. The minimum time between the two measurements was limited by the time required for the new test solution to fill the dead space in the microperfusion tubing. Since the current was at steady state while the cell was exposed to  $\beta$ -ionone, the concentration of cGMP should be constant, and the rates of its synthesis by cyclase and its hydrolysis by phosphodiesterase should be equal. The activation of cyclase as a result of treatment of the bleached cell with  $\beta$ -ionone then should be accompanied by a corresponding acceleration of phosphodiesterase.

Fig. 5 shows one example of an experiment in which the rate of phosphodiesterase was measured. In this case, cyclase was blocked by suddenly exposing the cell to a test solution in which  $\text{Li}^+$  was substituted for  $\text{Na}^+$  on an equimolar basis. The time course of the jump into  $\text{Li}^+$  solution is shown at the top of the figure. Fig. 5 (left) shows the current recordings from the cell; Fig. 5

(right) shows semi-logarithmic plots of the normalized current as a function of time. Here, the slope is proportional to the rate of the phosphodiesterase (Eq. 5). As with cyclase, bleaching produced an acceleration of phosphodiesterase (Fig. 5 B), and subsequent treatment with  $\beta$ -ionone resulted in an even higher enzymatic rate (Fig. 5 C). Again, the effect of  $\beta$ -ionone was completely reversible upon return of the cell to normal saline solution (Fig. 5 D). Thus,  $\beta$ -ionone caused a reversible acceleration of cGMP phosphodiesterase in a bleach-adapted rod.

The effect of  $\beta$ -ionone on phosphodiesterase was measured under the same bleaching conditions and the same  $\beta$ -ionone concentrations as cyclase (see the previous section) in a total of 30 cells. In 25 cells,  $\beta$ -ionone caused acceleration of phosphodiesterase and in the remaining 5 either no effect or a small deceleration was observed. However, in 4 of these 5 cells the fraction of the bleached pigment was 40% or higher. We interpret the inconsistency in these cells as being due to the inability of the  $\text{Li}^+$  jump method to resolve the high rate of phosphodiesterase after large bleaches (see MATERIALS AND METHODS). This speculation is supported by the fact that in contrast to phosphodiesterase, cyclase was accelerated in these cells and there was a decrease in both the dark current and the sensitivity as a result of the treatment with  $\beta$ -ionone. In the remaining 25 cells, the acceleration of phosphodiesterase was higher after smaller bleaches for each  $\beta$ -ionone concentration. In 17 experiments similar to those presented in Fig. 5, we examined the effect of 10  $\mu$ M  $\beta$ -ionone after a 20% pigment bleach. In these cells, the rate of phosphodiesterase in the presence of  $\beta$ -ionone was  $1.7 \pm 0.2$  (SEM,  $n = 17$ ) times higher than in the corresponding bleach-adapted state.

#### Phototransduction in a Dark-adapted Rod in the Presence of $\beta$ -Ionone

Experiments were designed to investigate the possible site of action of  $\beta$ -ionone on phototransduction. Specifically, we were interested in testing the hypothesis that  $\beta$ -ionone activates transduction by binding directly in the chromophore pocket of opsin. To test this idea, we compared the effect of  $\beta$ -ionone in a dark-adapted and background light-adapted rod with its effect after a bleach. As a first step, we examined the effect of  $\beta$ -ionone in dark-adapted cells. A total of 16 cells were tested. In a small fraction of these, treatment with  $\beta$ -ionone resulted in a slight activation of phototransduction. The decrease in the dark current, the loss of sensitivity, and the acceleration of cyclase and phosphodiesterase produced by  $\beta$ -ionone in these dark-adapted rods, although significant, was small compared with the effect of  $\beta$ -ionone seen after 20% or higher pigment bleaches. The observed acceleration of phototransduction in

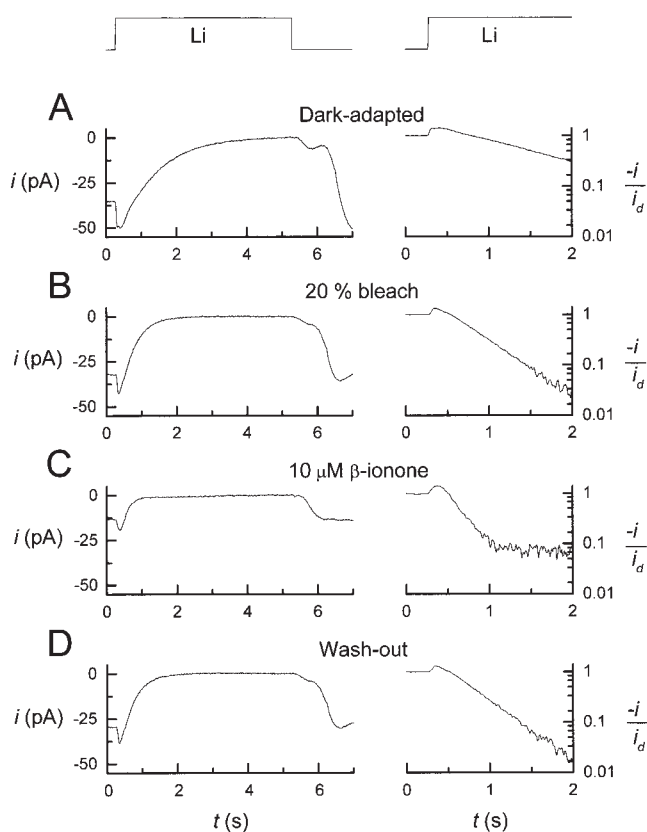


FIGURE 5. Effect of  $\beta$ -ionone on the rate of cGMP phosphodiesterase in a rod. The left panels show the current from a rod during steps into  $\text{Li}^+$  solution. The corresponding right panels show the normalized current on a semilogarithmic plot. The time course of the solution step is shown at the top. Recordings were made first in the dark-adapted state (A), after a 20% bleach (B), in  $10 \mu\text{M}$   $\beta$ -ionone solution (C), and then after washing out  $\beta$ -ionone (D). Each current trace is the average of six measurements. All recordings were done in the same cell.

some of the control experiments could be due to the fact that some of the cells were not completely dark adapted and contained some small fraction of bleached pigment. In accord with this notion, we observed that  $\beta$ -ionone activates phototransduction even after bleaches as low as 3%. Because the cells were exposed to dim red and infrared light during preparation, it is possible that in some cases a small but significant fraction of pigment was bleached. This idea is supported by the observation that the sensitivity of the cells slightly increased during the first 30–60 min after the dissection (data not shown), despite the fact that the animals were dark-adapted overnight. The interaction of the pigment bleached during the dissection with  $\beta$ -ionone could produce the observed activation of phototransduction.

To test this hypothesis directly, two dark-adapted cells that showed some acceleration of the transduction cascade during exposure to  $\beta$ -ionone were treated with

11-cis retinal in an effort to regenerate any residual bleached pigment. In both cells, about 1 h after addition of 11-cis retinal, the effect of  $\beta$ -ionone on sensitivity, dim flash kinetics, and cyclase and phosphodiesterase velocities was either significantly reduced or absent. Thus, complete regeneration of the pigment abolished the effect of  $\beta$ -ionone. Another argument, consistent with this notion, is based on the following observation: a hemisected eyecup was dark adapted for 24 h at  $4^\circ\text{C}$  after the dissection. Treatment of five cells from this preparation with  $\beta$ -ionone produced no effect, presumably because the pigment bleached during the dissection was regenerated in the intact retina during this additional dark period. Taken together, these observations indicate that treatment with  $\beta$ -ionone produces a small effect on transduction in freshly dissected dark-adapted cells and no effect in fully dark-adapted cells where all of the pigment has been regenerated.

#### *Rate of Guanylyl Cyclase and Phosphodiesterase in Bleached vs. Light-adapted Cell*

As a second step in investigating the possible site of action of  $\beta$ -ionone, we compared its effect in a background light-adapted rod with its effect after a bleach. In both cases, the level of phototransduction activity is higher than in the dark-adapted state. However, the background light intensity required to produce activation comparable with that of a significant pigment bleach is so low that it only photoactivates a negligible fraction of the pigment. For instance, a steady background producing activation of cyclase (phosphodiesterase) equivalent to a 20% bleach ( $12.8 \text{ photons } \mu\text{m}^{-2} \text{ s}^{-1}$ , see Fig. 6) would bleach  $<0.03\%$  of the pigment in 1 h. Thus, even though both bleaching and background light produce elevation of the rate of phototransduction, the retinal binding pocket on opsin may be free and available for binding only in the bleached state.

In four rods, the effect of  $\beta$ -ionone on cyclase in background light was directly compared with its effect on cyclase after a bleach. Each cell was first exposed to a series of backgrounds of increasing intensity. For each background, the rate of cyclase was measured in both saline solution and in  $\beta$ -ionone solution. An example at one background is shown in Fig. 6, A (saline solution) and B ( $\beta$ -ionone). The cell was then bleached and allowed to recover to a steady state. The rate of cyclase was measured once again in saline solution (Fig. 6 C), and then in  $\beta$ -ionone (D). Comparison between Fig. 6, A and C, shows that one of the backgrounds induced exactly the same activation of cyclase as the subsequent bleaching.  $\beta$ -Ionone failed to produce any effect on the rate of cyclase in the background light-adapted state (Fig. 6 B), but in contrast increased sig-



nificantly the rate of cyclase after the cell was bleached (D). This result indicates that the activation of phototransduction is not sufficient to induce acceleration of cyclase by  $\beta$ -ionone.

In an experiment similar to the one just described, the effects of  $\beta$ -ionone on the rate of phosphodiesterase in the light- and bleach-adapted states were also compared. In the two tested rods,  $\beta$ -ionone only accelerated phosphodiesterase in the bleach-adapted state, but did not affect the background light-adapted state. This result complements the result for cyclase described in the previous paragraph. Taken together, they indicate that  $\beta$ -ionone activates phototransduction only in the presence of bleached pigment. This observation supports the hypothesis that  $\beta$ -ionone affects phototransduction by interacting selectively with bleached pigment.

#### Time Course of the Effect of $\beta$ -Ionone After a Bleach

The results illustrated in Figs. 7 and 8 are from an experiment designed to determine the time course of on-

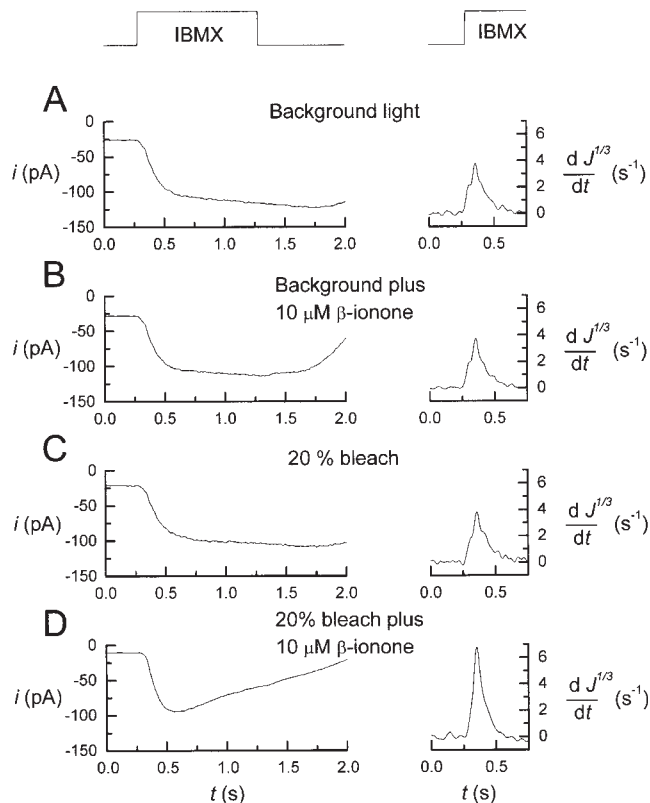


FIGURE 6. Comparison of the effects of  $\beta$ -ionone on cyclase rate in a rod exposed to dim background light and after a bleach. Figure details as in Fig. 4. Recordings are shown from a cell when exposed to background light (A), to  $10 \mu\text{M}$   $\beta$ -ionone solution while in the background (B), after a 20% bleach (C), and in  $10 \mu\text{M}$   $\beta$ -ionone solution (D). The intensity of the background was  $12.8 \text{ photons } \mu\text{m}^{-2} \text{ s}^{-1}$ . All recordings were done in the same cell.

set of transduction activation triggered by  $\beta$ -ionone. Since for the first few minutes after bleaching the rates of both cyclase and phosphodiesterase are too high to be measured reliably with a solution step, we chose the amplitude of the dark current as an index of the level of transduction. The cell was exposed to saturating test flashes to measure the dark current first in saline solution and then 25 s after exposure to  $\beta$ -ionone solution. The fact that the effect of  $\beta$ -ionone is completely reversible allowed us to do repetitive measurements in the course of recovery of the cell after the bleach. Current recordings from one such experiment are presented in Fig. 7. Fig. 7 A shows recordings from one rod in its dark-adapted state and at different times after the bleach. In the dark-adapted cell, a jump into  $\beta$ -ionone caused only a small junction current shift, but not a change in the amplitude of the saturating photoreponse. Thus, as described above,  $\beta$ -ionone did not affect the light-sensitive current in the dark-adapted cell. After bleaching 20% of the pigment, the current was initially completely suppressed, and then started to rapidly recover. During this period when the dark current was increasing quickly, estimation of the effect of  $\beta$ -ionone was rendered unreliable by the fact that measurements of the current in saline solution and in  $\beta$ -ionone were made 45 s apart. Several minutes after the bleach, the recovery of the cell slowed down sufficiently to allow reliable comparison of the dark current in saline solution and in  $\beta$ -ionone solution without any significant drift in the current during the 45 s between test flashes. Jumping into  $\beta$ -ionone solution produced a drop in the current whose total amplitude increased with time after the bleach. The kinetics of the decrease in the current caused by  $\beta$ -ionone were carefully examined. The effect of  $\beta$ -ionone on the current at different times after the bleach can be seen in Fig. 7 B. These recordings are from the same cell as Fig. 7 A, but are superimposed and plotted on a different time scale. For each of the traces recorded after the bleach, the current decrease caused by  $\beta$ -ionone could be described by a single exponential decay function. For the cell shown on Fig. 7, the time constant of these exponential functions changed from 12 s, measured 13 min after the bleach, to 3.7 s 1-h later. The time constant of the exponential current decay decreased with time after the bleach in all nine rods tested. On average, the decrease of the current caused by  $\beta$ -ionone shortly after the bleach was two times slower than the decrease in the current recorded 1-h later.

Fig. 8 shows the time course of the effect of  $\beta$ -ionone on the dark current amplitude for the same cell as a function of time after the bleach. Fig. 8 A presents the amplitude of the current in saline solution and in  $\beta$ -ionone in the dark-adapted cell and at different time points after the bleach. As pointed out above, the current im-

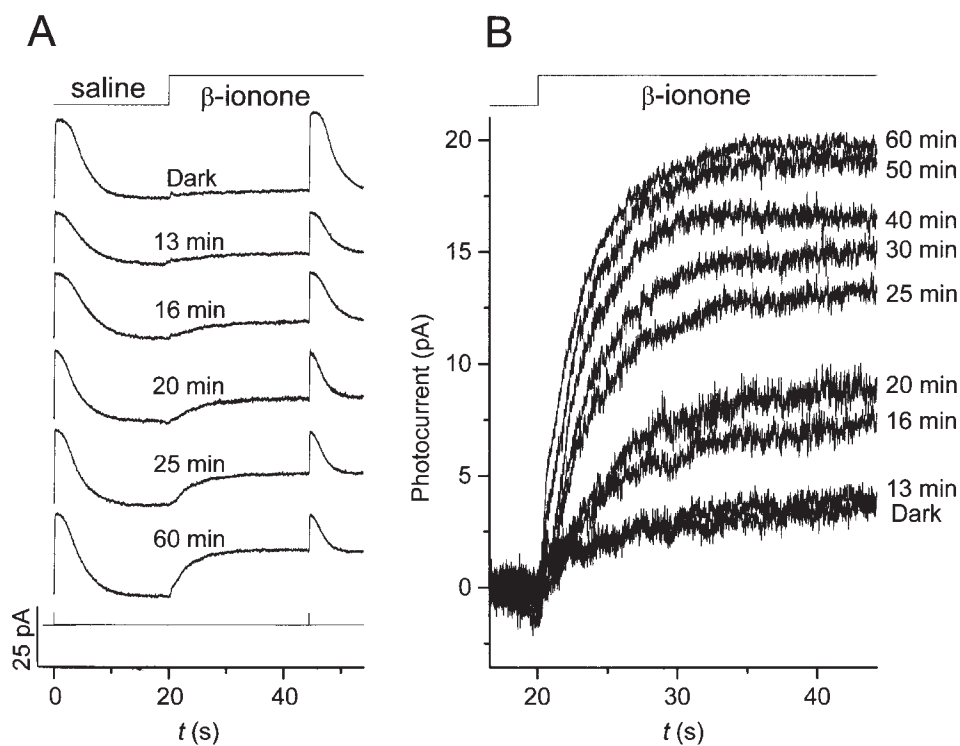


FIGURE 7. Change in the effect of  $\beta$ -ionone on the dark current after the bleach. Current recordings from a cell treated repeatedly with  $\beta$ -ionone during recovery from a 20% bleach. For each trace, the cell was given a saturating test flash first in saline solution, and then after a step into  $\beta$ -ionone solution. Both panels show recordings from the cell in its dark-adapted state and at different times after the bleach. The time course of the step into  $\beta$ -ionone solution is displayed on the top of each panel. In A, the time course of the test flashes is shown on the bottom. B shows in detail the changes in the current caused by the step in  $\beta$ -ionone.

mediately after the bleach was completely suppressed. The amplitude of the current in  $\beta$ -ionone 7 min after the bleach was slightly higher than the corresponding amplitude in saline solution measured 45 s earlier. As discussed above, we interpret this observation not to be a result of the treatment with  $\beta$ -ionone, but rather to follow from the fast recovery of the cell during that period. 13 min after the bleach, the amplitude of the current in  $\beta$ -ionone was already clearly smaller than the corresponding current in saline solution and with time that difference became more pronounced. To estimate the rate of the increase of this effect, we calculated the relative decrease in the current caused by  $\beta$ -ionone as a function of time after the bleach. Fig. 8 B shows the ratio of the amplitudes of the current in  $\beta$ -ionone and in saline solution from A, plotted against the time after the bleach. The decrease of that ratio could be fit by a single exponential decay function with a time constant of  $13 \pm 1.1$  (SEM) min. The average time constant for all cells tested in this way was  $13 \pm 2.1$  (SEM,  $n = 8$ ) min. This rate of increase of the effect of  $\beta$ -ionone as a function of time after a bleach is of the same order as the rate of decay of photoactivated pigment to free opsin and all-trans retinol derived from spectrophotometric experiments (Baumann, 1972; Brin and Ripps, 1977) or with early receptor potential measurements (Cone and Cobbs, 1969). The fact that the amplitude of the effect of  $\beta$ -ionone correlates with the concentration of free opsin in the cell provides another argument in

support of the notion that  $\beta$ -ionone exerts its effect by interacting exclusively with free opsin.

## DISCUSSION

### *Effect of $\beta$ -Ionone on Phototransduction in a Bleach-adapted Rod*

Our experiments show that in bleach-adapted rods  $\beta$ -ionone causes (a) a decrease in the dark current, (b) a decrease in the sensitivity, (c) an acceleration of the dim flash photoresponse, and (d) an increase in the rates of guanylyl cyclase and cGMP phosphodiesterase. Together, these results indicate that in bleach-adapted rods  $\beta$ -ionone acts as an agonist and activates phototransduction in the dark.  $\beta$ -Ionone produces no effect in fully dark-adapted cells, where the basal level of transduction is low and the chromophore pockets of the opsin molecules are still occupied by native 11-cis retinal. Furthermore,  $\beta$ -ionone produces no effect in background light-adapted cells, where the level of transduction is high but still most of the chromophore pockets are occupied by 11-cis retinal. The rate at which the amplitude of the observed effect increases in time after the bleach is of the same order as the rate of decay of photoactivated rod pigment to free opsin. Based on these observations, we conclude that the effect of  $\beta$ -ionone is the result of its direct interaction with the free opsin molecules produced by the bleaching light.

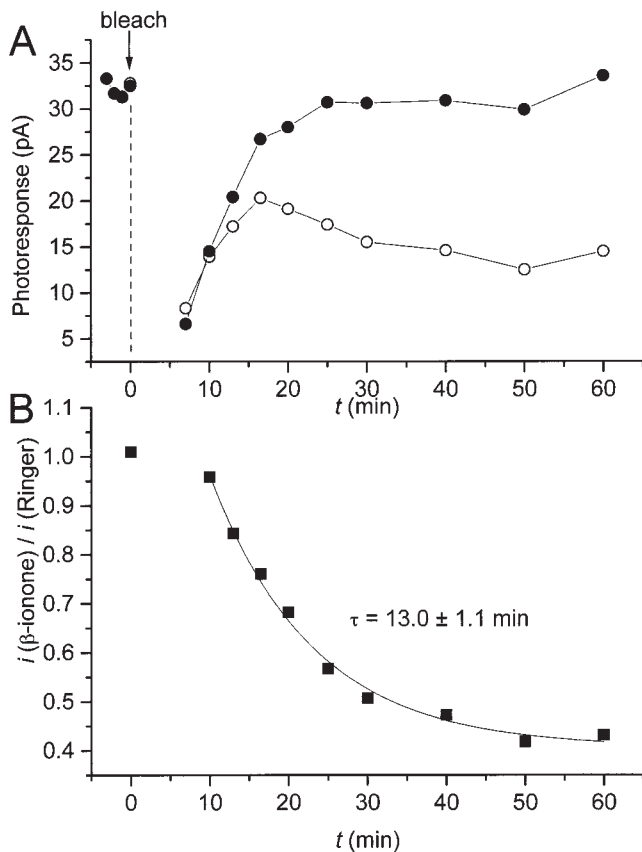


FIGURE 8. Time course of the change in the effect of  $\beta$ -ionone on the current derived from the cell in Fig. 7. A shows the current in saline solution (●) and in  $\beta$ -ionone (○) measured before and at different times after the bleach. B shows the ratio of the current in  $\beta$ -ionone and in saline solution for each time point in A. The data is fitted by a first order exponential decay function.

We suggest that  $\beta$ -ionone exerts its physiological effect by binding directly in the chromophore pocket of free opsin. Fig. 9 presents a model of the proposed mechanism of activation of phototransduction by  $\beta$ -ionone in rods. The chromophore pocket of opsin is shown schematically and the points of interaction of opsin with retinal or with  $\beta$ -ionone are indicated by a star. For each intermediate state, the relative enzymatic activity is indicated by the amplitude of the vertical bar to the right. In the dark-adapted state, 11-cis retinal is bound in the chromophore pocket of opsin and is covalently attached to it via a Schiff base linkage (Fig. 9 A). The dark-adapted pigment is not capable of activating phototransduction beyond its basal level, as indicated by the absence of a vertical bar on the right. Absorption of a photon by the pigment molecule results in the photoisomerization of retinal and the full activation of the complex (Fig. 9 B). Decay of this photoactivated rhodopsin produces all-trans retinol and free opsin. The bleached pigment exhibits low catalytic activity

(Fig. 9 C), resulting in a level of transduction in the bleach-adapted state higher than in the dark-adapted state. Binding of  $\beta$ -ionone in the free chromophore pocket of opsin produces a complex with catalytic activity that is higher than that of free opsin alone (Fig. 9 D). The upregulation of phototransduction caused by this increase in the catalytic activity of the bleached pigment can be observed physiologically and is reported here. As expected for the noncovalent type of interaction between  $\beta$ -ionone and opsin, the binding of  $\beta$ -ionone is reversible and its removal from the pocket results in restoration of the bleach-adapted catalytic activity of free opsin (Fig. 9 C).

#### Contrast between the Effect of $\beta$ -Ionone in Rods and Cones

The effect of  $\beta$ -ionone in bleach-adapted salamander cones has been studied previously (Jin et al., 1993; Cornwall et al., 1995). In cones,  $\beta$ -ionone partially reverses the effect of bleaching and downregulates pho-

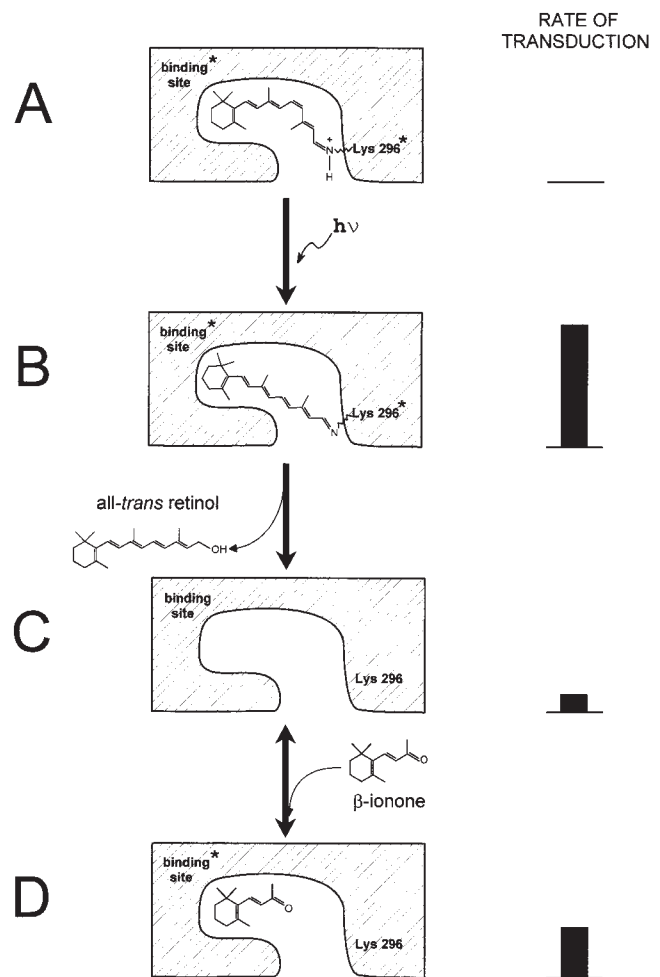


FIGURE 9. Model of the mechanism of activation of phototransduction by  $\beta$ -ionone in rods.

totransduction. In other words, administration of  $\beta$ -ionone to a bleach-adapted cone restores some of the large loss in sensitivity produced by the bleach and reverses the acceleration of the dim flash response. The dark current recovers partially. Treatment of bleach-adapted cones with  $\beta$ -ionone also causes a decrease in the rate of guanylyl cyclase. These results on cones are in direct contrast with the results reported here for rods. Thus, the interaction of  $\beta$ -ionone with the chromophore-binding pocket of rod and cone opsins leads to opposite physiological effects. This observation resolves the current discrepancy between biochemical and physiological data and suggests that the biochemical observation that opsin is activated by the binding of retinoid in the chromophore pocket is correct but applies only to rod opsin rather than to both rod and cone opsin.

The contrast between the chromophore pockets of rods and cones is not limited to the interaction with  $\beta$ -ionone. Jones et al. (1989) observed a significant decrease in the sensitivity of bleach-adapted rods treated with 11-cis retinol. In the same study, 11-cis retinol caused recovery of sensitivity in bleach-adapted cones. Together, these observations indicate that the contrast between rods and cones outlined here may be a fundamental feature of the interaction of the two types of opsin with their native chromophore.

#### *Nature of Bleaching Adaptation*

The state of opsin that is responsible for bleaching adaptation is a matter of controversy. According to one model, the free opsin produced after bleaching exhibits a low catalytic activity that sustains an elevated level of transduction in the bleached cell (Cornwall and

Fain, 1994; Cornwall et al., 1995). An alternative view is that interaction of all-trans retinal with free opsin is the factor causing bleaching adaptation (Fukada and Yoshizawa, 1981; Hofmann et al., 1992; Palczewski et al., 1994). A strong argument for a free opsin mechanism of bleaching adaptation deriving from our findings is that the occupancy of the chromophore pocket by  $\beta$ -ionone produces opposite effects in rods and cones. The noncovalent binding of all-trans retinal in the pocket would also be expected to produce opposite effects in rods and cones and therefore could not account for the bleaching adaptation in both photoreceptor types. In contrast, the sustained catalytic activity of free opsin could explain the elevated rate of phototransduction observed in both rods and cones after a bleach. Thus, the results from our experiments make it unlikely that bleaching adaptation is the result of the interaction of all-trans retinal with the chromophore pocket of free opsin.

Finally, our observations of the effect of  $\beta$ -ionone in bleach-adapted rods may have important implications for the physiology of regeneration of rhodopsin in the intact retina. By analogy with the results reported here, binding of the native 11-cis retinal in the chromophore pocket of rod opsin might be expected to form an active noncovalent complex that results in transient up-regulation of phototransduction and transient reduction in the sensitivity of the rod. In contrast, binding of retinal in the chromophore pocket of cone opsin might be expected to downregulate transduction, and thus contribute to the partial recovery of the sensitivity of the cone even before the formation of the Schiff base linkage and the regeneration of the pigment. Verification of this model will require further investigation.

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