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Light-mediated retinoic acid production

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ABSTRACT **Retinoids serve two main functions in biology: retinaldehyde forms the chromophore bound to opsins, and retinoic acid (RA) is the activating ligand of transcription factors. These two functions are linked in the vertebrate eye: we describe here that illumination of the retina results in an increase in RA synthesis, as detected with a RA bioassay and by HPLC. The synthesis is mediated by retinaldehyde dehydrogenases which convert some of the chromophore all-trans retinaldehyde, released from bleached rhodopsin, into RA. As the eye contains high levels of retinaldehyde dehydrogenases, and as the oxidation of retinaldehyde is an irreversible reaction, RA production has to be considered an unavoidable by-product of light. Through RA synthesis, light can thus directly influence gene transcription in the eye, which provides a plausible mechanism for light effects that cannot be explained by electric activity. Whereas the function of retinaldehyde as chromophore is conserved from bacteria to mammals, RA-mediated transcription is fully evolved only in vertebrates. Invertebrates differ from vertebrates in the mechanism of chromophore regeneration: while in the invertebrate visual cycle the chromophore remains bound, it is released as free all-trans retinaldehyde from illuminated vertebrate rhodopsin. RA synthesis occurring as corollary of dark regeneration in the vertebrate visual cycle may have given rise to the expansion of RA-mediated transcriptional regulation.**

Retinoids are essential for survival in all vertebrates, and most of their biological effects are mediated by retinoic acid (RA) (1). RA is required for many functions in the adult organism, and during embryonic development both deficit and excess of retinoids cause severe malformations (2–4). Development of the eyes is particularly vulnerable: partial vitamin A deprivation leads to micro- or anophthalmia in otherwise relatively normal offspring (2, 5). RA exerts its effects through binding to nuclear receptors, which belong to the transcription-factor family that includes also the receptors for steroids, thyroid hormone, vitamin D, and a diverse group of orphan receptors whose ligands are not known or which do not use ligands (6, 7). In vertebrates, expression of many proteins is known to be critically dependent on RA (8). Whereas invertebrates have homologs to most of these proteins, invertebrate expression is not regulated by RA (6).

While the retinoids are named after the retina, where much of the fundamental research was done (9), the RA field originated in transcriptional control studies in molecular biology (7), and the origin of the name is largely regarded as historical coincidence. Here we show that illumination of the vertebrate eye causes an increase in RA synthesis, which gives light a direct access to transcriptional regulation. This suggests that the two biological usages of retinoids could be linked evolutionarily: that the role of RA as transcriptional activator may have evolved in the vicinity and as a consequence of visual processes.

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MATERIALS AND METHODS

Tissue Preparations. All experiments were done on black mice derived from a B6/D2 outbred colony. Embryonic mice were staged according to Theiler (10), with day of conception defined as embryonic day 0 (E0) and day of birth as postnatal day 0 (P0). The mice were raised under normal cycling light conditions, and prior to the experiments they were dark-adapted for several hours. They were rapidly killed by decapitation, and their eyes were dissected in ice-cold tissue culture medium; in most cases, dissections were performed under dim red light (>600 nm, RoscoLux filter #27). The neural retinas can be easily dissected from mice of all ages, but clean dissections of retinal pigment epithelium (RPE) are only possible in young mice. Exposures to light were done either on dissected tissues or on live, awake mice. For detection of *in vitro* light effects (see Figs. 1–3), half of the samples were kept in the dark, and the other half was placed for 10 min onto a graphics light plate under bright room light. For detection of *in vivo* light effects (see Fig. 4), some of the mice were kept in the dark, and their littermates were placed onto the light plate. After different amounts of time, both groups of mice were rapidly killed and their eyes dissected under dim red light.

Measurements Based on RA Reporter Cells. For all measurements on small tissue samples we used a sensitive RA reporter cell line that responds to changes in RA with proportional changes in β-galactosidase synthesis (11): the cells detect mainly all-trans RA, and over a limited linear range they respond to a 10-fold increase in RA with a doubling in colorimetric reaction. As the reactive disposition of the reporter cells can vary between experiments, only comparative measurements of simultaneously processed samples were taken as reliable, and no attempts were made to obtain absolute RA values.

As described previously (12, 13), the cells were used for two types of assay: (*i*) detection of RA synthesized from endogenous precursors in isolated, cultured tissue samples, and (*ii*) detection of retinaldehyde dehydrogenase activity in protein fractions; in some experiments, both of these assays were done consecutively on the same samples (see Fig. 1*B*). For the first assay of RA levels, similar amounts of tissues were cultured overnight; the protein contents of the samples were determined (MicroBCA kit, Pierce); and the synthesized RA, released into the medium, was measured in culture supernatant volumes normalized for protein content. The second assay for retinaldehyde dehydrogenase activities was done, following a published protocol (12), on protein fractions separated by isoelectric focusing, which were tested for capacity to convert added retinaldehyde to RA.

HPLC Measurements. All tissue preparations and extractions were performed under red or amber light. The tissues were sonicated in 0.7 ml hypotonic buffer (10 mM phosphate buffer, pH 7.2/30 mM NaCl). The homogenates were suspended in 10 ml of 0.05 M KOH/50% ethanol plus 0.1% butylated hydroxytoluene antioxidant and extracted with 10 ml hexane. This extract containing mostly neutral lipids was discarded. The remaining aqueous phase was acidified with

Abbreviations: RA, retinoic acid; RPE, retinal pigment epithelium.

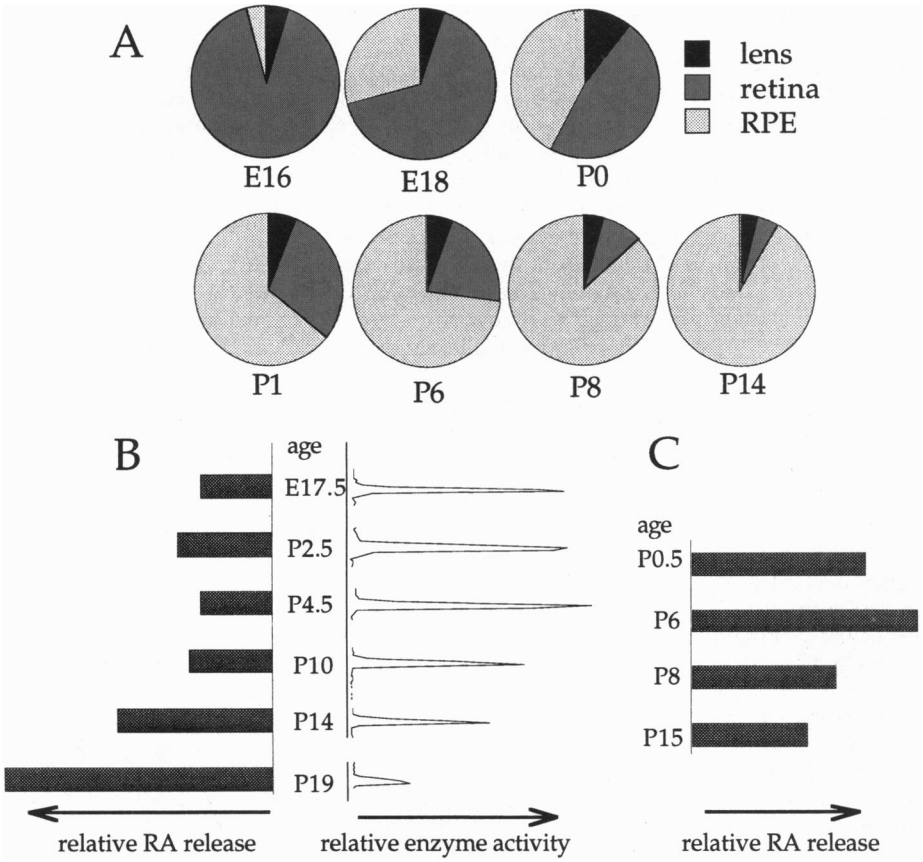

FIG. 1. (*A*) Developmental changes in relative RA amounts released from the neural retina, the RPE, and the lens. Note the developmental increase in relative RA amounts synthesized by the RPE. (*B*) Comparison of RA amounts released from RPE samples from mice of different ages (*Left*) and levels of retinaldehyde dehydrogenase (*Right*) measured in the same samples; all of these samples were dissected from light-adapted mouse pups under normal light conditions. Note that the older RPE samples synthesize relatively too much RA. (*C*) Measurements of RA amounts released from RPE samples of different ages dissected from dark-adapted mouse pups under dim yellow light. Note that under these conditions the RA levels decrease with age, similar to the enzyme activities.

100 μl 4 N HCl and extracted four times with 10 ml hexane/0.1% butylated hydroxytoluene; the extracts were pooled in 50-ml polypropylene tubes, evaporated under N_2, reconstituted with 5× 200 μl hexane and transferred into 2 ml vials. The solvent was again evaporated and the samples were reconstituted in 50-μl mobile phase for HPLC injection. Reversed-phase HPLC analysis was performed on a C18 column, with a linear gradient rising from 75% methanol/25% 40 mM ammonium acetate to 100% methanol in 20 min at a flow rate of 1 ml/min. Retinoids were detected at 340 nm. The

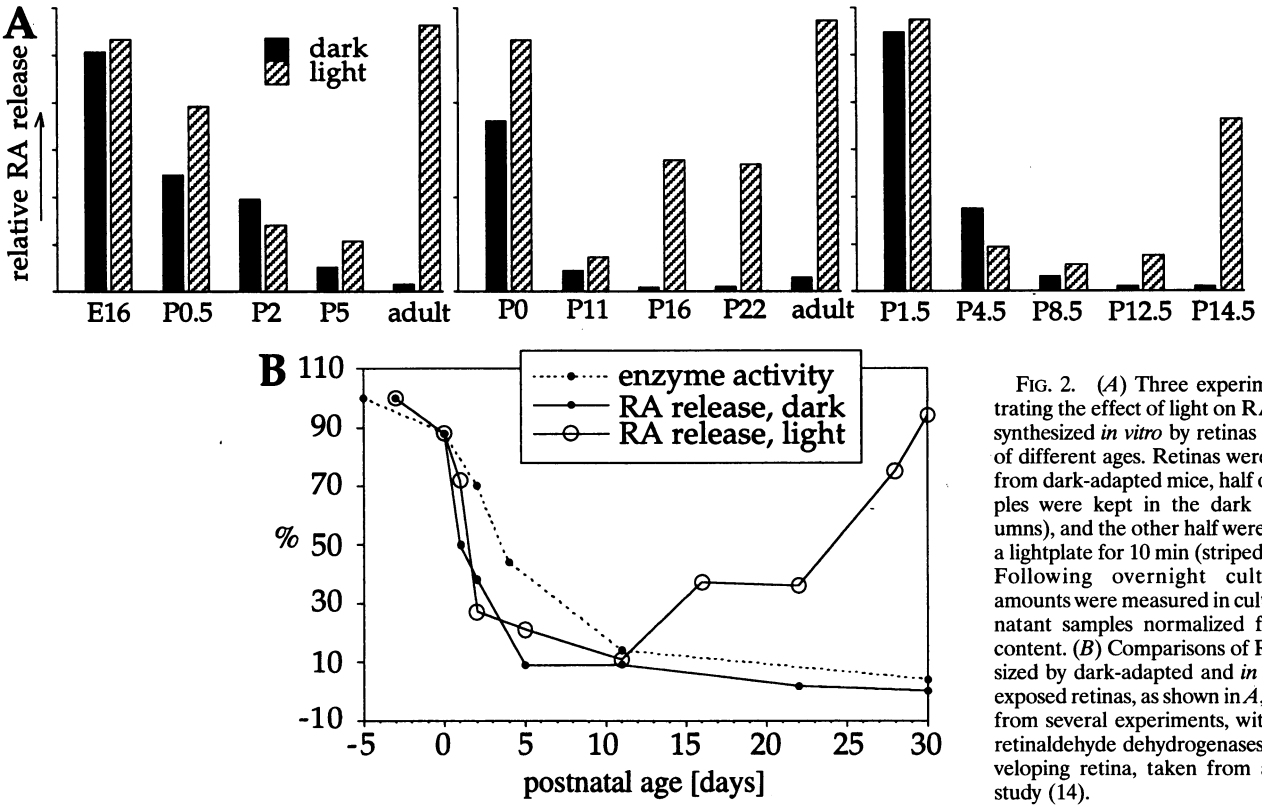

FIG. 2. (*A*) Three experiments illustrating the effect of light on RA amounts synthesized *in vitro* by retinas from mice of different ages. Retinas were dissected from dark-adapted mice, half of the samples were kept in the dark (solid columns), and the other half were placed on a lightplate for 10 min (striped columns). Following overnight culture, RA amounts were measured in culture supernatant samples normalized for protein content. (*B*) Comparisons of RA synthesized by dark-adapted and *in vitro* light-exposed retinas, as shown in *A*, combined from several experiments, with levels of retinaldehyde dehydrogenases in the developing retina, taken from a previous study (14).

identification of the synthesized substance as all-trans RA was based on its retention time, and this was confirmed by a normal phase, isocratic HPLC protocol using a poly(vinyl alcohol) silica column (25 cm × 0.6 mm, 5 μm, 100 Å) with a mobile phase consisting of 5% dioxane and 95% hexane. The flow rate was 2 ml/min. In addition, 200-μl fractions of the reversed-phased HPLC eluate were collected every 0.2 min and tested for biological activity, by diluting 4 μl of each fraction to 5% in L15 medium and incubating it with the reporter cells overnight.

RESULTS

Initially we set out to answer the question: what are the relative RA levels in different parts of the developing eye? The measurements were done on dissected, cultured ocular tissues with RA reporter cells (11). Comparisons of relative RA amounts generated by the different tissues show a systematic shift with developmental age: Fig. 1*A* illustrates how the ratios of relative RA levels in retina, RPE, and lens change from embryonic day 16 (E16) to postnatal day 14 (P14). In the embryo, the neural retina is by far the RA richest ocular tissue, but perinatally the RPE takes over. This arrangement will create an outside-in RA diffusion gradient in the postnatal retina, with highest levels at the photoreceptors and lowest at the ganglion cell layer.

To test whether the shift in RA ratios reflects merely the postnatal decline in retinaldehyde dehydrogenases described previously (14), we measured the developmental changes in the RPE. Fig. 1*B* illustrates that postnatally the retinaldehyde dehydrogenase levels in the RPE increase up to a maximum during the first week, but then decline; RA levels in the RPE, however, continue to increase. One explanation for this discrepancy could be abnormally high substrate levels for the retinaldehyde dehydrogenase. To test the possibility that these were generated by the light under which the samples were dissected, we dark-adapted mouse pups and dissected their RPE under very dim yellow light: RA measurements in these RPE samples show a decline with age, similar to the retinaldehyde dehydrogenase levels (Fig. 1*C*).

A similar discrepancy of even larger amplitude became apparent in comparisons of developing retinas. From dark-adapted mice of different ages the retinas were dissected under far-red light, half of the samples were exposed to bright room light for 10 min, the retinas were cultured overnight, and RA released into the supernatants was measured. The experiments in Fig. 2*A* show a large RA increase in the older retinas following illumination. A comparison of the developmental changes of RA release and dehydrogenase activity levels in the retina (14), combined from several experiments, is shown in Fig. 2*B*: dark RA levels follow the developmental changes in enzyme activities, and light-induced RA increases around the time when the eyes become functional.

To determine whether the increase in the RA reporter-cell response was due to light-released retinaldehyde converted into RA, rather than retinaldehyde itself, we did a reporter cell-based assay that exploits the fact that the only retinaldehyde dehydrogenase expressed in the adult retina is the AHD2 enzyme restricted to dorsal retina (12). When dark-adapted adult retinas were cut into dorsal and ventral halves under dim red light and exposed to bright light for 10 min, almost all RA increase in the cultured samples was detected in the dorsal samples (not shown). In addition, we did HPLC measurements (Fig. 3): retinas were dissected from dark-adapted adult mice, illuminated for 10 min, cultured in the dark for varying times, and assayed by reversed-phase HPLC. Drops of the HPLC eluate were collected at 0.2-min intervals and assayed with the reporter cells. Practically all detectable reporter response was present in a peak whose amplitude increased following illumination of the retinas (arrows); this peak comigrated with the all-trans RA standard.

The light-induced increase in RA synthesis described so far (Figs. 1–3) was detected in dissected, isolated tissue samples maintained in culture, an arrangement dictated by the measurements with the RA reporter cells that require tissue culture. Under the closed conditions the observed effects were very large, because the light-generated free retinaldehyde was trapped in the culture system. In the live eye with intact blood circulation the retinoids can diffuse away. Moreover, the dissections disrupt the functional integrity of the eye: separating the retinas from the RPE eliminates the interphotoreceptor space and its contents, which are known to be essential for retinoid transport. To address the possibility that in the intact eye the released chromophore is so efficiently shielded from the retinaldehyde dehydrogenases that no RA can be generated, we did two types of experiments on live, awake mice, one with the reporter cells, the other by HPLC.

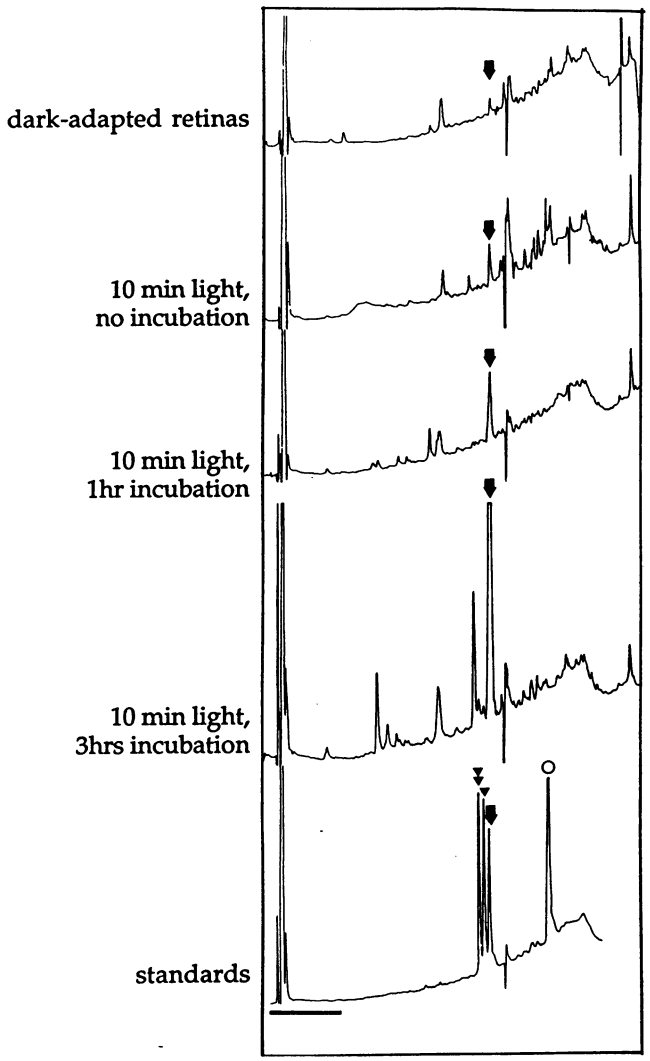

FIG. 3. HPLC traces of elution profiles from dark-adapted and *in vitro*-illuminated retinas, cultured for different times, and of retinoid standards. Each sample represents 10 adult retinas. The amount of all-trans RA (fat arrows) detected in the extracts was higher in light-exposed retinas, and it increased further with time of incubation. Standards represent 13-*cis* RA (2 triangles), 9-*cis* RA (1 triangle), all-trans RA (fat arrow), and all-trans retinaldehyde (circle). (Bar = 10 min run time.) A rough estimate of the all-trans RA levels/retina are as follows: dark-adapted, 2 pmol; 10-min light, no incubation, 5.6 pmol; 10-min light, 1-hr incubation, 10 pmol; 10-min light, 3-hr incubation, ≫47 pmol.

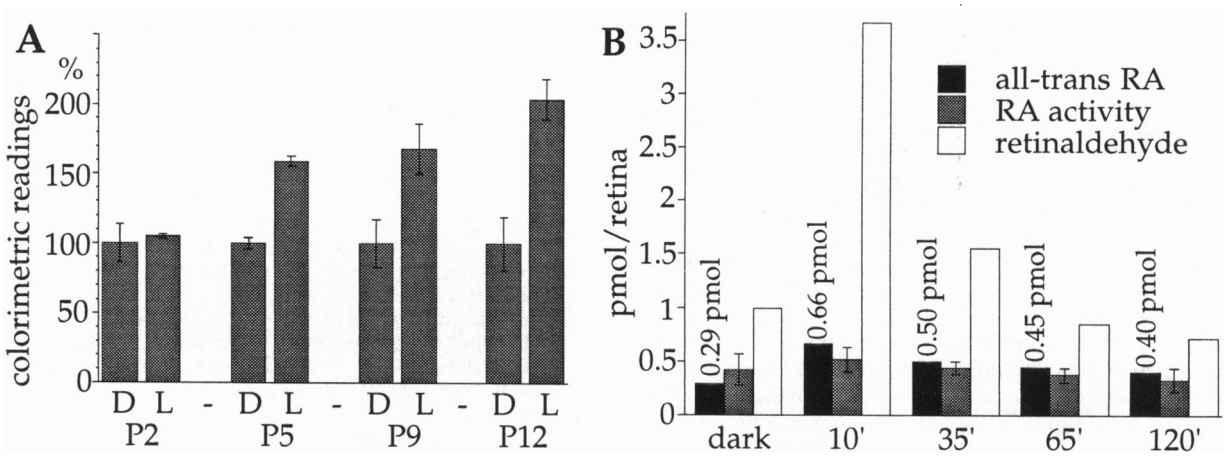

FIG. 4. (*A*) Comparisons of RA synthesized *in vitro* by RPE samples dissected from mouse pups of different ages which were dark- (D) and light-adapted (L) for 2 hr before sacrifice. The dark-adapted RA amounts are taken as 100%. The dissections from both groups of mice were done under far-red illumination; the difference thus reflects the light levels experienced by the live mice. Error bars represent SEM for two (P2&5) and four (P9&12) independent experiments. (*B*) HPLC measurements on eyecups of adult mice that were exposed to light for different amounts of time. Ten minutes of light exposure caused a doubling in the amounts of all-trans RA (amounts/eyecup indicated over the bars) and a quadrupling in retinaldehyde. The "RA activity" bars represent reporter cell activities of the all-trans RA fractions collected from the HPLC eluate, in colorimetric values on an arbitrary scale; error bars represent SEM from three measurements. Similar results to the experiment shown were obtained in three different series of HPLC measurements on mice exposed to light for varying amounts of time.

For the first set of experiments, mouse pups of different ages were used, half of which were placed in the dark for 2 hr and the other half exposed to bright light. Although the eyelids of the pups were still closed, the skin is transparent enough to allow some light to reach the retina. Both groups of mice were killed under dim red light, their RPEs were dissected free, washed, and cultured for detection of RA synthesis. In the older samples, the light-adapted RPE samples synthesized significantly more RA than the dark-adapted RPEs, with the difference increasing with age (Fig. 4*A*). This result indicates that the known effects of light-induced release of retinaldehyde and retinoid shift to the RPE (15) created a substrate pool for the retinaldehyde dehydrogenase in the RPE, which remained bound through the dissection procedure, a phenomenon likely to be operative *in vivo*.

For the second set of experiments, adult mice were dark-adapted and exposed to bright light for different amounts of time. Their eyecups (i.e., eyes without corneas and lenses) were dissected under red light and processed for HPLC measurements; 10 eyecups were used per time point. The results from one experiment are shown in Fig. 4*B*: after 10 min of light the amount of all-trans RA (0.66 pmol/eyecup) was about double the dark value (0.29 pmol), and it remained higher than the dark values but decreased gradually through the subsequent time points. Also plotted here is the known light-generated increase in retinaldehyde. These observations on adult eyecups illustrate clearly that light causes an increase in all-trans RA content in the live eye. Determination of the effective magnitude of the increase was hampered by the poor resolution of the measurement technique; the increase is at least 2-fold, but it may well be transiently and locally higher.

DISCUSSION

We have shown here two characteristics of ocular RA synthesis: (*i*) in the postnatal eye the RPE represents the site of highest retinaldehyde dehydrogenase activity, and (*ii*) RA synthesis is increased by light. The first observation indicates that RA may be a significant mediator of the known RPE/retina interactions (16). One process found to be stimulated by RA in the early postnatal retina is the formation of rod photoreceptors (17). Our observations make it likely that the RA for rod differentiation is supplied by the RPE. The second observation on light-mediated increase in RA synthesis, a phenomenon limited to the eye (unpublished observations), could be detected both in the retina and the RPE. Both increases are probably due to bleached rhodopsin releasing all-trans retinaldehyde, some of which becomes accessible to the retinaldehyde dehydrogenases. The retinaldehyde for the RA synthesized in the RPE probably originates in photoreceptors, forming a minor component of the known light-regulated retinoid fluxes between retina and RPE (15). As the

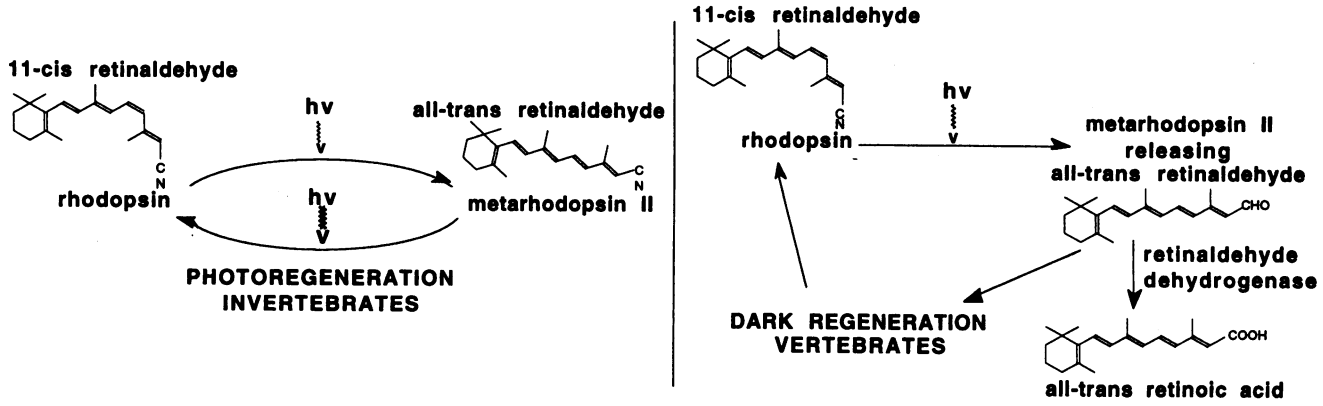

FIG. 5. Schematic of the visual cycle in invertebrates and vertebrates.

light-related effect in the RPE was resistant to the extensive washes during the dissections, it may be mediated by an all-trans retinaldehyde-binding opsin of unknown function that is expressed at very high levels (3% of protein) in the RPE (18). While most of the released retinaldehyde ought to be captured by the binding proteins that are involved in the regeneration of the 11-*cis* chromophore (15), bright light is likely to saturate the regeneration pathway, making some retinaldehyde available to the cytosolic dehydrogenases.

Although light-mediated RA production has not been observed before, light is known to have effects that cannot be explained by light-evoked electrical activity. A range of diverse physiological processes in the eye are responsive to ambient light levels, including melatonin production, expression of several phototransduction proteins and RPE-assisted photoreceptor turnover; and a circadian clock that can be reset by light has been localized to the photoreceptors (19). For several of the light-sensitive factors the regulation has been shown to take place at the transcriptional level, including rhodopsin, iodopsin, transducin, and arrestin (20–25). Our observations provide a plausible mechanism to directly link light to transcriptional regulation, with the prediction that RA can substitute for some of the light effects. Initial tests on arrestin confirm this prediction: light-induced increase in arrestin mRNA levels can be mimicked in the dark by intraperitoneal RA injections (26).

Light-mediated RA production may be relevant to the puzzle that RA-dependent transcriptional regulation seems to have evolved only relatively recently. In *Drosophila* no RA receptor homologs have been detected, and, in general, no RA-responsive transcription is believed to occur (6, 7). It has been reported, however, that transcription of *Drosophila* opsin is influenced by RA (27). Flies do express a range of nuclear receptors other than RA receptors, such as the ecdysone and several orphan receptors, including *seven-up* related to the COUP orphan receptor (28), and *ultraspiracle*, an RXR-related receptor that does not respond to RA, including 9-cis RA (29). It is thought that transcriptional regulation by nuclear receptors in general preceded phylogenetic appearance of RA receptors (6). The metabolic use of nonspecific aldehyde dehydrogenases is even more ancient than the use of nuclear receptors, as several are already present in bacteria and yeast, and all eukaryotic cells are likely to express a range of different isoforms (30).

Retinoid-mediated photosensitive processes, from bacteria to mammals, are based on retinaldehyde, bound covalently via Schiff-base linkage to opsins, and light-triggered retinaldehyde isomerization (15). Whereas in the rhabdomeric eyes of invertebrates the isomerized retinaldehyde remains bound in the stable metarhodopsin-II complex and is directly regenerated by light, the vertebrate visual cycle requires retinaldehyde regeneration at a site different from rhodopsin (Fig. 5): bleaching of vertebrate rhodopsin causes hydrolysis of the Schiff bond and release of free retinaldehyde (15). The observations presented here suggest the hypothesis that the evolution of RA-mediated transcription took off with the invention of dark regeneration and as a consequence of the phylogenetic appearance of unstable metarhodopsin II, at the transition from invertebrate to vertebrate vision. When light causes a burst of free retinaldehyde, some of it will meet an aldehyde dehydrogenase able to oxidize it to RA. The burst of RA diffusing away is likely to randomly associate with orphan receptors present. Due to its stiff conformation, RA may reversibly lock one of the receptors into an allosteric shape with significantly higher DNA affinity than the random shapes, conferring an advantage that can be evolutionarily selected for.

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