11-*cis*-Acyl-CoA:Retinol *O*-Acyltransferase Activity in the Primary Culture of Chicken Muller Cells†

Alberto Muniz, Elia T. Villazana-Espinoza, Bridget Thackeray, and Andrew T. C. Tsin*

Department of Biology, University of Texas at San Antonio, San Antonio, Texas 78249

Received May 10, 2006; Revised Manuscript Received August 11, 2006

ABSTRACT: A novel retinoid cycle has recently been identified in the cone-dominated chicken retina, and this cone cycle accumulates 11-*cis*-retinyl esters upon light adaptation. The purpose of this study is to investigate how 11-*cis*-retinyl esters are formed in the retina. Primary cultures of chicken Muller cells and cell membrane were incubated with all-*trans*- or 11-*cis*-retinol to study retinyl ester synthesis. In Muller cells, esterification of 11-*cis*-retinol was four times greater than esterification of all-*trans*-retinol. In the presence of palmitoyl-CoA and CRALBP, Muller cell membranes synthesized 11-*cis*-retinyl ester from 11-*cis*-retinol at a rate which was 20-fold higher than that of all-*trans*-retinyl ester. In the absence of CRALBP, 11-*cis*-retinyl ester synthesis was greatly reduced (by 7-fold). In the absence of palmitoyl-CoA, retinyl ester synthesis was not observed. Muller cell membranes incubated with radiolabeled palmitoyl-CoA resulted in the transfer of the labeled acyl group to retinol. This acyl transfer was greatly reduced in the presence of progesterone, a known ARAT inhibitor. 11-*cis*-ARAT activity remained unchanged when assayed in the presence of all-*trans*-retinol, suggesting a distinct catalytic activity from that of all-*trans*-ARAT. Apparent kinetic rates for 11-*cis*-ARAT were 0.135 nmol min^{-1} mg^{-1} (V_{max}) and 11.25 μM (K_M) and for all-*trans*-ARAT were 0.0065 nmol min^{-1} mg^{-1} (V_{max}) and 28.88 μM (K_M). Our data indicate that Muller cells in the chicken retina possess 11-*cis*-ARAT activity, thus providing an explanation for the accumulation of 11-*cis*-retinyl esters in the cone cycle.

The formation of a visual image is initiated by the photoisomerization of 11-*cis*-retinaldehyde to the all-*trans* isomer in the photoreceptors. To sustain this visual function, all-*trans*-retinaldehyde is reisomerized to the 11-*cis* isomer for visual pigment regeneration. This retinoid recycling pathway is known as the visual or retinoid cycle, and it has been well described for rod photoreceptors (*1*). The storage form of vitamin A in the eye is the ester form, and retinyl esters make up the largest pool of retinoids in the eye (*2*, *3*). It has been demonstrated that retinyl esters in the retinal pigment epithelium (RPE)[1] are the substrate for the isomerase enzyme (*4*, *5*), a key protein in the visual cycle, now identified as RPE65 (*6*−*8*).

Recently attention has been focused on a visual pathway which supplies vitamin A to cone photoreceptors. Cone pigment regeneration is severalfold faster than rod pigment regeneration in light conditions (*9*). Cones but not rods from isolated frog retina can spontaneously regenerate visual pigments (*10*, *11*), and isolated cones from salamander can recover visual sensitivity from 11-*cis*-retinol and 11-*cis*-retinaldehyde while rods can only recover visual sensitivity from 11-*cis*-retinaldehyde (*12*). It has also been shown that retinas of cone-dominated species such as chicken and ground squirrel contain a larger amount of retinyl esters than the RPE and that the majority of these retinyl esters are in the 11-*cis* conformation (*13*−*15*). More recently, the accumulation and depletion of 11-*cis*-retinyl esters, under light and dark conditions respectively, have been demonstrated in the cone-dominated chicken retina (*16*), and recently Mata demonstrated that the retinas of cone-dominated species possess retinoid processing enzymes, including a retinyl ester synthase (*17*).

Lecithin−retinol acyltransferase (LRAT), which transfers the acyl group from the *sn*-1 position of phosphatidylcholine to all-*trans*-retinol (*18*), is found in the liver and RPE (*17*). Acyl-CoA:retinol *O*-acyltransferase- (ARAT-) like activity, in which the acyl group of acyl-CoA is used to esterify retinol (*19*), has also recently been localized in these tissues (*20*−*22*). ARAT has not been purified or cloned, but its activity can be inhibited with progesterone (*21*). Furthermore, the retinyl ester synthase activities described by Mata et al. (*14*) in the retinas of cone-dominated species use palmitoyl-CoA as an acyl donor.

Muller cells in culture have been reported to produce all-*trans*-retinyl esters and 11-*cis*-retinol from all-*trans*-retinol (*14*). It has also been suggested that Muller cells may participate in visual pigment regeneration (*14*, *15*). Vitamin A metabolism in support of visual chromophore regeneration in Muller cells is conceivable since Muller cells are the main glia in the neural retina (*23*) and are in close proximity to cones (*24*). In the present study, we have examined the ability

† Supported by NIH Grant GM08194.

* Corresponding author. Phone: (210) 458-4480. Fax: (210) 458-4478. E-mail: atsin@utsa.edu.

[1] Abbreviations: CRALBP, cellular retinaldehyde binding protein; RPE, retinal pigment epithelium; LRAT, lecithin−retinol acyltransferase; ARAT, acyl-CoA:retinol *O*-acyltransferase; HBSS, Hank's balanced salt solution; MEM, minimal essential medium; PBS, phosphate-buffered saline; PBST, PBS containing 0.2% Triton X-100; BSA, bovine serum albumin; DPM, disintegrations per minute; DGAT1, acyl-CoA:diacylglycerol acyltransferase 1.

10.1021/bi060928p CCC: $33.50
Published on Web 09/14/2006

of Muller cells from the cone-dominated chicken retina to esterify retinol. We show that Muller cell membranes have the ability to esterify retinol and that this esterification is 11-*cis*-specific and it occurs through an ARAT activity. This is the first study to show an 11-*cis*-ARAT activity in Muller cell specific for 11-*cis*-retinol, and our results provide an explanation for 11-*cis*-retinyl ester accumulation in the cone-dominated retina.

MATERIALS AND METHODS

Primary Muller Cell Culture. Primary chicken Muller cell culture was established according to Das et al. (*14*) with minor modifications. Freshly severed chicken heads were collected on ice (Tyson Foods, Inc., Seguin, TX) and immediately transported to the laboratory. Eyes were enucleated within 3 h post-mortem and sterilized with 10% Wescodyne for 5 min. Eyes were sectioned at the level of the ora serrata, the vitreous was removed, and the retina was then dissected from the eye. After being rinsed in 1% antibiotic/antimycotic in Hank's balanced salt solution (HBSS), the retinas were incubated for 45 min at 37 °C in HBSS containing 5.0 mg/mL papain. The retinas were then rinsed three times in HBSS, and tissue was collected by centrifugation (1500*g*). Cells were then seeded in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and 5 mg/mL glucose and incubated at 37 °C and 5% CO_2. The medium was changed 24 h later and every 48 h thereafter until cultured cells were confluent. Cells from two to three retinas were seeded into each T75 flask. Cells were cultured until confluent between 14 and 19 days.

Immunochemistry. Primary Muller cells were prepared for culture as described above and seeded in Lab-Tek chamber slides. Cells were rinsed in phosphate-buffered saline (PBS) and fixed in 10% paraformaldahyde for 15 min. Following fixation, the cells were rinsed once more. To remove background level fluorescence, the fixed cells were exposed to 600 mW of ultraviolet light using a Bio-Rad GS gene linker. Muller cells were also collected and checked for endogenous retinoids by HPLC (10 T75 flasks at 3×10^6 cells/flask). The cells were permeabilized using PBST for 30 min at room temperature. Cells were incubated for 1 h in PBS containing 1% fatty acid free BSA (Sigma Aldrich) to block nonspecific binding. Incubation with the primary antibodies mouse anti-CRALBP (a gift from Dr. Jack Saari) and rabbit anti-GFAP (obtained from Dakocytomation, Glostrup, Denmark) was performed overnight at a 1:1000 dilution at 4 °C in PBS containing 1% BSA. After being rinsed three times with PBS containing 0.2% Triton X-100 (PBST), the cells were incubated with FITC-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Sigma) at a dilution of 1:200 for 60 min. Finally, the cells were rinsed with PBST three times and then mounted. As negative controls, some cells were processed without incubation with primary antibodies.

ARAT Activity in Primary Muller Cells. All procedures with vitamin A were performed under dim red light. Primary Muller cells were cultured as described above, except they were cultured in T25 flasks. Upon reaching confluence, the cells were serum starved for 6 h. The cultures were then incubated overnight in 5 mL of serum-free media containing 10 μM 11-*cis*-retinol or 10 μM all-*trans*-retinol and 1% BSA. The cells were then harvested and glass−glass homogenized. Retinoids were extracted with hexane and analyzed by high-performance liquid chromatography (HPLC). HPLC was performed using a Beckman System Gold with Agilents Zorbax RX-SIL 5 μM 4.5 × 250 mm column, part number 880975−901; the mobile phase used was 0.2% HPLC-grade dioxane/hexane at a flow rate of 2 mL/min. In all procedures, retinyl esters were identified by comparison to authentic retinoid standards at absorbance wavelengths of 318 nm for 11-*cis*-retinoids and 325 nm for all-*trans*-retinoids and by on-line photodiode array absorption spectra. Retinoids were quantified by comparing the experimental peak area to a retinoid standard curve.

Primary Muller Cell Membrane Preparation. Whole cell membranes were prepared from primary chicken Muller cells, fresh rat liver, and fresh rat muscle. All procedures were performed at 4 °C. Muller cells were collected and glass−glass homogenized in buffer containing 10 mM Tris, pH 7.5, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 1 mM DTT. The homogenate was centrifuged at 250000*g* for 30 min at 4 °C and the pellet resuspended in the same buffer.

Rat muscle and rat liver membranes were prepared according to Ross (*18*). Briefly, fresh tissue was collected, and the rat liver was perfused overnight at 4 °C in 250 mM sucrose and 10 mM potassium phosphate solution. The tissues were then minced and glass−glass homogenized in buffer containing 150 mM potassium phosphate and 1 mM DTT, pH 7.4. The liver homogenate was centrifuged at 725*g* for 10 min; the supernatant was then centrifuged at 13000*g*, after which floating fat was skimmed. To prepare microsomes, the supernatant was centrifuged at 250000*g* for 30 min. Rat muscle tissue was prepared by glass−glass homogenization and then centrifuged at 250000*g* for 30 min. The liver and muscle pellets were resuspended and fast-frozen in 150 mM potassium phosphate and 1 mM DTT buffer (pH 7.4) using acetone and dry ice. The Bradford assay was used to determine protein concentrations.

ARAT Assays. Membrane protein in increasing amounts was preincubated with palmitoyl-CoA in 100 mM Tris, pH 8, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ (reaction buffer) for 5 min at room temperature. The substrate, 11-*cis*-retinol or all-*trans*-retinol, was delivered in 2 μL of ethanol to the reaction buffer containing either cellular retinaldehyde binding protein (CRALBP) (a gift from Dr. Kris Palczewski), bovine serum albumin (BSA), or reaction buffer alone and preincubated for 5 min at room temperature. The protein samples and retinol samples were then combined and incubated at 37 °C for 30 min. The final reaction mixture was 100 μM palmitoyl-CoA, 30 μM CRALBP or 1% BSA, 10 μM retinol, and protein in increasing amounts (between 10 and 50 μg). The total reaction volume was 1 mL. The reaction was stopped with 1 mL of ice-cold ethanol. Retinoids were then extracted with hexane and analyzed by HPLC.

Transfer of the Labeled Acyl Group from ^{14}C-Labeled Palmitoyl-CoA to Retinol To Form Retinyl Esters. To show direct transfer of the radiolabeled acyl group from palmitoyl-CoA onto retinol to form retinyl palmitate, radiolabeled palmitoyl-CoA (100 μM, specific activity 4442 DPM/nmol) was preincubated with 30 μg of Muller cell membranes as described above. Retinol was delivered in ethanol as described above and preincubated with CRALBP. The protein sample and retinol sample were then combined and

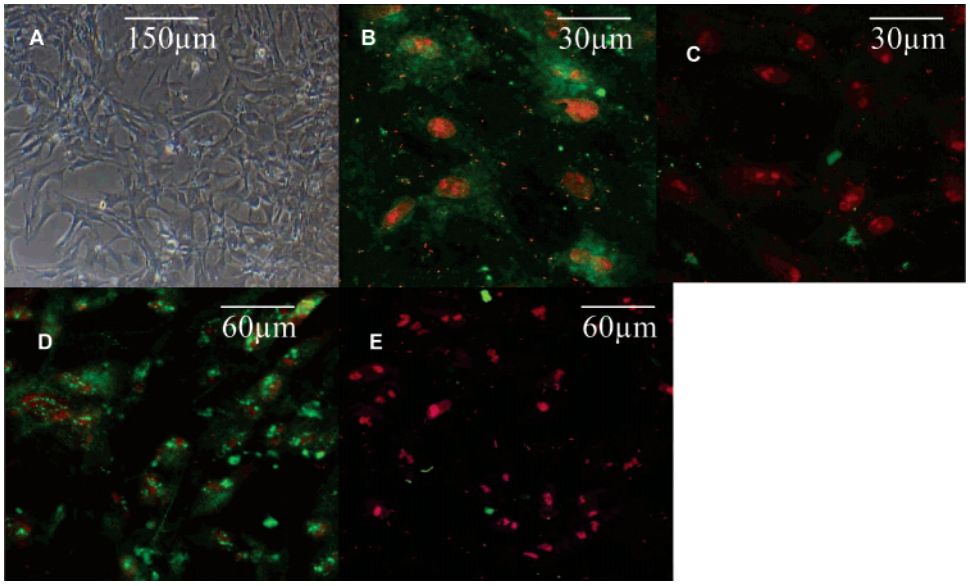

FIGURE 1: Photomicrograph of a primary culture of chicken Muller cells. (A) Muller cells freshly explanted from adult chicken eyes and cultured for 10 days (approximately 80% confluent; 4×). (B) Muller cells stained with anti-CRALBP monoclonal antibody and visualized by FITC-conjugated secondary antibody (green). Propidium iodide (red) was used to counterstain for cell nuclei (20×). (C) Negative control without anti-CRALBP. (D) Muller cells stained with anti-GFAP polyclonal antibody, visualized with FITC- (green) conjugated secondary antibody. Propidium iodide (red) was used to counterstain for cell nuclei (10×). (E) Negative control without anti-GFAP. Both primary antibodies were incubated at a 1:1000 dilution overnight at 4 °C.

incubated at 37 °C for 30 min, and the reaction was stopped with 1 mL of ice-cold ethanol. Retinoids were then extracted and analyzed by HPLC as described in the previous section and by liquid scintillation counting [results in disintegration per minute (DPM) at a counting efficiency of 100% for ^{14}C]. Radiolabeled authentic retinoid standards were used to determine the elution time of experimental retinoid products.

Rat liver and leg muscle membranes were used as positive and negative controls, respectively. Protein (30 μg) was preincubated with 100 μM radiolabeled palmitoyl-CoA (specific activity 4442 DPM/nmol) in buffer containing 150 mM K_2HPO_4, 1 mM DTT, and 1% BSA at pH 7.4 for 5 min at room temperature. 11-*cis*- or all-*trans*-retinol was then delivered to the mixture in 2 μL of ethanol to a final concentration of 10 μM. A final volume of 1 mL was then incubated, quenched, and analyzed as described above.

Inhibition of ARAT Activity by Progesterone. Muller cell membranes were preincubated with radiolabeled palmitoyl-CoA (100 μM, specific activity 1111 DPM/nmol) and progesterone (200 μM) delivered in 4 μL of ethanol for 5 min at room temperature in reaction buffer. 11-*cis*-Retinol was delivered as described above. The reaction mixture was then incubated at 37 °C for 30 min before being quenched with ice-cold ethanol.

For rat liver, membranes were preincubated with radiolabeled palmitoyl-CoA (100 μM, specific activity 1111 DPM/nmol) and progesterone (Sigma-Aldrich) (200 μM) delivered in 4 μL of ethanol for 5 min at room temperature in buffer containing 150 mM K_2HPO_4, 1 mM DTT, and 1% BSA at pH 7.4. All-*trans*-retinol (10 μM) was then delivered to the reaction mixture and incubated at 37 °C for 30 min. The reaction was then quenched with ice-cold ethanol. Retinoids were extracted using hexane and analyzed using HPLC and scintillation counting.

Muller cell and rat liver membranes were also incubated with labeled palmitoyl-CoA in the presence of 4 μL of ethanol as controls.

Kinetics of 11-cis- and All-trans-ARAT Activity in Membranes of the Primary Chicken Muller Cell. Muller cell membrane protein (30 μg) was preincubated with 100 μM palmitoyl-CoA. CRALBP (30 μM) was preincubated with 11-*cis*-retinol in increasing concentrations (0−20 μM). For all-*trans*-ARAT activity, 1% BSA was preincubated with all-*trans*-retinol. The protein and retinol samples were then combined and incubated at 37 °C for 30 min. The reaction was quenched using ice-cold ethanol. Retinoids were extracted with hexane and analyzed by HPLC. The Eadie−Hofstee plot was constructed to determine kinetic parameters.

RESULTS

Cell Culture and Immunochemistry. Primary chicken Muller cell cultures were established according to the methods of Das et al. (*14*). Figure 1A shows the morphology of freshly explanted Muller cells 10 days after seeding at approximately 80% confluence in a T75 flask. Confluence of 100% was reached between 14 and 19 days with 3×10^6 cells per T75 flask. Confluent Muller cell cultures (10 T75 flasks at 3×10^6 cells/flask) showed no detectable retinoids when analyzed by HPLC (data not shown).

To establish the identity of Muller cells in culture, we performed immunochemical studies with both Muller and glial cell markers. Figure 1B shows a primary Muller cell culture (20×) stained with anti-CRALBP antibody. CRALBP is expressed exclusively by Muller cells in the retina (*25*). Figure 1D shows a primary Muller cell culture (10×) stained with anti-GFAP antibody. GFAP is commonly used as a glial cell marker, and Muller cells are the main glia in the retina. Both anti-CRALBP and anti-GFAP staining were visualized using a FITC-conjugated secondary antibody.

Formation of Retinyl Esters by Primary Muller Cell Cultures from Exogenous Retinol. Primary Muller cells cultured in T25 flasks, about 10^6 cells/flask, were incubated overnight with either 10 μM 11-*cis*-retinol or 10 μM all-

Table 1: Formation of Retinyl Esters from Exogenous Retinol by Primary Chicken Muller Cells[a]

	11-*cis*-retinyl ester (nmol/10^6 cells)	all-*trans*-retinyl ester (nmol/10^6 cells)
11-*cis*-retinol	0.18 ± 0.06	0.02 ± 0.02
all-*trans*-retinol	0.0 ± 0.0	0.022 ± 0.02

[a] Primary Muller cells were cultured in T25 flasks. At confluence, each flask contained 10^6 cells. The cells were then incubated with either 10 μM 11-*cis*-retinol or 10 μM all-*trans*-retinol in 5 mL of media overnight. Cells were collected and retinoids extracted and analyzed by HPLC. Muller cells incubated with 11-*cis*-retinol synthesized 11-*cis*-retinyl esters 8.0-fold more than the amount of all-*trans*-retinyl ester synthesized from added all-*trans*-retinol. Statistical analysis (Student's *t* test) on the amount of 11-*cis*-retinyl esters from 11-*cis*-retinol and the amount of all-*trans*-retinyl esters from all-*trans*-retinol yields a *p* value >0.05, suggesting a significant difference between the synthesis of 11-*cis*- and all-*trans*-retinyl esters. Four separate experiments ($n = 4$; mean $\pm$ standard error) were conducted.

trans-retinol in 5 mL of media. Cells incubated with 11-*cis*-retinol had 11-*cis*-retinyl esters which were 8.0-fold the amount of all-*trans*-retinyl esters produced by cells incubated with all-*trans*-retinol (Table 1). Minimal 11-*cis* ester synthesis was observed in cells incubated with all-*trans*-retinol, and no all-*trans* esters were observed in cells incubated with 11-*cis*-retinol. Controls for thermoisomerization and esterification were performed by incubating retinol with culture media in the absence of cells.

Formation of Retinyl Esters from Exogenous Retinol by Cultured Chicken Muller Cell Membranes. Whole membrane preparations were made from primary chicken Muller cell and incubated with 11-*cis*-retinol in the presence and absence of CRALBP and in the presence and absence of palmitoyl-CoA. CRALBP is specific for 11-*cis*-retinoids (*26*); therefore, in these samples, we facilitated delivery of all-*trans*-retinol to cell membranes with BSA in our reaction mixture. Figure 2 shows the data for experiments testing several conditions under which Muller cell membranes synthesized retinyl esters. In the presence of 11-*cis*-retinol, palmitoyl-CoA, and CRALBP (condition 1), Muller cell membranes catalyzed the synthesis of 11-*cis*-retinyl ester, from 11-*cis*-retinol, at a rate of 0.166 nmol min^{-1} mg^{-1}, which was 20-fold higher than that for all-*trans*-retinyl ester synthesis (0.007 nmol min^{-1} mg^{-1}), from all-*trans*-retinol, in the presence of palmitoyl-CoA and BSA (condition 3). In the absence of CRALBP (condition 2), 11-*cis*-retinyl ester synthesis was greatly reduced to 0.023 nmol min^{-1} mg^{-1}. In the absence of palmitoyl-CoA, retinyl ester was not synthesized (data not shown). Similarly, all-*trans*-retinyl ester was not synthesized from all-*trans*-retinol in the presence or absence of CRALBP and absence of BSA (data not shown). Two experiments were conducted with similar results; the difference in retention time for peak 1 (Figures 2A and 2B) is due to mobile phase variability between experiments.

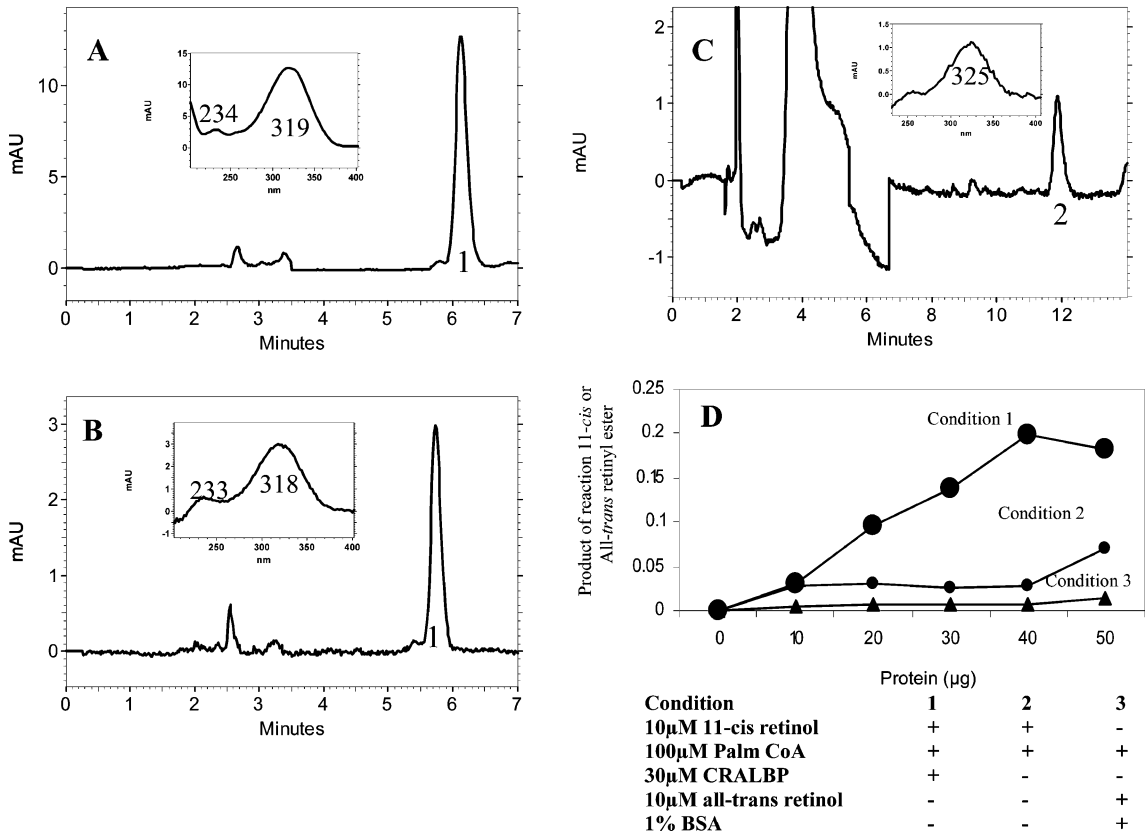

FIGURE 2: Formation of retinyl esters from exogenous retinol by membranes from cultured chicken Muller cells. Whole membranes were prepared from primary cultures of chicken Muller cells. HPLC chromatograms of retinyl esters extracted from membranes incubated with (A) 11-*cis*-retinol, CRALBP, and palmitoyl-CoA for 30 min (condition 1) detected at 318 nm and without (B) CRALBP (condition 2) detected at 318 nm. (C) HPLC chromatogram of retinyl esters extracted from membranes incubated with all-*trans*-retinol, palmitoyl-CoA, and BSA for 30 min (condition 3) detected at 325 nm. Peak 1: 11-*cis*-retinyl esters. Peak 2: all-*trans*-retinyl esters. (D) Summary of production of retinyl esters by Muller cell membranes under conditions 1, 2, and 3. Insets are the spectra for 11-*cis*- or all-*trans*-retinyl esters in the HPLC chromatograms. In the absence of palmitoyl-CoA no retinyl esters were formed (data not shown). Means and standard errors of duplicate determinations are indicated in the diagram. Two experiments were performed with similar results. Key: (●) 11-*cis*-retinyl esters; (▲) all-*trans*-retinyl esters.

Table 2: Transfer of the Labeled Acyl Group from ^{14}C-Labeled Palmitoyl-CoA to Retinol To Form Retinyl Ester by Primary Chicken Muller Cell Membrane[a]

	DPM on labeled retinyl esters		
radiolabeled retinyl esters	Muller cell membrane	rat liver membrane	rat muscle membrane
11-*cis*-RP	2800 ± 1290	72 ± 3	52 ± 17
ATRP	842 ± 409	713 ± 346	139 ± 65

[a] Whole membrane preparations were made from primary chicken Muller cell, rat liver, and rat leg muscle. 30 μg of each of the membranes was then incubated with 10 μM 11-*cis*-retinol in the presence of CRALBP and ^{14}C-labeled palmitoyl-CoA (specific activity = 4442 DPM/nmol) for 30 min (total volume 1 mL) to form 11-*cis*-retinyl palmitate (baseline levels of DPM were observed indicating minimal all-*trans*-retinyl palmitate synthesized from 11-*cis*-retinol). These cell membranes were also incubated with 10 μM all-*trans*-retinol in the presence of BSA and ^{14}C-labeled palmitoyl-CoA (specific activity = 4442 DPM/nmol) for 30 min to form all-*trans*-retinyl palmitate (baseline levels of DPM were observed indicating minimal 11-*cis*-retinyl palmitate synthesis from all-*trans*-retinol). Reactions was quenched with ice-cold ethanol and analyzed by HPLC and scintillation counting as described above. The formation of labeled 11-*cis*-retinyl palmitate by Muller cell membrane indicates a significant level of acyl transfer. In contrast, low levels of labeled 11-*cis*-retinyl palmitate associated with rat liver and muscle membranes suggest minimal acyl-transfer activity in these cells. Muller cell membranes also synthesized three times the labeled 11-*cis*-retinyl palmitate than all-*trans*-retinyl palmitate. Muller cell membranes and rat liver membranes synthesized similar amounts of all-*trans*-retinyl palmitate while the muscle membranes only produced baseline levels of DPM indicating minimal ester synthesis. Three experiments were conducted. Standard errors are indicated in the table.

Transfer of the Labeled Acyl Group from ^{14}C-Labeled Palmitoyl-CoA to Retinol To Form Retinyl Esters. To verify acyl transfer of ARAT activity in primary chicken Muller cell membranes, we performed an experiment to show direct transfer of a labeled acyl group from palmitoyl-CoA onto retinol to form labeled retinyl esters. Table 2 shows that Muller cell membranes transferred the labeled acyl group to 11-*cis*-retinol forming labeled 11-*cis*-retinyl palmitate (2800 DPM), while rat liver and rat muscle had relatively low activity. Muller cell membranes also transferred three times the amount of labeled acyl group to 11-*cis*-retinol, forming 11-*cis*-retinyl palmitate, than to all-*trans*-retinol to form all-*trans*-retinyl palmitate (2800 vs 842 DPM, respectively). Muller cell membranes and rat liver membranes synthesized similar amounts of labeled all-*trans*-retinyl palmitate while muscle membranes exhibited a relatively low level of acyl-transfer activity.

Inhibition of ARAT Activity in Muller Cell Membranes. To further confirm the presence of ARAT activity, we included progesterone, a known ARAT inhibitor (*19*), in our reaction mixtures. The presence of progesterone reduced synthesis of labeled all-*trans*-retinyl palmitate from all-*trans*-retinol by rat liver membranes by 84% (Figure 3D), bringing DPM levels down to baseline. In the presence of progesterone, all-*trans*-retinyl palmitate synthesis was not completely abolished (Figure 3C). This remaining synthesis is most likely due to LRAT, which is the main retinol-esterifying enzyme in liver. Synthesis of labeled 11-*cis*-retinyl palmitate from 11-*cis*-retinol in Muller cell membranes was reduced by 64% in the presence of progesterone (Figure 3H), once again bringing DPM levels to baseline. Synthesis of 11-*cis*-retinyl palmitate was not completely abolished (Figure 3G). It is possible that progesterone is a specific inhibitor for all-*trans*-ARAT activity and may not function as well on 11-*cis*-ARAT activity located in the Muller cell.

Kinetics of ARAT Activity in Chicken Muller Cell Membrane. To determine the kinetics of 11-*cis*-ARAT activity in primary Muller cell membranes, membranes were incubated with fixed concentrations of palmitoyl-CoA and CRALBP; 11-*cis*-retinol was added to the reaction mixture in increasing concentrations. For all-*trans*-ARAT activity, Muller cell membranes were also incubated with fixed concentrations of palmitoyl-CoA and BSA; all-*trans*-retinol was added to reaction mixtures in increasing concentrations. We measured the initial rates of retinyl palmitate synthesis by HPLC. Eadie−Hofstee transformation of the data yielded apparent parameters for 11-*cis*-ARAT activity, $V_{max} = 0.135$ nmol min^{-1} mg^{-1} and $K_m = 11.25$ μM, and for all-*trans*-ARAT, $V_{max} = 0.0065$ nmol min^{-1} mg^{-1} and $K_m = 28.88$ μM.

DISCUSSION

Retinal Muller cells have been suggested to play an important role in vitamin A processing for visual chromophore regeneration (*14*, *15*). However, direct evidence in support of this hypothesis has not been available. In the present study, we investigated the ability of primary Muller cells to esterify retinol both in culture and in vitro. We first established primary Muller cell cultures from the cone-dominated chicken retina. Under light microscopy, our primary cells exhibited Muller cell morphology (*13*), and they stained positive for CRALBP, a marker for Muller cells in the neural retina (*27*), as well as for GFAP, a glia cell marker.

Incubation of Muller cells with 11-*cis*-retinol(s) resulted in the production of more 11-*cis*-retinyl esters than of all-*trans*-retinyl esters (from all-*trans*-retinol). This suggests that Muller cells in culture may selectively esterify 11-*cis*-retinol over all-*trans*-retinol. Retinyl esters in the RPE are found in the all-*trans* conformation, and these all-*trans*-retinyl esters are the substrate for the isomerase enzyme (*4*, *5*). Although the accumulation of 11-*cis*-retinyl esters in the cone-dominated chicken retina has been described (*16*), it was not clear which cell type in the retina synthesizes 11-*cis*-retinyl ester. As the Muller cells are the main glial cell in the retina, it is likely that they synthesized 11-*cis*-retinyl esters for visual chromophore regeneration. This suggestion is further supported by the localization of CRALBP, which has a high affinity for 11-*cis*-retinoids, in the Muller cell (*26*).

Results from the present study show that the production of retinyl esters by primary chicken Muller cell membranes was highest in the presence of CRALBP, palmitoyl-CoA, and 11-*cis*-retinol. The absence of CRALBP significantly reduced the synthesis of 11-*cis*-retinyl esters. Since CRALBP binds 11-*cis*-retinoids endogenously, it is a possibility that CRALBP delivered 11-*cis*-retinol in the Muller cell membrane. Synthesis of all-*trans*-retinyl esters was minimal and only occurred in the presence of palmitoyl-CoA and BSA (Figure 2; BSA was used to deliver all-*trans*-retinol to the Muller cell membrane). The difference in the efficiency of 11-*cis*- or all-*trans*-retinyl ester synthesis by Muller cell membranes may have two explanations. One is that one ester synthase exists and that this ester synthase can catalyze

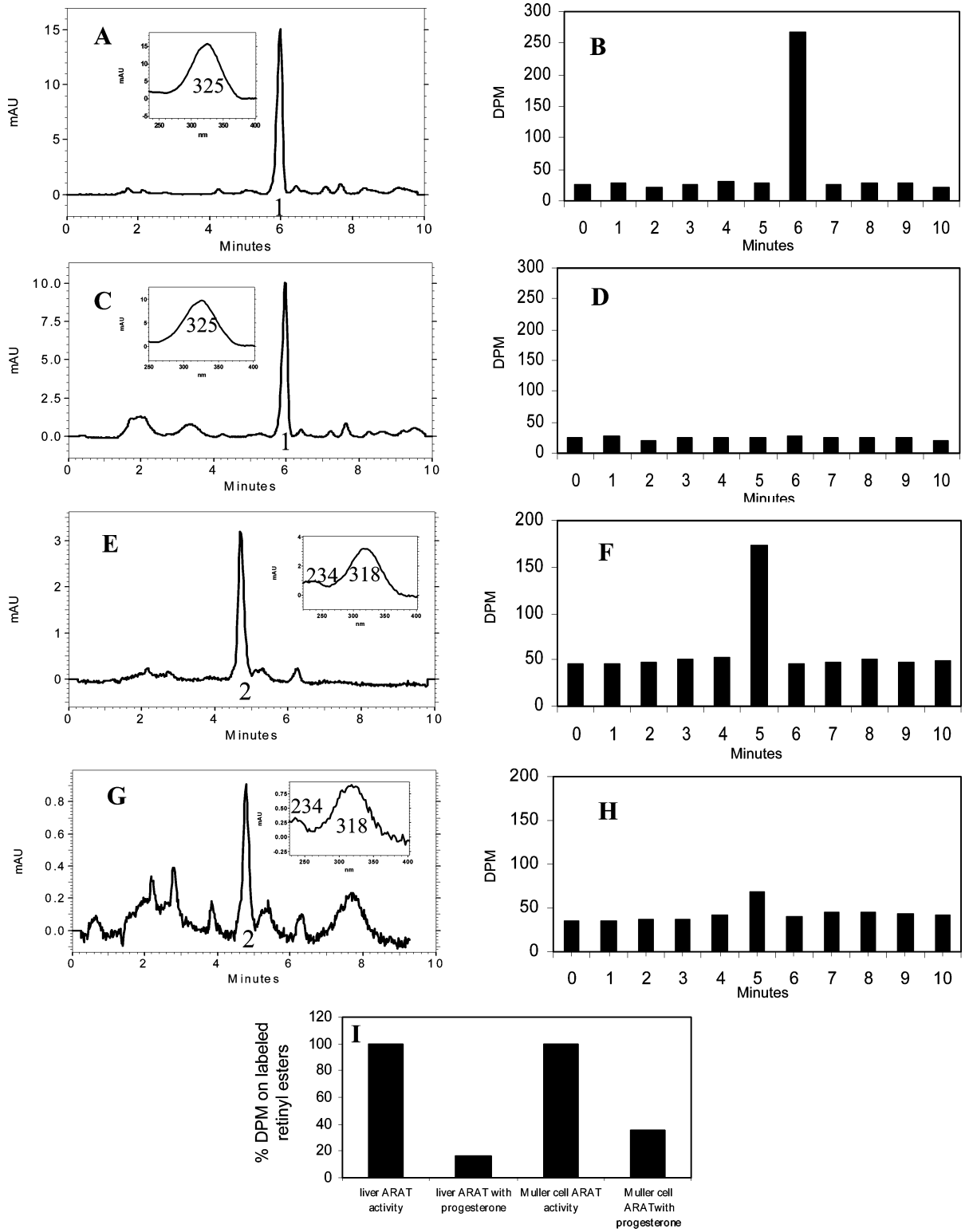

FIGURE 3: Inhibition of retinyl ester synthesis by liver and Muller cell membranes using progesterone. Whole membranes were prepared from rat liver and from the primary culture of chicken Muller cells. Membranes from liver and Muller cells (30 μg) were then incubated with 10 μM all-*trans*-retinol and 10 μM 11-*cis*-retinol, respectively, in the presence of ^{14}C-labeled palmitoyl-CoA (specific activity 1110 DPM/nmol) and progesterone (200 μM) for 30 min. Retinyl esters were extracted with hexane and analyzed by HPLC and a liquid scintillation counter for ^{14}C label. (A) and (C) are HPLC chromatograms, detected at 325 nm, of retinyl esters extracted from rat liver membranes incubated in the absence and presence of progesterone, respectively. (B) and (D) are DPM associated with retinyl esters produced by rat liver membranes incubated in the presence and absence of progesterone corresponding to (A) and (C). (E) and (G) are HPLC chromatograms, detected at 318 nm, of retinyl esters extracted from Muller cell membranes incubated in the absence and presence of progesterone, respectively. (F) and (H) are DPM associated with retinyl esters produced by Muller cell membranes incubated in the absence and presence of progesterone corresponding to (E) and (G), respectively. Insets are the spectra for 11-*cis*- or all-*trans*-retinyl esters in the HPLC chromatograms. (I) Summary of panels A−H. Progesterone reduced the amount of labeled all-*trans*-retinyl ester in rat liver by 84% and 11-*cis*-retinyl esters in Muller cell membranes by 64%, bringing DPM levels to baseline. Two experiments were performed with duplicate determinations. Peak 1: all-*trans*-retinyl ester. Peak 2: 11-*cis*-retinyl ester.

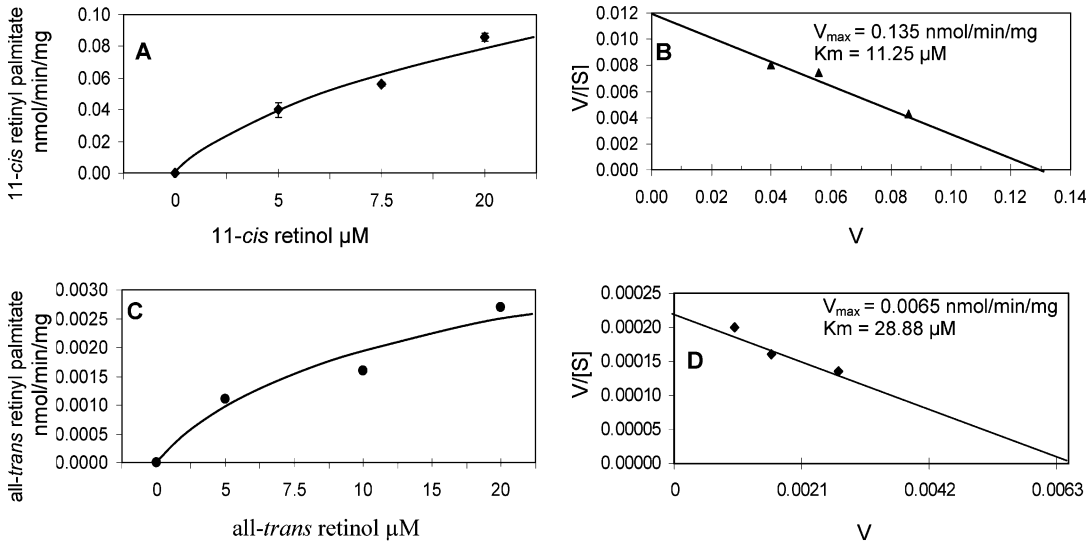

FIGURE 4: Kinetics of ARAT activity in chicken Muller cell membranes. Cell membranes were prepared from a primary culture of chicken Muller cells and incubated with increasing concentrations of 11-*cis*-retinol in the presence of 100 μM palmitoyl-CoA and 30 μM CRALBP for 30 min at 37 °C. (A) 11-*cis*-Retinol substrate saturation curve. (B) Eadie−Hofstee plot of the 11-*cis*-retinol substrate saturation curve yielding an apparent V_{max} of 0.135 nmol min^{-1} mg^{-1} and an apparent K_M value of 11.25 μM. (C) All-*trans*-retinol substrate saturation curve. (D) Eadie−Hofstee plot of the all-*trans*-retinol substrate saturation curve yielding an apparent V_{max} of 0.0065 nmol min^{-1} mg^{-1} and an apparent K_M value of 28.88 μM. For 11-*cis*-ARAT kinetics data, means and standard errors of triplicate determinations are indicated in the diagram. Three experiments were performed with similar results. For all-*trans*-ARAT kinetic data, two experiments was performed with single determinations.

esterification of both isomers of retinol, but is selective for the 11-*cis* conformation. The second explanation is that two distinct enzymes exist in the Muller cells, one that esterifies 11-*cis*-retinol and one that esterifies all-*trans*-retinol. The higher 11-*cis*-retinyl ester synthesis in Muller cells agrees with the recent discovery of a novel retinoid cycle in the retina of cone-dominated species in which 11-*cis*-retinyl esters are accumulated in the retina while all-*trans*-retinyl esters are accumulated in the RPE (*16*).

Since samples incubated in the absence of palmitoyl-CoA did not produce any esterification products, it strongly suggests that the esterification reaction is acyl-CoA dependent. An acyl-CoA-dependent ester synthase activity has previously been shown in the cone-dominated chicken retina (*15*). ARAT is an enzyme activity known to esterify retinol in an acyl-CoA-dependent manner (*19*). Recently, ARAT activity that can synthesize retinyl esters from both all-*trans*-retinol and 11-*cis*-retinol was reported in bovine RPE (*20*). However, this is the first study to show that an ARAT activity specific for 11-*cis*-retinol exists in Muller cells.

To fully establish ARAT enzyme activity, we have also demonstrated the acyl-transfer reaction by directly transferring the radiolabeled acyl group from palmitoyl-CoA onto retinol to form labeled retinyl esters. Data in Table 2 also show that Muller cell membranes transferred considerably more radioactive acyl groups to form labeled 11-*cis*-retinyl esters than labeled all-*trans*-retinyl esters and further support our results (using nonlabeled substrates) on 11-*cis*-ARAT activity. The acyl-transfer activity of all-*trans*-ARAT in Muller cell membranes was similar to that of liver membranes, suggesting that all-*trans*-ARAT activity in the Muller cells may be homologous to the hepatic ARAT. When progesterone, a specific ARAT inhibitor (*19*), was added to our reaction mixture, a significant reduction of retinyl ester synthesis (Figure 3) was noted in liver and Muller cell membrane. These data further verify the ARAT origin retinyl ester synthesis observed in Muller cell membranes.

To test the effect of all-*trans*-retinol on 11-*cis*-retinol esterification, we conducted a substrate competition assay. Results from this assay indicated that the 11-*cis*-ARAT activity remained the same in the presence of (equimolar of) all-*trans*-retinol (at 5, 10, and 20 μM retinol; data not shown). This suggests that 11-*cis*-ARAT activity in Muller cells membranes is distinct from the ARAT activity for all-*trans*-retinol.

K_m and V_{max} are important enzyme kinetic parameters. The K_m of an enzyme represents the substrate concentration that yields half the occupancy of catalytic sites. We determined the apparent K_m for 11-*cis*-retinol of the 11-*cis*-ARAT activity in Muller cell membranes to be 11.25 μM. This value is well between the documented K_m value for ARAT activity in RPE (4.1 μM) (*20*) and liver (30 μM) (*21*). The K_m value of the 11-*cis*-ARAT activity in Muller cells is also comparable to the value recently described for 11-*cis*-ARAT activity in RPE (8.7 μM) (*20*). We also determined an apparent K_m value for all-*trans*-ARAT activity in primary Muller cell membranes to be 28.88 μM, twice as high than that for 11-*cis*-retinol. This K_M value is very comparable to liver all-*trans*-ARAT activity, reported to be 30 μM (*18*). The lower K_m for 11-*cis*-ARAT activity in Muller cell membranes further suggests the specificity of this enzyme activity.

The V_{max} is representative of the activity of an enzyme at saturating substrate concentrations. Because V_{max} is influenced by the purity of the enzyme, we determined the apparent V_{max} value for 11-*cis*-ARAT activity for 11-*cis*-retinol and all-*trans*-retinol to be 0.135 and 0.0065 nmol min^{-1} mg^{-1} in whole Muller cell membranes. The 11-*cis*-ARAT activity from Muller cell membranes' apparent V_{max} value is below the documented ARAT activities for liver (0.3 nmol min^{-1} mg^{-1}) (*21*) and for 11-*cis*-ARAT in RPE (3.8 nmol min^{-1} mg^{-1}) (*20*). The V_{max} of all-*trans*-ARAT

activity in the Muller cell membranes is also lower than the reported liver ARAT activity. This lower V_{max} value for 11-*cis*- and all-*trans*-ARAT activities in our Muller cell samples is perhaps due to total membrane preparations from cell cultures used in the experiments rather than the microsomal membrane preparation from fresh tissue which would yield an enzyme preparation of higher purity.

The enzyme responsible for ARAT activity remains unknown. ARAT activity has recently been reported in 293T cells transfected with a cDNA library from chicken retina (*28*). This activity may be the result of a nonspecific palmitoyl-CoA-dependent enzyme known as DGAT1 (*29*). Future work will certainly establish whether this DGAT1 is the retinol-esterifying enzyme in the chicken retina.

Several lines of evidence show that the retina contains 11-*cis*-retinyl ester pools (*15*, *16*, *30*). However, the cellular location of these esters within the retina is unknown. To assay for the possibility that the Muller cell may be a storage site for 11-*cis*-retinyl esters, we have tested both confluent Muller cell cultures (approximately 15-day-old cultures) and freshly explanted and cultured cells (within 48 h) for endogenous retinoids by HPLC. We found the primary Muller cell cultures to contain no detectable level of retinoids. Two explanations may apply to these findings. The first is that retinoids may have been lost during culture preparation; the second is that the Muller cell is able to synthesize retinyl esters and secrete them.

The presence of 11-*cis*-specific ester synthase activity in the Muller cell suggests the presence of isomerase activity in this cell. Upon light exposure, all-*trans*-retinol is released by the photoreceptors and transferred to Muller cells. This all-*trans*-retinol would have to be isomerized, perhaps by a light-dependent isomerase, to the 11-*cis* conformation before being esterified by the 11-*cis*-ARAT activity observed in the present study of Muller cells. This would explain the large amount of 11-*cis*-retinyl ester recovered from cone-dominated retinas (*30*). Since cones are known to recover visual sensitivity from both 11-*cis*-retinol and 11-*cis*-retinaldehyde (*12*), 11-*cis*-retinyl esters may be transiently stored in the Muller cells to avoid retinol toxicity under high light illumination. Upon dark adaptation, esters are then hydrolyzed to 11-*cis*-retinol and transferred to the photoreceptor for pigment regeneration. Our results show no isomerase activity in the presence of all-*trans*-retinol. It is a possibility that the Muller cells in culture lose expression or activity of the enzyme responsible for isomerizing retinoids. It is also possible that the isomerase is light dependent and therefore not functional in our experimental conditions.

In summary, we have presented experimental evidence showing the presence of ARAT activity in the primary Muller cell culture from chicken retina. This activity is specific for 11-*cis*-retinol and results in the formation of 11-*cis*-retinyl ester. Our data provide the first experimental evidence to explain the accumulation of 11-*cis*-retinyl ester in cone-dominated chicken retina. Additional experiments will be needed to study the expression of this ARAT and how it supports the function of the cone visual cycle in the retina.

ACKNOWLEDGMENT

We thank Dr. D. Allen, Dr. N. L. Mata, and Mr. Simon Trevino for critical review of the manuscript. We also thank Dr. K. Palczewski for kindly donating CRALBP protein and Dr. J. Saari for kindly providing anti-CRALBP antibody. We thank Tyson Foods Co. for tissue donations and Ms. Sarika Jahagirdar for technical assistance.

REFERENCES

1. McBee, J. K., et al. (2001) Confronting complexity: the interlink of phototransduction and retinoid metabolism in the vertebrate retina, *Prog. Retinal Eye Res. 20*, 469−529.
2. Bridges, C. D., Alvarez, R. A., and Fong, S. L. (1982) Vitamin A in human eyes: amount, distribution, and composition, *Invest. Ophthalmol. Visual Sci. 22*, 706−714.
3. Flood, M. T., et al. (1983) Vitamin A utilization in human retinal pigment epithelial cells in vitro, *Invest. Ophthalmol. Visual Sci. 24*, 1227−1235.
4. Gollapalli, D. R., and Rando, R. R. (2003) All-*trans*-retinyl esters are the substrates for isomerization in the vertebrate visual cycle, *Biochemistry 42*, 5809−5818.
5. Moiseyev, G., et al. (2003) Retinyl esters are the substrate for isomerohydrolase, *Biochemistry 42*, 2229−2238.
6. Moiseyev, G., et al. (2005) RPE65 is the isomerohydrolase in the retinoid visual cycle, *Proc. Natl. Acad. Sci. U.S.A. 102*, 12413−12418.
7. Jin, M., et al. (2005) Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium, *Cell 122*, 449−459.
8. Redmond, T. M., et al. (2005) Mutation of key residues of RPE65 abolishes its enzymatic role as isomerohydrolase in the visual cycle, *Proc. Natl. Acad. Sci. U.S.A. 102*, 13658−13663.
9. Perry, R. J., and McNaughton, P. A. (1991) Response properties of cones from the retina of the tiger salamander, *J. Physiol. 433*, 561−587.
10. Goldstein, E. B. (1967) Early receptor potential of the isolated frog (*Rana pipiens*) retina, *Vision Res. 7*, 837−845.
11. Hood, D. C., and Hock, P. A. (1973) Recovery of cone receptor activity in the frog's isolated retina, *Vision Res. 13*, 1943−1951.
12. Jones, J. G., Crouch, K. R., Wiggert, B., Cornwall, M. C., and Chader, J. G. (1989) Retinoid requirements for recovery of sensitivity after visual-pigment bleaching in isolated photoreceptors, *Proc. Natl. Acad. Sci. U.S.A. 86*, 9606−9610.
13. Rodriguez, K. A., and Tsin, A. T. (1989) Retinyl esters in the vertebrate neuroretina, *Am. J. Physiol. 256* (Part 2), R255−R258.
14. Das, S. R., Bhardwaj, N., Kjeldbye, H., and Gouras, P. (1992) Muller cells of chicken retina synthesize 11-*cis*-retinol, *Biochem. J. 285*, 907−913.
15. Mata, N. L., et al. (2002) Isomerization and oxidation of vitamin a in cone-dominant retinas: a novel pathway for visual-pigment regeneration in daylight, *Neuron 36*, 69−80.
16. Trevino, S. G., et al. (2005) Retinoid cycles in the cone-dominated chicken retina, *J. Exp. Biol. 208* (Part 21), 4151−4157.
17. Mata, N. L., et al. (2005) Chicken retinas contain a retinoid isomerase activity that catalyzes the direct conversion of all-*trans*-retinol to 11-*cis*-retinol, *Biochemistry 44*, 11715−11721.
18. Saari, J. C., and Bredberg, D. L. (1989) Lecithin:retinol acyltransferase in retinal pigment epithelial microsomes, *J. Biol. Chem. 264*, 8636−8640.
19. Ross, A. C. (1982) Retinol esterification by rat liver microsomes. Evidence for a fatty acyl coenzyme A:retinol acyltransferase, *J. Biol. Chem. 257*, 2453−2459.
20. Kaschula, C. H., et al. (2006) Acyl CoA:retinol acyltransferase (ARAT) activity is present in bovine retinal pigment epithelium, *Exp. Eye Res. 82*, 111−121.
21. Randolph, R. K., Winkler, K. E., and Ross, A. C. (1991) Fatty acyl CoA-dependent and -independent retinol esterification by rat liver and lactating mammary gland microsomes, *Arch. Biochem. Biophys. 288*, 500−508.
22. Saari, J. C., and Bredberg, D. L. (1988) CoA- and non-CoA-dependent retinol esterification in retinal pigment epithelium, *J. Biol. Chem. 263*, 8084−8090.
23. Sarthy, V. H. R. (2001) *The Retinal Muller Cell Structure and Function* (Blakmore, C., Ed.) p 1, Plenum Publishers, New York.
24. Lee, E., et al. (2005) An ultramicroscopic study on the distribution of Muller cell processes in the outer retinal layers of the zebrafish, *Ann. Anat. 187*, 43−50.
25. Bunt-Milam, A. H., and Saari, J. C. (1983) Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina, *J. Cell Biol. 97*, 703−712.

26. Saari, J. C., Bredberg, L., and Garwin, G. G. (1982) Identification of the endogenous retinoids associated with three cellular retinoid-binding proteins from bovine retina and retinal pigment epithelium, *J. Biol. Chem. 257*, 13329−13333.
27. De Leeuw, A. M., et al. (1990) Immunolocalization of cellular retinol-, retinaldehyde- and retinoic acid-binding proteins in rat retina during pre- and postnatal development, *J. Neurocytol. 19*, 253−264.
28. Kaylor, J. J., M. J., Moghrabi, W., and Travis, G. H. (2006) Strategies for cloning the retinol isomerase in cone-dominant chicken retinas, in *IOVS*, Ft. Lauderdale, FL.
29. Orland, M. D., Anwar, K., et al. (2005) Acyl coenzyme A dependent retinol esterification by acyl coenzyme A: Diacylglycerol acyltransferase 1, *Biochim. Biophys. Acta 1731* (1), 76−82.
30. Villazana-Espinoza, E. T., Hatch, A. L., and Tsin, A. T. (2006) Effect of light exposure on the accumulation and depletion of retinyl ester in the chicken retina, *Exp. Eye Res. 83*, 871−876.

BI060928P