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Biosynthetic Studies of A2E, a Major Fluorophore of Retinal Pigment Epithelial Lipofuscin*

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We have examined questions related to the biosynthesis of A2E, a fluorophore that accumulates in retinal pigment epithelial cells with aging and in some retinal disorders. The use of *in vitro* preparations revealed that detectable levels of A2-PE, the A2E precursor, are formed within photoreceptor outer segments following light-induced release of endogenous all-*trans*-retinal. Moreover, experiments *in vivo* demonstrated that the formation of A2-PE in photoreceptor outer segment membrane was augmented by exposing rats to bright light. Whereas the generation of A2E from A2-PE by acid hydrolysis was found to occur very slowly, the detection in outer segments of a phosphodiesterase activity that can convert A2-PE to A2E may indicate that some portion of the A2-PE that forms in the outer segment membrane may undergo hydrolytic cleavage before internalization by the retinal pigment epithelial cell. The identities of additional minor components of retinal pigment epithelium lipofuscin, A2E isomers with *cis* olefins at positions other than the C13-C14 double bond, are also described.

The fluorophore A2E, a pyridinium bisretinoid (1, 2), accumulates in retinal pigment epithelial (RPE)[1] cells as a major component (3, 4) of the lipofuscin that is characteristic of senescence and some inherited retinal disorders. Recent evidence indicates that A2E has the potential for causing RPE cell death (5–9) and, as such, may contribute to the RPE cell atrophy that is observed in age-related macular degeneration (10) and retinal degenerative diseases such as Stargardt's disease (11, 12), Best's macular dystrophy (13), and cone-rod dystrophy (14).

The biosynthesis of A2E (4, 15) begins in the photoreceptor outer segment (ROS) membrane as a reaction between phosphatidylethanolamine and a single molecule of all-*trans*-retinal that generates a phosphatidylethanolamine-all-*trans*-retinal Schiff base conjugate (*N*-retinylidene-phosphatidylethanolamine). This adduct undergoes a [1,6]-proton tautomerization generating a phosphatidylethanolamine-retinyl enamine, which reacts with a second molecule of all-*trans*-retinal. After aza-6π-electrocyclization and auto-oxidation, the fluorescent phosphatidyl-pyridinium bisretinoid A2-PE is formed. A2-PE was established as the precursor of A2E by HPLC detection of A2E after enzyme-mediated hydrolysis of A2-PE, whereas the structure of A2-PE was confirmed by collision-induced dissociation mass spectrometric analysis (FAB collision-induced dissociation mass spectrometry/MS). The A2-PE that forms in the outer segments leads to the deposition of A2E in RPE cells because of the latter cell's role in phagocytosing the outer segment membrane that is discarded daily by the photoreceptor cell. The tendency for A2E to undergo photoisomerization was illustrated by the identification of *iso-A2E,* a Z-isomer, at the C13-C14 double bond of one of the hydrophobic retinal chains. In the HPLC profile of human RPE extracted in the dark, *iso-A2E* is a pigment that is slightly less polar than A2E. Using synthetic samples, *iso-A2E* has been shown to exist in photoequilibrium with A2E at a ratio of ~4:1, A2E:*iso-A2E*.

It was clear from the early work of Katz *et al.* (16, 17), which demonstrated that rats fed a diet lacking in retinoid precursors of 11-*cis*-retinal exhibited significantly reduced levels of RPE lipofuscin despite normal numbers of photoreceptor cells, that the constituents of RPE lipofuscin are predominantly vitamin A aldehyde-conjugate. Because the all-*trans*-retinal that is released from photoactivated rhodopsin and cone pigment is reduced to all-*trans*-retinol by an NADPH-dependent retinol dehydrogenase located in the photoreceptor outer segment, it is generally assumed that only all-*trans*-retinal that has avoided reduction is available to react with phosphatidylethanolamine. Correspondingly, conditions that allow the generation of all-*trans*-retinal to surpass its reduction by retinol dehydrogenase can lead to accelerated formation of A2-PE and an enhanced accumulation of A2E.

In vivo, A2-PE has been identified as the orange-colored fluorophore that accumulates in the photoreceptor outer segment debris of Royal College of Surgeon rats (15). Significant amounts of A2-PE were also detected in outer segments isolated from mice with a knockout mutation in the gene encoding for the photoreceptor-specific ATP-binding cassette RIM transporter (18). The latter protein, a member of the superfamily of ATP-binding cassette (ABC) proteins, is causative for Stargardt's disease, recessive cone-rod dystrophy, recessive retinitis pigmentosa, and some cases of atrophic age-related macular degeneration (19–24). It is postulated that the photoreceptor-specific ATP-binding cassette transporter makes all-*trans*-retinal available to all-*trans*-retinol dehydrogenase on the cytoplasmic side of the disc membrane (25–27). As a consequence of the loss of photoreceptor-specific ATP-binding cassette trans-

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[1] The abbreviations used are: RPE, retinal pigment epithelium; FAB, fast atom bombardment ionization; HPLC, high performance liquid chromatography; PLD, phospholipase D; ROS, photoreceptor outer segment(s); MS, mass spectroscopy.

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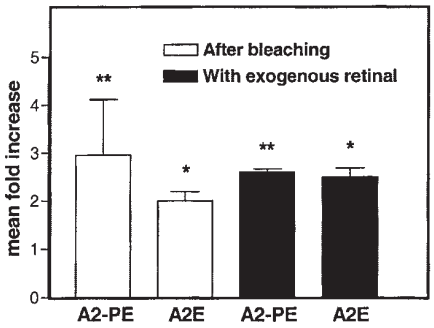

FIG. 1. **Quantitation of [$^{14}C_2$]A2PE and [$^{14}C_2$]A2E/*iso-A2E* formed in [$^{14}C_2$]ethanolamine-labeled excised whole retina in the presence of all-*trans*-retinal.** Values obtained after bleaching to release endogenous retinal (□) or after the addition of 50 μM exogenous retina (■) are presented as the fold increase above background radioactivity measured in the absence of bleaching and retinal. Each value represents the mean ± S.E. of two (exogenous retinal) or three (bleaching) independent experiments performed in duplicate. *, $p < 0.05$; **, $p < 0.01$, as determined by two-tailed t test when compared with background levels of radioactivity.

porter activity and the accumulation of A2-PE in outer segments, the levels of A2E in RPE cells of *abcr*−/− mice are reported to be 20 times greater than those in normal mice (25).

Determining the steps involved in the biosynthesis of A2E, together with the factors that influence its formation, is fundamental to our understanding of blinding retinal disorders associated with abnormal accumulations of A2E in RPE cells. In the present studies, we have examined several issues related to the biosynthesis of A2E including the mechanisms involved in the hydrolysis of A2-PE and the modulation of A2-PE accumulation by bright light exposure. We also report that previously unidentified components of hydrophobic extracts of human RPE are structurally related to A2E and *iso-A2E*.

EXPERIMENTAL PROCEDURES

Materials—[$^{14}C_2$]Ethanolamine (5 mCi/mmol) was purchased from PerkinElmer Life Sciences. Phospholipase D (type IV, *Streptomyces chromofofuscus*) and dipalmitoyl-L-α-phosphatidylethanolamine, α-tocopherol (vitamin E), calphostin C, and a protease inhibitor mixture (P-2714) were obtained from Sigma; Dulbecco's modified Eagle's medium, nonessential amino acids, and gentamycin sulfate were obtained from Invitrogen, and fetal bovine serum was acquired from Atlanta Biologicals (Norcross, GA); butylated hydroxytoluene was from Aldrich. All other chemicals were from Sigma. Glass-backed TLC plates (RP-C18) were purchased from Merck.

Synthesis and Purification of A2E and A2-PE—A2E was synthesized by incubating ethanolamine (4.75 mg, 77.5 μmol) and all-*trans*-retinal (50 mg, 176 μmol) in ethanol (3 ml) at room temperature for 2 days in the dark (4). A2-PE was prepared by reacting dipalmitoyl L-α-phosphatidylethanolamine (50 mg, 72 μmol) and all-*trans*-retinal (46 mg, 164 μmol) in chloroform (4 ml) at 37 °C for 3 days in the dark (15). A2E and A2-PE were purified by silica gel chromatography, and purity was confirmed by TLC and HPLC. HPLC-purified A2E was exposed to room light for varying time periods to provide mixtures of A2E-related photoisomers.

Excised Bovine Retina and Isolated ROS—Whole retinas were excised from bovine eyes under dim red light and incubated for 14 h in media consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 0.1 mM minimum Eagle's medium nonessential amino acids solution, and gentamycin sulfate (10 μg/ml) and containing [$^{14}C_2$]ethanolamine (1 μCi/ml, 5 mCi/mmol). After rinsing in Dulbecco's modified Eagle's medium, the retinas were either incubated in media containing exogenous all-*trans*-retinal (50 μM; 4 h) or exposed to white light delivered from a 60-W lamp at a distance of 20 cm for 30 min to release endogenous retinal from bleached rhodopsin, followed by additional incubation in the dark for 3.5 h. Subsequently, ROS were isolated by shaking bovine retinas in 35% (w/v) sucrose in 10 mM Tris buffer, pH 7.0 (2 retinas/30 ml), for ~1 min to detach the ROS (28). After centrifugation at 3500 × *g* for 15 min, the supernatant containing the isolated ROS was collected, and the lipids were extracted using chloroform: methanol (2:1, v/v) containing 0.1% trifluoroacetic acid. The organic phases were dried under argon, reconstituted in chloroform, and loaded onto glass-backed TLC plates (RP-C18) using the solvent system of methanol:chloroform (75:25) containing 2% trifluoroacetic acid. The samples were developed together with the synthetic compounds as a reference. The bands were scraped from the plate, and the radioactivity was measured by liquid scintillation counting and expressed relative to the quantity of protein measured before extraction. Alternatively, in some experiments, excised whole retinas were incubated in 50 μM all-*trans*-retinal without prior incorporation in [$^{14}C_2$]ethanolamine; ROS were isolated before bleaching under white light (60-W bulb, 30 min, 20-cm distance), and isolated ROS were exposed to light (300-W lamp, 2 min, 50-cm distance) delivered through a 500 nm band pass filter (29). In addition, synthetic A2-PE was incubated with isolated ROS in buffer containing 10 mM Hepes, 50 μmol of diethylenetriaminepentaacetic acid, 0.1 mmol of toluenensulfonyl fluoride, and 1 mmol of dithiothreitol (pH 6.6) for 3 h at 37 °C in the absence and presence of the putative PLD inhibitor calphostin C (5 μM) (30) or a protease inhibitor mixture containing 6.7 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 3.3 mM EDTA, 433 μM bestatin, 47 μM E-64, 3.3 μM leupeptin, and 1 μM aprotinin. To determine the amount of A2E formed, the latter was quantified by reverse phase HPLC, as described below, and for each experiment, the amount of A2E in the starting sample of A2-PE (0.3–1.3%) was subtracted from the amount present after incubation with ROS.

Extraction of Human RPE-choroid—RPE-choroid were isolated from human donor eyes (National Disease Research Exchange, Philadelphia, PA; age, 60–70 years), extracted in chloroform:methanol (2:1, v/v) containing 0.1% trifluoroacetic acid (4), and analyzed by normal and reverse phase HPLC, as described below.

Light Exposure in Rats—Sprague-Dawley rats (6–7 weeks of age) that had been maintained on a 12-h light/dark cycle were dark-adapted overnight. After pupillary dilation with cyclopentolate hydrochloride (0.5%; Alcon Laboratories, Fort Worth, TX) and tropicamide (1%; Alcon), 48 rats were exposed for 6 h to an illuminance of 350 foot-candles delivered from cool white fluorescent bulbs. A museum-grade plexiglass plate positioned between the light source and the animal cage eliminated UV light. The temperature in the transparent cages was 24 °C. After enucleation, the retinas were dissected into cold 20 mM Tris-HCl (pH 7.4) containing 20% sucrose, and ROS were isolated, extracted in chloroform:methanol (2:1, v/v) containing 0.1% trifluoroacetic acid, and analyzed by normal phase HPLC, as described below. ROS isolated from 48 age-matched Sprague-Dawley rats maintained under room lighting (<20 foot-candles) were also analyzed.

Enzymatic Digestion with Exogenous Phospholipase D—The lipid fraction containing A2-PE was reconstituted in Me_2SO and incubated in 40 mM 4-morpholinepropanesulfonic acid buffer, pH 6.5, containing 250 units/ml *S. chromofuscus* PLD and 15 mM $CaCl_2$. Incubation was carried out for 2 h at 37 °C in a shaking water bath. Chloroform:methanol (2:1, v/v) terminated the reaction and extracted the products.

HPLC—A Waters 600E HPLC equipped with Waters 996 photodiode array detector was used with a reverse phase C18 column (250 × 4.6 mm; Cosmosil 5C18; Nacalai Tesque, Kyoto, Japan). A2E and the photoisomer *iso-A2E* were eluted with the following gradients of acetonitrile in water (containing 0.1% trifluoroacetic acid): 85–96% (10 min), 96% (5 min), 96–100% (2 min), and 100% (13 min) (flow rate, 1 ml/min), and they were monitored at 430 nm. Analysis of pigment mixtures containing minor A2E-related components utilized a gradient of methanol in water containing 0.1% trifluoroacetic acid (linear gradient of 84–96% methanol over 10 min, followed by isocratic elution at 96% methanol for 5 min). Absorbance spectra of minor peaks with retention times similar to A2E in the HPLC profile were obtained by photodiode array detection at the appropriate retention times. Detection of A2-PE was carried out by normal phase HPLC (150 × 4.6 mm; YMC-Pack silica S-5 μm) with elution in a solvent system of hexane:isopropanol:ethanol:25 mM K_2PO_4 buffer:trifluoroacetic acid (123:95:25:6:0.07, v/v; pH 7.0). For A2E quantitation by reverse phase HPLC, the area of the A2E peak was normalized to the internal standard, A2-propylamine, which was synthesized from all-*trans*-retinal and propylamine.

Mass Spectrometry—FAB-MS was performed on a JEOL JMS-HX110A/110A tandem MS (Akishima, Tokyo, Japan) Xe beam FAB gun (6 kV) fitted with an MS-1 ion source. FAB collision-induced dissociation spectra were measured with 10 kV acceleration voltage (MS-1 and MS-2) on an electrically floated collision cell (8 kV). Helium was used as the collision gas with an attenuation of precursor ion intensity to ~30%. 3-Nitrobenzyl alcohol was used as matrix.

HPLC-electrospray ionization-MS was performed on a mixture of A2E photoisomers and on human eye extracts. These samples were

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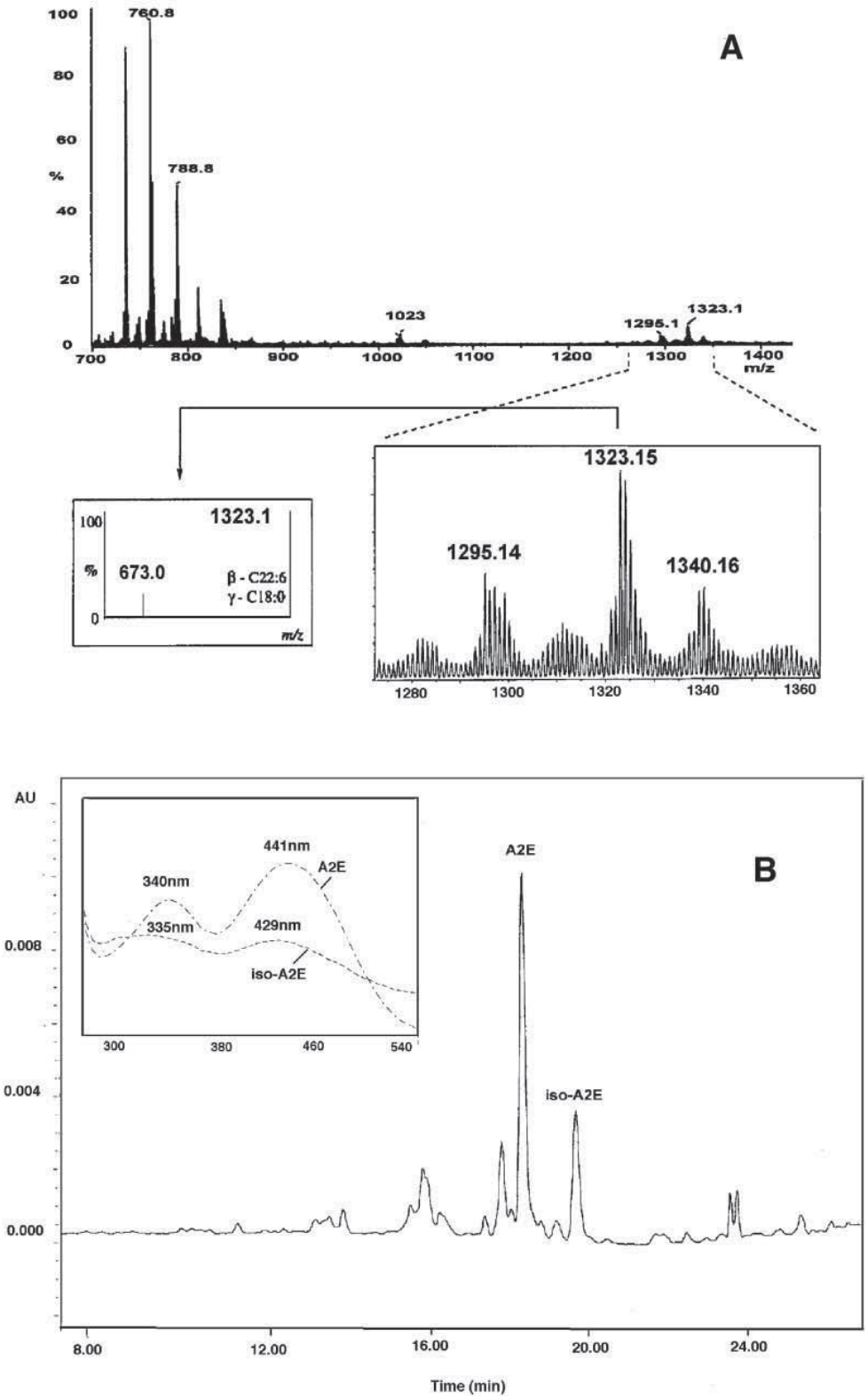

FIG. 2. **FAB-MS identification of A2-PE in ROS isolated from excised bovine retina after incubation with exogenous all-*trans*-retinal.** *A*, FAB-MS identification of A2-PE in the TLC fraction that co-migrated with authentic A2-PE. *Inset*, FAB collision-induced dissociation mass spectrometry of A2-PE representing m/z 673 product ion. *B*, the identification of A2-PE as the A2E precursor was confirmed by HPLC detection of A2E/*iso-A2E* after the TLC fraction in *A* was incubated with phospholipase D to hydrolyze A2-PE. Absorbance (*AU*) was monitored at 430 nm. *Inset*, UV spectra for A2E (−●−●−●−) and *iso-A2E* (—·).

separated using a Waters Alliance HPLC Model 2690 with a Cosmosil C18 (250 × 4.6 mm) column (Nacalai Tesque) utilizing a linear gradient from 84–96% methanol in water with 0.1% trifluoroacetic acid (10 min), followed by isocratic elution at 96% methanol at a flow rate of 0.3 ml/min. The presence of A2E and its isomers was observed using a Q-TOF (Micromass, Manchester, United Kingdom) equipped with a

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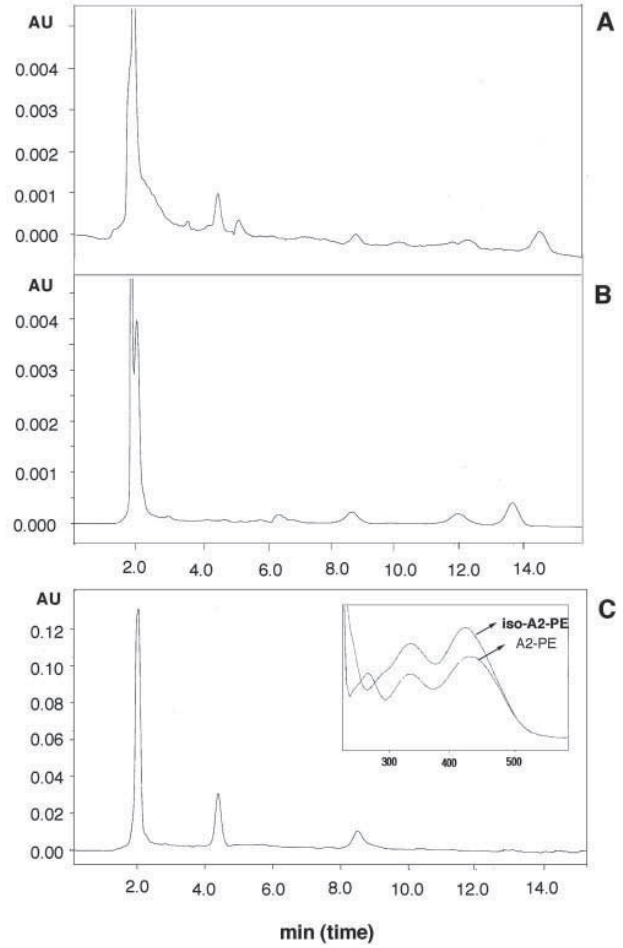

FIG. 3. **A2-PE is detected in ROS isolated from rat retinas after exposure *in vivo* to high intensity light.** Under conditions of bright light exposure (*A*), a peak at ~4 min, which is not present when rats are maintained under standard room light (*B*), was identified as A2-PE by co-injection of authentic A2-PE (*C*). The peak at ~8 min in *C* is probably iso-A2-PE. Normal phase HPLC profiles. Absorbance was monitored at 430 nm. *Inset*, UV spectra.

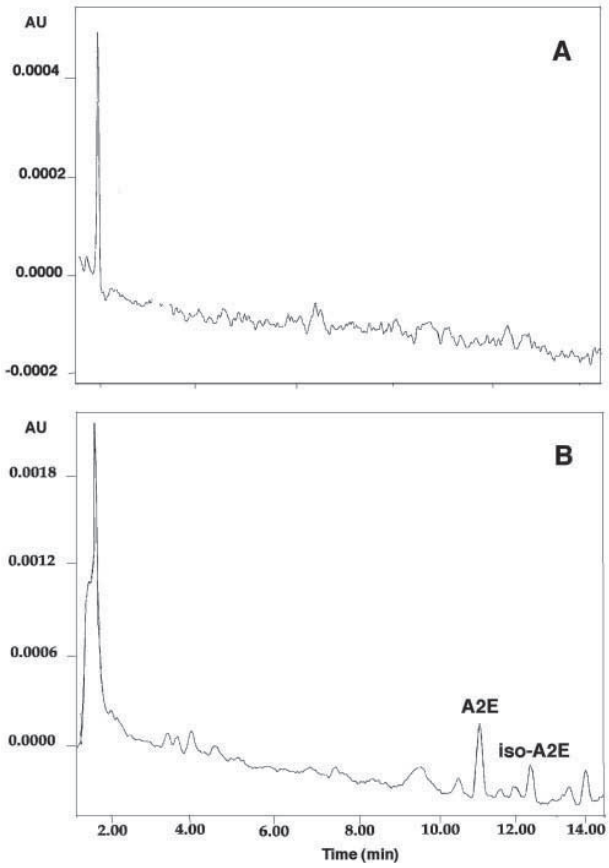

FIG. 4. **Reverse phase HPLC analysis after incubation of synthetic A2-PE at pH 5.5–6.0 for 24 h (*A*) and 7 days (*B*).** A2E/*iso-A2E* peaks appear in the HPLC profile, due to acid hydrolysis of A2-PE, only after a 7-day incubation in 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 5.5–6.0). These peaks are assumed to correspond to A2E and *iso-A2E* because of their retentions times; however, the inability to confirm their identification by UV indicates that they are present at very low abundance. Absorbance (*AU*) was monitored at 430 nm.

Z-spray electrospray ionization source. Electrospray ionization capillary voltage was set at 3.0 kV, and the source block temperature and desolvation temperature were set at 120 °C and 350 °C, respectively.

RESULTS

Biosynthesis of the Precursor A2-PE Is Dependent on Light-induced Release of all-trans-Retinal—To determine whether measurable quantities of A2-PE form in photoreceptor outer segments upon light exposure, excised bovine retinas that had incorporated [$^{14}C_2$]ethanolamine were illuminated to induce photoisomerization and release of all-*trans*-retinal. Accordingly, the formation of [$^{14}C_2$]A2-PE was observed at levels that were significantly elevated as compared with background radioactivity measured from retinas maintained in the dark (Fig. 1). Indeed the levels of [$^{14}C_2$]A2-PE generated by release of endogenous retinal were similar to that measured after incubating [$^{14}C_2$]ethanolamine-labeled retinas with 50 μM exogenous all-*trans*-retinal. To confirm the identification of A2-PE in these samples, ROS were isolated from excised retina that had been incubated with exogenous all-*trans*-retinal, and after extraction, the crude TLC fraction that co-migrated with authentic A2-PE (R_f 0.24; detection at 365 nm) was analyzed by FAB-MS. The relatively intense signals at m/z 1295 and 1323 corresponded to the molecular ions of $C_{83}H_{124}NO_8P$ and $C_{85}H_{128}NO_8P$ and thus were assigned to A2-PE (Fig. 2*A*). The FAB collision-induced dissociation spectra of the m/z 1323 peak revealed an intense product ion at m/z 673 that corroborated the presence of the phosphoryl-A2E group in A2-PE (Fig. 2*A*). The fluorescent band at R_F 0.24 also proved to be the precursor of A2E because incubation of this fraction with bacterial PLD resulted in peaks in the HPLC profile that were identified as A2E and *iso-A2E* on the basis of UV absorbance (λ_{max} 335 and 435) (Fig. 2*B*).

A2-PE Formation in Photoreceptor Outer Segments Is Augmented in Bright Light—The failure to detect A2-PE in control ROS maintained in the dark (Fig. 3; also see Liu *et al.,* Ref. 15), suggests that the level of detection afforded by HPLC (UV) is insufficient to monitor what must be a slow accretion of A2-PE in photoreceptor outer segments *in vivo*. Thus, to determine whether high levels of illumination can lead to the release of all-*trans*-retinal at levels sufficient to detect A2-PE in outer segment membrane, rats were dark-adapted overnight and then exposed to bright white light for 6 h. Subsequent HPLC analysis of extracts of ROS isolated from the illuminated rats revealed a peak that co-migrated with authentic A2-PE (Fig. 3). Conversely, this peak was not present in ROS isolated from control rats maintained under cyclic lighting conditions.

A2E Is Generated by Acid Hydrolysis of A2-PE at a Slow Rate—We have demonstrated repeatedly that in the presence of phospholipase D, A2-PE undergoes hydrolytic cleavage to generate A2E (Fig. 2; also see Liu *et al.,* Ref. 15). Nonetheless,

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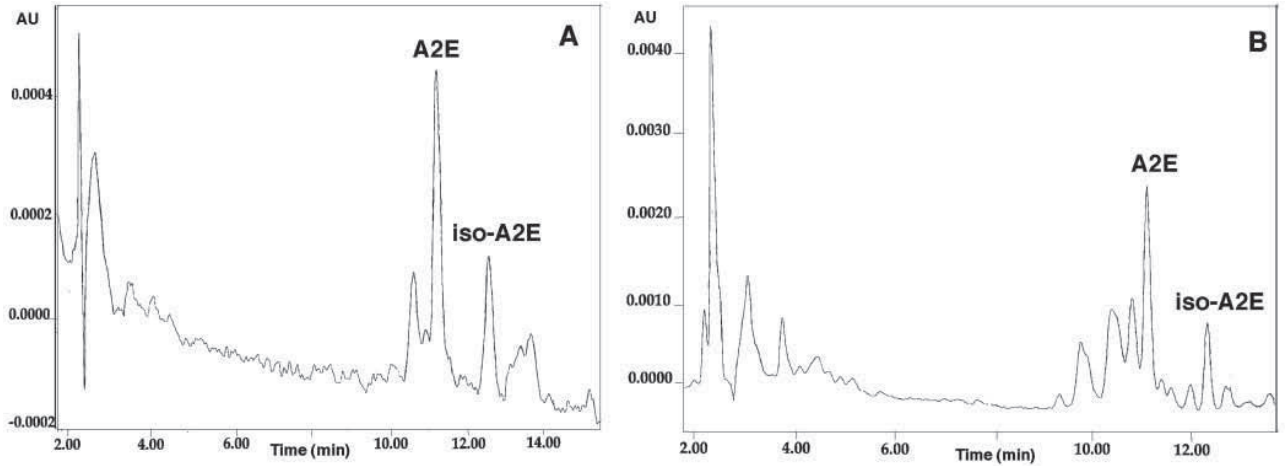

FIG. 5. **HPLC analysis of extracts of whole retina and isolated ROS after illumination to release endogenous all-*trans*-retinal.** After bleaching whole retina with white light (*A*) and isolated ROS with >500 nm light (*B*) followed by further incubation in the dark for 3.5 h, A2E/*iso-A2E* was detected by reverse phase HPLC. The chromatograms correspond to absorbances (*AU*) at 430 nm. The UV profiles of the HPLC peaks were similar to that in Fig. 2.

in addition to enzyme-mediated phosphate hydrolysis, it is conceivable that acid hydrolysis of A2-PE could occur. This prospect is especially significant because within RPE cells, A2-PE-containing outer segments could be expected to reside within the acidic environment of phagolysosomes. Thus, to investigate the possibility of acid-mediated hydrolysis of A2-PE, we incubated HPLC-purified A2-PE (0.1 mg) in 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 5.5–6.0 (37 °C), conditions that mimic the lysosomal compartment. No A2E was detectable after a 24-h period of incubation (Fig. 4*A*). After 1 week, the amount of A2E generated (Fig. 4*B*) was 7–8 ng, as measured by internal standard. This quantity of A2E, generated over the period of a week under acid conditions, represented ~0.01% of the original sample of A2-PE.

A Phosphodiesterase Activity That Can Generate A2E from A2-PE May Be Present in Outer Segments—In [$^{14}C_2$]ethanolamine-labeled excised retinas that were either bleached or incubated with exogenous retinal, the formation of [$^{14}C_2$]A2E was also observed (Fig. 1). We considered two explanations for this unexpected finding. One possibility was that despite careful washing after [$^{14}C_2$]ethanolamine incubation, much of the observed A2E had formed from reactions between residual free [$^{14}C_2$]ethanolamine and retinal. The alternative prospect was that A2E was generated by A2-PE hydrolysis within the outer segment membrane. Thus, to investigate these scenarios, several approaches were taken. First, by bleaching excised whole retina without prior incubation with [$^{14}C_2$]ethanolamine, we eliminated the possibility of A2E forming through direct reactions between retinal and exogenous free ethanolamine. Even in the absence of [$^{14}C_2$]ethanolamine, however, A2E/*iso-A2E* peaks appeared in the reverse phase HPLC profile when the retinas were illuminated and then incubated in the dark for an additional 4 h (Fig. 5*A*). A2E was similarly detected in extracts of ROS that were isolated and exposed to white light (data not shown) or irradiated through a 500 nm band pass filter, without prior incubation with [$^{14}C_2$]ethanolamine (Fig. 5*B*). To further investigate whether a phosphodiesterase activity in photoreceptor outer segments can release A2E from A2-PE, we also incubated synthetic A2-PE with isolated ROS, and by quantitative HPLC analysis (Fig. 6), we observed that A2E was generated from A2-PE. The production of A2E under these conditions was reduced by ~80% in the presence of a protease inhibitor mixture with PLD inhibitory activity (31) and when calphostin C, an inhibitor of PLD1 and PLD2 (30), was added.

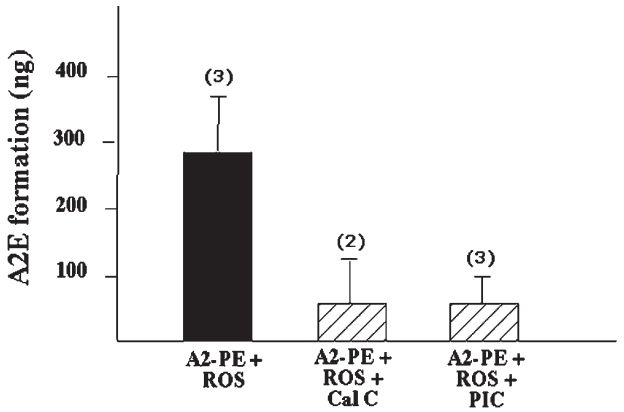

FIG. 6. **A2E is generated when the precursor A2-PE is incubated with bovine outer segments.** A2E formation from the incubation of A2-PE with bovine outer segments (*ROS*) with and without calphostin C (*Cal C*) and a protease inhibitor mixture (*PIC*) with PLD inhibitory activity. A2E was quantitated by reverse phase HPLC using internal standard. Values are the mean ± S.E. of the number of experiments presented in *parentheses*.

As part of our effort to track the formation and hydrolysis of A2-PE, we also attempted to identify A2-PE in human RPE. Although we are able to detect A2E in as little as 5% of the extract obtained from a single human eye (4), in RPE-choroid pooled from four human eyes (ranging in age from 60–70 years), A2-PE was undetectable by normal phase HPLC and FAB-MS (data not shown).

Additional Minor Components of Lipofuscin Extracts—In all experiments in which we have detected A2E and *iso-A2E* by reverse phase HPLC, including those samples obtained by A2-PE hydrolysis and ROS-retinal reaction (Figs. 2, 4, and 5), additional smaller peaks were present in the chromatograms. To begin to identify these compounds and their relationship to A2E, a sample of HPLC-purified A2E (Fig. 7*A*) was exposed to room light for 35 min. Subsequent HPLC analysis disclosed the presence of at least seven products, including A2E and *iso-A2E* (Fig. 7*B*), with the profile of peaks in the A2E region of the chromatogram matching the distribution observed in other experiments (*cf.* Figs. 2, 4, and 5). Whereas A2E (area = 71% at 430 nm) and *iso-A2E* (14%) were the most prominent pigments identified, four additional peaks (I, 9.5%; II, 3%; IV, 1%; V, 1%)

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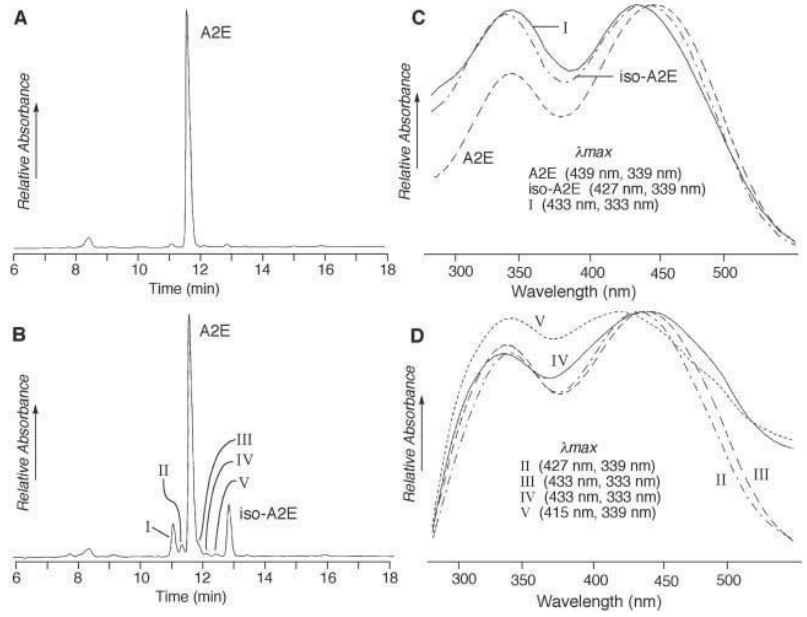

FIG. 7. **Photoisomerization of A2E.** *A*, prior to light exposure, HPLC-purified A2E was observed as a single peak in the chromatogram (430 nm detection, methanol:water gradient as described under "Experimental Procedures"). *B*, after exposure to room light for 35 min, a mixture of products was formed. *C* and *D*, analysis of each peak in *B* provided absorbance spectra that are analogous to that observed for A2E. The λ_{max} for each curve is indicated.

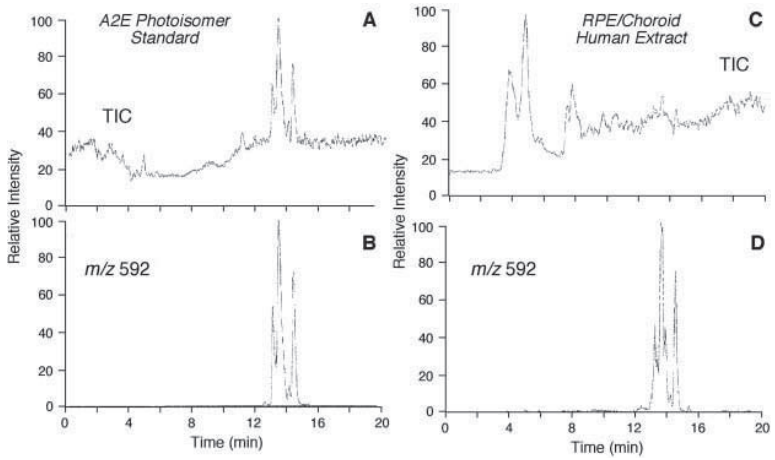

FIG. 8. **LC-MS analysis of photoequilibrated A2E pigment mixtures.** *A* and *B*, mass spectrometric analysis of a photoequilibrated A2E mixture generated as described in Fig. 7*B* (*A* and *B*) and a hydrophobic extract isolated from human eye (*C* and *D*) demonstrating total ion count (*A* and *C*) and chromatogram with detection at molecular weight 592 (*B* and *D*). The compounds present in both samples correspond to A2E, *iso-A2E*, and other A2E isomers.

and a shoulder on the A2E peak (III) were also generated during photoisomerization. Absorbance spectra obtained for each of the observed peaks (Fig. 7, *C* and *D*) revealed maxima that were generally blue-shifted in comparison with A2E. Although the relative intensities of the absorbances at approximately 330 and 440 nm varied among these fractions, in all cases, the general shape of the spectra was similar to that of A2E and *iso-A2E*. To determine whether these compounds were structurally related to A2E, mass spectrometry analysis was performed. Accordingly, LC-MS analysis indicated that all of the peaks in question had molecular weights of 592, corresponding to that of A2E (Fig. 8, *A* and *B*). Besides being present in light-exposed samples of synthetic A2E, these A2E isomers were also present in extracts of RPE-choroid harvested from human donor eyes (Fig. 8, *C* and *D*).

DISCUSSION

Whereas we have previously demonstrated the formation of A2-PE in isolated outer segments incubated in the presence of exogenous all-*trans*-retinal (15), in the present work we confirm that illumination of whole isolated retina to release endogenous retinal is sufficient to generate detectable levels of A2-PE. In addition, we have shown that the rate of formation of A2-PE in the outer segment membrane can be modulated by light intensity. The most parsimonious explanation for the production of detectable levels of A2-PE after bright light exposure *in vivo* was an increased availability of all-*trans*-retinal, with the rate of release of all-*trans*-retinal from photoactivated rhodopsin presumably exceeding the rate of reduction of all-*trans*-retinal by retinol dehydrogenase. On the other hand, we cannot rule out the possibility that a light-induced reduction in photoreceptor-specific ATP-binding cassette transporter activity may also have contributed to the accumulation of A2-PE under these conditions (32). The levels of [$^{14}C_2$]A2-PE formed in the excised whole retinas after illumination may have been accentuated in this preparation by an excess of all-*trans*-retinal stemming from a depletion of NADPH and consequent failure of all-*trans*-retinal reduction (33). Whether an insufficient supply of NADPH, such as that occurring with oxidative stress, contributes to A2-PE deposition *in vivo* is not known.

On the basis of their identical molecular weights, similar absorbance spectra, and HPLC retention times, we have also identified additional double bond isomers of A2E as components of RPE lipofuscin. Although Eldred and Katz (3) detected a number of fluorescent components in their original separation of RPE lipofuscin, A2E and *iso-A2E* are the only lipofuscin fluorophores to be structurally characterized (1, 4, 34).

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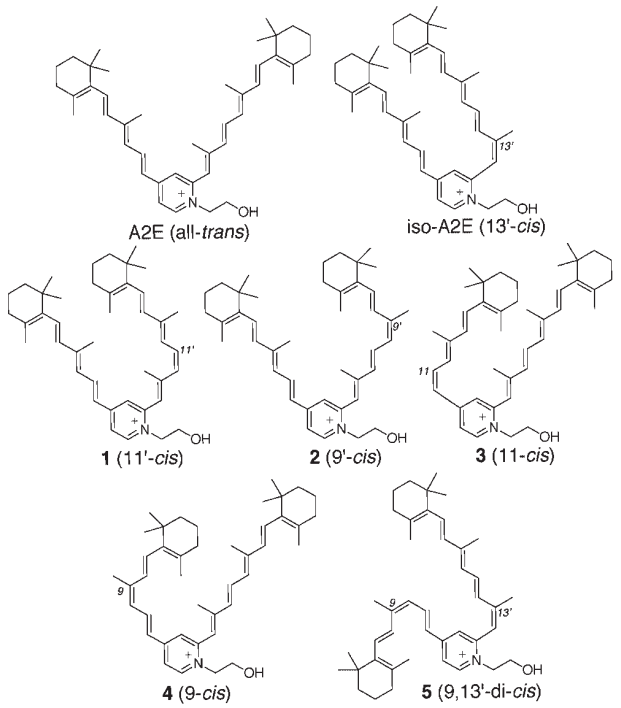

FIG. 9. **Structures of A2E, *iso-A2E,* and additional A2E-derived photoisomers are shown.**

Whereas all the double bonds of A2E assume the *trans* configuration, *iso-A2E* contains one *cis* double bond at position C13-C14 of one of the retinal chains. It is well known that all-*trans*-retinal also undergoes light-induced isomerization to form various double bond isomers, including 13-*cis,* 11-*cis,* 9-*cis,* and 9,–13-di-*cis*; generally, it is the C13-C14 double bond that is most prone to isomerization (35, 36). Despite the fact that A2E is formed from two molecules of retinal, only one of the retinal-derived chains retains a C13-C14 double bond that can isomerize. The carbon atoms derived from the C13-C14 positions of the second retinal are incorporated into the pyridinium ring system. Thus, a single C13-C14 double bond isomer, *iso-A2E,* is the most prominent isomer generated during light exposure. Clearly, however, the C11-C12 and C9-C10 double bonds of A2E are available for isomerization and could lead to four additional isomers, each containing a single *cis* double bond (Fig. 9, *1–4*). It is also likely that photoisomerization would produce A2E isomers containing two *cis* double bonds (Fig. 9, *5*). It would be expected that the additional *cis* double bonds would lead to absorbance spectra that are blue-shifted in relation to A2E; indeed, such a shift has been observed here. The range of A2E isomers we have detected could originate in photoreceptor outer segments upon illumination of all-*trans*-A2-PE. Alternatively, photoisomerization of A2E after phosphate ester hydrolysis of A2-PE would produce the same products. These minor A2E isomers are clearly generated *in vivo* as evidenced by the isolation of these materials from human RPE harvested under reduced lighting.

It has generally been assumed that the generation of A2E by phosphate hydrolysis of A2-PE occurs within the acidic conditions of RPE lysosomes (15, 18, 37). Contrary to expectations, we observed that at pH 5.5, an acidic environment similar to that in lysosomes (pH 5.5), A2E is generated from A2-PE at a slow rate. Only with an overnight incubation in 100 mM HCl is an entire sample of A2-PE converted to A2E (18). Although these results do not preclude the possibility that acid hydrolysis contributes to the generation of A2E from A2-PE, the finding that A2E is generated when A2-PE is incubated with phospholipase D (Fig. 3; see also Liu *et al.,* Ref. 15) indicates that enzyme-mediated mechanisms may play an important role. Moreover, the notion that at least some of the A2-PE that forms in the photoreceptor outer segment can undergo enzyme-mediated cleavage within the photoreceptor outer segment before internalization by RPE cells is supported by our observation of a phosphodiesterase activity in photoreceptor outer segments that is sensitive to inhibitors that act on PLD. The latter finding is consistent with previous reports of a PLD activity in outer segments isolated from bovine eyes (38). Also remarkable in this regard were the observations of Eldred and co-workers (39, 40) when they analyzed the orange-colored fluorophores present in the outer segment debris that accumulates in Royal College of Surgeons rat eyes. Thus by thin layer chromatography they detected, in addition to the fluorescent fraction later shown to be A2-PE (15, 41), a fluorophore with the same chromatographic mobility as the fluorophore they had observed in RPE lipofuscin (fraction VIII) (3) and that was later shown to be A2E (1, 34). Indeed, they speculated that the fluorophore with lower chromatographic mobility (A2E) was generated, within the outer segment debris, from a fluorophore demonstrating greater mobility (perhaps A2-PE) (37, 39, 40). Although our observations indicate that a phosphodiesterase activity in the outer segment may contribute to the hydrolysis of A2-PE, we do not doubt that hydrolytic cleavage of A2E can also occur within the RPE cell. The presence of A2-PE in RPE cells of *abcr*-null mutant mice supports to this assertion (18).

We and others (15, 18) have consistently observed that when normal ROS or whole retina is isolated in the dark, as opposed to bleaching conditions (41), A2-PE is not detectable. The complete replacement of the photoreceptor outer segment by the processes of disc assembly and shedding approximately every 10 days (42) is clearly a factor preventing the accretion of A2-PE in the membrane. Indeed, prevention of the latter accumulation may be one reason for the constant turnover of outer segment membrane. The consequences inherent in the failure to remove effete outer segment membrane at the RPE-photoreceptor interface are evident from the Royal College of Surgeons rat, in which the orange fluorophore A2-PE is amassed in abundance (15, 40). The accumulation of A2-PE within photoreceptor outer segment membrane is not just a generalized feature of degenerating retina, however, because in *rd*/*rd* mice, a blind strain that presents with a retinal degeneration caused by homozygous mutations in the gene encoding for the β subunit of rod cGMP-phosphodiesterase (43), A2-PE was not detected in retinal extracts by HPLC and FAB-MS.[2] Rather, these observations, together with the report that dark rearing of *abcr*−/− mice intervenes in the deposition of A2E (18), corroborate the dependence of A2-PE formation on light-induced release of all-*trans*-retinal and underscore the association between light exposure and the accumulation of lipofuscin by RPE cells.

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[2] Unpublished observation.

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