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Role of Noncovalent Binding of 11-*cis*-Retinal to Opsin in Dark Adaptation of Rod and Cone Photoreceptors

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Summary

Regeneration of visual pigments of vertebrate rod and cone photoreceptors occurs by the initial noncovalent binding of 11-*cis*-retinal to opsin, followed by the formation of a covalent bond between the ligand and the protein. Here, we show that the noncovalent interaction between 11-*cis*-retinal and opsin affects the rate of dark adaptation. In rods, 11-*cis*-retinal produces a transient activation of the phototransduction cascade that precedes sensitivity recovery, thus slowing dark adaptation. In cones, 11-*cis*-retinal immediately deactivates phototransduction. Thus, the initial binding of the same ligand to two very similar G protein receptors, the rod and cone opsins, activates one and deactivates the other, contributing to the remarkable difference in the rates of rod and cone dark adaptation.

Introduction

The visual pigments of vertebrate rod and cone photoreceptors consist of apoprotein, opsin, bound to the photoactivable ligand, 11-*cis*-retinal. These proteins are G protein receptors, with significant homology at the amino acid level (Xu et al., 1998). In the covalently bound form, which exists in darkness, the ligand acts as an inverse agonist, locking the protein in its inactive conformation. The role of light is to isomerize the 11-*cis* bond of the retinal to the all-*trans* form. The ligand in this state behaves as an agonist, activating the G protein receptor and initiating visual transduction. As decay of activated rhodopsin occurs, the ligand, in its all-*trans* form is removed to an adjacent tissue, the retinal pigment epithelium, where it is recycled back to the 11-*cis* form. The apoprotein itself has been demonstrated to have weak activity (Cornwall and Fain, 1994; Cornwall et al., 1995; Buczylko et al., 1996; Melia et al., 1997), a property common for many G protein receptors (de Ligt et al., 2000). Following the provision of fresh 11-*cis*-retinal from the pigment epithelium, the visual pigment is regenerated from its photobleached state back to one capable of photoactivation. This regeneration occurs by an initial noncovalent binding of 11-*cis*-retinal to the opsin (Matsumoto and Yoshizawa, 1975), followed by covalent attachment via a Schiff-base linkage (Bownds, 1967), which locks the protein back in its inactive conformation.

It has been known for 60 years that cone (photopic) dark adaptation occurs much faster than rod (scotopic) dark adaptation (Hecht et al., 1937), and this has been attributed until now only to the different rates of Schiff-base formation (Wald et al., 1955). Here, we test the hypothesis that the transient noncovalent retinal-opsin complex also affects the course of dark adaptation (Kefalov et al., 1999). To do that, we used single isolated amphibian rod and cone photoreceptors, which, in the absence of the pigment epithelium, are unable to regenerate their visual pigments until supplemented with the ligand chromophore, 11-*cis*-retinal (Pepperberg et al., 1978; Jones et al., 1989). This allows separation of the effects of light adaptation from those that occur as visual pigment is regenerated. Photobleaching a fraction of the pigment of isolated cells, and then treating with 11-*cis*-retinal, enabled us to directly observe the physiological effects of the noncovalent binding of retinal in the chromophore pocket of free opsin.

Results

11-*cis*-Retinal Causes a Transient Decrease in the Dark Current in Bleach-Adapted Rods

Membrane current was measured with the suction-pipette recording method from single isolated salamander rod and cone photoreceptors. Photoreceptors were briefly exposed to bright light to bleach a fraction of their visual pigment. After allowing sufficient time for the decay of the photoproducts to free opsin, the cells were exposed to 11-*cis*-retinal, and its effect on their physiology was monitored. Figure 1A shows respective responses from a rod to saturating flashes in the dark-adapted state, 40 min after a 20% pigment bleach, and at different times after exposure to lipid vesicles containing 11-*cis*-retinal. The reduced response amplitude after the bleach is consistent with the previous finding that bleach-adapted photoreceptors, which are unable to regenerate visual pigment, exhibit persistent activation of the phototransduction cascade (Cornwall and Fain, 1994; Cornwall et al., 1995). Surprisingly, after the exposure to 11-*cis*-retinal, we observed an additional decrease in amplitude of the saturating response (dark current). The reduction of the current was completed within several seconds upon adding 11-*cis*-retinal to the chamber. The current then recovered steadily to its dark-adapted level. Figure 1B shows the full time course of this experiment. Each point represents one measurement made with a saturating test flash. On average, 11-*cis*-retinal caused an initial decrease in the current amplitude to 59% ± 4% (S.E.M., n = 17) of that in the bleach-adapted state and a subsequent exponential recovery with a time constant of 4.4 ± 0.3 min (n = 10).

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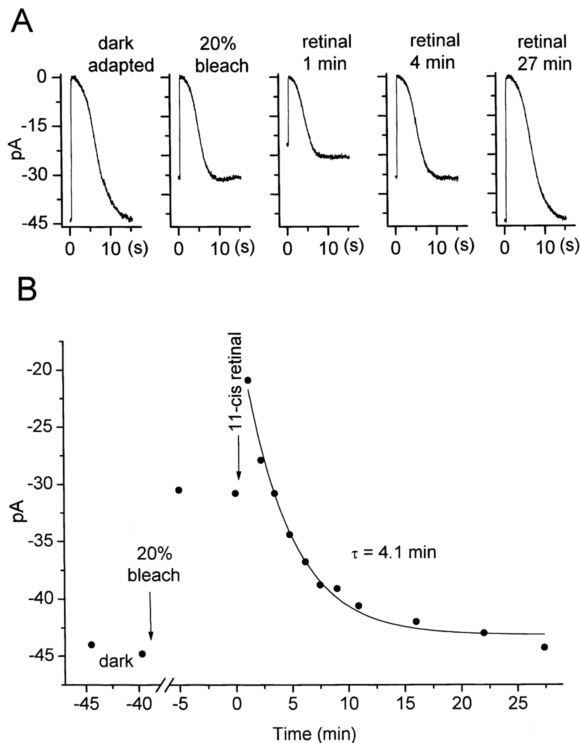

Figure 1. Effect of 11-*cis*-Retinal on the Dark Current in a Bleach-Adapted Rod

(A) Saturating flash responses from one rod in its dark-adapted state, following a 20% bleach and then 1 min, 4 min, and 27 min after treatment with 11-*cis*-retinal.

(B) Time course of the effect of 11-*cis*-retinal on the dark current in the same rod. Each data point represents the amplitude of the dark current as measured from (A). The recovery from the initial decrease of the current caused by 11-*cis*-retinal is fit by a single exponential function.

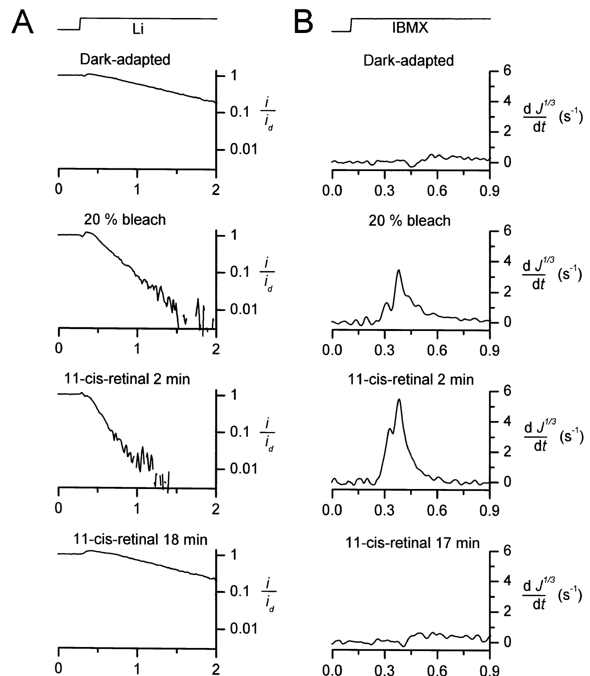

Figure 2. Measurements of the Activity of cGMP Phosphodiesterase and Guanylyl Cyclase in a Rod

(A) Effect of 11-*cis*-retinal on the activity of phosphodiesterase. Panels show the normalized current (i/i_{dark}), plotted logarithmically versus time, during steps into Li^+ solution from one cell in its dark-adapted state, following a bleach, and at two times after treatment with 11-*cis*-retinal.

(B) Effect of 11-*cis*-retinal on the activity of guanylyl cyclase. Panels show $d(J^{1/3})/dt$, where $J = i/i_{dark}$, during steps into IBMX solution from one cell in its dark-adapted state, following a bleach, and at two times after treatment with 11-*cis*-retinal. The time course of the solution step for both panels is shown at the top.

11-*cis*-Retinal Causes a Transient Activation of Phosphodiesterase and Guanylyl Cyclase in Bleach-Adapted Rods

We examined the possibility that the initial decrease in the rod dark current upon 11-*cis*-retinal treatment could be the result of a transient activation of the phototransduction cascade by the chromophore. We measured the effect of 11-*cis*-retinal on the activities of the two enzymes controlling the cytosolic level of cGMP, namely, guanylyl cyclase and cGMP phosphodiesterase. Light activates phosphodiesterase, which, through negative feedback involving Ca^{2+}, in turn increases guanylyl cyclase activity (Koch and Stryer, 1988). In steady state, the activities of phosphodiesterase and guanylyl cyclase are similar. The activity of phosphodiesterase in bleach-adapted rods was measured from the rate of decrease in dark current upon inhibition of guanylyl cyclase with a Ringer solution containing Li^+ instead of Na^+ (Hodgkin and Nunn, 1988). As a result of the continuing hydrolysis of cGMP by phosphodiesterase under these conditions, the current decayed exponentially, with a rate constant given by the slope of the normalized current traces plotted on a semilogarithmic scale (Figure 2A). The phosphodiesterase activity was 0.5 s^{-1} in the dark-adapted state (Figure 2A, top panel), and increased to 3.1 s^{-1} 37 min after a 20% bleach (Figure 2A, second panel from top). Previous measurements support the notion that this persistent elevated phosphodiesterase activity results from the presence of free opsin. Two minutes after exposure to 11-*cis*-retinal, the phosphodiesterase activity had increased further to 3.8 s^{-1} (Figure 2A, third panel from top) before decreasing gradually back to its dark-adapted value of 0.5 s^{-1} in 16 min (Figure 2A, bottom panel). In parallel, the sensitivity of the cell returned to its dark-adapted level (data not shown).

From six cells, the mean basal activity of phosphodiesterase in the dark-adapted state was 0.6 $\pm$ 0.1 s^{-1} and increased to 2.5 $\pm$ 0.3 s^{-1} as a result of the 20% bleach. The initial increase in phosphodiesterase activity caused by 11-*cis*-retinal was, on average, 50% $\pm$ 10% over its bleach-adapted value. At the time of the Li^+ jump, made approximately 2 min after the treatment with 11-*cis*-retinal, the amplitude of the dark current was 61% $\pm$ 4% of the bleach-adapted value.

Guanylyl cyclase activity was measured by exposing the cell to Ringer solution containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). As a result of the continuing synthesis of cGMP by guanylyl cyclase, the current rapidly increased. The maximum of the time derivative of the 3rd root of the normalized current provides a measure of the activity of guanylyl cyclase (Figure 2B; see Experimental Procedures for details). The guanylyl cyclase activity increased from 0.5

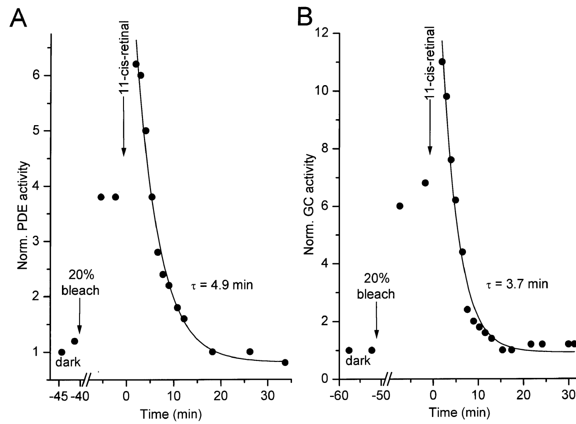

Figure 3. Time Course of the Effect of 11-*cis*-Retinal on Phosphodiesterase and Guanylyl Cyclase in a Rod

(A) Time course of the effect of 11-*cis*-retinal on the activity of phosphodiesterase in a rod. Each data point represents one measurement of the rate of phosphodiesterase (see Figure 2A). Shown are the dark-adapted activity of phosphodiesterase (dark), its steady state bleach-adapted activity after 20% bleach, and its change as a result of a treatment with 11-*cis*-retinal. The values of the dark current were: 45 pA in the dark adapted state; 40 pA in the bleach adapted state; 19 pA at the time of the first phosphodiesterase activity measurement in 11-*cis*-retinal; and 45 pA after full recovery.
(B) Time course of the effect of 11-*cis*-retinal on the activity of guanylyl cyclase in a rod. Each data point represents one measurement of the activity of guanylyl cyclase (see Figure 2B). Experimental conditions were as in Figure 3A. The values of the dark current were: 34 pA in the dark adapted state; 30 pA in the bleach adapted state; 14 pA at the time of the first guanylyl cyclase activity measurement in 11-*cis*-retinal; and 33 pA after full recovery. The recovery from the initial activation of phosphodiesterase and guanylyl cyclase by 11-*cis*-retinal is fit by a single exponential function. The changes in the dark current in both cells followed a pattern similar to the one shown on Figure 1B.

s^{-1} in the dark-adapted state (Figure 2B, top panel) to 3.4 s^{-1} 49 min after a 20% bleach (Figure 2B, second panel from top). As with phosphodiesterase, 11-*cis*-retinal caused an initial activation of guanylyl cyclase to 5.5 s^{-1}(Figure 2B, third panel from top), followed by recovery of guanylyl cyclase activity to its dark-adapted value of 0.5 s^{-1} in 15 min (Figure 2B, bottom panel).

From eleven cells, the mean basal activity of guanylyl cyclase in the dark-adapted state was 0.5 $\pm$ 0.1 s^{-1} (n = 11), and increased to 3.3 $\pm$ 0.8 s^{-1} (n = 5) as a result of a 20% bleach. The relative initial increase in guanylyl cyclase activity caused by 11-*cis*-retinal was independent of the fraction of pigment bleached from 5% to 90% and was, on average, 60% $\pm$ 10% (n = 11) over the bleach-adapted activity. At the time of the IBMX jump, approximately 2 min after the treatment with 11-*cis*-retinal, the amplitude of the dark current was 62% $\pm$ 5% (n = 11) of the bleach-adapted value.

The full time courses of recovery of phosphodiesterase and guanylyl cyclase activities in the presence of 11-*cis*-retinal from the two cells in Figure 2 are shown in Figure 3. Both could be fit by a single exponential function with time constants of 4.9 min and 3.7 min, respectively. Average values were 4.7 $\pm$ 0.3 min (n = 5) for phosphodiesterase and 4.6 $\pm$ 0.7 min (n = 6) for guanylyl cyclase. The actual initial activation of phosphodiesterase and guanylyl cyclase in the presence of

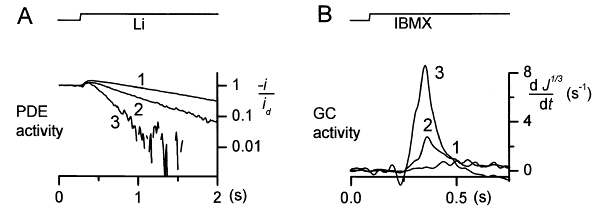

Figure 4. Effect of 11-*cis*-Retinol on the Activity of Phosphodiesterase (A) and Guanylyl Cyclase (B) in Two Bleach-Adapted Rods, Presented in a Way Similar to Figures 2A and 2B, Respectively.

1: dark-adapted; 2: 20% bleach; 3: 11-*cis*-retinol. The measured enzymatic activities with the corresponding dark current values in parentheses were as follows: A: dark-adapted 0.5 s^{-1} (45 pA); bleach-adapted 0.8 s^{-1} (36 pA); 11-*cis*-retinol 2.6 s^{-1} (16 pA); B: dark-adapted 0.8 s^{-1} (41 pA); bleach-adapted 2.8 s^{-1} (28 pA); 11-*cis*-retinol 8.6 s^{-1} (4 pA).

11-*cis*-retinal was probably higher than shown, since the first measurement of their activities in 11-*cis*-retinal was done about 2 min after adding the retinoid. Control experiments showed that 11-*cis*-retinal did not affect phosphodiesterase or guanylyl cyclase activities in dark-adapted rods or after complete pigment regeneration.

Taken together, our results demonstrate that 11-*cis*-retinal transiently activates the phototransduction cascade in bleach-adapted rods in the course of pigment regeneration. The absence of effect in dark-adapted cells indicates that the activation is caused by a specific interaction between retinal and free opsin.

The Transient Activation by 11-*cis*-Retinal Is Due to Its Noncovalent Interaction with Opsin

To determine if the transient activation by 11-*cis*-retinal is due to a noncovalent or covalent interaction with opsin, we measured phosphodiesterase and guanylyl cyclase activities in bleached rods following treatment with 11-*cis*-retinol, which binds to opsin noncovalently (Daemen, 1978) but is incapable of forming a Schiff-base linkage (Daemen et al., 1974). Treatment of bleached rods with 11-*cis*-retinol resulted within seconds in steady activation of both phosphodiesterase (Figure 4A) and guanylyl cyclase (Figure 4B), as well as a decrease of sensitivity and an acceleration of the dim-flash response (data not shown; see also Jones et al., 1989). Consistent with the inability of retinol to promote rod pigment regeneration, and in contrast to the case of 11-*cis*-retinal, little or no recovery occurred following this initial activation by retinol. Thus, the noncovalent binding of 11-*cis*-retinol in the chromophore pocket of free opsin activates the phototransduction cascade. We conclude that, similar to retinol, 11-*cis*-retinal activates phototransduction during its noncovalent interaction with opsin. Recent physiological (Kefalov et al., 1999) and biochemical studies (Buczylko et al., 1996; Tan et al., 1998) also support this notion.

11-*cis*-Retinal Causes Immediate Deactivation of Transduction in Bleach-Adapted Cones

For comparison, we also studied the effect of 11-*cis*-retinal on bleach-adapted cones. Because of the much faster cone response kinetics (Perry and McNaughton, 1991) and the limited time resolution of the phosphodi-

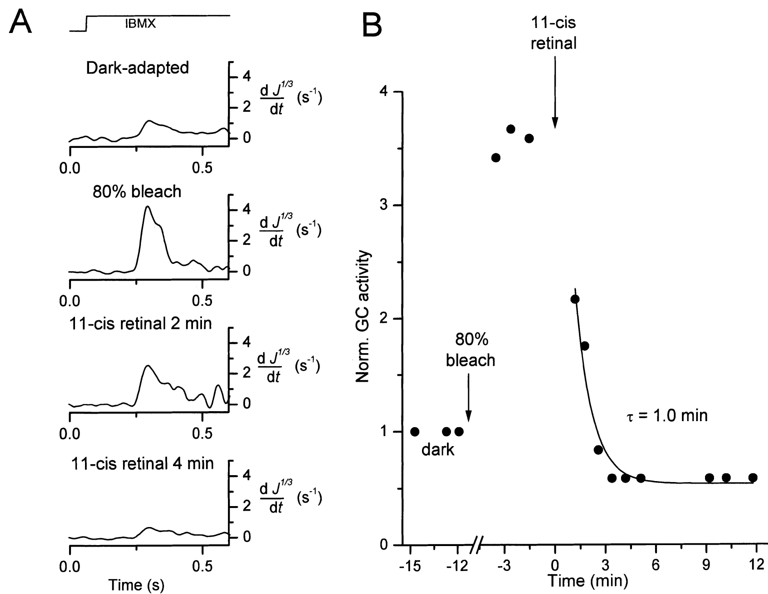

Figure 5. Effect of 11-*cis*-Retinal on the Activity of Guanylyl Cyclase in a Cone

(A) Panels show the derivative of the cube root of the normalized current, $d(J^{1/3})/dt$. See Figure 2A for details. The time course of the solution step is shown at the top.

(B) Time course of the effect of 11-*cis*-retinal on guanylyl cyclase in the same cone as in (A). Each data point represents one measurement of the rate of cyclase as in (A).

esterase measurements (Cornwall et al., 1995), we only measured the activity of guanylyl cyclase. In order to produce activation of the transduction cascade in the cones comparable to that of 20% bleach-adapted rods, at least 80% of the cone pigment had to be bleached. As pointed out above, the relative activation of phototransduction by 11-*cis*-retinal in bleach adapted rods is the same after bleaches from 5% to 90%, indicating that the effect is not dependent on the actual fraction of pigment bleached within the range used here. This allowed for a direct comparison between the effects of 11-*cis*-retinal in 20% bleached rods and 80% bleached cones.

In contrast to its effect in rods, 11-*cis*-retinal caused deactivation of the cone phototransduction cascade at the earliest time at which we could make measurements as demonstrated by the decrease in guanylyl cyclase activity (Figure 5), accompanied by an increase in dark current and sensitivity (data not shown). The activity of guanylyl cyclase then continued to decline exponentially with an average time constant of 1.0 ± 0.2 min ($n = 9$), until the cone dark-adapted completely. This recovery was several times faster than in rods, most likely reflecting the different kinetics of pigment formation in the two cell types (Wald et al., 1955).

Several arguments can be made against a possible transient activation of guanylyl cyclase in cones, too fast to be detected by our method. First, such activation would result in a transient decrease in the dark current, which was not observed. Second, previous studies have shown that the noncovalent binding of β-ionone and 9-*cis* C17 aldehyde to cone opsin deactivates phototransduction (Jin et al., 1993; Cornwall et al., 1995) in a way similar to the initial effect of 11-*cis*-retinal observed here. We did not study the effect of 11-*cis*-retinol on cones because of its ability to promote cone pigment regeneration (Jones et al., 1989), presumably via conversion to 11-*cis*-retinal, rendering impossible the separation of noncovalent from covalent interactions. A direct way of demonstrating the effect of the noncovalent binding of 11-*cis*-retinal in the chromophore pocket of cone opsin would be to use a microspectrophotometer to monitor the time course of pigment regeneration while following the dark adaptation of the cone with a suction electrode.

Effect of the Noncovalent Binding of 11-*cis*-Retinal to Opsin on the Kinetics of Dark Adaptation

To demonstrate directly the role of the noncovalent interaction between 11-*cis*-retinal and opsin in dark adaptation, we compared the rate of recovery of the dark current after a bleach in the absence and presence of retinal (Figure 6). The initial recovery of rods from a bleach was slower in the presence of 11-*cis*-retinal, presumably because of the transient activation by retinal. In contrast, cones recovered from a bleach significantly faster in the presence of 11-*cis*-retinal (Figure 6, inset). As expected, the recovery from the bleach was only partial in Ringer and complete in 11-*cis*-retinal for both photoreceptor types. Thus, the presence of 11-*cis*-retinal introduced a delay in the early recovery of rods from a bleach while accelerating the corresponding recovery in cones.

Discussion

The experiments described here demonstrate that in bleach-adapted rods, 11-*cis*-retinal causes a transient decrease in the dark current, a transient activation of cGMP phosphodiesterase, and a transient activation of guanylyl cyclase. In bleach-adapted cones, on the other hand, 11-*cis*-retinal causes immediate increase in the dark current and deactivation of guanylyl cyclase. Thus, in rods the noncovalent binding of 11-*cis*-retinal to opsin produces a transient activation of the phototransduction cascade. In cones, the noncovalent binding of 11-*cis*-retinal in the chromophore pocket most likely immediately deactivates transduction, though this remains to be shown directly.

Our results demonstrate that the noncovalent interac-

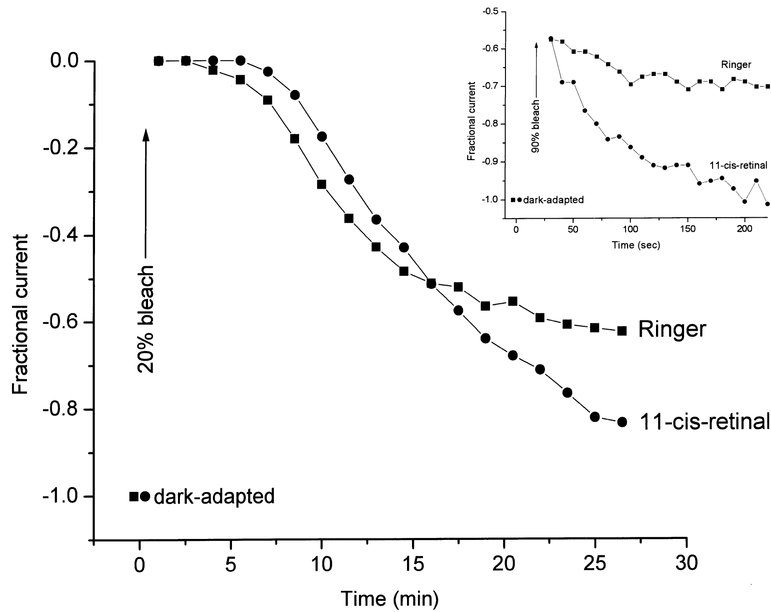

Figure 6. Effect of 11-*cis*-Retinal on the Rate of Recovery from a Bleach

Superimposed measurements of the current from two rods recovering from equal bleaches in Ringer (squares) and in 11-*cis*-retinal (circles). Inset shows the same for the case of cones. Retinal was added to the bath immediately before the bleach.

tion between 11-*cis*-retinal and opsin plays a pivotal role in determining the rate of dark adaptation in rod and cone photoreceptors. We show that this initial step of the ligand interaction with the protein in pigment regeneration affects the rates of rod and cone recovery from a bleach, contributing to the large difference in time required for the photopic and scotopic phases of dark adaptation. We propose that dark adaptation of bleach-adapted photoreceptors occurs in two stages (Figure 7). Following photoactivation and decay of the visual pigment to the free apoprotein, the noncovalent binding of 11-*cis*-retinal activates the rod and deactivates the cone phototransduction. Thus, the noncovalent binding of the same ligand, 11-*cis*-retinal, has opposite effects on two similar G protein receptors, the rod and cone opsins. In rods, 11-*cis*-retinal acts transiently as an agonist, activating the receptor and the phototransduction cascade in the dark, introducing a delay of minutes in dark adaptation. In cones, 11-*cis*-retinal directly binds as a reverse agonist, deactivating the transduction cascade, thus allowing the cones to regain sensitivity even before the regeneration of visual pigment. On formation of the protonated Schiff-base linkage between retinal and opsin, the ligand becomes an inverse agonist in both cell types completely inactivating the pigment molecules. As a result, the cells recover to their dark-adapted state.

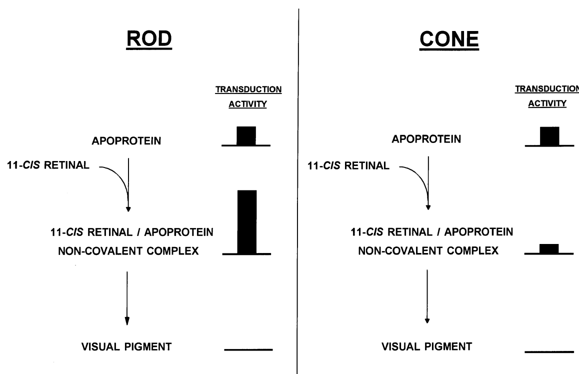

Figure 7. Schematic Representation of Changes in the Activity of the Transduction Cascade that Occur during Pigment Regeneration

See text for details.

One important question is what is the molecular mechanism by which the noncovalent binding of a retinoid in the chromophore pocket activates the rod opsin and inactivates the cone opsin? There is a body of evidence that the activity of the visual pigment is controlled by the state of protonation of the α-amino group of Lys-296. Disruption of the salt bridge between Lys-296 and its counterion Glu-113 and the resulting deprotonation of the Schiff-base triggered by the photoisomerization of 11-*cis*-retinal converts the pigment to its active form, Meta II (Doukas et al., 1978). Reprotonation of the Schiff-base during the transition from Meta II to Meta III renders the molecule inactive once again (Bennett, 1980). A weak salt bridge between Lys-296 and Glu-113, confining opsin in a low-activity state, exists in the apoprotein as well (Robinson et al., 1992). It is possible that the noncovalent binding of the retinoid disrupts the pocket environment enough to either weaken or completely break the salt bridge between Lys-296 and Glu-113, thus inducing a conformational change in the protein toward a more active state. The subsequent covalent attachment of 11-*cis*-retinal to Lys-296 will bring Glu-113 and Lys-296 close to each other and thus induce the formation of the salt bridge and the inactivation of the complex. The diametrically opposite effects of the occupancy of the chromophore pockets of rod and cone opsins is most likely the result of the significantly different pocket structure and ionic environment in the two proteins. Specifically, the weaker hydrogen bonds network of the cone pigment chromophore pocket (Imai et al., 1995) and the difference in the net charge of the pocket (Okano et al., 1992; Imai et al., 1995; Imai et al., 1997) may explain the

opposite physiological effects of the binding of a retinoid in the chromophore pocket of rod and cone opsins.

It is surprising that such a fundamental property of 11-*cis*-retinal as its ability to activate rod opsin during pigment regeneration has not been discovered until now. Probably the two major reasons for that are its transient occurrence and the unexpected activation instead of inactivation that the noncovalent binding of retinal causes. Interestingly, Buczylko et al. (1996) reported that in vitro 11-*cis*-retinal stimulates opsin phosphorylation very briefly. However, the low time resolution of their assay did not allow investigation of any transient effects occurring in the first few minutes of regeneration.

It is possible that the transient activation of rod opsin upon binding of retinal is the cost for ultimately forming the extremely thermally stable rod pigment (Baylor et al., 1980), rendering the rod very sensitive but slow to dark-adapt. In contrast, the noncovalent binding of retinal to cone opsin allows the cone to recover quickly and remain sensitive even in bright light but at the cost of low thermal pigment stability resulting in higher noise level and lower sensitivity of the cone (Rieke and Baylor, 2000). Thus, by having two different types of photoreceptors the visual system is able to achieve both high sensitivity and rapid adaptation for a wide range of light intensities.

In addition to providing an explanation for the differences in dark adaptation of the two types of photoreceptors, these experiments demonstrate the exquisite control that the same ligand, retinal, has in controlling two different G protein receptors that have a fair amount of sequence homology. Without the ligand present, both of these receptors have some basal activity, presumably from not being locked in a specific conformation. When the ligand is covalently bound as occurs in darkness, the receptors are firmly locked in their ground or inactive state. The light-activated form of the ligand, all-*trans*-retinal, converts the receptors to their active state. This process is analogous to that which occurs with a large array of G protein receptors that are generally activated or inactivated by binding with a ligand (Morris and Malbon, 1999). In this report, we have shown that intermediate interactions of the ligand with these receptors during the binding process can produce profound and different changes in their activity. These results introduce the possibility that such transient states are possible in other G protein receptors as well.

Experimental Procedures

Electrophysiology

A dark-adapted larval tiger salamander (*Ambystoma tigrinum*) was decapitated, the head and body pithed, and the eyes were enucleated in Ringer solution containing 110 mM NaCl, 2.5 mM KCl, 1.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM dextrose, 10 mM HEPES, pH 7.8, and bovine serum albumin (100 mg/l). Photoreceptors were mechanically isolated from the dark-adapted retinas and placed in a gravity-fed superfusion chamber on the stage of an inverted microscope (Cornwall et al., 1990). Individual cells in the suspension were viewed using an infrared television camera/monitor system fitted to the microscope. Measurements of membrane current were made extracellularly as described previously (Baylor et al., 1979). An intact rod or cone photoreceptor was drawn, inner segment first, into the tip of a tight-fitting glass recording pipette that contained Ringer solution. The recording pipette was connected to the head stage of a patch-clamp amplifier. The current recorded from the cell was converted to voltage, amplified, low pass filtered with an active 8-pole filter (20 Hz cut-off frequency), digitized at 250 Hz, and stored on a computer for subsequent analysis.

Light Stimulation

An optical stimulator provided test flashes (20 ms duration) as well as bleaching light (Cornwall et al., 1990). Wavelength (520 nm for rods and 600 nm for cones) and intensity of the stimulating light were set with calibrated narrow band interference and neutral density filters, respectively. The absolute intensity of the test flash/bleaching beam used in the rod experiments was 1.36×10^7 photons $\mu m^{-2}\ s^{-1}$ (520 nm); the absolute intensity for the cone experiments beam was 3.43×10^7 photons $\mu m^{-2}\ s^{-1}$ (600 nm). 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 light intensity in photons $\mu m^{-1}\ s^{-1}$, and t is the duration of light exposure in seconds. The value of the photosensitivity, P, used was $6.2 \times 10^{-9}\ \mu m^2$ for rods (Jones, 1995) and $6.0 \times 10^{-9}\ \mu m^2$ for cones (Jones et al., 1993).

Retinoids/Phospholipid Vesicles

11-*cis*-Retinal was purified by HPLC and stored at $-70°C$ under argon. 11-*cis*-Retinol was prepared by reduction of 11-*cis*-retinal with $LiAlH_4$. Both retinoids were delivered to the cells using phospholipid vesicles prepared by methods described previously (Yoshikami and Noll, 1982; Cornwall et al., 2000). The concentration of retinoid was determined spectrophotometrically and adjusted to 100 μM. 0.5 ml of the respective retinoid solution, was added to the experimental chamber using a pipetter.

Phosphodiesterase and Guanylyl Cyclase Activity Analysis

To estimate the activities of phosphodiesterase and guanylyl cyclase, we used a method originally devised by Hodgkin and Nunn (1988), and most recently used in a modified form by Kefalov et al. (1999). Briefly, the concentration of cGMP in the cell is controlled by the balance between its synthesis by guanylyl cyclase (with activity α) and its hydrolysis by phosphodiesterase (with activity β). Sudden block of guanylyl cyclase ($\alpha \cong 0$) or phosphodiesterase ($\beta \cong 0$) allows derivation of a formula for the activity of the other enzyme as a function of the cGMP-dependent current. Guanylyl cyclase was inhibited by rapidly exposing the cell to Ringer solution in which Li^+ is substituted for Na^+ in order to prevent the removal of calcium from the cell by the $Na^+/K^+,Ca^{2+}$ exchanger (Yau and Nakatani, 1984; Hodgkin et al., 1985). The turnover of Ca^{2+} in the outer segment is so rapid that the cessation of Ca^{2+} efflux causes a sudden rise in $[Ca^{2+}]_i$, which inhibits guanylyl cyclase (Koch and Stryer, 1988). The relative activity of phosphodiesterase compared to that of the dark-adapted cell in this case is given by $\beta/\beta_D = \ln(J)/\ln(J_D)$, where J is the normalized current i/i_{dark}, and D indicates the parameters of the cell in its dark-adapted state. After subtracting the junction current, β was estimated from the slope of the normalized current plotted on a semilogarithmic graph and then the ratio β/β_D was calculated. Alternatively, the cell was exposed to Ringer solution containing the phosphodiesterase inhibitor IBMX (500 μM). The relative activity of guanylyl cyclase in this case is given by $\alpha/\alpha_D = (K_{1/2}\ d\ i^{1/3}/dt)/(K^D_{1/2}\ d\ i_D^{1/3}/dt)$, where $K_{1/2}$ is the Michaelis constant for binding of cGMP to the light-sensitive channels. The time derivative of $i^{1/3}$ was calculated using the Savitzki-Golay method (Press et al., 1992), and its maximum was taken. The ratio $K_{1/2}/K^D_{1/2}$ was estimated from the corresponding change in the current (Nakatani et al., 1995; see Figure 6), and then the ratio α/α_D was calculated. Test solutions were delivered to the region surrounding the cells by a rapid microperfusion system (Cornwall and Fain, 1994). The solution change was over 90% complete within 25 ms, as estimated from the junction current recorded while the cell was exposed to bright light.

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References

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

Baylor, D.A., Matthews, G., and Yau, K.W. (1980). Two components of electrical dark noise in toad retinal rod outer segments. J. Physiol. (Lond.) *309*, 591–621.

Bennett, N. (1980). Optical study of the light-induced protonation changes associated with the metarhodopson II intermediate in rod-outer-segment membranes. Eur. J. Biochem. *111*, 99–103.

Bownds, D. (1967). Site of attachment of retinal in rhodopsin. Nature *216*, 1178–1181.

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

Cornwall, M.C., and Fain, G.L. (1994). Bleached pigment activates transduction in isolated rods of the salamander retina. J. Physiol. (Lond.) *480*, 261–279.

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

Cornwall, M.C., Jones, G.J., Kefalov, V.J., Fain, G.L., and Matthews, H.R. (2000). Electrophysiological methods for measurement of activation of phototransduction by bleached visual pigment in salamander photoreceptors. Methods Enzymol. *316*, 224–252.

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

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

Daemen, F.J., Rotmans, J.P., and Bonting, S.L. (1974). On the rhodopsin cycle. Exp. Eye Res. *18*, 97–103.

de Ligt, R.A., Kourounakis, A.P., and IJzerman, A.P. (2000). Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. Br. J. Pharmacol. *130*, 1–12.

Doukas, A.G., Aton, B., Callender, R.H., and Ebrey, T.G. (1978). Resonance Raman studies of bovine metarhodopsin I and metarhodopsin II. Biochemistry *17*, 2430–2435.

Hecht, S., Haig, C., and Chase, A.M. (1937). Rod and cone adaptation. J. Gen. Physiol. *20*, 831–850.

Hodgkin, A.L., McNaughton, P.A., and Nunn, B.J. (1985). The ionic selectivity and calcium dependence of the light-sensitive pathway in toad rods. J. Physiol. (Lond.) *358*, 447–468.

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

Imai, H., Imamoto, Y., Yoshizawa, T., and Shichida, Y. (1995). Difference in molecular properties between chicken green and rhodopsin as related to the functional difference between cone and rod photoreceptor cells. Biochemistry *34*, 10525–10531.

Imai, H., Terakita, A., Tachibanaki, S., Imamoto, Y., Yoshizawa, T., and Shichida, Y. (1997). Photochemical and biochemical properties of chicken blue-sensitive cone visual pigment. Biochemistry *36*, 12773–12779.

Jin, J., Crouch, R.K., Corson, D.W., Katz, B.M., MacNichol, E.F., and Cornwall, M.C. (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. (Lond.) *487*, 441–451.

Jones, G.J., Crouch, R.K., Wiggert, B., Cornwall, M.C., and Chader, G.J. (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., Fein, A., MacNichol, E.F., Jr., and Cornwall, M.C. (1993). Visual pigment bleaching in isolated salamander retinal cones. Microspectrophotometry and light adaptation. J. Gen. Physiol. *102*, 483–502.

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

Koch, K.W., and Stryer, L. (1988). Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions. Nature *334*, 64–66.

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

Melia, T.J., Jr., Cowan, C.W., Angleson, J.K., and Wensel, T.G. (1997). A comparison of the efficiency of G protein activation by ligand-free and light-activated forms of rhodopsin. Biophys. J. *73*, 3182–3191.

Morris, A.J., and Malbon, C.C. (1999). Physiological regulation of G protein-linked signaling. Physiol. Rev. *79*, 1373–1430.

Nakatani, K., Koutalos, Y., and Yau, K.W. (1995). Ca2+ modulation of the cGMP-gated channel of bullfrog retinal rod photoreceptors. J. Physiol. (Lond.) *484*, 69–76.

Okano, T., Kojima, D., Fukada, Y., Shichida, Y., and Yoshizawa, T. (1992). Primary structures of chicken cone visual pigments: vertebrate rhodopsins have evolved out of cone visual pigments. Proc. Natl. Acad. Sci. USA *89*, 5932–5936.

Pepperberg, D.R., Brown, P.K., Lurie, M., and Dowling, J.E. (1978). Visual pigment and photoreceptor sensitivity in the isolated skate retina. J. Gen. Physiol. *71*, 369–396.

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

Press, W.H., Teuklsky, S.A., Vetterling, W.T., and Flannery, B.P. (1992). Numerical Recipes in C: The Art of Scientific Computing, Second Edition (Cambridge: Cambridge University Press).

Rieke, F., and Baylor, D.A. (2000). Origin and functional impact of dark noise in retinal cones. Neuron *26*, 181–186.

Robinson, P.R., Cohen, G.B., Zhukovsky, E.A., and Oprian, D.D. (1992). Constitutively active mutants of rhodopsin. Neuron *9*, 719–725.

Tan, Q., Nakanishi, K., and Crouch, R.K. (1998). Mechanism of transient dark activity of 13-desmethylretinal/rod opsin complex. J. Am. Chem. Soc. *120*, 12357–12358.

Wald, G., Brown, P.K., and Smith, P.H. (1955). Iodopsin. J. Gen. Physiol. *38*, 623–681.

Xu, L., Hazard, E.S., 3rd, Lockman, D.K., Crouch, R.K., and Ma, J. (1998). Molecular cloning of the salamander red and blue cone visual pigments. Mol. Vis. *4*, 10.

Yau, K.W., and Nakatani, K. (1984). Electrogenic Na-Ca exchange in retinal rod outer segment. Nature *311*, 661–663.

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