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Retinoid requirements for recovery of sensitivity after visual-pigment bleaching in isolated photoreceptors

(vision/dark adaptation/11-*cis* retinoid/interphotoreceptor retinoid-binding protein)

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ABSTRACT **After visual-pigment bleaching, single isolated rod photoreceptors of *Ambystoma tigrinum* recover their sensitivity to light when supplied with 11-*cis*-retinal from liposomes or with 11-*cis*-retinal bound to interphotoreceptor retinoid-binding protein. Bleached rods do not recover sensitivity, or do so only very slowly, after exposure to 11-*cis*-retinol. The latter retinoid is "toxic" in that rods actually lose sensitivity in its presence. In contrast, bleached isolated cone cells recover sensitivity when either retinoid is supplied. It is suggested that the major pathway for rhodopsin regeneration during dark adaptation in the intact eye is transport of 11-*cis*-retinal from the pigment epithelium to the retina. The results also suggest that there may be separate pathways for visual-pigment regeneration in rods and cones during dark adaptation.**

Regeneration of visual pigment during dark adaptation or during maintained illumination requires retinoid isomerization from trans to cis form and conversion from alcohol to aldehyde before retinoid can be bound to opsin to reconstitute active pigment (1). The pigment epithelium (PE) has long been known to be involved in regeneration (2), implying a "visual cycle" that involves shuttling of retinoid from retina to PE and back again during cycles of light and dark. During light adaptation, there is indeed a progressive loss of retinoid from the retina, and an increase in the retinoid content of the PE. During darkness, the retinoid flow is reversed (3). Fulton and Rando (4) have presented strong evidence for localization of the retinoid-isomerizing system in PE rather than retina, but it remains uncertain which cells are involved in effecting the alcohol-to-aldehyde change that must take place during the visual cycle.

We now report a difference in the use of retinol and retinal by rod and cone cells, and a "toxic" effect of retinol on rod cell function. We suggest that 11-*cis*-retinal is the major form of retinoid transported from PE to retina. Alternatively, if the alcohol form is transported it must be converted to the aldehyde in a cell type other than the rod photoreceptor (which may be cones, or possibly Müller cells).

Pepperberg *et al.* (5) showed that the ordinarily permanent desensitization due to bleaching in isolated retina could be reversed by treatment with 11-*cis*-retinal. In their work, retinal was applied in ethanolic solution. Subsequently, it was shown (6) that liposomes could also be used to deliver retinoid. Our studies on isolated photoreceptors have employed both liposomes and interphotoreceptor retinoid-binding protein (IRBP). IRBP is a protein found at high concentration in the interphotoreceptor matrix, where its unique location and retinoid-binding properties (7, 8) make it likely that it is involved in retinoid movement between retina and PE. In support of this, we demonstrate here the transfer of retinoid between IRBP and photoreceptor cells.

MATERIALS AND METHODS

Isolated photoreceptor cells from dark-adapted, larval tiger salamanders (*Ambystoma tigrinum*) were prepared under infrared illumination from small pieces of retina by gentle trituration. Membrane currents were recorded using suction electrodes (9), with cells oriented to expose the outer segments (10). Suction electrodes were filled with physiological solution and connected via a Ag/AgCl pellet to a current-to-voltage converter. The reference electrode was an agar bridge filled with physiological solution, connected via a second Ag/AgCl pellet. Light stimulation (diffuse spots, unpolarized light) was through interference filters and neutral density filters and was electronically timed. Green light, 540 nm, was used to stimulate rods; this wavelength is close to the isosbestic point for native red rod porphyropsin of larval tiger salamander rods and the rhodopsin formed after regeneration with 11-*cis*-retinal. Yellow light, 580 nm, was used for bleaching, and the extent of pigment bleaching was taken from previous results linking rise in response threshold with fractional pigment bleach (11). For cones, 600-nm light was used for stimulation and bleaching; thus only red-absorbing cones were studied. The extent of bleaching was calculated by taking the *in situ* photosensitivity of the red cone visual pigment to be the same, after correction for dichroism, as that for 3-dehydroretinal-based visual pigment in solution (table 1 in ref. 12). Measurements of bleaching in these red cones by microspectrophotometry support this assumption (G.J.J., unpublished data obtained in collaboration with E. F. MacNichol, Jr.). Cone cells were identified by their narrow, tapering outer segments and round cell bodies.

The experimental chamber (1.5 ml) was formed by the space between a coverslip and the glass surface of a custom-made, water-immersion cap on a ×10, 0.25-n.a. objective lens used as a condenser on an inverted microscope. Bath perfusion was gravity-fed at about 0.5 ml per min. The physiological solution contained (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; Hepes, 10 (pH 7.8). All experiments were at room temperature (18–22°C).

Crystalline 11-*cis*-retinal was a gift from P. K. Brown (Harvard University) or from P. Sorter (Hoffman–La Roche). 11-*cis*-Retinol was prepared by reduction with $LiAlH_4$ in tetrahydrofuran, purified by HPLC, and stored under argon

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Abbreviations: PE, pigment epithelium; IRBP, interphotoreceptor retinoid-binding protein; CRALBP, cellular retinal-binding protein.

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at −70°C. Bovine IRBP was prepared as described (13). The IRBP was concentrated, dialyzed against physiological solution (with glucose omitted), and stored at −70°C. Concentrations were obtained by using extinction coefficients of 25,000 and 35,000 $M^{-1}\cdot cm^{-1}$ for 11-*cis*-retinal (375 nm) and 11-*cis*-retinol (320 nm), respectively (14), and 120,000 $M^{-1}\cdot cm^{-1}$ for IRBP. It was assumed that retinoid binding to protein does not significantly alter the extinction coefficient of either species (15).

For liposome preparation (16), phosphatidylcholine (25 mg, Sigma, type V-E) was dried (O_2-free N_2), lyophilized, and sonicated with 10 ml of physiological solution at 45 W (1-cm probe; Sonics and Materials, Danbury, CT) in an ice/water bath for 15 min. Retinoids were not sonicated with the phospholipid, since this can produce considerable degradation. Instead, retinoids were dried in a small glass vial, a liposome aliquot was added, and the vial was gently agitated overnight at 4°C. Retinoid uptake into the hydrophobic phase and its integrity were monitored by UV absorption spectroscopy after extraction into ethanol. IRBP was loaded with retinoid in the same way and UV absorption spectra were routinely measured.

Cells were exposed to the retinoids by adding 100 μl of stock solution into the bath after halting the bath perfusion. Dye experiments indicated equilibration with the total bath volume within a few seconds. The total amount of retinoid added, several nanomoles, was many times higher than the amount of opsin, a few femtomoles, in the outer segment of the single bleached rod or cone cell in each experiment.

RESULTS

Rod Cells and 11-*cis*-Retinal. Fig. 1 shows the light-evoked membrane current responses in an isolated rod cell before and after bleaching, and after recovery of the cell with 11-*cis*-retinal. After bleaching, at first no responses to light can be recorded. With time, however, responses return, though with a reduced maximum amplitude. A steady state is reached with a reduced sensitivity; i.e., the flash intensity that produces a small criterion response (threshold) is shifted to a much higher value. Upon exposure of the bleached rod cell to 11-*cis*-retinal, both the desensitization and the reduction in response maximum are reversed. In all experiments described here, the bleaching in rods produced a steady desensitization of 2.5–3 logarithmic units, indicating loss of 50–75% of visual-pigment content (11).

In Fig. 2, the sensitivity recovery of rods with 11-*cis*-retinal (2–5 μM) using liposomes as carrier is compared with recovery using IRBP (2 μM) as carrier. For both conditions, the total recovery time is about 1 hr, similar to the time for dark adaptation in the intact eye (2). There is a difference in the kinetics of recovery, however: an S-shaped curve is observed with IRBP, but not with liposomes as carrier (Fig. 2). This characteristic is more evident at lower IRBP and 11-*cis*-retinal concentrations: in two experiments, with IRBP at 0.3 and 1 μM and 11-*cis*-retinal at 0.6 and 2 μM, respectively, there was an initial delay of 20–30 min before any resensitization occurred. Recovery began immediately in two other experiments using liposomes with 11-*cis*-retinal at 0.8 and 1.3 μM. The total recovery time in all four experiments was about 2 hr.

Rod Cells and 11-*cis*-Retinol. Bleached rods show no or very little recovery in sensitivity when exposed to 11-*cis*-retinol, with either IRBP or liposomes as carrier. On the contrary, this retinoid causes a loss in sensitivity (Fig. 3 *A* and *B* and Fig. 4) and also reduces the maximum amplitude of the responses (in some experiments to zero). The maximum amplitude probably represents the level of circulating dark current in the bleached rod.

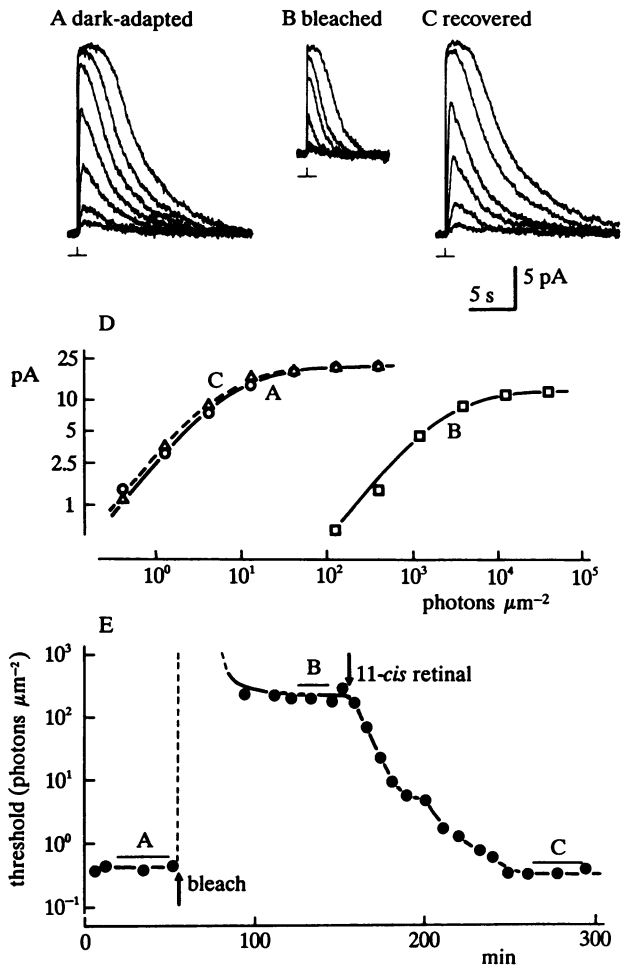

FIG. 1. Bleaching and recovery in an isolated salamander rod photoreceptor. (*A–C*) Membrane current responses to 10-ms, 540-nm flashes recorded before and after bleaching and after recovery with 11-*cis*-retinal. Each trace is an average of five responses. Bleaching light was a 20-s light step, 580 nm, 2.3×10^8 photons $\mu m^{-2}\cdot s^{-1}$. (*D*) Peak amplitudes of responses in *A–C* plotted against light intensity. Sensitivity is measured as the flash intensity that corresponds to a 1-pA response; these intensities are plotted as thresholds in *E* and Figs. 2–5. (*E*) Complete time course of sensitivity changes in this cell. Horizontal bars indicate when responses in *A–C* were recorded. Bath perfusion was halted from just before addition of 11-*cis*-retinal to just before bar marked C. 11-*cis*-Retinal (5 μM) was delivered by liposomes.

This "toxic" effect of 11-*cis*-retinol was very variable from one cell to another. In eight experiments with bleached rods, desensitization 20–30 min after exposure to 11-*cis*-retinol ranged from 1.5 log units (Fig. 3*A*) to >3.5 log units for the four cells that no longer showed responses to light flashes (Fig. 4). The effect was observed also in dark-adapted rods, though less prominently (Fig. 3*B*). In three experiments, desensitization by 11-*cis*-retinol 20–30 min after exposure was by 0, 0.5, and 1.0 log unit before bleaching and by 1.5, 1.5, and >3.5 log units in the same cells after bleaching. Control experiments (Fig. 3 *B* and *C*) showed that the desensitization was not due to halting of superfusion for extended periods of time.

The toxic effect of 11-*cis*-retinol can be reversed by washing (Fig. 3 *A* and *B*), and a similar recovery occurs on addition of an excess of IRBP (Fig. 4). For both cases, reversal is probably due to removal of retinol from the rod. It has been shown that retinol, but not retinal, can be removed from disc membranes by washing (17) and that the equilibrium transfer

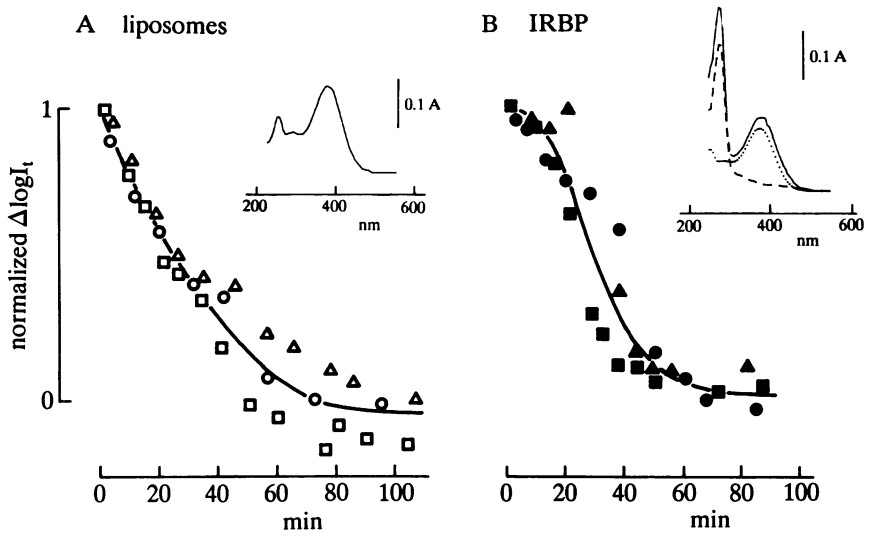

FIG. 2. Time course of recovery of sensitivity of bleached rods with 11-*cis*-retinal (2–5 μM) supplied by liposomes (*A*) or with 11-*cis*-retinal supplied by IRBP (2 μM) as carrier. Each symbol type represents a different cell. Data are normalized so that the logarithm of the threshold change from the initial dark-adapted level ($\Delta\log I_t$) is unity for bleached cells. (*A Inset*) UV absorption spectrum of 11-*cis*-retinal from one of the experiments after 10-fold dilution into ethanol. (*B Inset*) UV absorption spectrum of IRBP with bound 11-*cis*-retinal from one of the experiments after 10-fold dilution; dashed and dotted curves are the absorption spectra of IRBP and 11-*cis*-retinal assumed to underlie the composite spectrum.

of retinol from liposomes to disc membranes is considerably reduced by IRBP (18).

Reversal of the toxic effect of 11-*cis*-retinol by washing (six experiments) or by excess IRBP (two experiments) returned sensitivity only to close to the bleached level. The rods remained viable, however, since subsequent application of 11-*cis*-retinal returned the sensitivity fully back to its original, dark-adapted level (Figs. 3*A* and 4). This shows that exposure of rods to 11-*cis*-retinol results in no long-lasting desensitization that could mask a resensitization from the bleached level due to regeneration of visual pigment. Any such regeneration, if present, must be very low. In these eight experiments, the mean sensitivity 100 min after initial exposure to 11-*cis*-retinol was, relative to the previous bleached levels, −0.17 log unit (SD, ±0.34; range, −0.68 to +0.21), not significantly different from zero. Two cells were tested up to 150 min after exposure to 11-*cis*-retinol and showed sensitivities of +0.22 and +0.14 log unit relative to the previous bleached levels.

Cone Cells and 11-*cis* Retinoid. In contrast to the situation in rods, bleached isolated cone cells clearly recover sensitivity on exposure to 11-*cis*-retinol. The results of three experiments are shown in Fig. 5 (solid symbols). The visual-pigment content of three cones was bleached to three different levels (80%, 95% and >99% bleaching, see *Materials and Methods*). Cone cells adapt very quickly, within a few seconds, to the loss of visual pigment. Thresholds, except for very strong bleaching, then remain elevated and stable for long periods. The reason for the slow increase in sensitivity after a strong bleaching (Fig. 5, circles) is not known. This behavior may be due to there being a small amount of endogenous 11-*cis* retinoid in isolated cone cells. It is not seen when cells are bleached to the same extent in steps (G.J.J., unpublished data). All three cells recovered full sensitivity within 30–40 min when supplied with 11-*cis*-retinol by using IRBP as carrier. After bleaching, cone cells also recover sensitivity with 11-*cis*-retinal, using either liposomes (Fig. 5) or IRBP (two experiments, not illustrated) as carrier.

To rule out the possibility that the recovery of bleached cone cells with IRBP and 11-*cis*-retinol is due to the binding of endogenous retinal (from cellular debris in the experimental chamber) to IRBP and its subsequent transfer to the bleached cell, IRBP with no added retinoid was applied to bleached cone cells. No effect was seen in two experiments (Fig. 5, open symbols).

DISCUSSION

Isolated salamander rod cells can use 11-*cis*-retinal to recover sensitivity after visual-pigment bleaching, and, in our conditions, this occurs in times similar to dark-adaptation times in the intact eye. These rod cells either are unable to use 11-*cis*-retinol to recover sensitivity or do so only very slowly. No resensitization of bleached rods was found up to 100 min after exposure to 11-*cis*-retinol. A possible small resensitization was found after 150 min, but this amounted to a decrease in threshold of at most 0.2 log unit, which translates according to the data of Liebovic *et al.* (11) into a pigment regeneration rate of 0.04% min^{-1}. This is 10 times lower than the maximal regeneration rates found with 11-*cis*-retinol in the intact retina (6, 16). Full visual-pigment regeneration at this rate would take several days.

Since the total visual pigment of the retina is predominantly in rod outer segments (>95% in salamander retina; G.J.J., unpublished data), a major pathway for recovery after bleaching may be transport of 11-*cis*-retinal, rather than 11-*cis*-retinol, from PE to retina. Scattered evidence supports this idea. The retinoid isomerase system of bovine PE produces 11-*cis*-retinol from all-*trans*-retinol when purified (4), but 11-*cis*-retinal is also produced in membranes from frog and bovine PE (19, 20). The bovine PE retinol oxidoreductase is known to act preferentially on 11-*cis* retinoid (21), and cellular retinal-binding protein (CRALBP), which preferentially binds *cis* retinoids, is found in bovine PE cells to carry exclusively 11-*cis*-retinal (22). Furthermore, Adler and Evans (23) have reported an increase in the amount of 11-*cis*-retinal bound to IRBP within the bovine interphotoreceptor matrix during dark adaptation.

After bleaching of the isolated retina, 11-*cis*-retinol has been found to promote recovery of rod sensitivity or visual-pigment regeneration in the amphibian retina but not in the mammalian retina; 11-*cis*-retinal is effective in both cases (6, 16, 24). This result can be reconciled with the present data only if the amphibian retina is able to convert 11-*cis*-retinol to retinal within cells other than the rod photoreceptors. One

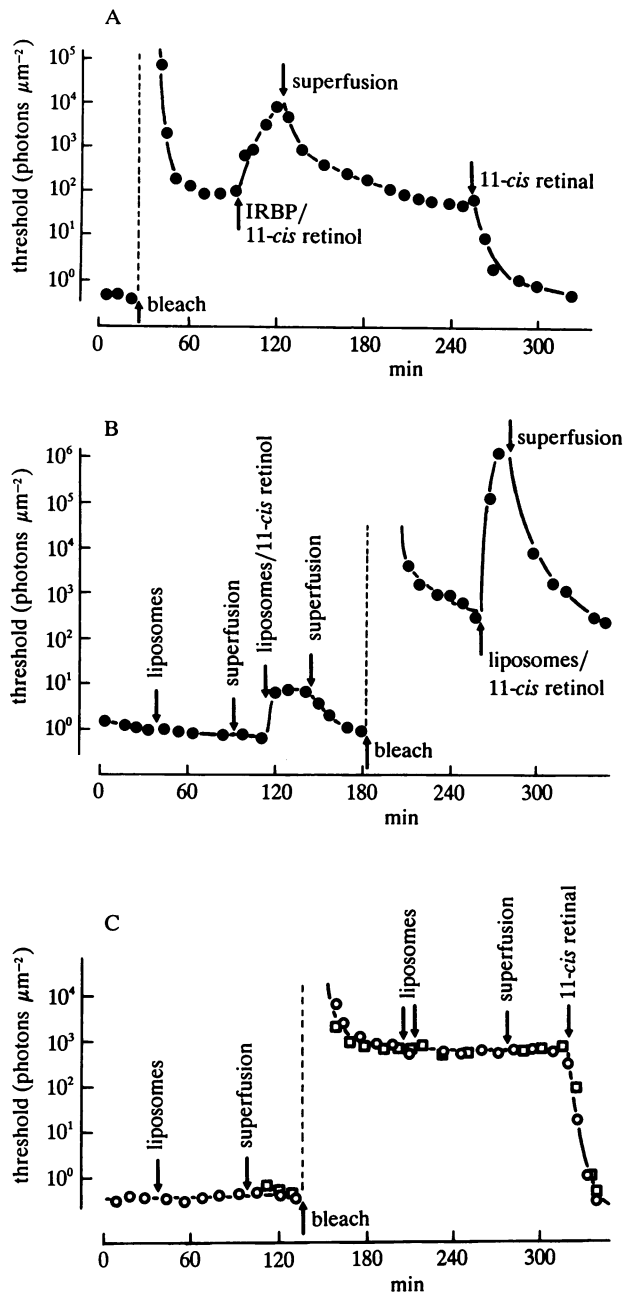

FIG. 3. Toxic effect of 11-*cis*-retinol on rod photoreceptors. (*A*) Effect of IRBP (2 μM)/11-*cis*-retinol (4 μM) on a bleached rod. The loss in sensitivity was reversed by washing, but only to close to the bleached level. Subsequent application of 11-*cis*-retinal (10 μM) with liposomes produced full recovery. Bath perfusion was halted just before both applications of retinoids. (*B*) Loss in sensitivity of dark-adapted and bleached rod on application of 11-*cis*-retinol. Liposomes without retinoid were also tested on this cell. 11-*cis*-Retinol concentration, 6 μM. Bath perfusion was halted just before all three applications of liposomes. (*C*) Two control experiments showing that halting of perfusion and addition of liposomes have no effect on sensitivity of dark-adapted and bleached rods.

possibility arising from the present work is that this could occur within cones. Alternatively, CRALBP found in the Müller cells of the bovine retina (7) has been observed to carry both 11-*cis*-retinol and 11-*cis*-retinal (22), so one could envisage conversion from alcohol to aldehyde occurring at this site. Contamination of the isolated frog retina by processes of the PE, a possibility demonstrated to occur in mammalian retinae by Bunt-Milam and Saari (7), could also explain

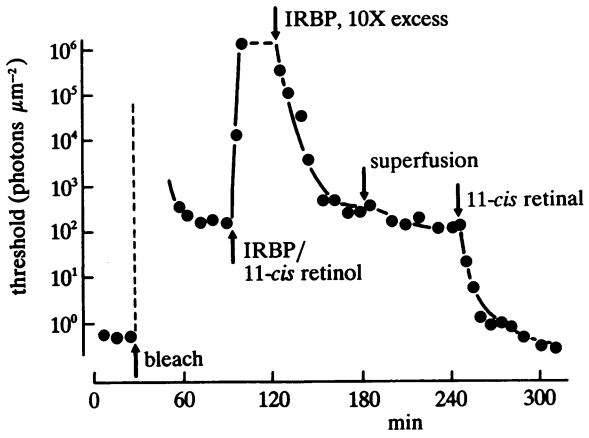

FIG. 4. Reversal of the toxic effect of 11-*cis*-retinol on a rod cell by addition of excess IRBP. Initial IRBP concentration was 0.2 μM, with 0.4 μM 11-*cis*-retinol. Second IRBP concentration was 2 μM, with no retinoid. Superfusion was halted just before the first addition of IRBP. Dashed horizontal line indicates period when no responses to flashes could be obtained and the circulating dark current was apparently zero. 11-*cis*-Retinal (15 μM) was delivered by liposomes.

the conflict between the present results and previous work, but it is less clear how this could explain why studies with isolated outer segments indicate that frog rod outer segments are able to convert 11-*cis*-retinol to the aldehyde form (16, 25) whereas bovine rods cannot (21).

It is possible that bleached salamander rods do not recover sensitivity upon exposure to 11-*cis*-retinol because they require 11-*cis*-3-dehydroretinol rather than 11-*cis*-retinol. We think this unlikely, however, since there is no discrimination between these two retinoids in the mixed rhodopsin/porphyropsin systems of freshwater fish (26) and of the bullfrog (27). Also, that salamander cones recover with 11-*cis*-retinol argues against a requirement for dehydroretinol in the salamander retina.

Cone cells in the isolated frog retina recover sensitivity after bleaching (28–30), whereas rods do not. Isolated salamander cone cells do not significantly recover (see Fig. 5). This suggests there is a pool of 11-*cis* retinoid in the retina that is available to promote cone recovery. There is evidence for such a store from a study of pigment regeneration in the human fovea (31), and 11-*cis* retinoid is found in the bovine retina bound to CRALBP in Müller cells (7, 22). The results of our experiments suggest the possibility of more than one pathway for return of 11-*cis* retinoid from PE to the two types of photoreceptor, since IRBP within the bovine interphotoreceptor matrix carries 11-*cis*-retinol as well as 11-*cis*-retinal (23, 32, 33). Separate pathways for recovery in the two photoreceptor types might be necessary to facilitate cone recovery under conditions of bright, maintained illumination. Although the affinity of cone opsin for 11-*cis*-retinal is much greater than that of rod opsin (34), this may well be insufficient to promote cone recovery in the face of a large pool of bleached opsin in rods.

Finally, the present results support the hypothesis that IRBP participates in retinoid movement between PE and retina (35), showing that transfer of retinoid from IRBP to photoreceptors occurs and promotes recovery after bleaching in intact functioning cells. Furthermore, with concentrations of IRBP and retinoid in the micromolar range, recovery occurs in times that are not markedly different from those in the intact eye. Of particular further interest is the finding that the toxic effect of 11-*cis*-retinol on rod photoreceptors that we describe here is much reduced on addition of excess IRBP. Thus, IRBP could also have a role of buffering the rods from a reversible, deleterious effect of retinol. Such a role for

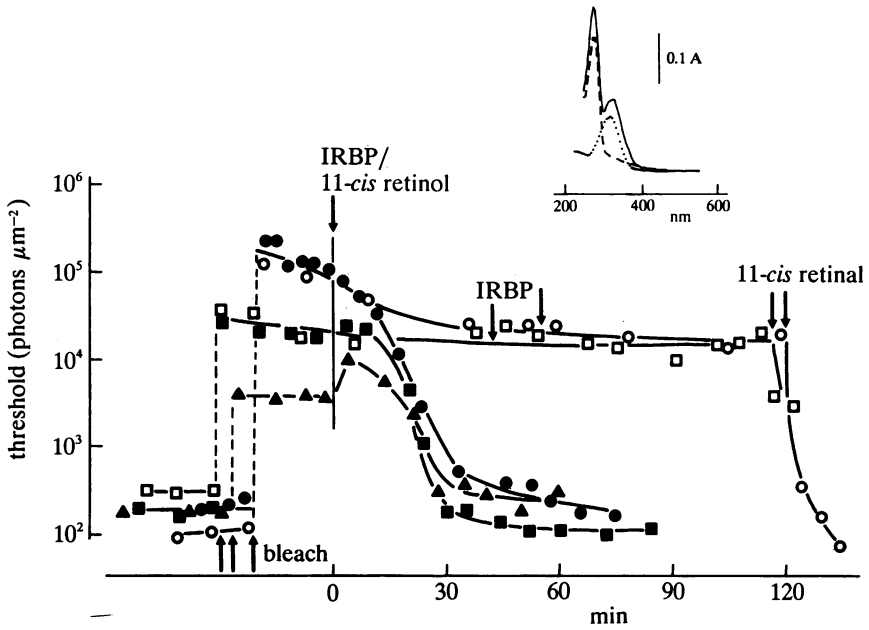

FIG. 5. Recovery of sensitivity after bleaching in salamander cone photoreceptors. Solid symbols show changes in thresholds for three cone cells, before and after bleaching and during recovery with 11-*cis*-retinol using IRBP as carrier. Open symbols represent two control experiments with bleached cones exposed to IRBP without retinoid, and then to 11-*cis*-retinal. Thresholds correspond to 1-pA responses for 10-ms, 600-nm flashes. Bleaching light was 600-nm steps, 2.5×10^7 photons $\mu m^{-2} \cdot s^{-1}$ lasting 10–44 s, and calculated to bleach 80%, 95%, and >99% of visual-pigment content (see *Materials and Methods*). Bath perfusion was halted just before all applications of IRBP and was resumed when full recovery was obtained, or after 30–40 min for IRBP alone. 11-*cis*-Retinal (11 μM) was delivered by liposomes. (*Inset*) UV absorption spectrum of IRBP with bound 11-*cis*-retinol used in the experiments, after 10-fold dilution. Dashed and dotted curves are the absorption spectra of IRBP and 11-*cis*-retinol assumed to underlie the composite spectrum.

IRBP was suggested by the experiments of Ho *et al.* (18), who showed that IRBP actually hinders the transfer of retinol between liposomes and rod disc membranes.

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