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Interphotoreceptor retinoid-binding protein promotes rhodopsin regeneration in toad photoreceptors

(visual pigment/retinal pigment epithelium/vision/*Bufo marinus*)

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ABSTRACT **Interphotoreceptor retinoid-binding protein (IRBP) has been hypothesized to function as an intercellular shuttle in the vertebrate eye, serving to transport retinoids between the retinal pigment epithelium (RPE) and photoreceptors in the process by which visual pigment is regenerated after photolysis. This hypothesis was tested in preparations utilizing the toad (*Bufo marinus*) eye and purified, initially ligand-free IRBP obtained from the bovine eye. Rod outer segments (ROS) or neural retinas were isolated and bleached, then incubated with native RPE (RPE-eyecup) in the presence or absence of IRBP. The amount of rhodopsin present after incubation was determined by spectrophotometric analysis and compared with that in control preparations receiving bovine serum albumin or Ringer's solution only. Supplementation with IRBP enhanced the formation of rhodopsin in both the ROS/RPE-eyecup and retina/RPE-eyecup preparations. Regeneration in ROS/RPE-eyecups receiving IRBP (1.8 nmol) increased in a roughly linear manner with the period of incubation (0–4 hr), at a rate of 0.44 nmol/hr. The extent of regeneration was graded with the quantities of IRBP and opsin introduced into the RPE-eyecup. With increasing amounts of IRBP (up to 5.2 nmol) or of initially available opsin (up to 15.6 nmol), the amount of rhodopsin formed (3-hr incubation) approached the same plateau value, about 2.5 nmol. Analysis of IRBP-supplemented Ringer's solution incubated in the RPE-eyecup showed 11-*cis*-retinal to be virtually the only retinoid withdrawn from the RPE. With large quantities of IRBP (3.2–9.2 nmol), the amount of 11-*cis*-retinal (2.7 ± 0.5 nmol) withdrawn from the RPE during a 3-hr incubation was similar to the plateau value of rhodopsin formed in the ROS/RPE-eyecup. No 11-*cis*-retinal was observed in albumin-supplemented Ringer's solution (0.4–11.2 nmol of bovine serum albumin) or in Ringer's alone after similar incubation in the RPE-eyecup. The results suggest that an IRBP-mediated transfer of 11-*cis*-retinal from the RPE to the rods supports rhodopsin regeneration *in vivo*.**

The regeneration of rhodopsin in vertebrate rod photoreceptors requires the supply of 11-*cis*-retinal to opsin contained in the rod outer segments (ROS) (1). A dependence on the retinal pigment epithelium (RPE) was demonstrated more than a century ago by Kühne (2), who showed that contact with the RPE is essential for the regeneration of visual pigment to take place in a previously bleached retina. Although it is clear that the 11-*cis* configuration of the retinoid is formed within the RPE (3–9), neither the identity of the 11-*cis* retinoid returned from the RPE to the photoreceptors nor the mode of intercellular transfer has been firmly established. Also uncertain is the role in this process of interphotoreceptor retinoid-binding protein (IRBP), a soluble protein that readily binds different chemical and isomeric forms of retinoid and is uniquely localized to the interphotoreceptor matrix compartment that separates the retina and the apical surface of the RPE (10–14).

We recently showed that IRBP supports the delivery of all-*trans*-retinol to the RPE, consistent with a carrier function for IRBP in the intact eye (15). Here we report that, in reconstituted systems consisting of native RPE, initially ligand-free IRBP, and bleached ROS or retina, IRBP promotes the formation of rhodopsin in the photoreceptors. Moreover, the process utilizes 11-*cis*-retinal specifically withdrawn from the RPE. The results provide evidence for a direct involvement of IRBP in the regeneration of visual pigment *in vivo* and link the result of Kühne's classic experiment with the activity of IRBP. They furthermore indicate that 11-*cis*-retinal, rather than a precursor, is the principal 11-*cis* retinoid transferred to the photoreceptors during regeneration.

METHODS

IRBP was purified from light-adapted bovine eyes (16) and stored at −20°C. Delipidated bovine serum albumin (BSA), nicotinamide adenine dinucleotide phosphate ($NADP^+$), polyoxyethylene 10 tridecyl ether, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), potassium borohydride, and the all-*trans* isomers of retinal, retinol, and retinyl palmitate were obtained from Sigma. 11-*cis*-Retinal was kindly provided by Paul K. Brown of Harvard University; 11-*cis*-retinol was prepared by borohydride reduction of the 11-*cis*-retinal (7). *n*-Hexane and dioxane (American Burdick & Jackson, Muskegon, MI) were of high-performance liquid chromatography (HPLC) grade. The Ringer's solution contained (in mM) NaCl, 111; KCl, 2.5; $CaCl_2$, 0.86; $MgCl_2$, 1.6; glucose, 5.6; and Hepes, 3.0, at pH 7.8 (17).

All tissues were obtained from the eyes of toads (*Bufo marinus*) that were fully dark-adapted (≥6 hr) at the time of sacrifice; animals were killed by double pithing. Under dim red light, the enucleated eye was hemisected and drained of vitreous humor. The retina was gently removed from the posterior hemisphere, and the vitreal cavity of the resulting "RPE-eyecup" (15) was washed 20 times with 1 ml of Ringer's. ROS purified by sucrose flotation (18) and suspended in Ringer's solution were exposed for 10–15 min to intense green light that bleached ≥80% of the available rhodopsin. Aliquots of the unbleached and bleached suspension were analyzed for rhodopsin to determine baseline (i.e., preincubation) levels of remaining rhodopsin and of free

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Abbreviations: IRBP, interphotoreceptor retinoid-binding protein; RPE, retinal pigment epithelium; ROS, rod outer segment(s); BSA, bovine serum albumin.

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opsin available for regeneration (initially available opsin). Portions of the suspension of bleached ROS were supplemented with IRBP or BSA dissolved in Ringer's solution, or with Ringer's only. Incubations of the supplemented, bleached ROS with the RPE-eyecup (21–23°C, in darkness) were initiated by delivering 250 μl of the suspension to the vitreal cavity of the RPE-eyecup and terminated by withdrawal of the ROS suspension. An aliquot of the recovered suspension, typically 40 μl, was solubilized [1% (vol/vol) polyoxyethylene 10 tridecyl ether] and supplemented with hydroxylamine (final concentration, 100 mM). Absorbance spectra (Perkin–Elmer Lambda-7 spectrophotometer) recorded before and after intense irradiation were used to determine the hydroxylamine difference spectrum of the sample (6); the concentration of rhodopsin was determined from the absorbance difference at 500 nm (ΔA_{500}), using an extinction coefficient of 42,000 $M^{-1}\cdot cm^{-1}$ (19). Amounts of rhodopsin formed in the incubated preparations were determined by subtracting the baseline rhodopsin level (see above) from levels measured after incubation. Quoted molar levels of opsin and rhodopsin refer to those contained in the 250 μl incubated in the RPE-eyecup.

Isolated retinas to be incubated with RPE-eyecups were first bleached (20-min irradiation) and washed seven times with 3 ml of Ringer's solution. A 20-μl aliquot of test solution consisting of 65 μM IRBP in Ringer's, or 65 μM BSA in Ringer's, or Ringer's only, was delivered to the apical surface of the washed RPE-eyecup immediately before the retina was replaced, photoreceptor side down, on the RPE. The procedure tended to disperse the 20 μl of test solution over the region where the two tissues came in contact. The retina was removed from the RPE-eyecup after 3 hr of incubation; ROS were prepared from each retina, suspended in 400 μl of buffer, and analyzed for both rhodopsin and protein. Protein concentrations were determined using a modified Bradford's assay and BSA standards (20).

Analyses of solutions (IRBP-supplemented and BSA-supplemented Ringer's, and Ringer's only) that had been incubated in the RPE-eyecup were initiated immediately after the incubation period. Unless otherwise noted, the following procedure was used for extraction and spectrophotometric determination of retinoid contained in the incubated solution. Ethanol (200 μl) was thoroughly mixed with the 250 μl of solution withdrawn from the RPE-eyecup; the sample was further supplemented with 600 μl of hexane, and the resulting mixture was vigorously shaken. The upper (predominantly hexane) phase of this final mixture was recovered and an absorbance spectrum was obtained. The extinction coefficient (365 nm) of 11-*cis*-retinal in this extract was taken as $2.6 \times 10^4\ M^{-1}\cdot cm^{-1}$, the value in hexane (21). For HPLC analysis of the extracted retinoid, the hexane-dominated solvent was evaporated under nitrogen, and the residue was dissolved in hexane. The HPLC determinations (15) (Waters spherical silica column, Resolve Si; column dimensions, 3.9 × 150 mm; absorbance detection at 320 and 370 nm) employed a step gradient of dioxane/hexane similar to that described by Tsin *et al.* (22).

RESULTS

Dependence of Regeneration on IRBP and RPE. The formation of a substantial amount of rhodopsin in previously bleached ROS required incubation of the ROS with both IRBP and the RPE. In the experiment described by Fig. 1, portions of a suspension of bleached ROS were supplemented with either IRBP (7.0 μM) or with Ringer's only, incubated for 3 hr in an RPE-eyecup, and then analyzed for the level of light-sensitive pigment (hydroxylamine difference spectrum). The spectrum obtained from the Ringer's-supplemented ROS/RPE-eyecup showed a ΔA_{500} value of 0.10 (Fig. 1, ■). This spectrum was similar to the baseline difference spectrum obtained from the ROS immediately after the preparative bleaching irradiation (data not illustrated; $\Delta A_{500} = 0.08$); the similarity indicated that the final level of pigment measured in the Ringer's-supplemented ROS/RPE-eyecup was due largely to native rhodopsin remaining after the initial bleaching exposure. By contrast, the difference spectrum obtained from the IRBP-supplemented RPE-eyecup showed the formation of a large quantity of light-sensitive pigment (Fig. 1, ●; $\Delta A_{500} = 0.36$); this pigment was identified as rhodopsin, based on the correspondence of its hydroxylamine difference spectrum with that of native rhodopsin (Fig. 1, △). Additional aliquots of the bleached ROS suspension were supplemented with 7.0 μM IRBP or with Ringer's only, incubated for 3 hr in a test tube (i.e., in the absence of the RPE), and then analyzed. The spectra obtained from these controls were

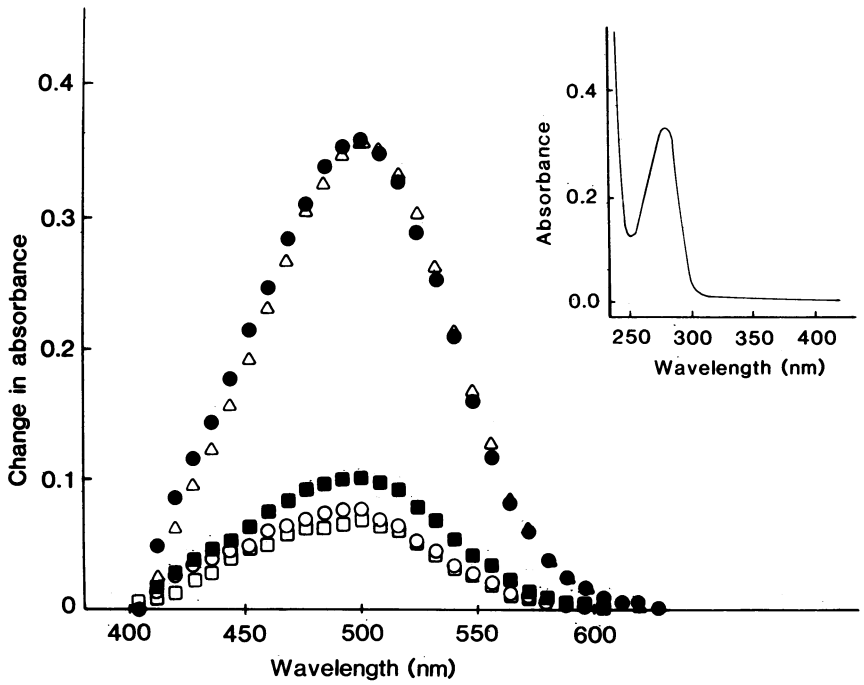

FIG. 1. Hydroxylamine difference spectra of ROS. The bleached ROS were supplemented with IRBP (final concentration, 7.0 μM), or with Ringer's only, and then incubated for 3 hr in an RPE-eyecup or test tube. The RPE-eyecups were obtained from the same animal. ●, IRBP, RPE-eyecup; ■, Ringer's, RPE-eyecup; ○, IRBP, test tube; □, Ringer's, test tube. △, Relative difference spectrum of native rhodopsin in the parent ROS suspension; these data have been scaled downward by a factor of 1.6 for comparison with results obtained from the IRBP-supplemented RPE-eyecup. (*Inset*) Absorbance spectrum of IRBP in Ringer's solution.

similar to the baseline difference spectrum (ΔA_{500} values of 0.08 and 0.07; Fig. 1, ○ and □). This finding is consistent with spectrophotometric (Fig. 1 *Inset*) and spectrofluorometric data (not shown) indicating that the IRBP was essentially free of endogenous retinoid. Together, the data of Fig. 1 indicate both a carrier role for IRBP and the involvement of the RPE as a source of 11-*cis* retinoid in the regeneration observed in the IRBP-supplemented ROS/RPE-eyecup.

Dependence on Incubation Period, Level of Available Opsin, and IRBP Concentration. The time course of regeneration was examined in a group of 12 RPE-eyecups that had received aliquots of the same IRBP-supplemented, bleached ROS suspension; each added aliquot contained 1.8 nmol of IRBP and 3.0 nmol of free opsin. The amount of rhodopsin that was formed increased in roughly linear fashion with the period of incubation (0–4 hr), at a rate of 0.44 nmol/hr (Fig. 2). In similar experiments, groups of 11 RPE-eyecups were incubated with bleached ROS that had been supplemented with 1.8 nmol of BSA or with Ringer's only. Rates of rhodopsin formation among the BSA- and Ringer's-supplemented preparations were 0.07 and 0.14 nmol/hr, respectively (data not illustrated).

The amount of rhodopsin formed in the ROS/RPE-eyecup was graded with the level of opsin initially available for regeneration (Fig. 3*A*). Incubation with relatively small quantities of opsin (≤1 nmol) gave rise to nearly complete regeneration. That is, the molar amount of rhodopsin formed during the 3-hr incubation approached that of the available opsin (compare Fig. 3*A* data with dashed line representing 100% regeneration). However, with increasing levels of initially available opsin, the quantity of rhodopsin formed during the 3-hr incubation approached a plateau value. The extent of regeneration during this incubation period also increased with the amount of IRBP introduced into the RPE-eyecup (Fig. 3*B*), and it too approached a plateau value. It is noteworthy that the plateau levels observed in both types of experiment were similar, representing the formation of about 2.5 nmol of rhodopsin (see *Discussion*).

Retinoid Released from the RPE. Incubation of the RPE-eyecup with 250 μl of IRBP-supplemented medium (i.e., no ROS or retina introduced) led to the appearance, in the IRBP solution, of material absorbing maximally near 380 nm (Fig. 4*A*). Treatment of this solution with ethanol and hexane extracted the 380-nm-absorbing material into the resulting hexane-dominated phase. The principal retinoid extracted

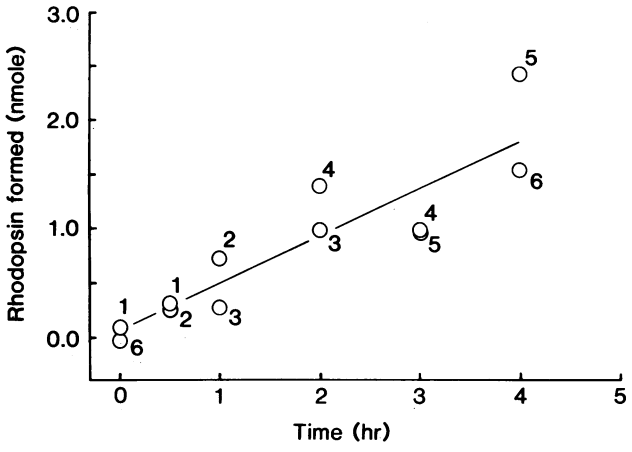

FIG. 2. Time course of rhodopsin formation. Data obtained from 12 RPE-eyecups that received aliquots of the same IRBP-supplemented ROS suspension (1.8 nmol of IRBP); numbers identify pairs of RPE-eyecups obtained from the same animal. Initially available opsin delivered to each RPE-eyecup was 3.0 nmol. The slope of the line fitted by linear regression to all data points is 0.44 nmol/hr.

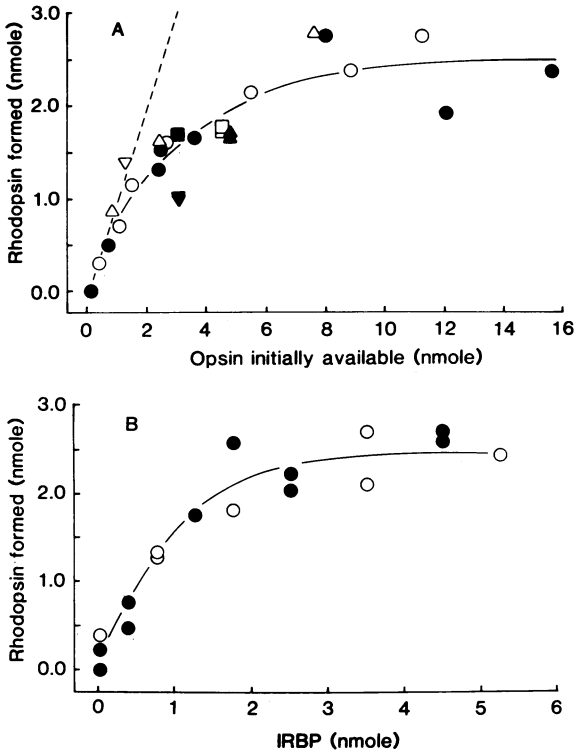

FIG. 3. (*A*) Rhodopsin formed (3-hr incubation) vs. concentration of initially available opsin. In each experiment, a suspension of bleached ROS was diluted to varying extent in Ringer's solution, then supplemented with IRBP and delivered to the RPE-eyecup. Identical symbols represent data obtained from the same suspension of bleached ROS. Each RPE-eyecup received 1.8 nmol of IRBP. The dashed line corresponds to complete regeneration of the available opsin. (*B*) Rhodopsin formed (3-hr incubation) vs. concentration of IRBP. Each RPE-eyecup received 3.2 nmol of available opsin and the indicated quantity of IRBP. Identical symbols represent data obtained from the same preparation of bleached ROS.

was identified as 11-*cis*-retinal on the basis of its chromatographic behavior (Fig. 4*B*) and its absorbance spectrum (Fig. 4*A Inset*). Interestingly, no 11-*cis*-retinol was detected in the extracted incubation medium; the absence of this retinoid is consistent with recent evidence that externally applied 11-*cis*-retinol does not support the regeneration of rhodopsin in isolated, intact rod photoreceptors of the amphibian retina (23).

Spectrophotometric data obtained in the experiment shown in Fig. 4, which involved incubation with 2.8 nmol of IRBP, indicated that the quantity of 11-*cis*-retinal withdrawn from the RPE was 2.6 nmol. In six additional experiments, RPE-eyecups were incubated with Ringer's solution containing 3.2–9.2 nmol of IRBP; together, the spectrophotometric data obtained from these preparations indicated the presence of 2.7 ± 0.5 nmol of 11-*cis*-retinal in the extracellular medium after 3 hr of incubation (Table 1). As controls, RPE-eyecups were incubated with BSA-supplemented Ringer's (0.4–11.2 nmol of BSA) or with Ringer's alone. In each of these experiments, no retinal was detected spectrophotometrically after 3 hr of incubation; if present in the incubation medium, the amount of retinal was <0.1 nmol (Table 1). Thus, the release of 11-*cis*-retinal was highly dependent on the presence of IRBP.

Regeneration in Bleached Retina. The effects of incubation with IRBP or BSA were compared in three experiments, each of which employed the two RPE-eyecups and retinas obtained from a single animal. The previously isolated and bleached retinas were incubated for 3 hr in RPE-eyecups that had received a 20-μl aliquot of either 65 μM IRBP or 65 μM BSA. Determination of protein concentrations in the ROS

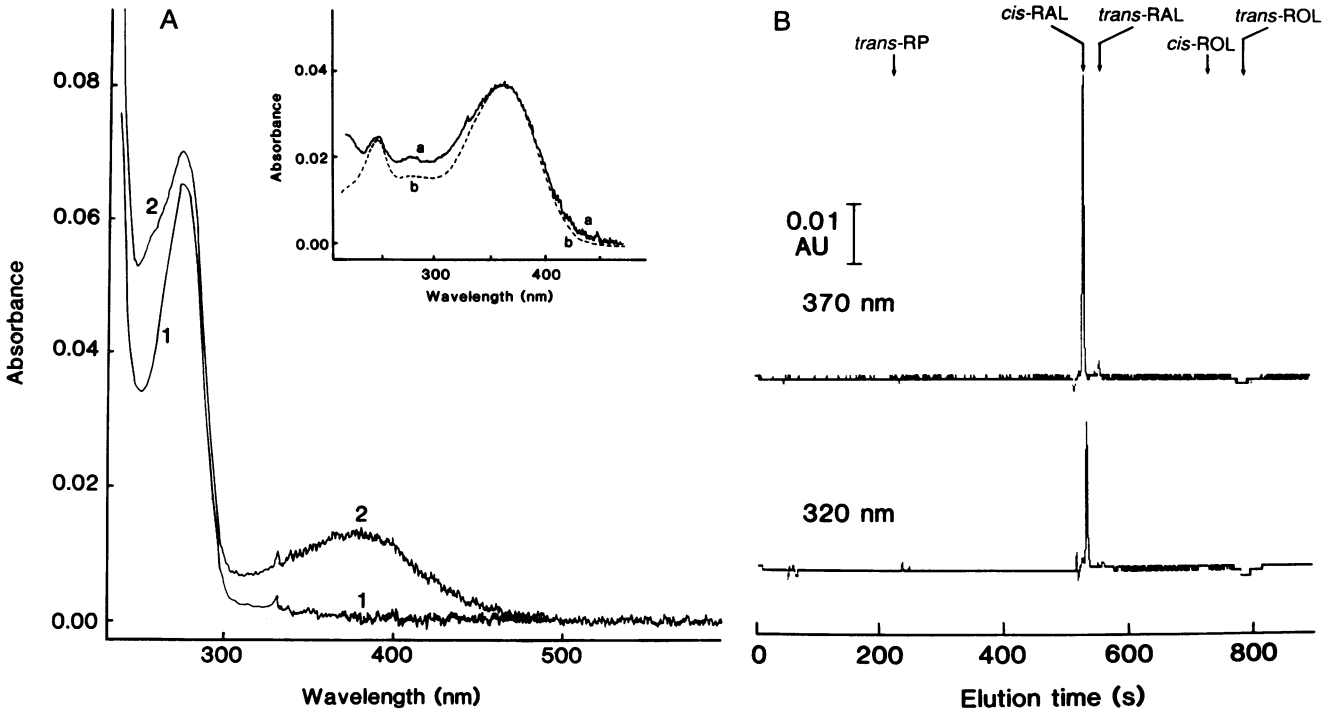

FIG. 4. (*A*) Absorbance spectra of IRBP-supplemented Ringer's solution. A 250-μl aliquot (sample 1) containing 2.8 nmol of IRBP was delivered to an RPE-eyecup and then immediately withdrawn. An identical aliquot (sample 2) then was delivered to the same RPE-eyecup and incubated for 3 hr. Spectra 1 and 2 were obtained, respectively, from the zero-time control and the incubated sample, after 20-fold dilution of each in Ringer's. (*Inset*) An aliquot (100 μl) of sample 2 was supplemented with 100 μl of ethanol; 160 μl of this mixture was further supplemented with 600 μl of hexane and vigorously shaken. Solid curve (a), absorbance spectrum of material extracted from sample 2 into the hexane-dominated phase. Dashed curve (b), absorbance spectrum of 11-*cis*-retinal dissolved in hexane. (*B*) HPLC profiles at 370 nm (upper profile) and 320 nm (lower profile) of material extracted from sample 2. Arrows identify the elution times of all-*trans*-retinyl palmitate (*trans*-RP), 11-*cis*-retinal (*cis*-RAL), all-*trans*-retinal (*trans*-RAL), 11-*cis*-retinol (*cis*-ROL), and all-*trans*-retinol (*trans*-ROL). Vertical marker: absorbance scale (in absorbance units, AU) applicable to both profiles.

obtained from a given pair of incubated retinas ($[prot]_{IRBP}$ and $[prot]_{BSA}$, respectively) yielded a value of $[prot]_{IRBP}/[prot]_{BSA}$ that, among the three experiments, differed from unity by only 15% on average (1.15 ± 0.10; mean ± SD), indicating roughly similar recovery of the ROS from the two retinas. By contrast, the ratio of rhodopsin concentrations for the ROS obtained in a given experiment, $[rho]_{IRBP}/[rho]_{BSA}$, was substantially greater than unity (2.24 ± 0.16), indicating a marked effect of IRBP.

DISCUSSION

The results show that IRBP and the RPE together promote a substantial regeneration of rhodopsin in previously bleached toad rods and that the RPE serves as the source of the 11-*cis* retinoid required for regeneration. The dependence of regeneration on IRBP, interpreted in the context of previous data (10–15), indicates that IRBP functions as a carrier of 11-*cis* retinoid in the present experimental system and strongly suggests a similar role for the protein *in vivo*.

Table 1. 11-*cis*-Retinal contained in incubation medium after 3 hr

Supplementation of medium delivered to RPE-eyecup		11-*cis*-Retinal,* nmol
Protein	Amount, nmol	
IRBP	3.2	1.9
	3.3	3.0
	3.9	2.7
	5.5	3.2
	5.5	3.0
	9.2	2.4
		(mean ± SD: 2.7 ± 0.5)
BSA	0.4, 0.9, 2.4, 3.8, 11.2	All <0.1
None†	—	All <0.1

*Based on spectrophotometry of extracted retinoid (see *Methods*).
†Five preparations.

The present findings also provide direct evidence that 11-*cis*-retinal, rather than a precursor, is the principal retinoid transferred from the RPE to the rods. We base this conclusion on two lines of evidence. First, retinoid extracted from IRBP-supplemented medium that had been incubated in the RPE-eyecup consisted almost entirely of 11-*cis*-retinal (Fig. 4). Second, the quantity of 11-*cis*-retinal (about 2.7 nmol) released by the RPE into Ringer's solution containing relatively large amounts of IRBP (Table 1) was similar to the quantity of rhodopsin regenerated (about 2.5 nmol) in ROS/RPE-eyecups containing high concentrations of either opsin or IRBP (Fig. 3). That is, the amount of 11-*cis*-retinal "releasable" by the RPE accounts well for the maximum level of rhodopsin regeneration.

The conclusion that 11-*cis*-retinal is released by the RPE is consistent with earlier evidence that 11-*cis*-retinal is a major endogenous ligand of IRBP isolated from the dark-adapted eye (14, 24, 25) and is both synthesized and released by the RPE in tissue culture (26, 27). This finding is also in accord with the observation (data not illustrated) that $NADP^+$ (160 μM), a cofactor in the enzymatic oxidation of 11-*cis*-retinol to 11-*cis*-retinal (28), lacks a stimulatory effect on regeneration in the IRBP-supplemented ROS/RPE-eyecup. Together with previous biochemical and electrophysiological data (3–9, 15, 23, 29–32), the present findings favor a scheme in which all-*trans*-retinol and 11-*cis*-retinal are the principal retinoids transferred, respectively, to the RPE from bleached rods and to the rods from the RPE. This view supposes that the

RPE-mediated support of rhodopsin regeneration in the amphibian eye does not involve the oxidation of 11-*cis*-retinol to 11-*cis*-retinal within the rods, despite the presence in ROS of an oxidoreductase with demonstrated activity toward 11-*cis*-retinol (7, 28). The *in vivo* role of this oxidoreductase thus remains an interesting, open question.

Whether the 11-*cis*-retinal provided by the RPE in the present experiments derives from a preexisting store of 11-*cis* retinoid in the RPE or from the isomerization of all-*trans* retinoid during the incubation period also remains to be determined. The nearly equivalent plateau levels of rhodopsin formed at high IRBP and opsin concentrations (Fig. 3) suggest that maximal regeneration under the present conditions is limited by a process independent of the amounts of IRBP and opsin in the RPE-eyecup. This process could be the formation of 11-*cis*-retinal in the RPE or its release to the extracellular medium. The precise mechanism by which IRBP delivers its 11-*cis*-retinal ligand to the photoreceptors *in vivo* is also yet to be determined. Available data (14, 24), indicating a relatively low affinity of IRBP for retinoid, raise the possibility of a direct transfer of the lipophilic 11-*cis*-retinal from IRBP to the photoreceptor plasma membrane but do not exclude a receptor-mediated transfer, i.e., delivery of the 11-*cis*-retinal to a specific protein on the plasma membrane. *In vivo*, the transfer of 11-*cis*-retinal to bleached rods could occur in concert with the transfer of all-*trans*-retinol (bleaching product) from the rods to IRBP (14).

The activity of IRBP greatly exceeded that of BSA and of Ringer's alone, with respect to promoting both the withdrawal of 11-*cis*-retinal from the RPE and the regeneration of rhodopsin. However, although no 11-*cis*-retinal was observed in unsupplemented or BSA-supplemented Ringer's after incubation in the RPE-eyecup (Table 1), ROS/RPE-eyecup preparations incubated with these solutions exhibited small amounts of rhodopsin regeneration (text accompanying Fig. 2). It is possible that the regeneration observed in ROS/RPE-eyecups receiving unsupplemented or BSA-supplemented Ringer's reflected an ability of the ROS themselves to induce the release of 11-*cis*-retinal from the RPE. That is, free opsin in the bleached ROS (a "trapping" agent for 11-*cis*-retinal) or the ROS membranes (a trap for lipophilic retinoid) may have promoted the transfer of relatively small amounts of 11-*cis*-retinal from the RPE to the ROS through the aqueous extracellular medium. It is equally possible that the transfer was mediated by small quantities of endogenous IRBP copurifying with the ROS.

The present experiments relate directly to, and explain at a molecular level, Kühne's pioneering observation (2). Using our reconstituted system consisting of native RPE, bleached visual pigment (ROS or retina), and IRBP from an external source, we have obtained a remarkably similar result, one indicating that IRBP participates in the transfer of retinoid between the RPE and the retinal photoreceptors.

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