Muller cells of chicken retina synthesize 11-cis-retinol

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The amounts of endogenous retinyl palmitate, retinol and retinaldehyde were measured in the neural retina and retinal pigment epithelium (RPE) of predominantly cone (chicken), rod (rat) and more mixed (cat, human) retinae. The ratio of 11-cis to all-trans isomers of retinyl palmitate and retinol in the neural retina and the RPE increases progressively with the increase in diurnality of the species from rat to chicken. The membrane fractions of both chicken and bovine RPE enzymically isomerize all-trans retinol to 11-cis-retinol. Chicken neural retina membranes enzymically form 11-cis-retinol and all-trans-retinyl palmitate from all-trans-retinol. Light and electron microscopy revealed no contamination of chicken neural retina by RPE. Muller cells from chicken retina were isolated, cultured and characterized by immunocytochemical localization of cellular retinaldehyde-binding protein. Cultured chicken Muller cells form all-trans-retinyl palmitate, 11-cis-retinol and 11-cis-retinyl palmitate from all-trans-retinol and release most of the 11-cis-retinol into the medium. The results indicate that chicken neural retina and Muller cells in particular synthesize 11-cis-retinoids from all-trans-retinol.

INTRODUCTION

The retinal epithelium enzymically synthesizes 11-cis-retinoids from all-trans-retinol and therefore plays a key role in the vitamin A cycle of vision (Rando et al., 1991). Attempts to show such isomerization in the neural retina have been unsuccessful (Bernstein et al., 1987). The fact that Muller cells contain 11-cis-retinaldehyde-binding protein (CRALBP) (Bunt-Milam & Saari, 1983), which specifically binds 11-cis-retinol, suggests that there is a certain degree of 11-cis-retinoid metabolism in the neural retina that goes beyond what would be expected if the epithelium were the sole site of isomerization. In addition, we (Das et al., 1991) and others (Tsin et al., 1984, 1988; Bridges et al., 1987; Rodriguez & Tsin, 1989) have shown the presence of 11-cis and all-trans-retinyl esters and the existence of 11-cis-retinyl palmitate hydrolyase activity in different vertebrate neural retinæ (Tsin & Lam, 1986; Rodriguez & Tsin, 1989).

In order to examine further the role of the neural retina in the regeneration of visual pigment, we have studied the retinoid content and its metabolism in the neural retina and the epithelial layer in predominantly rod (rat) and cone (chicken) as well as more mixed (cat, bovine, human) retinaæ. The results indicate that there is a greater proportion of 11-cis-retinoid pools in both the neural and epithelial compartments of cone than of rod retinaæ. Chicken retina was found to isomerize and esterify enzymically all-trans-retinol in its neural as well as its epithelial compartments. To confirm that it is the neural retina and not the possible retinal pigment epithelium (RPE) contamination that is responsible for the isomerization and esterification observed in chicken, we have cultured Muller cells from chicken neural retina, characterized them by immunocytochemical localization of CRALBP and studied the metabolism of all-trans-retinol in these cultures. Results from these experiments show that cultured chicken Muller cells can take up supplied all-trans-retinol, esterify it to all-trans-retinyl palmitate and convert it into 11-cis-retinol, most of which is secreted into the medium in a time-dependent manner. Formation of small amounts of 11-cis-retinyl palmitate was also detected.

EXPERIMENTAL

Materials

Chicken eyes were obtained from freshly slaughtered birds supplied by Dominick Live Poultry, Tarrytown, NY, U.S.A. Human donor eyes were received through the New York Eye Bank for Sight Restoration and the National Disease Research Interchange. Bovine eyes were supplied by Max Insel Cohen Inc., Livingston, NJ, U.S.A. Cat and rat eyes were obtained from animals purchased through Institutional Animal Care and Use Committee at Columbia University Health Sciences Division, New York, NY, U.S.A. Leupeptin, glutaraldehyde, Triton X-100, laminin and papain were from Sigma Chemical Co, St. Louis, MO, U.S.A. Rabbit anti-(bovine CRALBP) was a generous gift from Dr. John C. Saari of University of Washington, Seattle, WA, U.S.A. The h.p.l.c. column used for the analysis of the retinoids was supplied by Supelco Inc, Bellefonte, PA, U.S.A. 3H-labelled 11,12-all-trans-retinol was from New England Nuclear Research Products, Boston, MA, U.S.A. Authentic retinoids used as standards were a gift from Hoffmann–LaRoche, Nutley, NJ, U.S.A. Sources of other chemicals and solvents were as described previously (Das & Gouras, 1988; Das et al, 1990).

Methods

Studies of endogenous retinoids. For this study, human retinal epithelial cells and neural retina were used from eyes with maximum post-mortem time of 24 h. In the study of bovine and chicken, eyes were used within 6 h of death. Post-mortem time for feline and rat eyes was less than 1 h. Eyes from each species were enucleated immediately after death and kept on ice to avoid variation of retinoid concentrations. Chickens were decapitated before being scalded, a common practice for feathering birds. The eyes were enucleated and cut open around the ora serata. The anterior segment and vitreous were removed and eye cups soaked in Hanks’ balanced salt solution for 30 min; the neural retina was gently placed with fine-tipped forceps under a dissecting microscope (Carl Zeiss), cut at the optic disc and removed from the eye cup. Any neural retina with visible pigmented material was discarded to avoid contamination with RPE cells. These steps were performed with the eyes on a bed of ice. Pieces of the neural retina were dropped into a solution of 3% (v/v) glutaraldehyde in Earle’s buffer (Gibco) for histological examination. Pigment epithelial cells were dissociated with trypsin as described elsewhere (Flood et al., 1980). For every experiment, one pair each of human and feline, three pairs of chicken and five pairs of rat eyes were used. The ratios of 11-cis- to all-trans-retinoids presented in the Results section are from three such

Abbreviations used: RPE, retinal pigment epithelium; CRALBP, 11-cis-retinaldehyde-binding protein.
experiments. Neural retina from individual species were pooled, mixed with 2 ml of cold 50 mm-Tris/HCl, pH 7, and homogenized in a Dounce glass/glass tissue grinder as described previously (Das & Gouras, 1988). A sample of homogenate was used to determine the protein content, by using a Bio-Rad microassay kit. The concentration of pigment epithelial cells in the pools of each species was determined with a haemocytometer before homogenization by the procedure described above. These steps were performed under normal laboratory light.

Extraction of retinoids from the tissue homogenates was carried out under dim red light in amber glassware with acetone and n-hexane; retinoids were analysed and identified by h.p.l.c. as described elsewhere (Das et al., 1990). H.p.l.c. analysis of retinoids was carried out on a normal phase Supelcosil LC-Si column (4.6 mm × 15 cm) packed with spherical silica (3 μm particle size) in a Waters h.p.l.c. system. The mobile phase used for the analysis of retinyl ester was 0.3% diethyl ether/n-hexane at 0.4 ml/min. For identification of retinol, the mobile phase was used by 6% dioxan/n-hexane at 0.5 ml/min. Retinoldehyde was eluted with 3% ethyl acetate/0.1% propan-2-ol (final concentrations) in n-hexane at 0.4 ml/min. The separation of cis and trans isomers of authentic retinoids attained by using this h.p.l.c. system was similar to that previously described (Das et al., 1990). Lowering the collection time from 0.5 min to 0.25 min per fraction enabled us to improve the resolution of the radiolabelled retinoids. Quantification of the endogenous retinoids was performed by injecting known amounts of authentic all-trans-retinol, all-trans-retinyl palmitate and all-trans-retinaldehyde. A linear relationship was obtained between quantity injected and integrated peak area. A standard curve for each retinoid was used to determine its amount.

Preparation of the total membrane fraction for retinol isomerization activity. The neural retinae were isolated as already described. The pigment epithelial layer along with choroid was peeled from the eye cup and placed on ice. Neural retina and RPE cell layers from individual species were pooled separately. Membrane components for the retinol-isomerization study were prepared from both chicken and bovine RPE and neural retina following the procedure described by Fulton & Rando (1987). All buffers used for the isomerization study contained 0.01% (v/v) leupeptin to prevent proteolytic degradation of the enzymes. The resuspended membrane components were then centrifuged at 175000 g for 30 min and the supernatant was discarded. This was repeated four to five times in order to remove NAD cofactors so that the redox activity in the membrane was reduced. Very little or no isomerization activity was detected in the experiments where unwashed membrane preparations were used (not shown in the results) because most of the all-trans-retinol was oxidized to all-trans-retinaldehyde. Depending on the amount of tissue, the membrane preparations were resuspended in 1–3 ml of the phosphate buffer and stored at −70°C in small portions. A portion of the resuspended membrane component was used to determine the protein content.

Isomerization assay. Taking 1 μCi of [3H]-labelled all-trans-retinol (specific radioactivity 55.7 Ci/mmol) per assay, a stock solution for all the assay points was prepared. Depending on the nature of the experiments, the required amounts of radiolabelled all-trans-retinol was evaporated to dryness in a conical amber glass tube under N2. It was then redissolved in 1–2 μl of ethanol, and at 5% (w/v) fatty-acid-free BSA solution was added to it so that radioactive concentration of the resultant solution was 1 μCi/20 μl. The stock solution was vortex-mixed and left at 4°C for 15 min before the assay was performed. Membrane components from either neural retina or RPE were sonicated by using a Branson sonifier cell disrupter (model 200) immediately before the assay with 30 s bursts with pauses for cooling.

Finely suspended membrane components were placed in a Beckman quartz cuvette and u.v.-irradiated with Blak Ray UVL-56 (Ultra Violet Products Inc., San Gabriel, CA, U.S.A.) at a distance of 10 cm for 15 min. This eliminates most of the endogenous retinoid pool making the supplied radiolabelled all-trans-retinol more accessible to the enzymes. Under dim red light and at 0°C, 20 μl of the radiolabelled all-trans-retinol solution was placed in a 1.5 ml polypropylene microcentrifuge (Sarstedt Inc., Pennsauken, NJ, U.S.A.) tube. Dulbecco’s phosphate buffer (pH 7.0) and 750 μg of membrane protein (except in the protein-dependent experiment, where different amounts of protein were used) were added to a final volume of 250 μl. Depending on the nature of the experiment, this assay mixture was incubated at 37°C in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago, IL, U.S.A.) for different time-periods. Quenching and extraction of the assay mixture for retinol-isomerization activity was carried out as described by Fulton & Rando (1987). The extracted retinoids from the assay mixture were stored in micro amber Reacti-vials (Pierce Chemical Co, Rockford, IL, U.S.A.) at −20°C before being analysed by h.p.l.c.

Radioactive reaction products were mixed with authentic unlabelled retinol or retinyl palmitate isomer mixtures and analysed by h.p.l.c. for either retinol or retinyl palmitate. The eluates from the h.p.l.c. column were collected at the authentic retinoid peaks in 7 ml-capacity polystyrene scintillation vials (Fisher Scientific, Fairlawn, N.J., U.S.A.) by using a Gilson FC 203 programmable fraction collector (Gilson Medical Electronics, Middletown, WI, U.S.A.) at 0.25 min intervals. A 4 ml portion of Betafluor (National Diagnostics, Manville, NJ, U.S.A.) was then added to each vial, and radioactivity was counted in an LKB 1217 Rackbeta liquid-scintillation counter.

Light and electron microscopy. For histological studies of the glutaraldehyde-fixed neural retina, it was first dehydrated and embedded in Epon (Electron Microscope Sciences, Fort Washington, PA, U.S.A.). Embedded tissue was then sectioned on a Sorvall ultramicrotome and examined using Zeiss light and electron microscopes.

Chicken Müller cell culture. Neural retinae from freshly enucleated chicken eyes (eight to ten eyes per preparation) were dissected as already described, rinsed with 20 ml of 1% (v/v) antibiotic-antimycotic (Gibco) in Ca2+/Mg2+-free Hanks’ balanced salt solution and broken into small pieces with fine forceps. Up to three to four pooled retinae were digested with 1 mg of papain/ml at 37°C for 45 min. The tissue was resuspended in Eagle’s minimal essential medium containing 10% (v/v) fetal bovine serum, 5 mg of glucose/ml and 3.5 mg of NaHCO3/ml, agitated with a wide-bore Pasteur pipette and centrifuged at 50 g for 3 min in a standard Dynac centrifuge with angled heads (Clay Adams, Parsippany, N.J., U.S.A.) The supernatant was removed, discarded and the procedure repeated twice. The resulting pellet was resuspended in 12 ml of Eagle’s minimal essential medium containing 10% (v/v) fetal bovine serum, 5 mg of glucose/ml, and 5 mg of NaHCO3/ml and cultured in 2 ml portions per 35 mm × 10 mm culture plates by the procedure of RPE culture described by Flood et al. (1980). The age of these cultures used in the retinoid metabolism study was between 2 and 3 weeks. Retinol supplementation of these cells followed the procedure described for RPE cells by Das & Gouras (1988).

For immunocytochemical characterization of these cells, they were plated out in a two-well Lab-Tek chamber slide (Nunc), precoated with 25 mg of laminin/ml in phosphate-buffered saline (PBS) (Gibco).

Immunocytochemistry. Media from the cells grown for 2 weeks in Lab-Tek chamber slides were removed, thoroughly rinsed with 10 mg of BSA/ml of PBS and fixed for 30 min at 20°C with
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Fig. 1. Ratios of 11-cis to all-trans isomers of retinyl palmitate, retinol and retinaldehyde in neural retina (a) and RPE cells (b)

Ratios of endogenous 11-cis to all-trans isomers of retinyl palmitate (■), retinol (□) and retinaldehyde (■) in the neural retina and RPE of chicken, human, cat and rat are shown. Results are means ± S.D. for three replicate experiments.

Fig. 2. Time-dependent formation of 11-cis-retinol (a) and all-trans-retinyl palmitate (b) by chicken and bovine neural retina and RPE cell total membrane fractions after incubation with 3H-labelled all-trans-retinol

The incubation of the tissue with radiolabelled all-trans-retinol was as described in the Experimental section. Results are means ± S.D. for four replicate experiments. ●, Chicken neural retina; ○, chicken RPE; ■, bovine neural retina; □, bovine RPE.

Fig. 3. Protein-dependent formation of 11-cis-retinol (a) and all-trans-retinyl palmitate (b) by chicken neural retina (●) and RPE (○) cell membrane fractions on incubation with 3H-labelled all-trans-retinol

The procedure was as described in the Experimental section.

10 % (v/v) p-formaldehyde in PBS. Cells were then treated with PBS containing 0.03 % (v/v) Triton X-100 and 10 mg of BSA/ml (washing buffer) and 1 ml of non-immune bovine serum for 30 min at 20°C. After removal of the non-immune serum from one chamber, cells were incubated overnight with 150 μl of anti-

CRALBP diluted 1:200 in washing buffer at 4°C. The cells in the other chamber were used as control for immunostaining. Cells were washed with washing buffer and incubated for 1 h at 20°C with 500 μl of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:20 in the washing buffer. Cells were then washed with the washing buffer, and shaken dry. A drop of glycerol was added, and the cells were covered with a glass coverslip and viewed under a Zeiss inverted-phase microscope equipped for epifluorescence.
RESULTS

The amounts of endogenous retinyl palmitate, retinol and retinaldehyde in the chicken, human, cat and rat neural retina and the RPE cells were measured as described in the Experimental section. The amounts of retinoids in the neural retinæ were calculated as ng/mg of protein and in the RPE cells as ng/10¹⁰ cells, so that retinoids from eyes of different sizes could be compared. Fig. 1 illustrates the ratios of 11-cis to all-trans isomers for endogenous retinyl palmitate, retinol and retinaldehyde present in the neural retinæ (Fig. 1a) and RPE cells (Fig. 1b) of chicken, human, cat and rat. These results show that there is a higher proportion of 11-cis than the corresponding all-trans isomer of retinyl palmitate present in the chicken neural retina than in the other three species. There is a progressive increase in the ratios of 11-cis to all-trans isomer of retinyl palmitate and retinol in the neural retinæ with increasing diurnality of the species. A similar pattern is seen in the corresponding retinal epithelia.

Since the transfer of the retinyl esters between pigment epithelium and neural retina does not seem to occur, we determined whether chicken neural retina converts all-trans-retinoid into the 11-cis isomer. Total membrane fractions from both neural retina and RPE cells were incubated with ³H-labelled all-trans-retinol over a period of 2 h as described in the Experimental section. Membrane preparations from bovine neural retina and RPE cells were also incubated with radiolabelled all-trans-retinol for comparison. The amounts of 11-cis-retinol formed by different tissues are shown in Fig. 2(a). Both bovine and chicken RPE membrane fractions could convert all-trans-retinol into 11-cis-retinol. Only chicken neural retina, however, formed significant amounts of 11-cis-retinol from supplied all-trans-retinol. The amounts formed by the chicken neural retina ranged between 49 and 69% of that formed by RPE. No 11-cis-retinol was detected in control experiments where no tissue was added. Some 13-cis-retinol formed non-enzymically was detected but not illustrated. Fig. 2(b) shows that both bovine and chicken RPE esterified all-trans-retinol, but only chicken neural retina formed significant amounts of retinyl palmitate. Both isomerizing and esterifying activities were eliminated by boiling the tissue at 100°C for 30 min (results not shown), implying that the reaction is enzymic.

Fig. 3 shows that the rate of formation of 11-cis-retinol (Fig. 3a) and all-trans-retinyl palmitate (Fig. 3b) depends on the quantity of protein added to the assay mixture. The amounts of all-trans-retinol isomerized to 11-cis-retinol and esterified to all-

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**Fig. 4.** Light micrographs of the chicken retina before (a) and after (b) dissection for biochemical assay

The procedure was as described in the Experimental section. Magnification 400 x.

**Fig. 5.** Electron micrographs of the chicken retina before (a) and after (b) dissection for biochemical assay

The tissue was processed for electron microscopy as described in the Experimental section. Magnification is 1850 x.
trans-retinyl palmitate increased with increasing amounts of added protein. The increase is similar in pattern but less in degree for neural retina than for RPE. The amounts of 11-cis-retinol and all-trans-retinyl palmitate formed after incubating the tissues at 37°C with 3H-labelled all-trans-retinol for 1 h are expressed as percentages of the amounts of all-trans-retinol incubated with the tissue.

In order to assess whether there was contamination of chicken neural retina with RPE, we examined the neural retina by light and electron microscopy. Fig. 4(a) shows the chicken retina with its heavily pigmented epithelial layer. The pigmentation extends into the thin apical processes but is absent from the finer extensions which reach as far as the inner segments. Fig. 4(b) shows that after dissection of the neural retina there is no evidence of epithelial cells and their characteristic pigmentation. Fig. 5(a) shows an electron micrograph of the retina. The pigmented apical processes of the epithelial cells contact the inner segments of the photoreceptors. They reach the processes of Muller cells which also extend as far as the inner segments. Fig. 5(b) shows that the dissected neural retina contains no pigmentation but only membranous debris.

We then determined whether Muller cells from the chicken retina could convert all-trans-retinol into the 11-cis isomer. Chicken retinal Muller cells were grown as primary cultures. Fig. 6 shows this to be a homogeneous population of cells which show the characteristic appearance of Muller cells in vitro (Lewis et al., 1988) free of RPE contamination. These cells were characterized by immunocytochemistry as described in the Experimental section.
section. Fig. 7 shows specific labelling with anti-CRALBP-produced fluorescence in the cytoplasm which extends up to the processes, confirming the presence of CRALBP in these cells from chicken neural retina and supporting our conclusion that they are Muller cells (Saari, 1990).

Primary cultures of chicken Muller cells supplemented with 

\[ {}^3\text{H}\text{-labelled all-trans-retinol} \] incorporated all-trans-retinol, synthesized 11-cis-retinol, all-trans-retinyl palmitate and 11-cis-retinyl palmitate and released 11-cis-retinol into the medium. Fig. 8 shows the time-dependent formation and release of these retinoids. All-trans-retinol incorporation reached a maximum at 1 h and then decreased with time. Much more 11-cis-retinol was detected in the medium than in the cells, implying a rapid release of this retinoid by the Muller cells. Isomerization did not occur in the experiments where all-trans-retinol was incubated in the absence of Muller cells (results not shown). Small amounts of 11-cis-retinyl palmitate were also detected in the cells but 11-cis-retinaldehyde was not detected in either the cells or the medium.

DISCUSSION

We began these experiments by examining the retinoid pools in retinae of animals with different ratios of rods to cones, from nocturnal rats to diurnal chickens. The results demonstrated a progressive increase in the ratio of 11-cis to all-trans isomers of retinyl palmitate stores in the neural retina with increasing proportions of cones. The relatively high content of 11-cis-retinyl palmitate in chicken retina has already been noted by others (Bridges et al., 1987; Rodriguez & Tsai, 1989). This led us to determine whether the chicken neural retina is capable of isomerizing all-trans- to 11-cis-retinol, even though attempts to demonstrate such activity in bovine (Fulton & Rando, 1987) and frog neural retina produced negative results (Bernstein et al., 1987). Our results show that such isomerization is detectable in washed membrane fraction of chicken but not bovine neural retina. In order to determine whether this isomerizing activity was due to contamination from the epithelial cells remaining with the dissected neural retina, we used both light and electron microscopy to examine the tissue. We found no evidence of pigmented cells adhering to the neural retina, but electron microscopy did reveal traces of membranous material around the chicken outer segments which could be material from the epithelial layer. We therefore concluded that if this isomerization was due to the contamination of neural retina by RPE, there must be relatively high isomerizing activity in the apical processes of chicken retinal epithelium.

In order to pursue this question further, we cultured Muller cells from chicken retina. These cells are a good candidate for possible isomerization activity because they contain CRALBP which specifically binds 11-cis-retinol and they have processes that come in contact with the photoreceptors. In addition, they are one of the few cell types in the neural retina that can be grown in culture. The cells that we isolated resembled Muller cells in appearance and stained positively for CRALBP, a marker for Muller cells in the neural retina (Saari, 1990). When all-trans-retinol was supplied to these cells in culture, we found that they were able to synthesize 11-cis-retinoids. Relatively large amounts of 11-cis-retinol were released into the medium and smaller amounts of 11-cis-retinol and retinyl palmitate were detected in the cells. This distribution of 11-cis-retinoids is in striking contrast with the 11-cis compounds synthesized by human retinal epi-
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thelium in culture. The latter produce relatively large amounts of 11-cis-retinaldehyde which they release into the medium (Das et al., 1990; Flannery et al., 1990). This is an interesting point because experiments with isolated photoreceptors (Jones et al., 1989) showed that the cones but not the rods are capable of incorporating 11-cis-retinol and oxidizing it to the aldehyde form. There are several reports that the cone (Goldstein, 1970; Hood & Hock, 1973) and cone-like photopigments (Goldstein & Wolf, 1973) are capable of regeneration after photic bleaching without any interaction with the retinal epithelium. This is in contrast with rhodopsin regeneration which appears never to occur if the neural retina is not in contact with the retinal epithelium (Kuhne, 1876; Reuter, 1966; Bridges, 1973). The only report that some rhodopsin regeneration can occur in the isolated neural retina (Cone & Brown, 1969) might be explained by the fact that only a small patch of retina was examined in which endogenous stores of 11-cis-retinaldehyde might have allowed a limited amount of regeneration. If Muller cells are capable of isomerizing all-trans- to 11-cis-retinol, it is still surprising that duplex retinae such as bovine or frog show no detectable activity, although they contain diurnal cones. This could be explained by a limitation of the assay conditions under which isomerizing activity was studied. Another approach to this problem may be to examine the ability of the cultured Muller cells from such retinae to isomerize retinoids.

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