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Sensitization of bleached rod photoreceptors by 11-*cis*-locked analogues of retinal

(rhodopsin/retinoids/dark adaptation/visual pigment/vision)

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ABSTRACT **Photoactivation of rhodopsin initiates both excitation and adaptation in vertebrate rod photoreceptors. Bleaching of rhodopsin to free opsin and all-*trans*-retinal in isolated rods produces a stable desensitization (bleaching adaptation) that is much larger than expected from pigment depletion alone. In our experiments, a 93% bleach produced a 500-fold increase in the light intensity required for saturation of the light response. This component of adaptation was 32-fold larger than the 16-fold increase expected from pigment depletion alone. 11-*cis*-Retinal, when delivered to isolated rods from liposomes, combines with free opsin to form a bleachable photopigment that fully restores sensitivity. 11-*cis*-Locked analogues of retinal combine with opsin to form unbleachable pigments in isolated bleached rods from the tiger salamander. They restore sensitivity to a substantial (16- to 25-fold) but incomplete extent. The analogues apparently relieve a stable component of adaptation when they interact with opsin. Because these analogues do not detectably excite rods, the structural requirements of both retinal and opsin for the relief of adaptation are different from those of excitation. The biochemical basis of light adaptation resulting from pigment bleaching and the minimum structural requirements of retinal for its relief remain to be determined.**

When bright light bleaches a substantial fraction of rhodopsin in rods that have been isolated from their normal source of the native chromophore 11-*cis*-retinal, the bleaching produces a stable physiological loss in sensitivity that is much greater than the loss of sensitivity due to pigment depletion alone (1–3). The stable loss of sensitivity due to both of these components taken together is commonly referred to as bleaching adaptation (2, 4). Application of 11-*cis*-retinal (Fig. 1, analogue **1**) and other photochemically active isomers relieves both of these components at the same time (7–9). The origin of the stable physiological component of bleaching adaptation and the nature of the structural requirements of retinal and opsin for its relief are fundamental questions in visual physiology.

11-*cis*-Locked analogues of retinal (Fig. 1, analogues **3** and **4**) cannot isomerize about the 11,12 bond (6, 10). These analogues combine with the apoprotein opsin to produce pigments that do not form bathorhodopsin photointermediates and that do not bleach (6, 10–12). The capacity of analogue **3** for excitation or relief of adaptation was originally explored by Crouch *et al.* (13), but no physiologial activity was evident after administration of analogue **3** to vitamin A-deprived rats. We decided to investigate recovery from bleaching adaptation by using current recordings from isolated rods (4, 14) that were extensively bleached and then exposed to lipid vesicles containing analogue at high concentration (15, 16).

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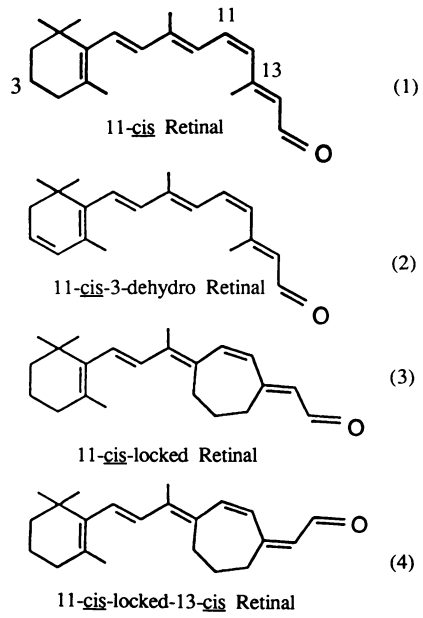

FIG. 1. Structures of retinal analogues used to probe excitation and adaptation in rod photoreceptors. 11-*cis*-Retinal, **1**, is the native chromophore of the visual pigment of the principal rods of the adult tiger salamander (peak at 502 nm; ref. 5). 11-*cis*-3-Dehydroretinal, **2**, is the principal chromophore of the visual pigment of the larval salamander used in these studies (5). Both 11-*cis*-locked retinal, **3**, and 11-*cis*-locked 13-*cis*-retinal, **4**, form unbleachable pigments with absorption maxima at 490 and 488 nm, respectively, when combined with bovine opsin *in vitro* (6).

MATERIALS AND METHODS

11-*cis*-Locked retinals (analogues **3** and **4**) were prepared by the method of Akita *et al.* (6), stored at −80°C under argon gas, and checked for chemical and isomeric purity by HPLC [μPorasil (Waters), hexane/ethyl acetate solvents] before use. 11-*cis*-Retinal was a generous gift from P. Sorter (Hoffmann–La Roche). Liposomes were prepared from 25 mg of phosphatidylcholine (Sigma, type V-E), which was dried, lyophilized, and sonicated with 10 ml of physiological saline until a translucent mixture was obtained (17). Retinal analogues were incorporated into the liposomes by drying an ethanolic solution of analogue under nitrogen or argon gas in the bottom of a conical vial (Kontes) in sufficient quantity (usually a 5-fold excess) to yield a concentration of ≈100 μg/ml when mixed with vesicles and stirred with a conical Teflon stirrer for 1 hr.

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Retinas from dark-adapted larval tiger salamanders (*Ambystoma tigrinum*) were harvested under infrared illumination, and rods were isolated from fragments of retina by trituration. Extracellular membrane current was recorded from the inner segment with a suction electrode connected to a current-to-voltage converter (4, 14). Except during exposure to analogues, rods were superfused with physiological saline containing (in mM) NaCl, 108; KCl, 2.4; $CaCl_2$, 1.6; $MgCl_2$, 1.2; NaH_2PO_4, 1.0; $NaHCO_3$, 0.5; glucose, 5; and Hepes, 10 (pH 7.8) at room temperature (18–22°C).

To evaluate sensitivity changes in the experiments described below, we take the sensitivity S to be the ratio of the incremental response amplitude ΔR in picoamperes to the incremental flash intensity ΔI in photons per μm^2 at vanishing flash intensities (3, 18):

$$S = \lim_{I \to 0}(\Delta R/\Delta I). \quad [1]$$

This quantity may be conveniently estimated from peak response data collected over the entire range of responses by plotting the peaks of the flash responses against flash intensity and fitting a stimulus–response function (Eq. 2) having a variable steepness of saturation and linear low-intensity asymptote as described by Bäckström and Hemilä (19, 20):

$$R(I) = R_{max}\frac{I}{I + I_s\dfrac{1 + (2 - Z)I/I_s}{1 + ZI/I_s}}. \quad [2]$$

The parameters in this function are the response maximum, R_{max} (pA); a semisaturating flash intensity, I_s (photons per μm^2); and Z, a dimensionless parameter that controls the steepness with which the function saturates and the fraction of R_{max} at which I_s occurs (19, 20). Note that the semisaturation intensity I_s is operationally defined by Eq. 2 and is a semisaturation constant in its broader meaning of approximately rather than exactly one-half maximum. The derivative of Eq. 2 as flash intensity approaches zero is a linear slope given by R_{max}/I_s and hence these two parameters may be used to estimate sensitivity as given in Eq. 1. In the experiments described below, we will examine the sensitivity changes due to shifts in the semisaturation intensity I_s separately from those due to changes in maximal response amplitude R_{max}. It is convenient to think of log R_{max} and $\log(1/I_s)$ as being additive components of sensitivity on a logarithmic scale. The present sensitivity measurements may be compared with earlier measures of threshold elevation by recalling that "threshold" intensity (I_t) is proportional to the reciprocal of sensitivity, i.e., I_s/R_{max} (7–9). For these experiments, the average value of Z was 1.4 ± 0.2 (n = 15 cells).

Spectroscopic measurements of visual pigment absorption spectra were made with a photon-counting microspectrophotometer (21, 22) on intact rods and rod outer segments suspended in a 70-μm-thick chamber bounded by two glass coverslips. The fraction of pigment bleached in the physiological experiments was estimated by bleaching rods and intact outer segments in the microspectrophotometer under similar conditions within a beam of 520-nm light adjusted to the same intensity as that used in the physiological measurements. Spectral sensitivity measurements were made with narrow-bandpass interference filters and neutral density filters calibrated with a PIN photodiode radiometer (United Detector Technology, Santa Monica, CA) (23).

RESULTS

In isolated rods from the tiger salamander, we first confirm earlier findings that substantial bleaching produces a stable desensitization by reducing both the reciprocal of the semisaturation constant, $1/I_s$ (2.7 ± 0.3 log units, n = 11), and the maximum response amplitude, R_{max} (0.6 ± 0.2 log unit), and that, of the two, changes in $1/I_s$ are by far the larger (2, 7–9, 16). A control experiment illustrating these changes in sensitivity in response to a 5-min bleach and the method used to estimate them by application of Eq. 2 is shown in Fig. 2. This experiment also illustrates the restoration of sensitivity by 11-*cis*-retinal and the lack of sensitization by the bleaching product, all-*trans*-retinal. In the experiments that follow we will consider the amounts of pigment bleached and the effects of retinal analogues in restoring sensitivity to bleached cells. We will focus attention on the change in $1/I_s$ because it is the larger factor in bleaching adaptation and because it may be directly compared with the physical loss of sensitivity resulting from loss of photon absorption.

In each experiment and the control illustrated in Fig. 3 the initial semisaturation intensity, $I_s(0)$, of an unbleached rod was measured at the start of the experiment. A bleaching light (520 nm, wide field) was then applied for 5 min to stably lower $1/I_s$ by ≈2.5 log units. After the cell had recovered to a stable level of sensitivity, the superfusion was stopped and a 100-μl aliquot of lipid vesicles in physiological saline containing analogue (open symbols) or all-*trans*-retinal, a control (filled symbols), at ≈100 μg/ml displaced the residual bath volume of ≈50 μl of saline (first upward arrow). To maximize the stability and longevity of cells, superfusion was resumed either immediately after an increase in sensitivity was observed or after 1 hr for the control. Addition of analogue **3** or **4** (Fig. 3, open squares and triangles, respectively) restored >1 log unit of the component of sensitivity measured by $1/I_s$; however, this component reached a plateau about 1 log unit below its original level. The time course of resensitization with analogues is comparable to the time course of resensitization with 11-*cis*-retinal (analogue **1**). As measured by

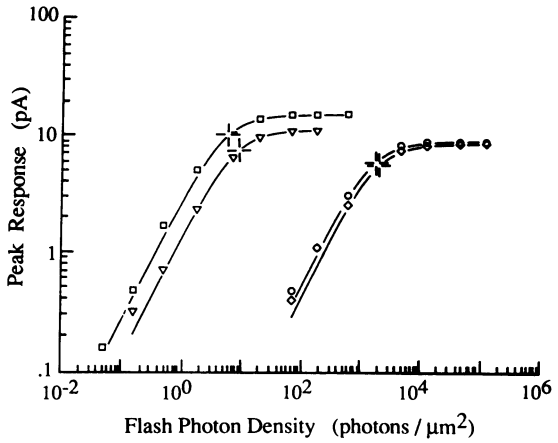

FIG. 2. Shifts of stimulus–response functions induced by bleaching and by the addition of 11-*cis*-retinal. Peak current responses (open symbols) are plotted against flash intensity, and a stimulus–response function (solid line, Eq. 2) is fitted to the data. The open cross indicates the position of the semisaturating light intensity, I_s. Horizontal movement of the cross indicates changes in sensitivity due to changes in I_s. Vertical movements indicate changes in sensitivity due to changes in R_{max}. Bleaching with 520-nm light of high intensity (6.6×10^7 photons per μm^2 per sec^1) for 5 min shifts the position of the curve from the dark-adapted position (open squares) to the right by 2.5 log units and down by 0.2 log unit (open diamonds). Following the addition of all-*trans*-retinal (open circles) there is little recovery in the position of the response curve (<0.1 log unit for both I_s and R_{max}). Addition of 11-*cis*-retinal substantially restores sensitivity by shifting the response curve back to the left by 2.3 log units and up by 0.1 log unit (open triangles). The data points are averages of three responses obtained at four times during the control experiment whose time course is illustrated in Fig. 3.

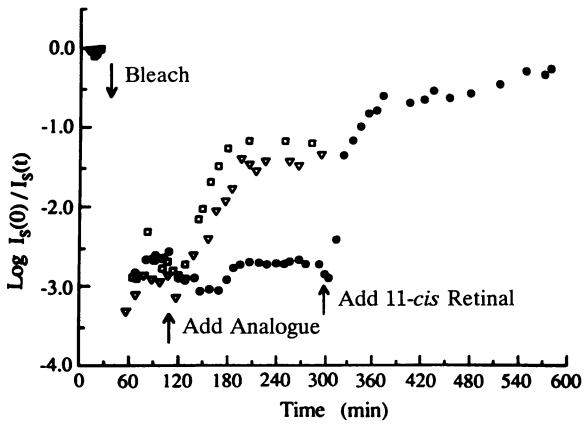

FIG. 3. Time course of changes in the major component of sensitivity during bleaching and resensitization with 11-*cis*-locked analogues of retinal. The logarithm of the semisaturation constant at time t, $I_s(t)$, relative to $I_s(0)$ is plotted against time in minutes. A 520-nm bleaching light ($I = 6.6 \times 10^7$ photons per μm^2 per sec^1) was applied for a total of 5 min at the time indicated by the downward arrow (23). After the sensitivity of a cell had stabilized, analogue was added at the time indicated by the first upward arrow. The analogues were **3** (open triangles), **4** (open squares), or all-*trans*-retinal, a control (filled circles). 11-*cis*-Retinal, **1**, was added to the control cell at the time indicated by the second upward arrow.

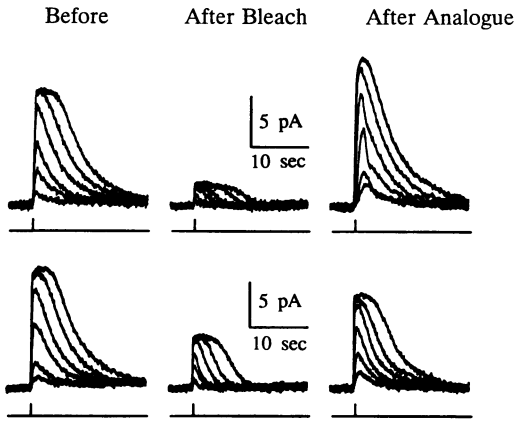

FIG. 4. Restoration of response amplitude and kinetics following resensitization with locked analogues **3** and **4** (*Lower* and *Upper*, respectively). (*Left*) In the control (Before), a series of responses to 20-msec flashes beginning at −6.5 log units and ascending in 0.5-log-unit steps of intensity are shown superimposed. The light monitor marks the occurrence of the test flash. (*Center*) After a bleach, the light intensity required to elicit a comparable series of responses is elevated by 2.5 log units (see legend to Fig. 1), and the amplitudes of the responses are depressed. (*Right*) After application of analogues, the amplitudes of the responses recover to near their dark-adapted level; however, the light intensity required to elicit a comparable series remains elevated by ≈1 log unit.

shifts in $1/I_s$, seven cells exposed to analogue **3** recovered 1.2 ± 0.4 (mean ± SD) of 2.7 ± 0.3 log units of sensitivity lost in bleaching. With analogue **4**, applied to four cells, the recovery was 1.4 ± 0.2 of 2.6 ± 0.2 log units.

In contrast, the control records (filled circles) in Fig. 3 show that all-*trans*-retinal did not restore any sensitivity over the course of 3 hr. The temporary desensitization of a few tenths of a log unit after addition of all-*trans*-retinal was reversed when the superfusion was restarted at the end of an hour. The observation of a stable desensitization confirms earlier observations (2, 7–9, 16) and extends the period of observed stability to >4 hr beyond the bleach. As further shown in Fig. 3, subsequent application of 11-*cis*-retinal (at the time indicated by the second upward arrow) restored the component of sensitivity measured by $1/I_s$ close to its original value and largely within the first hour.

In addition to partially raising $1/I_s$, both locked analogues restored the maximum response amplitude close to its pre-bleach state (Fig. 4). Bleaching significant amounts of pigment reduces R_{max}, and resensitization with 11-*cis*-retinal is known to reverse this effect (7–9, 16). In the seven cells measured above, analogue **3** restored 0.4 ± 0.2 of 0.6 ± 0.2 log unit of sensitivity lost to bleach-induced reduction in R_{max}. For analogue **4** the increase in sensitivity due to an increase in R_{max} was 0.3 ± 0.4 of 0.6 ± 0.1 log unit lost (n = 4). These changes in response amplitude contribute to increases in sensitivity, but their contribution to the analogue-induced increase in sensitivity is only 5% of the total ($100 \times 10^{0.4}/10^{0.4+1.3}$; n = 11) and is only loosely coupled to the analogue-induced increases in log $1/I_s$ (correlation coefficient = 0.45).

We next determined by microspectrophotometry (21, 22) whether the locked analogues could form visual pigments in these cells. For reference, a control measurement of the absorptance spectrum of the native pigment (small dots) is shown at the right in Fig. 5*A* (23). It is well fitted by an 11-*cis*-3-dehydroretinal (analogue **2**) pigment absorptance template (smooth curve) peaking at 519 nm (23). Because there is a small and variable amount of 11-*cis*-retinal (analogue **1**) in larval animals, the spectral peak has been reported to range between 523 and 516 nm (5, 23). Three cells bleached under conditions equivalent to those of the physiological experiments contained only 7 ± 1% of their pigment 30 min after bleaching. It is likely that some of this residual pigment was freshly regenerated from cellular stores of retinal **2**, which can replenish a small percentage of the pigment (24). Control application of retinal **1** to bleached cells produced a pigment with a distinct absorptance spectrum that is well fitted by a retinal **1**-based pigment template that peaks at 505 nm (left absorptance spectrum and fitted curve, Fig. 5*A*).

Application of analogue **3** or **4** to bleached cells produced closely overlapping absorptance spectra that had an average maximal absorptance of 0.26 ± 0.03 and 0.26 ± 0.03 (n = 5 for each), respectively, and that are well fitted by a retinal **1** absorptance template with a peak at 496 nm (small dots, Fig. 5*B*). However, these locked analogue pigments appeared to be unbleachable in that, on average, they did not lose >5% of their initial density (2 ± 4% for analogue **3**, 5 ± 2% for analogue **4**) after three exposures to white light, which bleached 84 ± 3% (n = 4) of the native pigment in other cells on the first exposure. The small loss in density occurred primarily after the first light exposure of the analogue pigments and is consistent with the loss expected from a small fraction of residual native pigment.

To test whether the locked analogue pigments were capable of excitation, we measured the spectral sensitivities of resensitized cells. Control measurements show that the spectral sensitivity of a cell is primarily determined by the dominant photoactive pigment (Fig. 5*A*). In unbleached cells (open symbols), this is the native pigment. In cells bleached and resensitized with retinal **1**, the spectral sensitivity (filled symbols) shifts to match the absorptance spectrum of the new photoactive pigment, as expected. However, in cells partially resensitized with analogue **3** (Fig. 5*B*, open symbols) or **4** (Fig. 5*B*, filled symbols), the spectral sensitivity maximum remains at 519 nm and does not shift to the absorptance spectrum maximum of the new analogue pigment.

DISCUSSION

The failure of the spectral sensitivity to shift to the absorptance spectrum of the locked analogue pigments following partial resensitization with the analogues indicates that the

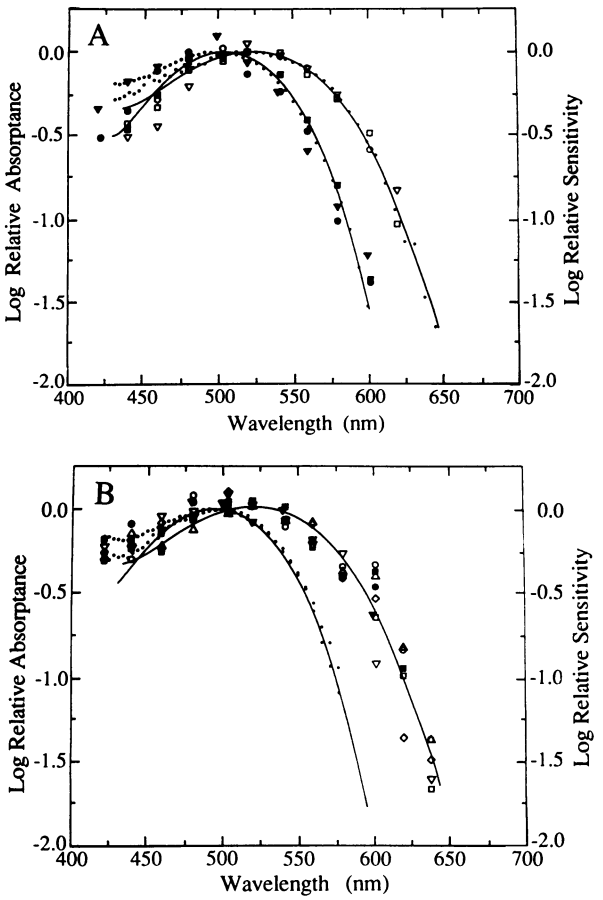

FIG. 5. Measurements of visual pigment absorptance and spectral sensitivity in salamander rods. (*A*) Control measurements of the spectral sensitivity and absorptance spectrum of native and retinal **1**-based pigments in isolated cells confirm that spectral sensitivity closely matches the absorptance spectrum of the predominant photoactive pigment under our experimental conditions. Here the small dots show the absorptance spectra of cells measured by microspectrophotometry (23). Absorptance = $1 - 10^{-OD}$, where OD = optical density. The absorptance spectra are fitted with a visual-pigment absorptance template derived from a wavelength-compensated polynomial (G. Jones, personal communication). For the native pigment based on retinal **2** the template peaks at 519 nm (spectrum at right, data from ref. 23); for retinal **1** the peak is at 505 nm (spectrum at left, average of four cells). The open symbols are measurements of spectral sensitivity of the native retinal **2** pigment in three cells, which show that spectral sensitivity matches the absorptance spectrum of the native pigment in unbleached cells. The filled symbols represent spectral sensitivity measurements following resensitization with 11-*cis*-retinal in three cells and show that the spectral sensitivity shifts to match the absorptance spectrum of the new photoactive pigment. (*B*) Experimental measurements of absorptance and spectral sensitivity in bleached cells following exposure to locked analogues. The dots show that the locked analogues produce overlapping absorptance spectra which are similar in shape to that of retinal **1**-based pigment but which peak at 496 nm (spectrum at left). Unlike cells resensitized with retinal **1**, cells resensitized with either analogue **3** (open symbols, five cells) or **4** (filled symbols, three cells) do not show a shift in spectral sensitivity to the new absorptance peak.

dominant sensitivity arises from residual native pigment. The present results rule out efficient photoexcitation by 11-*cis*-locked retinals in rods. This is in contrast to results with *Chlamydomonas*, where analogue **4** was reported to produce sensitivity 55 times greater than analogue **3** and 1.8 times greater than retinal **1** (25–27).

We suggest that the locked analogues partially resensitize rods by relieving a stable inhibitory constraint on some component of the cascade of excitation initiated by the residual native pigment. The remaining desensitization (1.4 ± 0.4 log units, $n = 11$), not restored by the analogues, is consistent with a 1.2-log-unit loss of sensitivity expected from the reduction of the absorptance of photoactive native pigment to 7% of its original value.

The biochemical source of bleaching adaptation has yet to be identified. It is unlikely to be any of the short-lived bleaching intermediates of rhodopsin, because this inhibition persists undiminished for up to 4 hr (Fig. 3) and is resistant to hydroxylamine (28, 29). Nor is it likely that free all-*trans*-retinal or one of its metabolites depresses sensitivity, as neither addition (ref. 6 and Fig. 3) nor depletion (28, 29) of all-*trans*-retinal alters the level of bleaching adaptation.

The suggestion that free opsin desensitizes rods beyond the extent predicted by pigment depletion originates in the quantitative measurements of Dowling (30) and Rushton (31). Two competing physiological explanations of the densitization have emerged. One is that free opsin sends out a continuous inhibitory signal that reduces amplification in the transduction cascade below its dark adapted level (3, 32). The other is that free opsin sends out a continuous excitatory signal in darkness identical to that of photoexcitation and that this "dark light" in turn produces both input noise and adaptation equivalent to an adapting background light (33–35). The former explanation seems more likely on a number of other grounds reviewed by Ripps and Pepperberg (3). However, a recent paper by Okada *et al.* (36) lends biochemical support to the hypothesis of a continuous residual activation of transducin by opsin.

If opsin is indeed the source of bleaching adaptation, it does not yet appear to be possible to assign this role to incomplete phosphorylation of opsin. ATP, the source of phosphate for opsin phosphorylation, appears to be required for deactivation of transduction in functioning rod outer segments (37), and bright lights may temporarily overload the phosphorylation capacity of rods and thus produce a transient elevation of light-induced dark noise of the type considered by Lamb (34) and Lisman (38). However, the stability of bleaching adaptation in darkness, the small increase in maximum response amplitude on addition of locked analogues, and the weak coupling of changes in response maximum to changes in the semisaturation intensity observed on addition of locked analogues argue against direct residual activation of transducin by incompletely phosphorylated opsin as the main mechanism of bleaching adaptation.

We note here that one historical difficulty with opsin as a source of bleaching adaptation was the paradoxical observation of Rushton (39) that fast photoreversal of bleaching did not appear to alter the time course of dark adaptation in the human eye (40). However, in a careful study, Catt *et al.* (41) could not reproduce Rushton's observations in the isolated retina, and their study raises questions about the accuracy of the original densitometric measurements in the human subjects.

11-*cis*-Locked analogues provide a means of isolating the physiological component of bleaching adaptation in rods. We expect the identification of the biochemical basis of bleaching adaptation and the structural requirements of retinal and opsin for its relief will give new insights into the function of rhodopsin and related receptor molecules.

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