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11-*cis* ARAT Activity in Primary Culture of Chicken Muller Cells

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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 4 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/mg ($V_{\max}$) and 11.25 μ M (K_M); and for all-*trans* ARAT, 0.0065 nmol/min/mg ($V_{\max}$) and 28.88 μ M (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 ester in the retina 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. In order to sustain this visual function, all-*trans* retinaldehyde is re-isomerized 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) are the substrate for the isomerase enzyme [4,5], a key visual 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 several fold 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

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[16], and recently Mata demonstrated that the retinas of cone-dominated species possess retinoid processing enzymes, including a retinyl ester synthase [17].

Lecithin retinol acyl transferase (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 retinol acyl transferase (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 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. 1992 with minor modifications. Freshly severed chicken heads were collected on ice (Tyson Foods, Inc., Seguin, Texas) and immediately transported to the laboratory. Eyes were enucleated within three hours post-mortem and sterilized with 10 % Wescodyne for 5 minutes. Eyes were sectioned at the level of the ora serrata, the vitreous removed and the retina was then dissected from the eye. After being rinsed in 1% antibiotic/antimycotic in Hanks 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 collected by centrifugation (1500 × g). Cells were then seeded in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and 5 mg/ml of glucose and incubated at 37° C and 5% CO₂. The media was changed 24hrs later and every 48 hrs thereafter until cultured cells were confluent. Cells from 2–3 retinas were seeded into each T75 flask. Cells were cultured until confluent between 14–19 days.

Immunocytochemistry

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% paraformaldehyde for 15 minutes. 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 Biorad 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 minutes at room temperature. Cells were incubated for one hour in PBS containing 1% fatty acid free BSA (Sigma Aldrich) to block nonspecific binding. Incubation with primary antibodies mouse anti-CRALBP (a gift from Dr. Jack Saari) and rabbit anti GFAP (obtained from Dakocytomation, Denmark) was performed overnight at a 1:1000 dilution at 4°C in PBS containing 1% BSA. After rinsing 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 minutes. 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 cell

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 hours. The cultures were then incubated overnight in 5ml serum free media containing 10 μ M 11-*cis* retinol or 10 μ M 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 μ M 4.5 $\times$ 250 mm column, part number 880975–901; the mobile phase used was 0.2% HPLC grade dioxane/hexane at a flow rate of 2ml/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 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 10mM tris pH 7.5, 2mM CaCl₂, 2mM MgCl₂, and 1mM DTT. The homogenate was centrifuged at 250,000 $\times$ g for 30 minutes at 4° C and the pellet re-suspended in the same buffer.

Rat muscle and rat liver membranes were prepared according to Ross 1982 [18]. Briefly, fresh tissue was collected, and the rat liver was perfused overnight at 4° C in 250 mM sucrose and 10mM 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 $\times$ g for 10 minutes; the supernatant was then centrifuged at 13,000 $\times$ g after which floating fat was skimmed. To prepare microsomes, the supernatant was centrifuged at 250,000 $\times$ g for 30 minutes. Rat muscle tissue was prepared by glass-glass homogenization and then centrifuged at 250,000 $\times$ g for 30 minutes. The liver and muscle pellets were re-suspended 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 pre-incubated with palmitoyl CoA in 100 mM Tris pH 8, 2 mM CaCl₂, 2 mM MgCl₂, (reaction buffer) for 5 minutes at room temperature. The substrate, 11-*cis* retinol or all-*trans* retinol, was delivered in 2 μ l 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 pre-incubated for 5 minutes at room temperature. The protein samples and retinol samples were then combined and incubated at 37° C for 30 minutes. The final reaction mixture was 100 μ M palmitoyl CoA, 30 μ M CRALBP or 1% BSA, 10 μ M retinol and protein in increasing amounts (between 10 and 50 μ g). Total reaction volume was 1ml. The reaction was stopped with 1ml ice cold ethanol. Retinoids were then extracted with hexane and analyzed by HPLC.

Transfer of labeled acyl group from ¹⁴C 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 μ M, specific activity 4,442 DPM/nmol was

pre-incubated with 30µg Muller cell membranes as described above. Retinol was delivered in ethanol as described above and pre-incubated with CRALBP. The protein sample and retinol sample were then combined and incubated at 37° C for 30 minutes and the reaction was stopped with 1ml 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 ¹⁴C). Radiolabeled authentic retinoid standards were used to determine elution time of experimental retinoid products.

Rat liver and leg muscle membranes were used as positive and negative controls, respectively. 30µg of protein were pre-incubated with 100µM radiolabeled palmitoyl CoA (specific activity 4,442 DPM/ nmol) in buffer containing 150mM K₂PO₄, 1mM DTT, and 1% BSA at pH 7.4 for 5 minutes at room temperature. 11-*cis* or all-*trans* retinol was then delivered to the mixture in 2 µl ethanol to a final concentration of 10 µM. A final volume of 1ml was then incubated, quenched, and analyzed as described above.

Inhibition of ARAT activity by progesterone

Muller cell membranes were pre-incubated with radiolabeled palmitoyl CoA (100 µM, specific activity 1,111 DPM/nmol) and progesterone (200µM) delivered in 4 µl of ethanol for 5 minutes 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 pre-incubated with radiolabeled palmitoyl CoA (100 µM, specific activity 1,111 DPM/nmol) and progesterone (Sigma-Aldrich) (200µM) delivered in 4 µl of ethanol for 5 minutes at room temperature in buffer containing 150mM K₂PO₄, 1mM DTT and 1% BSA at pH 7.4. 10 µM all-*trans* retinol was then delivered to the reaction mixture and incubated at 37° C for 30 minutes. 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 primary chicken Muller cell

30 µg of Muller cell membrane protein was pre-incubated with 100µM palmitoyl CoA. 30 µM CRALBP was pre-incubated with 11-*cis* retinol in increasing concentrations (0–20µM). 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 minutes. 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 Immunocytochemistry

Primary chicken Muller cell cultures were established according to the methods of Das, et al., 1992. Figure 1A shows the morphology of freshly explanted Muller cells 10 days after seeding at approximately 80% confluence in a T75 flask. 100% confluence was reached between 14 to 19 days with 3 × 10⁶ cells per T75 flask. Confluent Muller cell cultures (10 T75 flasks at 3 × 10⁶ cells/flask) showed no detectable retinoids when analyzed by HPLC (data not shown).

To establish the identity of Muller cells in culture, we performed immunocytochemical studies with both Muller and glial cell markers. Figure 1B shows a primary Muller cell culture (40x) stained with anti-CRALBP antibody. CRALBP is expressed exclusively by Muller cells in the retina

[25]. Figure 1D shows a primary Muller cell culture (20x) 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 $10\mu\text{M}$ 11-*cis* retinol or $10\mu\text{M}$ all-*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). No 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 thermo isomerization 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 cells 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/mg which was 20-fold higher than that for all-*trans* retinyl ester synthesis (0.007 nmol/min/mg), from of 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/mg . 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).

Transfer of labeled acyl group from C¹⁴ labeled palmitoyl CoA to retinol to form retinyl esters

In order 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 (2,800 DPM), while rat liver and rat muscle had relatively low activity. Muller cell membranes also transferred 3 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 (2,800 DPM vs. 842 DPM, respectively). Muller cell membranes and rat liver membranes synthesized similar amounts of labeled all-*trans* retinyl palmitate while muscle membranes exhibited 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% (Fig. 3D) bringing DPM levels down to baseline. In the presence of progesterone, all-*trans* retinyl palmitate synthesis was not completely abolished (Fig. 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 (Fig. 3H), once again bringing DPM levels to base line. Synthesis of 11-*cis* retinyl palmitate was not completely abolished (Fig. 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/mg and $K_m = 11.25$ μ M and for all-*trans* ARAT: $V_{\max} = 0.0065$ nmol/min/mg and $K_m = 28.88$ μ M.

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 (Fig.2; BSA was used to deliver all-*trans* retinol to 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 esterification of both isomers of retinol, but is selective for 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 supports our results (using non-labeled 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 (Fig. 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 (equi-molar of) all-*trans* retinol (at 5, 10 and 20 μ M of 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 μ M. This value is well between the documented K_m value for ARAT activity in RPE (4.1 μ M) [20] and liver (30 μ M) [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 μ M) [20]. We also determined an apparent K_m value for all-*trans* ARAT activity in primary Muller cell membranes to be 28.88 μ M, and 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 μ M [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 nmol/min/mg and 0.0065 nmol/min/mg in whole Muller cell membranes. The 11-*cis* ARAT activity from Muller cells membranes' apparent V_{max} value is below the documented ARAT activities for liver (0.3 nmol/min/mg) [21] and for 11-*cis* ARAT in RPE (3.8 nmol/min/mg) [20]. 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 cells samples is perhaps due to total membrane preparations from cell cultures used in the experiments rather than 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. This activity may be the result of a nonspecific palmitoyl CoA dependent enzyme known as DGAT1[28] .

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 [15,16,29]. 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 hrs) 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, or second 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 [29]. 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 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.

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Abbreviations

CRALBP, cellular retinaldehyde binding protein
RPE, retinal pigment epithelium
LRAT, lecithin: retinol acyl-transferase
ARAT, acyl CoA: retinol acyl-transferase
HBSS, Hanks Balanced Salt Solution
MEM, minimal essential medium
PBS, phosphate buffer saline
PBST, PBS containing 0.2% Triton X-100
BSA, bovine serum albumin
DPM, disintegrations per minute

DGAT1, acyl CoA: diacylglycerol acyltransferase 1

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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; 4X). **B.** Muller cells stained with anti-CRALBP monoclonal antibody and visualized by FITC-conjugated secondary antibody (green). Propidium Iodide (red) was used to counter stain for cell nuclei (20X). **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 counter stain for cell nuclei (10X). **E.** Negative control without anti-GFAP. Both primary antibodies were incubated at a 1: 1000 dilution overnight at 4° C.

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 minutes (Condition 1) detected at 318nm; and **B.** without CRALBP (Condition 2) detected at 318nm. **C.** HPLC chromatogram of retinyl esters extracted from membranes incubated with all-*trans* retinol, palmitoyl CoA and BSA for 30 minutes (Condition 3) detected at 325nm. 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. • 11-*cis* retinyl esters, ▲ all-*trans* retinyl esters.

Figure 3.

Inhibition of retinyl ester synthesis by liver and Muller cell membranes using progesterone. Whole membranes were prepared from rat liver and from primary culture of chicken Muller cells. Membranes from liver and Muller cells (30 μ g) were then incubated with 10 μ M all-*trans* retinol and 10 μ M 11-*cis* retinol, respectively, in the presence of 14 C labeled palmitoyl CoA (specific activity 1,110 DPM/nmol) and progesterone (200 μ M) for 30 minutes. Retinyl esters were extracted with hexane and analyzed by HPLC and liquid scintillation counter for 14 C label. **A** and **C** are HPLC chromatograms, detected at 325nm, 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 318nm, 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 figures 3A-3H. 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 membrane. 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 minutes at 37° C. **A.** 11-*cis* retinol substrate saturation curve. **B.** Eadie-Hofstee plot of 11-*cis* retinol substrate saturation curve yielding an apparent V_{max} of 0.135 nmol/min/mg and apparent K_M value of 11.25 μM . **C.** all-*trans* retinol substrate saturation curve. **D.** Eadie Hofstee plot of all-*trans* retinol substrate saturation curve yielding an apparent V_{max} of .0065 nmol/min/mg and 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 kinetics data, two experiments was performed with single determinations.

Table 1**Formation of retinyl esters from exogenous retinol by primary chicken Muller cells**

Primary Muller cells were cultured in T25 flasks. At confluence, each flask contained 10^6 cells. The cells were then incubated with either 10 μ M 11-*cis* retinol or 10 μ M 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 (students 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 > .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.

Amount (nmol of retinyl esters) recovered from Muller cells incubated with retinol		
	11 — <i>cis</i> retinyl ester nmol/ 10^6 cells	All- <i>trans</i> retinyl ester nmol/ 10^6 cells
11 - <i>cis</i> retinol	0.18 $\pm$.06	0.02 $\pm$.02
All - <i>trans</i> retinol	0.0 $\pm$ 0.0	0.022 $\pm$.02

Table 2**Transfer of labeled acyl group from C¹⁴ labeled palmitoyl CoA to retinol to form retinyl ester by primary chicken Muller cell membrane**

Whole membrane preparations were made from primary chicken Muller cell, rat liver, and rat leg muscle. 30 µg of each of the membranes were then incubated with 10 µM 11-*cis* retinol in the presence of CRALBP and ¹⁴C labeled palmitoyl CoA (specific activity = 4,442 DPM/nmol) for 30 minutes (total volume 1ml) 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 ¹⁴C label palmitoyl CoA (specific activity = 4,442 DPM/nmol) for 30 minutes 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 3 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 on the table.

DPM on labeled retinyl esters			
Radiolabeled retinyl esters	Muller cell membrane	Rat liver membrane	Rat Muscle membrane
11- <i>cis</i> RP	2800 ± 1290	72 ± 3	52 ± 17
ATRP	842 ± 409	713 ± 346	139 ± 65