Comparison of retinyl ester hydrolase activities in bovine liver and retinal pigment epithelium

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Abstract Various properties of retinyl ester hydrolysis in the liver and the retinal pigment epithelium (RPE) have been studied, yet the relationship between the retinyl ester hydrolase (REH) activities in these tissues of the same species is not known. In the present study, REH activities in bovine liver and RPE microsomes were compared to explore potential biochemical relationships of retinyl ester metabolism in these tissues. Rates of [³H]all-*trans* retinyl palmitate hydrolysis by liver and RPE were comparable (i.e., $V_{max}^{app} \approx 300$ pmol/min per mg; $K_m^{app} \approx 30$ µM), while hydrolysis of [³H]11-*cis* palmitate by RPE was significantly higher ($V_{max}^{app} = 1{,}667$ pmol/min per mg). When equimolar amounts (10 µM) of either [¹⁴C]triolein or unlabeled 11-*cis* retinyl palmitate were added to [³H]all-*trans* REH assays, all-*trans* REH activities in liver and RPE demonstrated similar time-dependent inhibition profiles. In contrast, hydrolysis of [³H]11-*cis* retinyl palmitate by RPE was relatively unaffected by addition of either [¹⁴C]triolein or unlabeled all-*trans* retinyl palmitate. Additionally, modification of the microsomal proteins with *N*-ethylmaleimide produced profound, dose-dependent alterations in K_m^{app} values for all-*trans* retinyl ester hydrolysis, whereas K_m^{app} for 11-*cis* REH in the RPE was not significantly altered. ■ These results have elucidated common biochemical features of all-*trans* retinyl ester hydrolysis in liver and RPE. In contrast, hydrolysis of 11-*cis* retinyl ester in RPE is characterized by a distinctive substrate preference and unique biochemical properties.—Mata, N. L., J. R. Mata, and A. T. C. Tsin. Comparison of retinyl ester hydrolase activities in bovine liver and retinal pigment epithelium. *J. Lipid Res.* 1996. **37:** 1947–1952.

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Mobilization of retinoids from tissue storage sites is facilitated by retinyl ester hydrolase (REH) enzymes (reviewed in ref. 1). The physiological significance of all-*trans* REH activity in rat liver membranes has been particularly well documented (2–4). The retinal pigment epithelium (RPE) of the eye also possesses abundant stores of all-*trans* retinyl esters. In addition, unique enzymic properties inherent to RPE cells confer the capacity to generate 11-*cis* isomers of vitamin A (5, 6).

Thus, in the RPE, vitamin A is stored as all-*trans* and 11-*cis* retinyl esters (7–9). Because liver and RPE are the principal storage sites for retinyl esters, similar mechanisms may regulate retinyl ester metabolism in these tissues. However, it is not clear how all-*trans* REH activity in liver is related to all-*trans* and 11-*cis* REH activities in RPE. In fact, analysis of all-*trans* and 11-*cis* retinyl ester hydrolysis in the liver and the RPE of the same species has not been reported.

Previous reports from Blaner et al. (10), and from our laboratory (11) have identified distinct biochemical properties of all-*trans* and 11-*cis* REH activities. However, it is not clear whether the observed differences are due to specific enzymic mechanisms or to undefined physical interactions that allow the isomeric retinyl ester substrates to be presented in a different manner to broad specificity hydrolases (1).

In this investigation, we have analyzed REH activities in bovine liver and RPE in order to probe potential relationships of retinyl ester metabolism in these tissues. Our examination has revealed many biochemical similarities of all-*trans* retinyl palmitate hydrolysis. Characteristics of 11-*cis* retinyl palmitate hydrolysis by RPE were distinct from these common features and indicated the presence of a separate catalytic mechanism for 11-*cis* retinyl ester hydrolysis in the RPE.

MATERIALS AND METHODS

All-*trans*-retinyl palmitate, bovine serum albumin (BSA; fraction IV), dithiothreitol (DTT), disodium ethylenediaminetetraacetic acid (2Na-EDTA), all-*trans*-retinol, all-*trans*-retinyl palmitate, *N*-ethylmaleimide (NEM), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). [9,10-^{3}H]palmitic acid (specific activity 37 Ci/mmol) was obtained from DuPont-NEN (Rochester, NY). [^{14}C]triolein (carboxyl-^{14}C; specific activity 120 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Purified 11-*cis*-retinyl palmitate was a gift from Hoffman-La Roche (Nutley, NJ). All other retinoids were purified by high performance liquid chromatography (HPLC) and quantified by UV-visible spectrophotometry prior to use in REH assays. Econo-1 liquid scintillation cocktail and HPLC grade solvents were obtained from Fisher Scientific (Houston, TX). Quantitation of ^{3}H and ^{14}C was achieved with a Packard 2200CA liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL).

Preparation of [^{3}H]all-*trans*- and [^{3}H]11-*cis*-retinyl palmitate substrates

[^{3}H]all-*trans*- and [^{3}H]11-*cis*-retinyl palmitate substrates ([^{3}H]A*t*RP and [^{3}H]11*c*RP, respectively) were prepared by reacting the respective vitamin A alcohols with [9,10-^{3}H]palmitic acid anhydride (11). Specific activity of the retinyl ester substrates was adjusted to 65,000–75,000 dpm/nmol by addition of unlabeled, all-*trans* or 11-*cis* retinyl palmitate. Substrate was routinely added to reaction mixtures in 10 µl of ethanol.

Preparation of bovine RPE and liver microsomes

Preparation of bovine RPE microsomes has been previously described (11). All procedures were performed at 4°C. Briefly, RPE/choroid was removed from freshly enucleated bovine eyes and placed in buffer A (0.25 M sucrose, 10 mM Tris-acetate, 1 mM DTT, 2 mM 2Na-EDTA, pH 7.0) prior to transport by overnight courier to our laboratory. After a thawing period on ice, additional buffer A was added to the samples (final volume = 4 ml/RPE) and RPE/choroid tissue was agitated using a vortex mixer. Choroid tissue was removed with a dissecting needle and the resulting RPE cell suspension was homogenized (Polytron Homogenizer, Brinkman Instruments; setting 7 for 20 s). Bovine liver homogenates were prepared from 0.5–1.0 gm portions of fresh liver as follows. Liver samples were rinsed in ice-cold buffer A, blotted dry, weighed, and minced with scissors. Four volumes of buffer A was added, and the tissue was homogenized with a Polytron Homogenizer (setting 7 for 30 s). Microsomal protein was prepared from RPE and liver homogenates by differential centrifugation as previously described (11). Aliquots of the microsomal proteins were stored at -85°C. Protein determinations were made by the dye-binding method using BSA as a standard (12).

REH enzyme assay

The radiometric REH assay first described by Prystowsky, Smith, and Goodman (13) was used in the present study. In a routine REH assay, 2 nmol of [^{3}H]A*t*RP or [^{3}H]11*c*RP was delivered in 10 µl of ethanol to pre-incubated (1–2 min @ 37°C) reaction mixtures containing 50 mM Tris-acetate, pH 8.0, and 5–10 µg of microsomal protein (final reaction volume = 200 µl). After a timed incubation at 37°C, aqueous and lipid phases of the reaction mixtures were partitioned as described by Belfrage and Vaughn (14). One milliliter of the aqueous ([^{3}H]palmitic acid) phase was removed, placed in 10 ml Econo-1 scintillation cocktail and analyzed for ^{3}H using a liquid scintillation counter. Non-enzymic hydrolysis of the substrates was assessed in each analysis using heat-denatured microsomal protein. REH activity is represented as the molar amount of [^{3}H]palmitic acid liberated versus incubation time. Apparent kinetic constants (K_m^{app} and V_{max}^{app}) were determined from double-reciprocal (Lineweaver-Burk) plots of substrate saturation data. Each experiment was performed with triplicate samples and the results were confirmed in at least two separate trials. Statistical analyses were performed using the Student's *t*-test (Sigmaplot 5.0; Jandel Scientific, Corte Madera, CA).

Substrate specificity and competition

The effect of [^{14}C]triolein on REH activities was assessed using the aforementioned REH activity assay. In these studies, [^{14}C]triolein (10 µM; specific activity = 50,000 dpm/nmol) was included in reaction mixtures containing either 10 µM [^{3}H]A*t*RP or 10 µM [^{3}H]11*c*RP. The examined [^{3}H]retinyl ester substrate and [^{14}C]triolein were delivered simultaneously in a total volume of 10 µl ethanol. Time course profiles (0–7.5 min) for the REH activities in the absence and presence of [^{14}C]triolein were obtained and the REH activity (pmol [^{3}H]palmitic acid/min) in experimental and control samples was plotted. Liberation of [^{14}C]oleic acid from [^{14}C]triolein ester hydrolysis was also monitored but is not shown in order to facilitate clarity of Fig. 1. Competition studies in which the hydrolysis of [^{3}H]A*t*RP and [^{3}H]11*c*RP was challenged with unlabeled 11-*cis* and all-*trans* retinyl palmitate, respectively were performed as described above for [^{14}C]triolein assays. In preliminary studies, the REH reaction was determined to be linear over a 0–10 min time interval. Because the ob-

served rate changes are directly proportional to the initial velocity of the reaction during this period, time course profiles of the REH activities in experimental and control samples are reported.

Microsomal protein modification

Experiments were designed to assess the effects of a thiol hydrolase inhibitor, N-ethylmaleimide (NEM), on all-trans and 11-cis REH activities in the microsomal proteins. Stock solutions of NEM were prepared in DMSO at 20× the desired concentration (0.5–20 mM) immediately prior to use in REH assays. Varied concentrations of NEM were delivered to reaction mixtures containing 50 mM Tris-acetate, pH 8.0, and protein (5 μg). Control samples containing only DMSO (10 μl) were analyzed concurrently. Non-enzymic hydrolysis of the substrates was assessed in each analysis using heat-denatured microsomal protein. Samples were preincubated for 1–2 min at 37°C prior to addition of substrate (1–20 nmol of either [³H]AtRP or [³H]11cRP in 10 μl ethanol; final reaction vol = 200 μl). Incubation was resumed for 20 min at 37°C. Samples were then processed and REH activity was quantified as described above. Apparent kinetic constants (K_m^{app}, and K_i^{app}) were determined following Lineweaver-Burk transformation and Dixon plot of the substrate saturation data (15). In this study, samples were analyzed in triplicate in three separate trials.

RESULTS

Analysis of substrate concentration and hydrogen ion dependencies for all-trans REH activity in bovine liver and RPE microsomes revealed comparable V_{max}^{app} and K_m^{app} values ($\approx$ 300 pmol/min per mg and $\approx$ 30 μM, respectively), and similar hydrogen ion dependencies (maximal at pH 7–9). Although hydrolysis of [³H]11cRP by these tissues was also optimal at neutral to alkaline pH, V_{max}^{app} and K_m^{app} for 11-cis REH activity in liver (V_{max}^{app} = 526 pmol/min per mg and 45 μM, respectively) were lower than those determined for 11-cis REH in RPE (V_{max}^{app} = 1,667 pmol/min per mg and K_m^{app} = 58 μM). Because liver lacks an endogenous pool of 11-cis retinyl esters, the observed 11-cis REH activity is clearly non-physiologic and may be due to "nonspecific" mechanisms. Studies designed to address this possibility are delineated below.

Substrate specificity: effect of triolein on REH activity

Hydrolysis of [³H]AtRP and [³H]11cRP by liver and RPE microsomes was studied in the presence of [¹⁴C]triolein. Time course profiles for all-trans and 11-cis REH activities are given in Fig. 1. In the liver, inclusion of [¹⁴C]triolein resulted in significant inhibition of all-trans and 11-cis REH activities (56% inhibition, 1A; and 48% inhibition, 1B, respectively at t = 7.5 min). Although
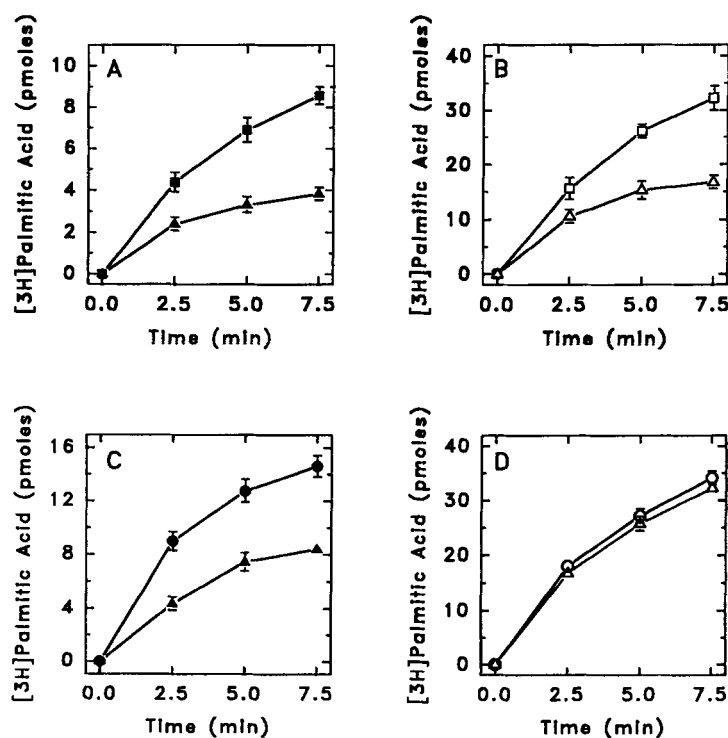

Fig. 1. Effect of triolein on REH activities in bovine liver and RPE microsomes. The hydrolysis of [³H]11-cis (open symbols) and [³H]all-trans (solid symbols) retinyl palmitate (10 μM) by bovine liver (panels A and B) and RPE (panels C and D) microsomes was monitored as a function of incubation time in the presence of 10 μM [¹⁴C]triolein (triangles). The routine REH assay system was utilized. Time course profiles (0–7.5 min) of the hydrolytic activities were obtained and REH activity (pmol [³H]palmitic acid/min) in experimental and control samples was plotted (mean ± SD, n = 6). At each time point indicated in panels A, B, and C, the group means of the experimental samples were determined to be significantly different than those of the control samples (α = 0.05).

similar inhibition was observed with all-*trans* REH activity in RPE (46% inhibition at t = 7.5 min, 1C), 11-*cis* retinyl ester hydrolysis in RPE was virtually unaffected by the addition of triolein (1D). Notably, a concomitant hydrolysis of [^{14}C]triolein was also observed but is not plotted in Fig. 1 for purposes of clarity. In fact, when triolein concentration was increased 2-fold relative to the retinyl ester substrate concentration, all-*trans* REH activities, and 11-*cis* REH activity in liver, were reduced proportionately (data not shown); this effect was not observed for 11-*cis* REH activity in the RPE.

Substrate competition: effect of *cis/trans* isomer competition on REH activity

The hydrolysis of [^{3}H]A*t*RP was also challenged with unlabeled 11-*cis* retinyl palmitate, while the hydrolysis of [^{3}H]11*c*RP was challenged with unlabeled all-*trans* retinyl palmitate. REH activities were monitored as described in Methods and are shown in **Fig. 2.** In the liver, [^{3}H]all-*trans* (2A) and [^{3}H]11-*cis* (2B) REH activities were similarly reduced in the presence of their respective, unlabeled, retinyl ester isomer ($\approx$50% inhibition). In RPE, however, only hydrolysis of [^{3}H]A*t*RP was diminished as result of similar substrate competition (45% inhibition, 2C). Hydrolysis of [^{3}H]11*c*RP by RPE microsomes was unaffected by the addition of unlabeled all-*trans* retinyl palmitate (2D).
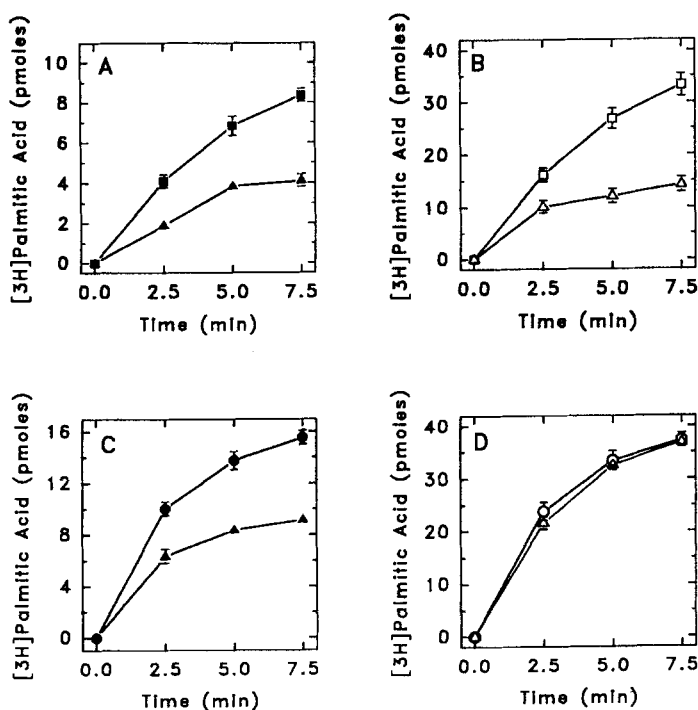
Chemical modification of REH proteins

In order to further resolve biochemical properties of 11-*cis* and all-*trans* REH activities in liver and RPE microsomes, a known inhibitor of thiol hydrolases, *N*-ethylmaleimide (NEM), was used to modify specific amino acid residues in the microsomal proteins. Specifically, NEM alkylates the -SH group of cysteine residues. Rate studies were performed with fixed protein (5 μg), increasing substrate (0–100 μM), and at several NEM concentrations (0–1 mM). Data from liver studies, shown in **Fig. 3A,** revealed a profound alteration of all-*trans* REH apparent enzyme-substrate affinity with increasing NEM concentration (K_i^{app} = 120 μM, 3A, solid symbols). Determination of the apparent inhibitor constant for hydrolysis of 11-*cis* retinyl palmitate by NEM-treated liver microsomes revealed a similar K_i^{app} of 200 μM (3A, open symbols). All-*trans* REH activity in RPE, shown in Fig. 3B, was also profoundly influenced by NEM as K_m^{app} was altered with increasing NEM concentration (K_i^{app} = 100 μM, 3B, solid symbols). In marked contrast, hydrolysis of 11-*cis* retinyl palmitate by RPE microsomes (3B, open symbols) was not significantly affected by NEM; K_m was relatively unchanged and the apparent inhibitor constant was several times higher than that observed for the other REH activities (K_i^{app} = 480 μM). It is important to note that variabilities in the data shown in Fig. 3 were too small to warrant the use of error bars.

Fig. 2. Analysis of retinyl ester substrate competition. Substrate competition studies in which the hydrolysis of [^{3}H]A*t*RP (solid symbols) and [^{3}H]11*c*RP (open symbols) by bovine liver (panels A and B) and RPE (panels C and D) microsomes was challenged with 10 μM of the respective unlabeled retinyl ester isomer (triangles) were performed as described for [^{14}C]triolein assays. A time course profile of the REH activity in experimental and control samples was plotted (mean ± SD, n = 8). At each time point indicated in panels A, B, and C, the group means of the experimental samples were determined to be significantly different than those of the control samples (α = 0.05).
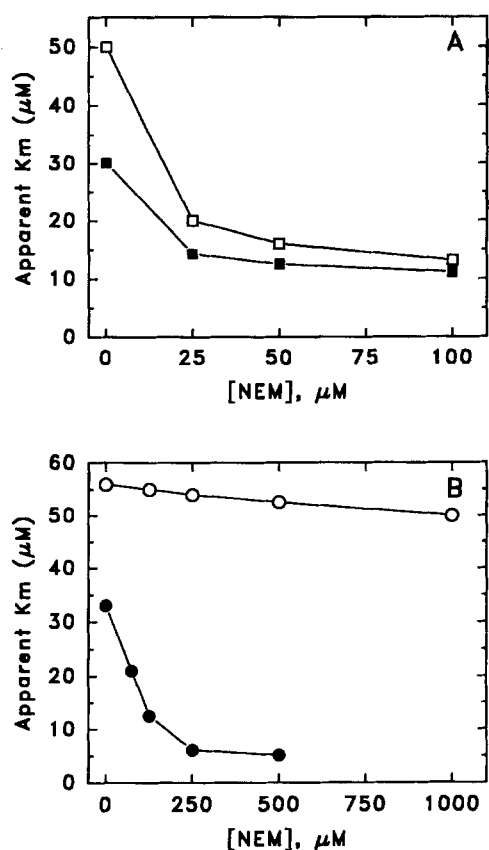
Fig. 3. Effect of *N*-ethylmaleimide on hydrolysis of 11-*cis* and all-*trans* retinyl palmitate by bovine liver and RPE microsomes. A series of substrate saturation studies were performed using microsomal protein which was preincubated (1–2 min @ 37°C) in the absence and presence of varied concentrations of NEM (0.025–1.0 mM). [³H]11-*cis* and [³H]all-*trans* retinyl palmitate concentrations ranged from 5 to 100 μM. REH activities were quantified as described in the text. The apparent rate constant for enzyme-substrate affinity (K_m^{app}) was determined from a secondary plot (Lineweaver-Burk) of substrate saturation rate data. The apparent inhibitor constants (K_i^{app}) were determined via Dixon plot in which $1/v_0$ is plotted against inhibitor concentration; the x-axis intercept of this plot gives $-K_i^{app}$ (20). Legend: solid squares, liver all-*trans* REH; open squares, liver 11-*cis* REH; solid circles, RPE all-*trans* REH; open circles, RPE 11-*cis* REH.

DISCUSSION

The focus of the present investigation was to study the relationship of retinyl ester hydrolysis in tissues that represent principal storage sites for all-*trans* and 11-*cis* retinyl esters. Specifically, we have attempted to identify similarities between all-*trans* and 11-*cis* REH activities in bovine liver and RPE microsomes. To this end, we have conducted studies that address REH substrate specificity and biochemical nature of the REH active site(s).

Prior to this report, very little information was available regarding the relationship of the physiologically relevant all-*trans* REH activity in liver to REH activities in RPE. Because RPE possesses abundant stores of

retinyl esters, the presence of similar REH activities seems likely. Moreover, biochemical characterization of membrane-associated REH activities in ocular and hepatic tissues of a single species has not been reported. Previous comparisons of this nature have typically utilized rat liver for study of hepatic REH activity while bovine RPE has been used for analysis of REH activity in the eye (16).

Results from the present study highlight many similarities between all-*trans* REH activities in bovine liver and RPE. Thus, all-*trans* REH activities associated with liver and RPE microsomal proteins demonstrate comparable V_{max}^{app} and K_m^{app} values, and are similarly reduced in the presence of physiological esters, such as triolein, or by competition with 11-*cis* retinyl palmitate. Additionally, each all-*trans* REH activity appears to rely to some extent on the presence of cysteine residues in the microsomal proteins. The fact that all-*trans* REH enzyme-substrate affinities are similarly altered as a function of increasing NEM concentration suggests that the modification did take place, or had a bearing on, similar catalytic sites within these tissues.

Although, it is difficult to justify a physiological role for all-*trans* REH activity in the visual cycle (17), mechanisms for release of retinol for metabolic functions such as retinoic acid synthesis and/or retinol/retinyl ester homeostasis must be present in all tissues. Based upon the present biochemical comparisons of all-*trans* REH in RPE and all-*trans* REH in liver, the possibility exists that these two all-*trans* REHs may be isozymes. Additional investigations will be required to consolidate this hypothesis.

We have also identified a hydrolytic activity against 11-*cis* retinyl palmitate in liver microsomes. Results from substrate competition studies indicate that a 1:1 molar competition with either triolein or all-*trans* retinyl palmitate reduces 11-*cis* REH activity in liver by ≈50%. It is noteworthy that the inhibition became more pronounced with increasing concentrations of competing substrate (data not shown). In contrast, 11-*cis* REH activity in RPE was not significantly affected by the inclusion of these competing substrates, although they were hydrolyzed to a great extent during the course of the reaction. These data suggest that the 11-*cis* ester may be "nonspecifically" hydrolyzed by broad-specificity hydrolases present in liver microsomes (e.g., triolein ester hydrolase).

Results from our studies also tend to abrogate the notion that differences in 11-*cis* and all-*trans* REH activities in RPE are due solely to 'undefined physical interactions.' The distinctive apparent inhibitor constants for NEM-induced inhibition of 11-*cis* REH activity in RPE (NEM K_i^{app} = 480 μM) versus the NEM K_i^{app} for the other REH activities (100–200 μM) are particularly interesting

as this result suggests a fundamental difference in the amino acid composition at the active site of the enzyme(s) that catalyze(s) hydrolysis of 11-*cis* and all-*trans* retinyl esters. It is conceivable that the presence of 11-*cis* retinyl esters in the RPE has necessitated the evolution of unique enzymic activities that are responsible for their metabolism.█

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