Effect of 11-Cis 13-Demethylretinal on Phototransduction in Bleach-adapted Rod and Cone Photoreceptors

D. Wesley Corson,*‡ Vladimir J. Kefalov,§ M. Carter Cornwall,§ and Rosalie K. Crouch*

From the *Department of Pathology & Laboratory Medicine, and ‡Department of Ophthalmology, Medical University of South Carolina, Charleston, South Carolina 29425; and §Department of Physiology, Boston University School of Medicine, Boston, Massachusetts 02118

abstract We used 11-cis 13-demethylretinal to examine the physiological consequences of retinal's noncovalent interaction with opsin in intact rod and cone photoreceptors during visual pigment regeneration. 11-Cis 13-demethylretinal is an analog of 11-cis retinal in which the 13 position methyl group has been removed. Biochemical experiments have shown that it is capable of binding in the chromophore pocket of opsin, forming a Schiff-base linkage with the protein to produce a pigment, but at a much slower rate than the native 11-cis retinal (Nelson, R., J. Kim deReil, and A. Kropf. 1970. *Proc. Nat. Acad. Sci. USA.* 66:531–538). Experimentally, this slow rate of pigment formation should allow separate physiological examination of the effects of the initial binding of retinal in the pocket and the subsequent formation of the protonated Schiff-base linkage. Currents from solitary rods and cones from the tiger salamander were recorded in darkness before and after bleaching and then after exposure to 11-cis 13-demethylretinal. In bleach-adapted rods, 11-cis 13-demethylretinal caused transient activation of phototransduction, as evidenced by a decrease of the dark current and sensitivity, acceleration of the dim flash responses, and activation of cGMP phosphodiesterase and guanylyl cyclase. The steady state of phototransduction activity was still higher than that of the bleach-adapted rod. In contrast, exposure of bleach-adapted cones to 11-cis 13-demethylretinal resulted in an immediate deactivation of transduction as measured by the same parameters. These results extend the validity of a model for the effects of the noncovalent binding of a retinoid in the chromophore pockets of rod and cone opsins to analogs capable of forming a Schiff-base and imply that the noncovalent binding by itself may play a role for the dark adaptation of photoreceptors.

key words: opsin • 11-cis retinal • photoreceptors • pigment regeneration • dark adaptation

INTRODUCTION

Light initiates a cascade of biochemical events in vertebrate photoreceptors that begins with the photoisomerization of the 11-cis retinal and results in the visual response (for reviews, see Stryer, 1991; Lagnado and Baylor, 1992; Pugh and Lamb, 1993; Yau, 1994). Photon absorption also initiates the destruction of visual pigment and activates a second biochemical pathway that regenerates bleached pigment and results in the restoration of sensitivity (Crouch et al., 1996). However, little is known about the dynamic relationship between the intermediate steps in visual pigment regeneration and dark adaptation.

The principal biochemical events that occur during dark adaptation are well established (for review, see Leibrock et al., 1998). After its release from opsin, all-trans retinal is reduced to all-trans retinol by retinol dehydrogenase (Saari et al., 1993), transported out of the rods and cones, and carried to the retinal pigment epithelium by interphotoreceptor matrix retinoid binding protein (IRBP) (Bok, 1990; Saari, 1994; Crouch et al., 1996). In the pigment epithelium, all-trans retinol is either converted back to 11-cis retinal or stored in one of the ester forms (Bernstein and Rando, 1986). The 11-cis retinal is then returned in association with IRBP through the interphotoreceptor matrix to the outer segments of photoreceptors (Okajima et al., 1990), where it condenses with opsin to produce new visual pigment.

Visual pigment formation has been proposed to occur in two steps (Matsumoto and Yoshizawa, 1975). In rods, the first step is a noncovalent interaction between the ring end of the chromophore and an anchoring site deep in the opsin pocket. The exact conformation of this site is unknown; however, methyl groups at the 1 and 5 positions on the ionone ring appear to be essential (Crouch and Or, 1983). The second step is the formation of a Schiff-base bond between a lysine and the terminal carbon of retinal. It is now generally accepted that, in rods at least, the recovery of sensitivity occurs as the Schiff-base is protonated and the transduction cascade is deactivated (Cohen et al., 1992).

Recent experiments have suggested that these two steps may have distinct physiological consequences and

that dark adaptation may be more complicated than previously thought (Jin et al., 1993; Buczylko et al., 1996; Kefalov et al., 1999). It now appears from biochemical as well as physiological experiments that the noncovalent binding of retinal to opsin that must occur before the formation of the Schiff-base bond may have important effects on dark adaptation. A number of derivatives of retinal have been found that bind to opsin, but by virtue of their shortened polyene chains, are unable to form a Schiff-base bond. These compounds, including 9-cis C-17 aldehyde, all-trans C-17 aldehyde, and β-ionone, have been shown by competitive binding studies to attach to the same site as 11-cis retinal (Matsumoto and Yoshizawa, 1975; Daemen, 1978; Towner et al., 1981; Crouch et al., 1982). Binding of these compounds to bleached visual pigment results in a complex that is capable of activating transducin (Fukada and Yoshizawa, 1981) as well as promoting opsin phosphorylation by rhodopsin kinase (Buczylko et al., 1996). The importance of these biochemical observations is underscored by recent physiological experiments in which both bleached rods and cones have been exposed to β-ionone. Treatment of bleached rods with β-ionone further activates the transduction cascade and increases their desensitization (Kefalov et al., 1999), whereas the same treatment of bleached cones causes recovery of sensitivity (Jin et al., 1993). These results suggest that not only are there important physiological consequences of the noncovalent binding of retinoids into the binding site of opsin, but that the effects on the sensitivity of the photoreceptor are quite different for rods and cones.

The experiments described here were designed to examine and compare the physiological effects of noncovalent and covalent interactions of chromophore and opsin that normally occur during dark adaptation. 11-Cis 13-demethylretinal is a useful tool to address this question. This retinoid has been shown to bind into the chromophore pocket of opsin and to form a Schiff-base linkage with opsin. However, the latter step occurs about nine times slower than with the native 11-cis retinal (Nelson et al., 1970). Thus, the lifetime of the 11-cis 13-demethylretinal • opsin noncovalent intermediate is several times longer that the corresponding 11-cis retinal • opsin complex. Experimentally, this slow rate of pigment formation allows sufficient time for physiological examination of the effects of the initial binding of the retinoid in the pocket and of the subsequent formation of the protonated Schiff-base linkage. Biochemical experiments have shown that the addition of 11-cis 13-demethylretinal to opsin initially activates transducin (Tan et al., 1998) and rhodopsin kinase (Buczylko et al., 1996). The effect is time dependent and decays in both cases with a time constant of ∼40 min. The inactivation is accompanied by the formation of 13-deme-

thyl rhodopsin, and is presumably due to the formation of a protonated Schiff-base linkage between the chromophore and the protein (Tan et al., 1998). 13-Demethyl rhodopsin has spectral properties almost identical to native rhodopsin (Nelson et al., 1970), it is capable of activating transducin in a light-dependent manner (Ebrey et al., 1980), and is phosphorylated at the same sites and with the same affinity by rhodopsin kinase as the native rhodopsin (Buczylko et al., 1996).

The first part of this article describes experiments that were designed to investigate the separate effects of the noncovalent and covalent interactions between retinal and rod opsin by studying the effect of introducing 11-cis 13-demethylretinal in bleach-adapted rods. The second part deals with the corresponding effects of 11-cis 13-demethylretinal in bleach-adapted cones.

Some of these experiments have been reported previously at meetings of the Biophysical Society (Corson et al., 1995), and the Association for Research in Vision and Ophthalmology (Cornwall et al., 1996; Corson et al., 1999).

MATERIALS AND METHODS

All experiments were performed according to procedures approved by the Animal Use and Care Committee of Boston University School of Medicine as being consistent with humane treatment of laboratory animals, and with standards set forth in the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act.

Rod photoreceptors were mechanically isolated from dark-adapted retinae of larval tiger salamander *(Ambystoma tigrinum)* by methods that have been described previously (Cornwall et al., 1990). After dark-adaption overnight, animals were decapitated, the head and body were pithed, and the eyes were enucleated in physiological saline solution. The eyes were hemisected and the retinae were removed and isolated from the pigment epithelium. Cells were mechanically isolated by chopping the retina into small pieces and passing the solution containing retinal fragments through a heat polished Pasteur pipette. A small portion of the resulting suspension was then placed in a chamber located on the stage of an inverted microscope (Invertescope D; Carl Zeiss, Inc.), where individual cells in the suspension were viewed using an infrared television camera and monitor fitted to the microscope.

Measurements of membrane current were made extracellularly by methods that have been described previously (Baylor et al., 1979; Cornwall et al., 1990). Individual rods were identified by their characteristic shape and were drawn, inner segment first, into the tip of a glass micropipette that contained physiological solution. Pipette tips were heat polished to have an internal diameter of ∼10 μm, just slightly smaller than the diameter of the ellipsoid portion of the photoreceptor. The physiological salt solution in the pipette was connected via a silver/silver chloride junction to the head stage of a patch clamp amplifier (EPC-7; List Electronic). The current recorded from the cell was converted to voltage, amplified, and low-pass filtered with an active eight-pole filter at 20 Hz cutoff frequency (902LPF; Frequency Devices Inc.). These data were digitized at 250 Hz, stored on a computer, and subsequently analyzed using the pCLAMP 6 data acquisition and analysis software (Axon Instruments, Inc.) and the Origin 4 graphics and data analysis software (Microcal Software).

Light Stimulation

An optical stimulator provided test flashes as well as bleaching lights (Cornwall et al., 1990). The light source was calibrated at the beginning of each experiment with a photometer (80X; United Detector Technology). The absolute intensity of the test flash/bleaching beam used in the rod experiments was 1.36×10^7 photons $\mu m^{-2} s^{-1}$ (500 nm); the absolute intensity for the cone experiments beam was 3.43×10^7 photons $\mu m^{-2} s^{-1}$ (600 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 $F = 1 - \exp(-IPt)$, where F is the fraction of bleached pigment, I is the absolute light intensity in photons $\mu m^{-2} s^{-1}$, and t is the duration of light exposure in seconds. The value used for the photosensitivity of the cell, P, was 6.2×10^{-9} μm^2 for rods (Jones, 1995) and $6.0 \times 10^{\times 9}$ μm^2 for cones (Jones et al., 1993).

Solutions

The recording chamber was perfused with a saline solution that contained (mM): 110 NaCl, 2.5 KCl, 1.6 MgCl$_2$, 1.0 CaCl$_2$, 10 dextrose, 10 HEPES, pH 7.8, and bovine serum albumin (100 mg liter^{-1}). Entry of solution into the chamber was via a gravity-fed inlet supplied from a reservoir; outflow occurred through a tissue wick located in a trough at one end the chamber.

Test solutions were delivered to the region surrounding the outer segment of the cell by a rapid microperfusion system. The construction and use of this system has been described previously (Cornwall and Fain, 1994; Kefalov et al., 1999). This device consisted of two pipettes, each having an inner diameter of ~90 μm, mounted on separate micromanipulators. The pipettes were positioned in the bath so that their tips were opposed, and ~200 μm apart. Solution was propelled from the end of one pipette, and aspirated into the tip of the other by gravity in such a way that a laminar plume of fluid with discrete boundaries was formed between them. The manipulators supporting these pipettes were mounted on a micrometer-driven platform whose lateral displacement was actuated by a computer-driven stepping motor. This arrangement allowed the movement of the plume of fluid laterally so as to surround the outer segment of the cell and expose it to a test solution for a predetermined time. The time required for a complete solution change, estimated from the junction current recorded while the cell was exposed to bright light, was ~25 ms. The test solutions (see below) were delivered to the inlet pipette from either of two reservoirs by a multiway valve. The time necessary for replacing the solution in the tubing and pipette was ~45 s, allowing exposure of the cell to two different test solutions within 1 min.

The test solution in which the rate constant of guanylyl cyclase was measured (3-isobutyl-1-methylxanthine, IBMX, solution)[1] was made by adding IBMX to normal saline solution to a final concentration of 500 μm. The solution in which the rate constant of phosphodiesterase was measured (Li$^+$ solution) was made by replacing NaCl in the saline solution with equimolar LiCl.

11-Cis 13-demethylretinal was synthesized as described previously (van den Tempel and Huisman, 1966). The product was purified by HPLC, characterized by absorbance and nuclear magnetic res-

---

onance spectra, and stored at $-70°C$ under argon until used. 13-Demethylretinal was delivered to the cells using phospholipid vesicles (Perlman et al., 1982; Yoshikami and Noll, 1982; Jones et al., 1989). Vesicles were prepared by placing 25 mg 1-α-phosphatidylcholine dissolved in a chloroform/methanol solution (Sigma-Aldrich) in a vial and evaporating the solvent under a stream of nitrogen gas. Any residual solvent was removed by lyophilizing the sample for 10 min. 10 ml of saline solution was added to the vial, and the contents were sonicated for 10 min in an ice bath (1 s on/1 s off) at 95 W using a 1.0-cm probe (Vibracell High Intensity Ultrasonic Processor; Sonics and Materials). 1.5 ml of the resulting vesicle solution was added to a vial containing about 300 μg 11-cis 13-demethylretinal and the contents were sonicated for 1.5 min at 30 W. The exact concentration of the retinoid was determined spectrophotometrically by making a 10-fold dilution in ethanol (molar extinction coefficients for 11-cis 13-demethylretinal of 12,600 M^{-1} cm^{-1} at 380 nm) and adjusted to 100 μm. 0.5 ml of the retinoid solution was added to the experimental chamber using a calibrated pipetter. No significant photoisomerization of the retinoid in the chamber was expected to occur because of the low intensity and unfavorable spectral composition of the test flashes. All manipulations were done under dim red/infrared light.

Fig. 1 shows an average absorbance spectrum of 11-cis 13-demethyl rhodopsin in isolated salamander rod outer segments measured using a photon-counting microspectrophotometer (Corson et al., 1994). In this experiment, rods were exposed to a bright light calculated to have bleached >99% of the rhodopsin, and then were treated with a vesicle solution containing 11-cis 13-demethylretinal. The continuous line fitted to the data is an exponential visual pigment template (Stavenga et al., 1993). The wavelength of peak absorbance of the fitted curve was 488 nm. The graph in the inset shows a normalized bleaching difference spectrum of a single rod outer segment loaded with 11-cis 13-demethyl rhodopsin and subsequently exposed to a bright bleaching light, and demonstrates that this analog visual pigment is photolabile.

Theory and Analysis of Data

To estimate the change in the rate constants (to be referred to simply as rates) of cGMP phosphodiesterase (PDE) and guanylyl cyclase (GC), we used a method originally devised by Hodgkin and Nunn (1988) and most recently used in a modified form by Kefalov et al. (1999) (see also Cornwall and Fain, 1994). In brief, the concentration of cGMP in the cell is controlled by the balance between its synthesis by guanylyl cyclase and its hydrolysis by phosphodiesterase (Eq. 1):

$$\frac{d}{dt}[cGMP] = \alpha[GTP] - \beta[cGMP], \qquad (1)$$

where α is the rate of GC, and β is the rate of PDE (Hodgkin and Nunn, 1988). Combining this expression with the relation between concentration of cGMP and current (Pugh and Lamb, 1990), one can derive a relation between the rates of GC and PDE and the amplitude of the current:

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

where i is the photocurrent, 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 corresponding Hill coefficient. Sudden block of GC ($\alpha \cong 0$) or of PDE ($\beta \cong 0$) by the corresponding test solution allows derivation from Eq. 2 of a formula for the rate of the other enzyme as a function of the cur-
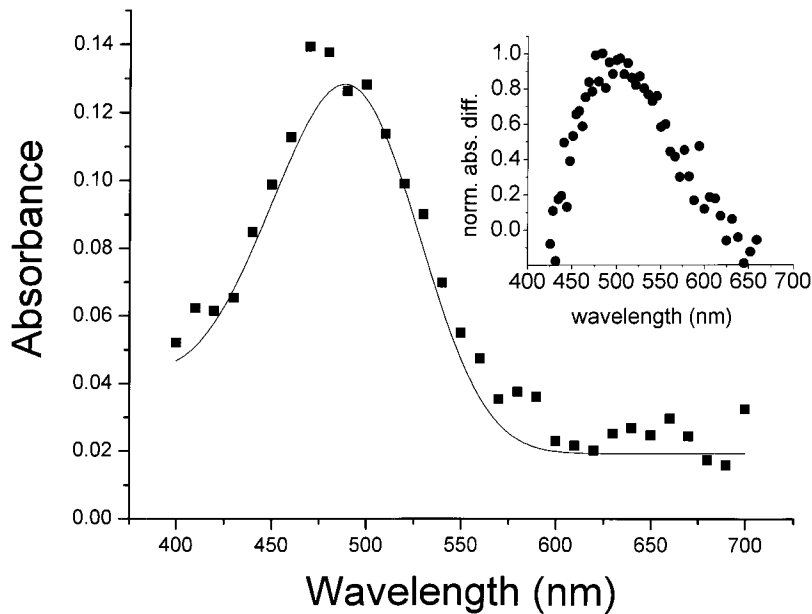
Figure 1. Absorbance spectrum of 11-cis 13-demethyl rhodopsin. Transverse absorbance measurements were averaged from four rods after bleach and regeneration with 11-cis 13-demethylretinal (■). A pigment absorbance template (smooth curve) was calculated by nonlinear curve-fitting of an exponential template for a vitamin A1 visual pigment to estimate the peak wavelength (488 nm), maximum pigment absorbance (0.11), and residual absorbance (0.02) at long wavelengths (Stavenga et al., 1993). (Inset) The normalized bleaching difference spectrum (●) of this photolabile pigment in a single cell in the presence of hydroxylamine.

rent. Thus, assuming that the channel characteristics ($K_{1/2}$ and N) and i_m do not change during the step in the test solution, one can obtain the relative rate of PDE compared with that of the dark-adapted cell (see Kefalov et al., 1999, for details) (Eq. 3):

$$\frac{\ln(J)}{\ln(J_D)} = \frac{\beta}{\beta_D}, \qquad (3)$$

where D indicates the parameters of the cell in its dark-adapted state and J is the normalized current, i/i_D, or the current during the solution change, expressed as a fraction of the steady state membrane current of the dark-adapted cell. After subtracting the junction current, β was estimated from the slope of the normalized current change plotted on a semilogarithmic graph and then the ratio β/β_D was taken. Using the same assumptions as in the case of PDE, the relative rate of GC (Eq. 4) can also be derived from Eq. 2:

$$\frac{K_{1/2}\frac{d}{dt}[i^{1/3}]}{K_{1/2}^D\frac{d}{dt}[(i^{DA})^{1/3}]} = \frac{\alpha}{\alpha_D}, \qquad (4)$$

where N was taken to be equal to 3 (Yau and Baylor, 1989) and i^{DA} is the membrane current during the solution change of the dark-adapted cell. The time derivative of the current di/dt was calculated using the Savitzki-Golay method (Press et al., 1992). The ratio $K_{1/2}/K_{1/2}^D$ was estimated from the corresponding change in the current (see Figure 6 of Nakatani et al., 1995) and then the ratio α/α_D was calculated.

RESULTS

Bleach-adapted Rods

Photocurrent and light sensitivity. Current recordings were made from isolated photoreceptors in their dark-adapted state, then after a bleach, and finally after application of 11-cis 13-demethylretinal. The experiment illustrated in Fig. 2 shows the effect of 11-cis 13-demethylretinal on the dark current and on the sensitivity of a solitary bleach-adapted rod. The cell was stimulated with a series of test flashes increasing in intensity in half log unit steps to monitor changes in the amplitude of the current and sensitivity. Fig. 2 A shows a series of photoresponses recorded from the dark-adapted rod. After bleaching 5% of the pigment, the cell was allowed 20 min to recover to a steady state and a second set of photoresponses was recorded (Fig. 2 B). It can be seen that the bleach caused a small reduction in the dark current of ∼5 pA. Next, 0.5 ml of saline solution containing vesicles loaded with 11-cis 13-demethylretinal (100 μm in bulk stock solution) was injected into the chamber and allowed to remain there for several minutes before being washed out. When the bleach-adapted rod was exposed to 11-cis 13-demethylretinal (11-cis 13-DM retinal), the current decreased within 20 s to a new lower steady state level (Fig. 2 C).

After the initial decrease in dark current and sensitivity caused by the treatment with 11-cis 13-demethylretinal, partial recovery was observed, even while the cell was still exposed to the retinoid solution. The final steady state dark current (not shown) was greater than the current immediately after exposing the cell to the 11-cis 13-demethylretinal, but it was still less than the current observed in the bleach-adapted state before the retinoid treatment. The kinetics of this partial recovery were studied in detail using repetitive measurements of the rate of guanylyl cyclase and will be discussed below. Unless otherwise specified, all subsequent measurements were made within 5 min of exposure to 11-cis 13-demethylretinal.

Peak photocurrent amplitudes for each set of photoresponses from Fig. 2, A–C, were plotted against flash photon density and fitted with a single hyperbolic satu-
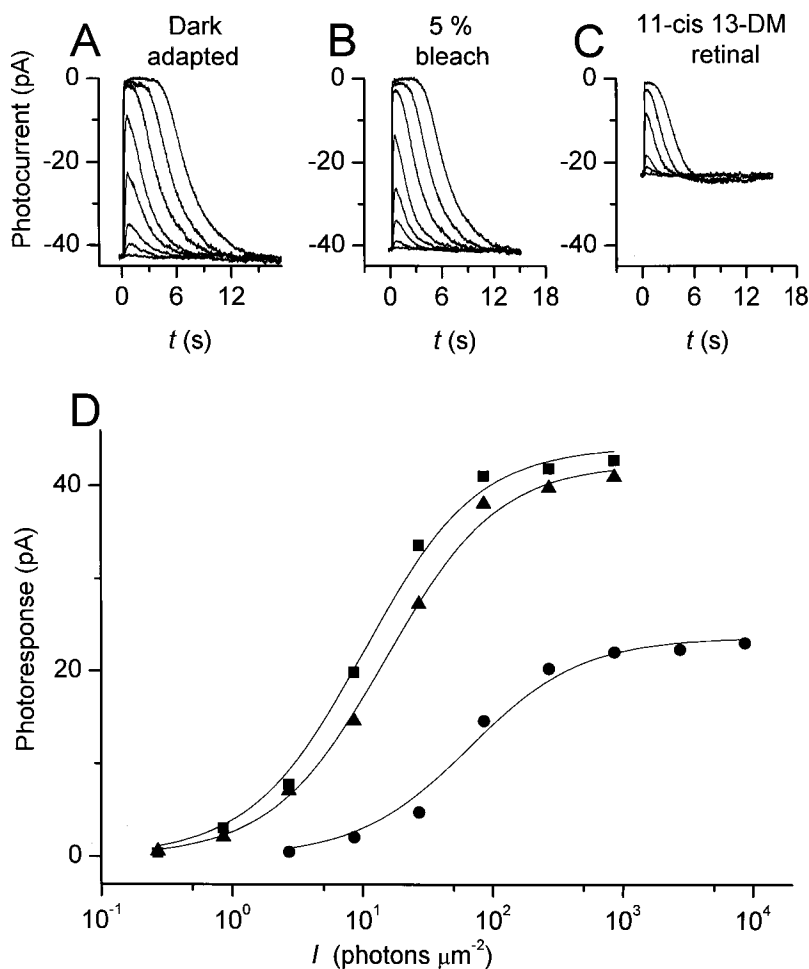
Figure 2. Effect of 11-cis 13-demethylretinal on the membrane dark current and on the stimulus–response relations in a rod. Shown is a series of flash responses from one cell in its dark-adapted state (A), after a 5% bleach (B), and in 100 μm 11-cis 13-demethylretinal solution (C). The corresponding intensity–response curves for the peak amplitude of the change in the dark current as a function of flash intensity are shown in D: dark-adapted (■), bleach adapted (▲), and in 11-cis 13-demethylretinal (●).

rating function (Naka and Rushton, 1966) (Fig. 2 D). As expected, the 5% bleach (▲) caused a small decrease in the sensitivity and dark current of the rod from the dark-adapted state (■). Treating the bleached rod with 11-cis 13-demethylretinal produced an additional large decrease in the sensitivity and dark current (●).

The effect of 11-cis 13-demethylretinal on the dark current and on the sensitivity was studied in a total of 27 bleach-adapted rods. All rods exhibited a decrease in both the dark current and in the sensitivity in the presence of 11-cis 13-demethylretinal. The bleaches used in these experiments varied from 5 to 20%. The effect of 11-cis 13-demethylretinal on the dark current and the sensitivity was studied in 22 cells in which 5% of the pigment was bleached. Under these conditions, the dark current recorded under steady state was reduced to $92 \pm 1\%$ (SEM, $n = 22$) of its dark-adapted value. Sensitivity was reduced by 0.30 ± 0.14 log units (SEM, $n = 18$), as measured from changes in the ratio of the response maximum to the semisaturating light intensity of the hyperbolic stimulus–response functions, $S = R_{max}/I_s$ (Baylor et al., 1979). Treatment with 11-cis 13-demethylretinal caused a further decrease in the dark current to $39 \pm 3\%$ (SEM, $n = 22$) of its

bleach-adapted value. The corresponding decrease in the sensitivity in the first 2 min after the treatment was on average 0.63 ± 0.34 log units (SEM, $n = 18$). In 20 of 22 cells, the initial decrease in the dark current was followed by a small recovery. The dark current in the steady state, measured several minutes after introducing the 11-cis 13-demethylretinal recovered to $59 \pm 2\%$ (SEM, $n = 20$) of the bleach-adapted value.

This recovery of the dark current was accompanied by a slight increase in the sensitivity. Due to the transient nature of the effect, and the fact that several minutes of measurements are required to estimate the sensitivity of the cell, the exact value of this increase could not be measured.

Seven cells were treated a second time with 11-cis 13-demethylretinal. In six of these, the dark current decreased once again to its value measured immediately after the first treatment with 11-cis 13-demethylretinal. In the seventh cell, the second treatment with the retinoid did not induce any change in the dark current.

The effect of 11-cis 13-demethylretinal on the dark current and the sensitivity was also studied in a small number of rods where 10 or 20% of the pigment was bleached. As expected, the decrease in the current

compared with the dark-adapted state was higher for the higher bleaches. In rods exposed to a 10% bleach, the dark current was reduced to $76.0 \pm 6.4\%$ (SEM, $n = 3$) of its dark-adapted value. After a 20% bleach, the dark current was reduced to $39.5 \pm 2.5\%$ (SEM, $n = 2$) of that observed before the bleach. However, the further decrease in the current from the bleach-adapted level caused by 11-cis 13-demethylretinal was comparable in the two cases, and was similar to that observed after the 5% bleach: $41.7 \pm 15.2\%$ (SEM, $n = 3$) for the 10% bleach; and $44.5 \pm 3.5\%$ (SEM, $n = 2$) for the 20% bleach. Thus, the percentage decrease of the dark current in the presence of 11-cis 13-demethylretinal was independent of the bleach fraction for the bleaches used in this study. This observation implies that the fraction of opsin bound to 11-cis 13-demethylretinal was the same in each case.

Dim flash kinetics. Analysis of the time course of responses to dim flashes when photoreceptors are in a different state of adaptation or after a drug treatment can provide information about kinetic changes taking place in the transduction cascade (Baylor and Hodgkin, 1974; Baylor et al., 1979; Jones et al., 1993). Accordingly, we investigated the effect of 11-cis 13-demethylretinal on the kinetics of the photoresponse in bleach-adapted rods. Fig. 3 shows normalized dim flash responses, elicited from one rod in its dark-adapted state, after a 5% bleach, and in the presence of 11-cis 13-demethylretinal. As expected, the bleach accelerated the photoresponse. Fig. 3 shows that the time to peak decreased to 74% of the corresponding dark-adapted time. Treatment with 11-cis 13-demethylretinal accelerated the response even further and the time to peak decreased to 53% of the dark-adapted value. No significant change in the kinetics of the photoresponse was observed once the cell was treated with 11-cis 13-demethylretinal, indicating that the effect of the retinal analog on response kinetics is not reversible. 11-Cis 13-demethylretinal caused acceleration of the dim flash response in all 22 rods tested.

Rate of guanylyl cyclase. We performed a series of experiments designed to look directly at the effect of 11-cis 13-demethylretinal on the rates of cyclase and phosphodiesterase in bleach-adapted rods. The methods used for measuring the rates of these two enzymes have been described previously (Hodgkin and Nunn, 1988; Cornwall and Fain, 1994; Kefalov et al., 1999). Fig. 4 shows an example of one such experiment in which guanylyl cyclase was studied. Using the microperfusion system, the rod was quickly exposed to saline test solution containing 500 μm IBMX 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 at the top. As argued in materials and methods, the time derivative

of $J^{1/3}$ is proportional to the rate of change in cGMP concentration and 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 rod, where it had only a low basal activity (Fig. 4 A). After a 5% bleach, and after a recovery period of several minutes, the rate of cyclase was only slightly higher (Fig. 4 B). Next, 0.5 ml of a vesicle solution containing 100 μm 11-cis 13-demethylretinal was injected into the chamber and allowed to remain there for several minutes before being washed out. Exposure of bleach-adapted rod to 11-cis 13-demethylretinal produced an initial sevenfold acceleration of cyclase (Fig. 4 C). The measurement of the rate of cyclase was done 1 min after the treatment with the retinoid. However, judging from the change in the dark current, this activation of the transduction cascade was completed within 10 s after the analog was added to the chamber. The recovery of the current after its initial increase during the jump in IBMX solution (Fig. 4 C, left) is most likely due to the action of the calcium-driven feedback on the activated phototransduction cascade.

After the initial large acceleration, the rate of cyclase gradually decreased over the next 40 min. The steady state rate of cyclase was significantly reduced compared with the rate immediately after the treatment and was only threefold higher than in the bleach-adapted state (Fig. 4 D).

Thus, treatment of bleach-adapted rods with 11-cis 13-demethylretinal resulted in an initial large acceleration of the cyclase rate that was followed by a partial recovery. The steady state rate after the treatment was always lower than the initial high rate, but still significantly higher than that of the bleach-adapted state.

The effect of 11-cis 13-demethylretinal on the rate of cyclase was studied in a total of 24 bleach-adapted rods. In all of these, retinal caused acceleration of guanylyl cyclase. The range of pigment bleaches was from 5 to 20%. Higher bleaches were not used because of the difficulty in estimating the significantly higher rates of cyclase and phosphodiesterase when these cells were exposed to 11-cis 13-demethylretinal.

The effect of 100 μm 11-cis 13-demethylretinal in rods after a 5% bleach was studied in detail. In a total of 17 cells under these conditions, the rate of cyclase after the bleach was on average 1.6 ± 0.1 (SEM, $n = 17$) times higher than in the corresponding dark-adapted state. Treatment initially accelerated cyclase to a rate that was, on average, 4.4 ± 0.4 (SEM, $n = 17$) times higher than in the bleach-adapted state. The steady state rate of cyclase in the presence of 11-cis 13-demethylretinal was $54 \pm 3\%$ (SEM, $n = 11$) of the rate of cyclase measured immediately after the addition of the retinoid, but still about twofold higher than the pretreatment, bleach-adapted rate of cyclase.
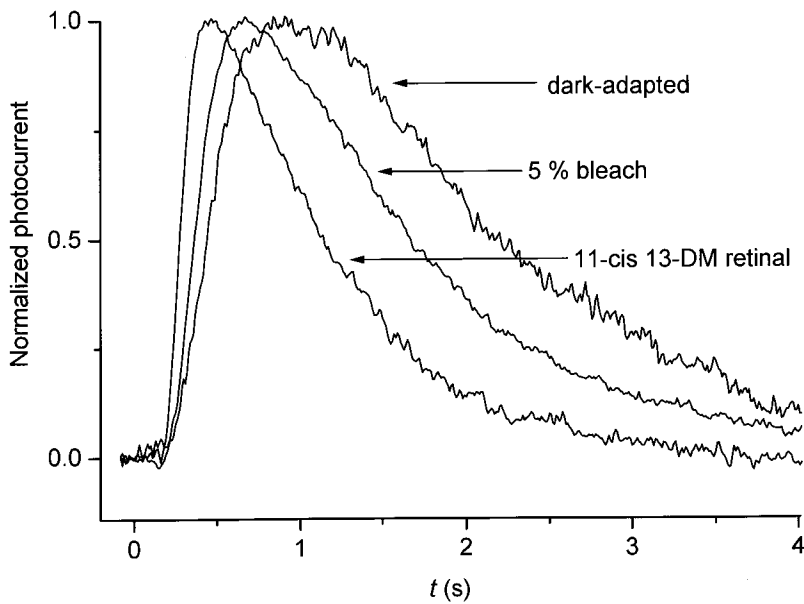
Figure 3. Effect of 11-cis 13-demethylretinal on the kinetics of dim flash responses in a rod. Normalized dim flash responses from one rod in a dark-adapted state, after a 5% bleach, and in the presence of 100 μm 11-cis 13-demethylretinal. The flash intensities were 0.9, 2.7, and 27 photons/μm^2 and the corresponding response amplitudes were 2.9, 6.4, and 4.4 pA, respectively.
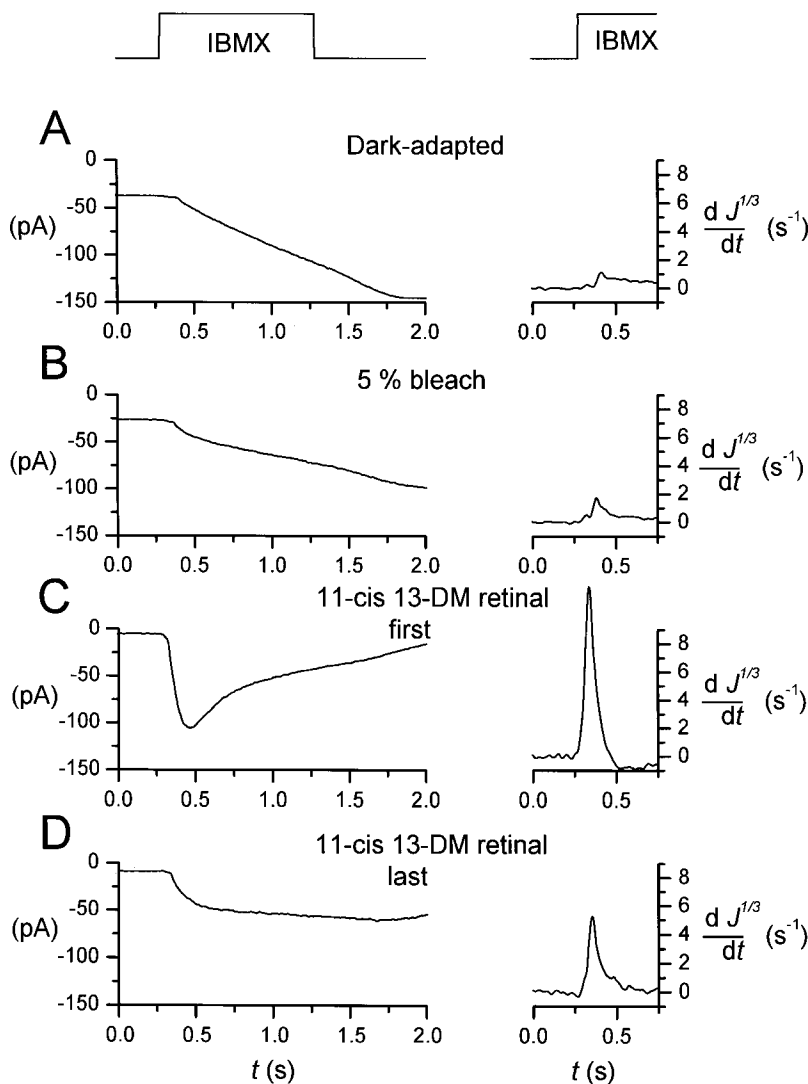

Figure 4. Effect of 11-cis 13-demethylretinal on the rate of guanylyl cyclase in a rod. (Left) The current recordings from a rod during steps into 0.5 mM IBMX solution. (Right) The derivative of the cube root of the normalized current, $d(J^{1/3})/dt$. The time course of the solution step for both panels is shown at the top. Recordings were made first in the dark-adapted state (A), then after a 5% bleach (B), immediately after exposing the cell to 100 μm 11-cis 13-demethylretinal solution (C), and 30 min after exposing the cell to 11-cis 13-demethylretinal (D). Each current trace is the average of six measurements. All recordings were done in the same cell.

We also studied the effect of 11-cis 13-demethylretinal after 10% bleach in five cells. The acceleration of cyclase caused by the 10% bleach was higher than that of the 5% bleach. The rate of cyclase after the bleach was on average 2.3 ± 0.5 (SEM, n = 5) times higher than in the dark-adapted state. Immediately after adding 11-cis 13-demethylretinal to the bleach-adapted rods, the rate of cyclase was accelerated further to 3.1 ± 0.9 (SEM, n = 5) times that of the bleach-adapted state. The steady state rate of cyclase in the presence of 11-cis 13-demethylretinal was still higher than the rate of the bleach-adapted cell, but it was reduced to 60 ± 10% (SEM, n = 2) of the rate of cyclase immediately after the addition of the retinoid.

In the two cells where the effect of the analog was studied after 20% bleach, the rate of cyclase was accelerated by the bleach on average to 3.5 ± 0.5 (SEM, n = 2) times the rate in the corresponding dark-adapted state. The treatment with 11-cis 13-demethylretinal initially accelerated cyclase 3.1 ± 0.7 (SEM, n = 2) times compared with the bleach-adapted state. The steady state rate of cyclase in the presence of 11-cis 13-demethylretinal was 60 ± 20% (SEM, n = 2) of the initial value, but still about twofold higher than the pretreatment, bleach-adapted steady state value.

The time course of deactivation of cyclase that followed the initial acceleration immediately upon treating the rod was studied in detail in seven cells where 5% of the pigment was bleached. One such experiment is shown on Fig. 5. The decrease in the rate of cyclase in each case could be expressed by a single exponential decay function. The time constant for the cell shown in Fig. 5 was 22.4 min. The rate of deceleration of cyclase varied significantly from cell to cell and on average it

was 17.0 ± 2.2 (SEM, n = 7). This is somewhat faster than the rate of inactivation expected from biochemical experiments (Buczylko et al., 1996; Tan et al., 1998).

The cause for the observed deactivation of the transduction cascade that followed the initial activation by 11-cis 13-demethylretinal was examined next. Because deactivation was observed whether or not excess 11-cis 13-demethylretinal was removed from the chamber, washout of the retinoid could be ruled out as the cause for the slow deactivation of transduction. Another possibility is that the reversal of the initial effect is due to the formation of covalent linkage between the retinoid and free opsin. If that were the case, at least a fraction of the free opsin produced by the bleach would be converted to 11-cis 13-demethyl rhodopsin and, as a result, the amount of free opsin in the rod would be reduced. Therefore, it would be expected that a second treatment with 11-cis 13-demethylretinal would produce a smaller effect or no effect at all, depending on the residual amount of free opsin.

The rate of cyclase was monitored in four bleached rods that were treated multiple times with 11-cis 13-demethylretinal. The retinoid solution was allowed to stay in the chamber for several minutes and then was washed out. Once the rod had reached a steady state after the addition of analog, a second dose of the vesicle solution was introduced into the chamber and its effect on the rate of cyclase was measured. In all experiments, the second treatment induced an additional acceleration of guanylyl cyclase from its steady state rate (Fig. 5, right arrow). The effect of the second treatment with 11-cis 13-demethylretinal on the rate of cyclase in three cells was smaller than the corresponding effect after the first treatment (Fig. 5). In the fourth cell, each of
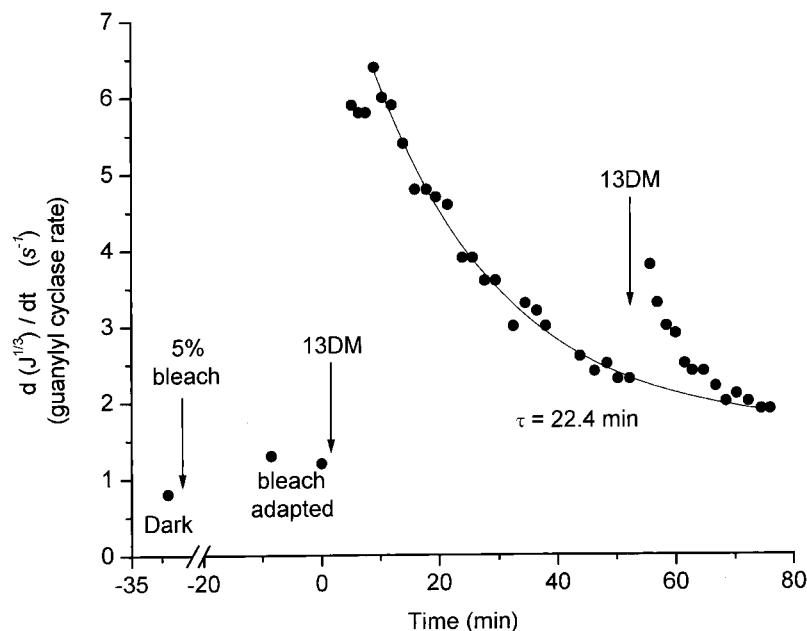

Figure 5. Time course of the recovery of guanylyl cyclase after its initial acceleration by 11-cis 13-demethylretinal in a rod. Each data point represents one measurement of the rate of cyclase done by exposing the cell to IBMX solution. Shown are the dark-adapted rate of cyclase (Dark), its steady state bleach-adapted rate after 5% bleach (bleach adapted) and its change as a result of two repetitive treatments with 11-cis 13-demethylretinal (13DM, arrows). The recovery from the initial activation of cyclase by the first treatment with 11-cis 13-demethylretinal is fit by a single exponential decay function with a time constant of 22.4 min.

four repetitive treatments with 11-cis 13-demethylretinal induced approximately the same effect on the rate of cyclase (data not shown). The result of this experiment supports the notion that the reversal of the initial effect is due to the formation of covalent linkage between the retinoid and free opsin. Also in support of this hypothesis is the observation that, similar to the relative rates of pigment regeneration measured in biochemical experiments (Nelson et al., 1970), the rate of deceleration of cyclase after treatment with 11-cis 13-demethylretinal is four to five times slower than the corresponding deceleration after treatment with 11-cis retinal (manuscript in preparation).

Rate of phosphodiesterase. The effect of 11-cis 13-demethylretinal on the rate of phosphodiesterase in bleach-adapted rods was also studied. The protocol for delivering the retinoid was the same as just described for the case of guanylyl cyclase. Fig. 6 shows one example where the effect of 100 μm 11-cis 13-demethylretinal on the rate of phosphodiesterase was studied in a rod after bleaching 5% of the pigment. Fig. 6 (left) shows current recordings during the solution change. Fig. 6 (right) plots J on a semilogarithmic scale. The slope of this trace, as argued in materials and methods is proportional to the rate of cGMP hydrolysis by phosphodiesterase in this case. Bleaching 5% of the pigment produced an acceleration of phosphodiesterase by 50% (compare Fig. 6, A and B). The first measurement of the rate of phosphodiesterase after treating the rod with the analog was performed about 3 min after adding the vesicle solution to the chamber. As a result of the treatment with the retinoid, the rate of phosphodiesterase was increased over threefold compared with the bleach-adapted rate (Fig. 6 C). Measurement of phosphodiesterase 50 min later revealed that the rate of phosphodiesterase has decreased and for this cell was about two times higher than the pretreatment, bleach-adapted rate (Fig. 6 D).

The effect of 11-cis 13-demethylretinal on the rate of phosphodiesterase in bleach-adapted rods was studied in a total of 20 cells. The bleach fraction varied from 5 to 20% and the concentration of 11-cis 13-demethylretinal in all experiments was 100 μm. In all 20 bleach-adapted rods tested, 11-cis 13-demethylretinal caused acceleration of phosphodiesterase. In the 16 cells where 5% of the pigment was bleached, the rate of phosphodiesterase in the bleach-adapted state was, on average, 1.8 $\pm$ 0.1 (SEM, $n = 16$) times higher than in the corresponding dark-adapted state. The rate of phosphodiesterase immediately after the addition of the retinoid solution to the bath was, on average, 2.4 $\pm$ 0.1 (SEM, $n = 16$) times higher than in the bleach-adapted state.

An attempt was made to study the time course of the recovery of phosphodiesterase after its initial accelera-
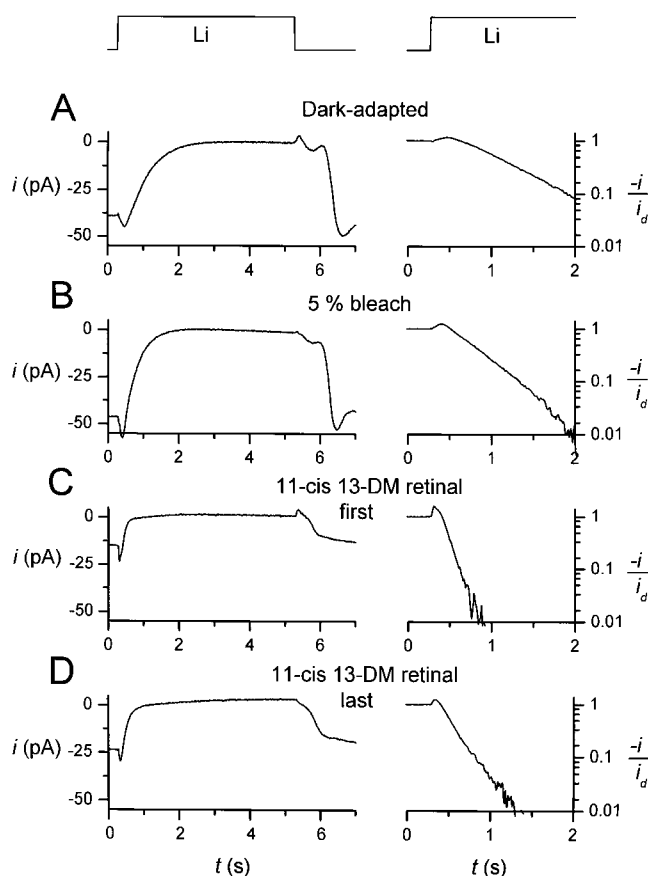

Figure 6. Effect of 11-cis 13-demethylretinal on the rate of cGMP phosphodiesterase in a rod. (Left) The current from a rod during steps into Li$^+$ solution. (Right) The normalized current on a semilogarithmic plot. The time course of the solution step is shown at the top. Recordings were made first in the dark-adapted state (A), and then after a 5% bleach (B), immediately after exposing the cell to 100 μm 11-cis 13-demethylretinal solution (C), and 30 min after exposing the cell to 11-cis 13-demethylretinal (D). Each current trace is the average of six measurements. All recordings were done in the same cell.

tion after treatment with 11-cis 13-demethylretinal. However, the rate of phosphodiesterase did not change significantly after its initial acceleration in the five rods that were studied. The reason for this lack of effect as compared with the experiments in which the cyclase rate was measured (see Fig. 5) is not known.

In the four cells where the bleach fraction was 10 or 20%, the acceleration of phosphodiesterase by both the bleach and the treatment with 11-cis 13-demethylretinal was higher than in the 5% bleach experiments. For the case of 10% bleach, the rate of phosphodiesterase in the bleach-adapted state was 2.0 $\pm$ 0.1 (SEM, $n = 2$) times higher than in the dark-adapted state. The rate of phosphodiesterase in the presence of 11-cis 13-demethylretinal was 2.5 $\pm$ 1.3 (SEM, $n = 2$) times higher than in the bleach-adapted state. For the 20% bleach, the corresponding increase was 3.4 $\pm$ 0.4

(SEM, $n = 2$) for the bleach-adapted to dark-adapted ratio, and 2.7 ± 1.3 (SEM, $n = 2$) for the acceleration caused by the retinoid.

Phototransduction in dark-adapted rods in the presence of 11-cis 13-demethylretinal. Control experiments were performed to see if the effect induced by 11-cis 13-demethylretinal in bleach-adapted rods is the result of its binding directly in the chromophore pocket of opsin. The effect of treatment with the retinoid was studied in four dark-adapted rods. In all of them, treatment with 11-cis 13-demethylretinal caused a slight acceleration of the transduction cascade, as evinced by the slight decrease in the dark current and in the sensitivity, as well as by the small acceleration of the dim flash response and of the rates of cyclase and phosphodiesterase (data not shown). However, the amplitude of the changes induced by this treatment was significantly smaller than the corresponding effects after bleaching as little as 5% of the pigment. The rate of cyclase increased on average by 60% and the rate of phosphodiesterase increased by 20%, compared with the severalfold increase in these rates caused by 11-cis 13-demethylretinal in bleach-adapted cells. The slight activation of the phototransduction cascade by 11-cis 13-demethylretinal in dark-adapted rods could be the result of the presence of a small amount of free opsin in rods of freshly dissected animals, as recently argued by Kefalov et al. (1999). We conclude that the major mechanism by which 11-cis 13-demethylretinal is affecting the level of transduction in rods is by interacting with free opsin, most likely by binding in its chromophore pocket.

Bleach-adapted Cones

The effect of 11-cis 13-demethylretinal on the activity of phosphodiesterase and cyclase in bleach-adapted red cones was also investigated. Red-sensitive cones were identified by their distinctive morphology and high sensitivity to test flashes at 600 nm. To produce activation of the transduction cascade in the cones comparable with that of 5–10% bleach-adapted rods, 90% of the cone pigment had to be bleached. The concentration of 11-cis 13-demethylretinal used was 100 μm. A total of five bleach-adapted cones were studied.

Photocurrent and light sensitivity. The experiment illustrated in Fig. 7 shows the effect that 11-cis 13-demethylretinal has on the dark current and sensitivity of a bleach-adapted cone. Fig. 7 A illustrates a series of superimposed flash responses recorded from the dark-adapted cone. During the exposure to brief bright light that bleached 90% of the pigment, the dark current was completely blocked. Within 10 s after the termination of the bleach, the current recovered to a steady state. The bleach caused a steady decrease in the dark current, as evinced by the decreased amplitude of the saturating flash response (Fig. 7 B). On average, bleach-

ing 90% of the pigment caused a decrease in the dark current to $65 \pm 5\%$ (SEM, $n = 5$) of its dark-adapted value.

When the bleach-adapted cone was exposed to vesicle solution containing 11-cis 13-demethylretinal, the dark current reached a new steady state within 3–5 s with a value greater than that of the bleach-adapted cone (Fig. 7 C). No transient decrease of the current upon adding 11-cis 13-demethylretinal was observed. After the treatment with the retinoid, the amplitude of the current in all of the cones studied was higher than the bleach-adapted state, but still significantly lower than the dark-adapted state of the cell and, on average, was increased to $78 \pm 4\%$ (SEM, $n = 5$) of the dark-adapted value. This effect was not transient and the current remained at this level after flushing the retinoid solution from the chamber. Thus, the treatment with 11-cis 13-demethylretinal caused a recovery of about one third of the dark current lost as a result of the bleach. Considering that, in the case of cones, significant activation of the transduction cascade produces relatively small changes in the dark current (see, for instance, Jones et al., 1993), the small recovery of the current caused by the retinoid could be the result of a significant deactivation of the cone phototransduction cascade.

The sets of photoresponses shown on Fig. 7, A–C, were used to construct the corresponding intensity–response curves and estimate the effect of 11-cis 13-demethylretinal on the photosensitivity of the bleach-adapted cone (Fig. 7 D). Bleaching 90% of the pigment resulted in an ~100-fold (2 log units) decrease in the sensitivity of the cone (Fig. 7 D, ■ and ▲). On average, the bleach caused a loss of sensitivity of 1.8 ± 0.2 (SEM, $n = 5$) log units. When the bleach-adapted cone was exposed to 11-cis 13-demethylretinal, the sensitivity increased but still remained lower than the sensitivity in the dark-adapted state (Fig. 7 D, ●). On average, the recovery of sensitivity induced by 11-cis 13-demethylretinal was 0.5 ± 0.1 (SEM, $n = 5$) log units. Thus, treatment of bleach-adapted cones with the retinoid caused recovery of about one third of the sensitivity lost as a result of the bleach.

Dim flash kinetics. The observation that 11-cis 13-demethylretinal increases the dark current and sensitivity of bleach-adapted cones suggests that the retinoid down regulates the reactions of the phototransduction cascade. As has been observed after treatment of bleached cones with β-ionone (Jin et al., 1993), deactivation of the transduction cascade by 11-cis 13-demethylretinal would be expected to slow down the kinetics of the dim flash response.

Fig. 8 shows normalized dim-flash responses from one cone, elicited in the dark-adapted state, after a 90% bleach, and then after treatment with 11-cis 13-
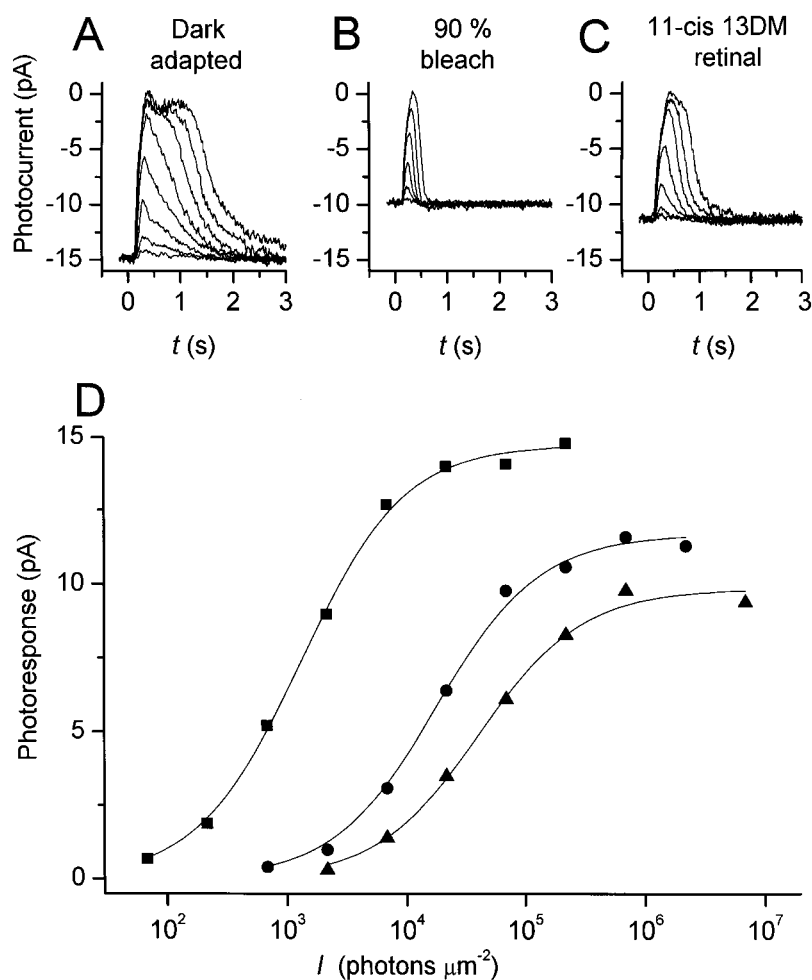
Figure 7. Effect of 11-cis 13-demethylretinal on the dark current and on the sensitivity in a cone. Shown is a series of flash responses from one cell in its dark-adapted state (A), after a 90% bleach (B), and in 100 μm 11-cis 13-demethylretinal solution (C). The corresponding intensity–response curves are shown in D: dark-adapted (■), bleach adapted (▲), and in 11-cis 13-demethylretinal (●).

demethylretinal. As a result of the bleach, the photoresponse was accelerated and peaked 40 ms earlier than the dark-adapted response. Treating the bleach-adapted cone with the retinoid partially reversed this acceleration and the response in the treated state peaked only 10 ms earlier than the response in the dark-adapted state. Similar results were obtained from all five tested cones.

Thus, treatment of bleach-adapted cones with 11-cis 13-demethylretinal caused a partial reversal of the acceleration of the dim flash response caused by the bleach. The effect was not transient and persisted even after washing out the retinoid from the chamber.

Rate of guanylyl cyclase. We also performed a series of experiments designed to study directly the effect of 11-cis 13-demethylretinal on the level of transduction in cones. Because of the much faster cone response kinetics (Perry and McNaughton, 1991) and the limited time resolution of the phosphodiesterase measurements (Cornwall et al., 1995), we only measured the rate of cone guanylyl cyclase. The results from one such experiment are shown in Fig 9. Cyclase was first measured in the dark-adapted state (Fig. 9 A), where only a

low basal activity was observed. Bleaching 90% of the pigment caused an over twofold increase in the rate of cyclase (Fig. 9 B). After exposure of the bleach-adapted cone to 11-cis 13-demethylretinal, the effect of the bleach on cyclase was partially reversed and the rate of cyclase was lower than in the bleach-adapted state, but still higher than in the dark-adapted state. 11-Cis 13-demethylretinal caused deceleration of cyclase in all five cones tested. On average, the increase of the rate of cyclase from the dark-adapted state caused by the 90% bleach was 2.8 ± 0.3 (SEM, $n = 5$). The treatment with 11-cis 13-demethylretinal reduced the rate of cyclase to 2.2 ± 0.3 (SEM, $n = 5$) times that of the dark-adapted state.

DISCUSSION

Our results demonstrate that treatment of bleach-adapted rods with a vesicle solution containing 11-cis 13-demethylretinal results in: (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 phos-
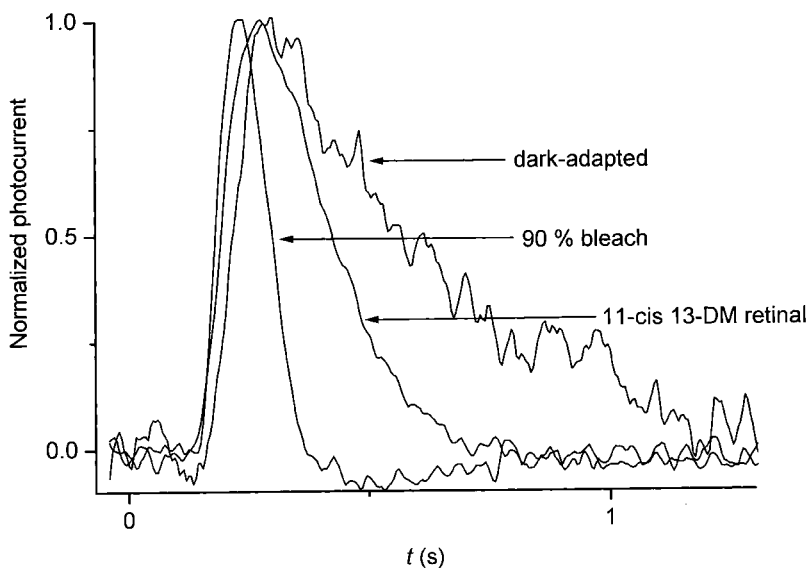
Figure 8. Effect of 11-cis 13-demethylretinal on the kinetics of dim-flash responses in a cone. Normalized dim-flash responses from one cone in a dark-adapted state, after a 90% bleach, and in the presence of 100 μm 11-cis 13-demethylretinal. The flash intensities were 70, 21,700, and 6,900 photons/μm², and the corresponding response amplitudes were 1.1, 2.6, and 2.9 pA, respectively.

phodiesterase. Control experiments on dark-adapted rods similarly treated showed that 11-cis 13-demethylretinal produces little or no effect on the level of transduction activity. Taken together, these results suggest

that, in bleach-adapted rods, 11-cis 13-demethylretinal combines with opsin and acts as an agonist to activate the phototransduction cascade in the dark in a manner similar to light. This activation is similar to that re-
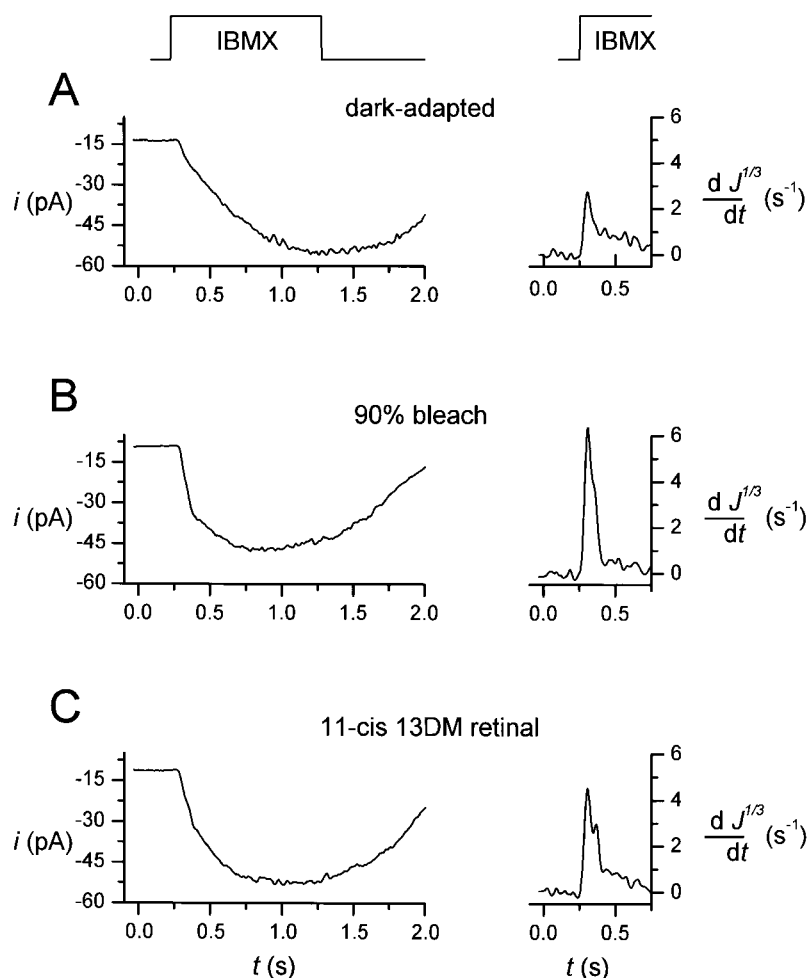

Figure 9. Effect of 11-cis 13-demethylretinal on the rate of guanylyl cyclase in a cone. (Left) The current recordings from a cone during steps into 0.5 mM IBMX solution. (Right) The derivative of the cube root of the normalized current, $d(J^{1/3})/dt$. The time course of the solution step for both panels is shown at the top. Recordings were made first in the dark-adapted state (A), then after a 90% bleach (B), and in 100 μm 11-cis 13-demethylretinal solution (C). Each current trace is the average of four measurements. All recordings were done in the same cell.

ported previously to occur in bleached rods exposed to β-ionone (Kefalov et al., 1999). We conclude that, similar to β-ionone, 11-cis 13-demethylretinal produces its physiological effect by binding directly in the chromophore pocket of free opsin and producing an active noncovalent complex. This effect is transient and, after the initial activation, the activity of the transduction cascade decreases gradually to a lower steady state level. The time course of the down regulation of the transduction cascade observed after the initial activation by 11-cis 13-demethylretinal is ~17 min. The steady state rates of cyclase and phosphodiesterase are lower than the initial activation observed immediately upon adding 11-cis 13-demethylretinal to the cell. However, they are still higher than the corresponding bleach-adapted rates before the treatment with the retinoid. In contrast to the effect produced by β-ionone, the effect of 11-cis 13-demethylretinal is not reversible and persists even after washing out the excess retinoid from the chamber.

These results are consistent with previous biochemical studies in which 11-cis 13-demethylretinal was found to activate opsin (Ebrey et al., 1980; Palczewski et al., 1994; Tan et al., 1998). Also in agreement with biochemical data (Palczewski et al., 1994) is our observation that the steady state level of transduction after treatment with 11-cis 13-demethylretinal is higher than the bleach-adapted state (free opsin state), but lower than the initial high level of transduction observed upon treatment with 11-cis 13-demethylretinal (noncovalent retinal • opsin complex).

Fig. 10 shows a proposed scheme by which 11-cis 13-demethylretinal may affect the level of transduction in bleach-adapted rods. We speculate that the initial high level of transduction in bleach-adapted rods observed after treatment with 11-cis 13-demethylretinal is the result of the noncovalent binding of the retinoid in the chromophore pocket of free opsin (Fig. 10 D). The subsequent partial recovery is due to the covalent attachment of the retinoid to opsin and the formation of 11-cis 13-demethyl rhodopsin. It is likely that Schiff-base formation between opsin and 11-cis 13-demethylretinal does not go to completion (Nelson et al., 1970) and that an equilibrium mixture of opsin, noncovalent 11-cis 13-demethylretinal • opsin complexes, and 11-cis 13-demethyl rhodopsin exists after treatment of the bleached rod with the retinoid (Fig. 10 E). Thus, the final level of activity will be a result of the combined effect of the three states (Fig 10 E).

The presence of residual amounts of free opsin according to this scheme provides an explanation for our observation that a second treatment with 11-cis 13-demethylretinal produces activation of the transduction cascade, but to a smaller extent than the first treatment. After each treatment, the excess retinoid was flushed from the chamber. The addition of a second dose of 11-cis 13-demethylretinal will result in an increased concentration of free retinoid in the cell. This higher concentration will lead to the increased binding of 11-cis 13-demethylretinal to free opsin, and thus to activation of the transduction cascade. According to this scheme, after the excess of the retinoid is removed, the concentration of free 11-cis 13-demethylretinal in the cell will decrease once again. As a result, the concentration of opsin with non–covalently bound retinoid should decline and the activity of the phototransduction cascade should return to its steady state.

Our studies of the effect of 11-cis 13-demethylretinal on the level of transduction in bleach-adapted cones, on the other hand, failed to demonstrate any activation by the retinoid. Instead, in bleach-adapted cones, we observed an immediate deactivation of transduction upon treatment with 11-cis 13-demethylretinal. Unfortunately, no biochemical studies on the ability of this retinoid to form a pigment with cone opsin have yet been reported. Thus, we cannot rule out the possibility that the observed deactivation of transduction in bleach-adapted cones is the result of the formation of a covalent Schiff-base linkage. However, based on the observation that 11-cis 13-demethyl rhodopsin is inactive in the dark (Buczylko et al., 1996), the formation of a new pigment with the retinal analog would be expected to reverse the effect of the bleach and inactivate transduction to its dark-adapted level. The observation that only a partial recovery was observed, together with the fact that the noncovalent binding of other retinoids in the chromophore pocket of cone opsin also causes only a partial reversal of bleaching adaptation (Jin et al., 1993; Cornwall et al., 1995), indicates that, most likely, pigment regeneration is not the major factor for the observed partial recovery from the bleach. Instead, we suggest that the deactivation of transduction in bleach-adapted cones is driven predominantly by the noncovalent binding of 11-cis 13-demethylretinal in the chromophore pocket.

Our results on the effect of 11-cis 13-demethylretinal on the level of transduction in bleach-adapted rod and cone photoreceptors complement previous observations of the effects of β-ionone on phototransduction in bleach-adapted rods and cones. They extend the validity of the models proposed for the effects of the noncovalent binding of a retinoid in the chromophore pockets of rod (Kefalov et al., 1999) and cone (Jin et al., 1993) opsins to retinoids that are capable of forming a Schiff-base. Taken together, these observations suggest that the noncovalent binding by itself may play a role for the dark adaptation of photoreceptors.

The dark adaptation of bleach-adapted rods and cones most likely occurs in two stages. In the first stage, the noncovalent binding of 11-cis retinal in the chromophore pocket of free rod opsin desensitizes the rod. The analogous reaction in cones results in partial re-
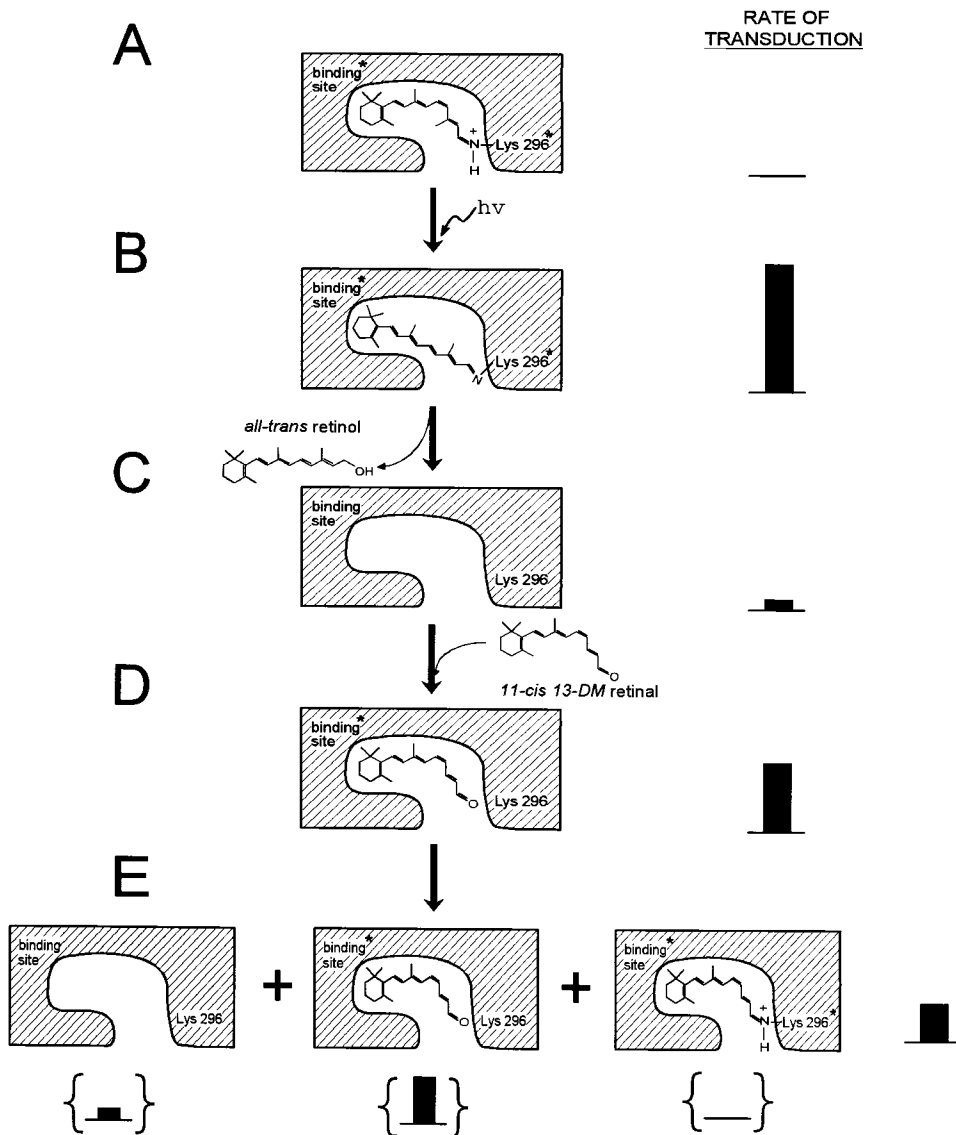
Figure 10. A model of the mechanism of activation by 11-cis 13-demethylretinal of phototransduction in rods. (A) rhodopsin, (B) photoactivated rhodopsin (Meta II), (C) free opsin, (D) noncovalent 11-cis 13-demethylretinal • opsin complex, (E) equilibrium mixture of free opsin, noncovalent 11-cis 13-demethylretinal • opsin complexes, and 11-cis 13-demethyl rhodopsin.

sensitization. The subsequent covalent attachment of retinal to opsin via a protonated Schiff-base in both cell types then completely inactivates the bleached pigment to complete dark adaptation. The opposite effects of the noncovalent binding of retinal to opsin in rods and cones may explain, in part, why dark adaptation of photopic color vision is so much more rapid than scotopic vision mediated by rods.

REFERENCES

Baylor, D.A., and A.L. Hodgkin. 1974. Changes in time scale and sensitivity in turtle photoreceptors. *J. Physiol.* 242:729–758.

Baylor, D.A., T.D. Lamb, and K.W. Yau. 1979. The membrane current of single rod outer segments. *J. Physiol.* 288:589–611.

Bernstein, P.S., and R.R. Rando. 1986. In vivo isomerization of all-trans- to 11-cis-retinoids in the eye occurs at the alcohol oxidation state. *Biochemistry.* 25:6473–6478.

Bok, D. 1990. Processing and transport of retinoids by the retinal pigment epithelium. *Eye.* 4:326–332.

Buczylko, J., J.C. Saari, R.K. Crouch, and K. Palczewski. 1996. Mechanisms of opsin activation. *J. Biol. Chem.* 271:20621–20630.

Cohen, G.B., D.D. Oprian, and P.R. Robinson. 1992. Mechanism of activation and inactivation of opsin: role of Glu113 and Lys296. *Biochemistry.* 31:12592–12601.

Cornwall, M.C., D.W. Corson, R.K. Crouch, and G.L. Fain. 1996. Activation of guanylyl cyclase in bleached salamander rods by 11-*cis*13-demethyl retinal. *Invest. Ophthalmol. Vis. Sci.* 37:S268.

Cornwall, M.C., and G.L. Fain. 1994. Bleached pigment activates transduction in isolated rods of the salamander retina. *J. Physiol.*

480:261–279.

Cornwall, M.C., A. Fein, and E.F. MacNichol, Jr. 1990. Cellular mechanisms that underlie bleaching and background adaptation. *J. Gen. Physiol.* 96:345–372.

Cornwall, M.C., H.R. Matthews, R.K. Crouch, and G.L. Fain. 1995. Bleached pigment activates transduction in salamander cones. *J. Gen. Physiol.* 106:543–557.

Corson, D.W., M.C. Cornwall, E.F. MacNichol, S. Tsang, F. Derguini, R.K. Crouch, and K. Nakanishi. 1994. Relief of opsin desensitization and prolonged excitation of rod photoreceptors by 9-desmethylretinal. *Proc. Natl. Acad. Sci. USA.* 91:6958–6962.

Corson, D.W., B. Katz, and R.K. Crouch. 1995. Desensitization of bleached rod photoreceptors by 11-*cis* 13 desmethyl-retinal. *Biophys. J.* 68:A333. (Abstr.)

Corson, D.W., V.J. Kefalov, R.K. Crouch, C. Rosebrock, and M.C. Cornwall. 1999. Opposite effects on activity of the transduction cascade in bleached rods and cones following treatment with 11-*cis* 13-demethyl retinal. *Invest. Ophthalmol. Vis. Sci.* 40:S239.

Crouch, R.K., G.J. Chader, B. Wiggert, and D.R. Pepperberg. 1996. Retinoids and the visual process. *Photochem. Photobiol.* 64:613–621.

Crouch, R.K., and Y.S. Or. 1983. Opsin pigments formed with acyclic retinal analogues. *FEBS Lett.* 158:139–142.

Crouch, R.K., C.D. Veronee, and M.E. Lacy. 1982. Inhibition of rhodopsin regeneration by cyclohexyl derivatives. *Vis. Res.* 22:1451–1456.

Daemen, F.J.M. 1978. The chromophore binding space of opsin. *Nature.* 276:847–848.

Ebrey, T., M. Tsuda, G. Sassenrath, J.L. West, and W.H. Waddell. 1980. Light activation of bovine rod phosphodiesterase by nonphysiological visual pigments. *FEBS Lett.* 116:217–219.

Fukada, Y., and T. Yoshizawa. 1981. Activation of phosphodiesterase in frog rod outer segment by an intermediate of rhodopsin photolysis. II. *Biochim. Biophys. Acta.* 675:195–200.

Hodgkin, A.L., and B.J. Nunn. 1988. Control of light-sensitive current in salamander rods. *J. Physiol.* 403:439–471.

Jin, J., R.K. Crouch, D.W. Corson, B.M. Katz, E.F. MacNichol, and M.C. Cornwall. 1993. Noncovalent occupancy of the retinal-binding pocket of opsin diminishes bleaching adaptation of retinal cones. *Neuron.* 11:513–522.

Jones, G.J. 1995. Light adaptation and the rising phase of the flash photocurrent of salamander retinal rods. *J. Physiol.* 487:441–451.

Jones, G.J., R.K. Crouch, B. Wiggert, M.C. Cornwall, and G.J. Chader. 1989. Retinoid requirements for recovery of sensitivity after visual-pigment bleaching in isolated photoreceptors. *Proc. Natl. Acad. Sci. USA.* 86:9606–9610.

Jones, G.J., A. Fein, E.F. MacNichol, Jr., and M.C. Cornwall. 1993. Visual pigment bleaching in isolated salamander retinal cones. Microspectrophotometry and light adaptation. *J. Gen. Physiol.* 102:483–502.

Kefalov, V.J., M.C. Cornwall, and R.K. Crouch. 1999. Occupancy of the chromophore binding site of opsin activates visual transduction in rod photoreceptors. *J. Gen. Physiol.* 113:491–503.

Lagnado, L., and D. Baylor. 1992. Signal flow in visual transduction. *Neuron.* 8:995–1002.

Leibrock, C.S., T. Reuter, and T.D. Lamb. 1998. Molecular basis of dark adaptation in rod photoreceptors. *Eye.* 12:511–520.

Matsumoto, H., and T. Yoshizawa. 1975. Existence of a beta-ionone ring-binding site in the rhodopsin molecule. *Nature.* 258:523–526.

Naka, K.I., and W.A. Rushton. 1966. S-potentials from colour units in the retina of fish (Cyprinidae). *J. Physiol.* 185:536–555.

Nakatani, K., Y. Koutalos, and K.W. Yau. 1995. Ca^{2+} modulation of the cGMP-gated channel of bullfrog retinal rod photoreceptors. *J. Physiol.* 484:69–76.

Nelson, R., J. Kim deReil, and A. Kropf. 1970. 13-Desmethyl rhodopsin and 13-desmethyl isorhodopsin: visual pigment analogues. *Proc. Natl. Acad. Sci. USA.* 66:531–538.

Okajima, T.I., D.R. Pepperberg, H. Ripps, B. Wiggert, and G.J. Chader. 1990. Interphotoreceptor retinoid-binding protein promotes rhodopsin regeneration in toad photoreceptors. *Proc. Natl. Acad. Sci. USA.* 87:6907–6911.

Palczewski, K., S. Jager, J. Buczylko, R.K. Crouch, D.L. Bredberg, K.P. Hofmann, M.A. Asson-Batres, and J.C. Saari. 1994. Rod outer segment retinol dehydrogenase: substrate specificity and role in phototransduction. *Biochemistry.* 33:13741–13750.

Perlman, J.I., B.R. Nodes, and D.R. Pepperberg. 1982. Utilization of retinoids in the bullfrog retina. *J. Gen. Physiol.* 80:885–913.

Perry, R.J., and P.A. McNaughton. 1991. Response properties of cones from the retina of the tiger salamander [published erratum appears in *J. Physiol.* 1991. 436:771]. *J Physiol.* 433:561–587.

Press, W.H., S.A. Teuklsky, W.T. Vetterling, and B.P. Flannery. 1992. Numerical Recipes in C: The Art of Scientific Computing. 2nd ed. W.H. Press, editor. Cambridge University Press, Cambridge, UK. 650–655.

Pugh, E.N., Jr., and T.D. Lamb. 1990. Cyclic GMP and calcium: the internal messengers of excitation and adaptation in vertebrate photoreceptors. *Vis. Res.* 30:1923–1948.

Pugh, E.N., Jr., and T.D. Lamb. 1993. Amplification and kinetics of the activation steps in phototransduction. *Biochim. Biophys. Acta.* 1141:111–149.

Saari, J.C. 1994. Retinoids in photosensitive systems. *In* The Retinoids: Biology, Chemistry, and Medicine. 2nd ed. M.B. Sporn, A.B. Roberts, and D.S. Goodman, editors. Raven Press, New York, NY. 331–385.

Saari, J.C., D.L. Bredberg, G.G. Garwin, J. Buczylko, T. Wheeler, and K. Palczewski. 1993. Assays of retinoid dehydrogenases by phase partition. *Anal. Biochem.* 213:128–132.

Stavenga, D.G., R.P. Smits, and B.J. Hoenders. 1993. Simple exponential functions describing the absorbance bands of visual pigment spectra. *Vis. Res.* 33:1011–1017.

Stryer, L. 1991. Visual excitation and recovery. *J. Biol. Chem.* 266:10711–10714.

Tan, Q., K. Nakanishi, and R.K. Crouch. 1998. Mechanism of transient dark activity of 13-desmethylretinal/rod opsin complex. *J. Am. Chem. Soc.* 47:12357–12358.

van den Tempel, P.J., and H.O. Huisman. 1966. Vitamin A analogues. V. Synthesis of 9-, 13-, and 9,13- desmethyl homologues of vitamin A. *Tetrahedron.* 22:293–299.

Towner, P., W. Gaertner, B. Walckhoff, D. Oesterhelt, and H. Hopf. 1981. Regeneration of rhodopsin and bacteriorhodopsin. The role of retinal analogues as inhibitors. *Eur. J. Biochem.* 117:353–359.

Yau, K.W. 1994. Phototransduction mechanism in retinal rods and cones. The Friedenwald Lecture. *Invest. Ophthalmol. Vis. Sci.* 35:9–32.

Yau, K.W., and D.A. Baylor. 1989. Cyclic GMP-activated conductance of retinal photoreceptor cells. *Annu. Rev. Neurosci.* 12:289–327.

Yoshikami, S., and G.N. Noll. 1982. Technique for introducing retinol analogs into the isolated retina. *Methods Enzymol.* 81:447–451.