Preparative and Biosynthetic Insights Into pdA2E and isopdA2E, Retinal-Derived Fluorophores of Retinal Pigment Epithelial Lipofuscin

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PURPOSE. Retinal-derived fluorophores that accumulate as RPE lipofuscin are implicated in pathological mechanisms of AMD. One component of RPE lipofuscin has been characterized as pdA2E, a pyridinium adduct derived from all-trans-retinal and excess ethanolamine. One-step preparation and biosynthetic studies of pdA2E and its novel isomer called isoPDa2E are reported.

METHODS. Biosynthetic reaction mixtures, RPE/choroids and neural retinas dissected from bovines, eyes harvested from Abca4+/Rdh8+/+ mice, irradiated samples, and enzyme-treated solutions were analyzed by HPLC, mass spectrometry, nuclear magnetic resonance spectroscopy, fluorescence spectrophotometry, and density functional theory (DFT).

RESULTS. Optimization of the in vitro synthesis of pdA2E resulted in a biomimetic preparation of this pigment in a yield of 15%; this protocol also allowed the identification of isoPDa2E, a double-bond isomer of pdA2E at the C13/C14 position in bovine RPE lipofuscin. Interconversion between these two molecules occurs when either pdA2E or isoPDa2E is exposed to light. A phospholipase D-based assay demonstrated the possibility of pdA2E-PE in the biosynthetic pathway. DFT calculations revealed that the 492-nm absorbance was assigned to the long arm of pdA2E and the 340/342-nm absorbance to the short arm. Fluorescence efficiency of pdA2E and isoPDa2E is very similar, but is much weaker in comparison with A2E, isoA2E, and isoA2E.

CONCLUSIONS. Our results facilitate the understanding of compositions and biosynthetic pathways of adverse RPE lipofuscin.

Keywords: retinal pigment epithelium, biomimetic synthesis, lipofuscin, biosynthetic pathway, biomarker, electronic transition
Because most of the biological properties of pdA2E and its relationship to AMD remain unclarified, the development of an efficient approach to prepare large quantities of pdA2E becomes extremely crucial for further biochemical studies of this adduct. Although pdA2E has been obtained through biomimetic synthesis,\textsuperscript{20} the yield was only approximately 7% based on the former route (run 4, Table). In the following, we report an optimized biomimetic protocol for the pdA2E preparation that significantly facilitated the production of this pigment. More importantly, biosynthetic and HPLC experiments have allowed for isolation and identification of a new fluorescent pigment from bovine RPE lipofuscin, a double-bond isomer of pdA2E (Fig. 1D). In addition, we provide evidence that pdA2E biosynthesis may involve the formation of the precursor, pdA2-PE, in neural retina. 

**MATERIALS AND METHODS**

**Eye Tissues**

A local slaughterhouse commercially provided fresh bovine eyes, from which RPE/choroids and neural retinas were dissected separately.

**Abca4/Rdh8 double knockout (Abca4\textsuperscript{-/-} Rdh8\textsuperscript{-/-}) mice\textsuperscript{22} were obtained from the Krzysztof Palczewski Laboratory (Case Western Reserve University, Cleveland, OH, USA) as a generous gift, and raised under 12-hour on/off cyclic lighting with an in-cage illuminance of 180 to 230 lx in the Laboratory Animal Center of Zhejiang University, Hangzhou, China. All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with approval from the Institutional Animal Care and Use Committee of Zhejiang University Medical School.

**Optimal Synthesis of pdA2E and isopdA2E**

Ethanolamine (64.7 mg, 1.06 mmol) was added to a solution of atRAL (51.1 mg, 0.18 mmol) in 3 mL ethanol, and were performed open to the air and under dim red light. The addition of 0.16 mmol AcOH (1 equivalent) is aimed to maintain the acidic reaction environment. The yields are calculated as TFA salts because the products were isolated by silica gel chromatography eluted with TFA-CH\textsubscript{3}OH-CH\textsubscript{2}Cl\textsubscript{2}. The percentages in parentheses denote the content of the pdA2E isomers in the resulting samples.

**TABLE. Optimization of the pdA2E Synthesis**

<table>
<thead>
<tr>
<th>Run*</th>
<th>Reactant Molar Ratio, atRAL:EA</th>
<th>Additives</th>
<th>Time, d</th>
<th>Results, %†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2:1</td>
<td>AcOH</td>
<td>2–4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2:1</td>
<td>None</td>
<td>2–3</td>
<td>0</td>
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<tr>
<td>3</td>
<td>2:1</td>
<td>None</td>
<td>4</td>
<td>Trace</td>
</tr>
<tr>
<td>4</td>
<td>1:1</td>
<td>None</td>
<td>4</td>
<td>7 (36)</td>
</tr>
<tr>
<td>5</td>
<td>1:4</td>
<td>None</td>
<td>3</td>
<td>5 (26)</td>
</tr>
<tr>
<td>6</td>
<td>1:4</td>
<td>None</td>
<td>7</td>
<td>11 (78)</td>
</tr>
<tr>
<td>7</td>
<td>1:6</td>
<td>AcOH</td>
<td>5</td>
<td>15 (45)</td>
</tr>
</tbody>
</table>

* All reactions include atRAL (0.18 mmol) and EA as reactants in 3 mL ethanol, and were performed open to the air and under dim red light. The addition of 0.16 mmol AcOH (1 equivalent) is aimed to maintain the acidic reaction environment.

† The yields are calculated as TFA salts because the products were isolated by silica gel chromatography eluted with TFA-CH\textsubscript{3}OH-CH\textsubscript{2}Cl\textsubscript{2}. The percentages in parentheses denote the content of the pdA2E isomers in the resulting samples.

**FIGURE 1.** A2E, isoA2E, pdA2E, and isopdA2E. (A–D) Planar structures, mass-to-charge ratio (m/z), UV-visible absorbance maxima (nm), and electronic transition assignments (\(\rightarrow\)). (E, F) Molecular modeling on pdA2E and isopdA2E was carried out by optimizing (Gaussian 03, B3LYP/6-31G (d)) initial conformers, which were constructed according to the NOESY data. S, short arm; L, long arm.

Because most of the biological properties of pdA2E and its relationship to AMD remain unclarified, the development of an efficient approach to prepare large quantities of pdA2E becomes extremely crucial for further biochemical studies of this adduct. Although pdA2E has been obtained through biomimetic synthesis,\textsuperscript{20} the yield was only approximately 7% based on the former route (run 4, Table). In the following, we report an optimized biomimetic protocol for the pdA2E preparation that significantly facilitated the production of this pigment. More importantly, biosynthetic and HPLC experiments have allowed for isolation and identification of a new fluorescent pigment from bovine RPE lipofuscin, a double-bond isomer of pdA2E (Fig. 1D). In addition, we provide evidence that pdA2E biosynthesis may involve the formation of the precursor, pdA2-PE, in neural retina.
Light-Mediated Isomerization

A solution of HPLC-purified pdA2E or isopdA2E in water with 2% dimethyl sulfoxide (DMSO) (200 μM) was subjected to monochromatic light (3500 lx) delivered from a 500-Watt xenon illuminant with a 490-nm optical filter. The isomerization extent at 10 minutes was analyzed by HPLC with a 45-μL injection volume. To test for the identity of compounds, synthetic standards were co-injected with irradiated samples, respectively. The dC18 column was used with a gradient mobile phase consisting of acetonitrile and water in the presence of 0.1% TFA (85%-100% acetonitrile, 0.8 mL/min, 0-15 minutes; 100% acetonitrile, 0.8 mL/min, 15-30 minutes; 100% acetonitrile, 0.8-1.2 mL/min, 30-40 minutes). The eluate was monitored at 490 nm.

Comparison of Fluorescence Efficiency

The A2E16 isoA2E16 isoA2E17 and atRAL dimer18 were synthesized as published. These compounds, together with pdA2E and isopdA2E, were individually prepared as a stock solution in DMSO (10 mM) and kept at −80°C in the dark. Methanol was used to dilute these stock solutions to a final concentration of 50 μM. Fluorescence emission spectra were monitored by a Hitachi F-4500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) with the excitation wavelength (λex) at 450 or 488 nm.

RESULTS

Optimization of the PdA2E Synthesis

A wide range of reaction conditions were used to optimize the yield of pdA2E from the reactants of atRAL and ethanolamine. Typical results are listed in the Table. The same conditions as for the optimal A2E synthesis16 gave no pdA2E (run 1), as previously described.20 Two equivalents of atRAL reacted with one equivalent of ethanolamine in the absence of acetic acid (AcOH) to generate no pdA2E within 3 days (run 2), although a trace amount of pdA2E was detected on the fourth day (run 5). We previously reported a biomimetic protocol (run 4) that included atRAL and ethanolamine at a molar ratio of 1:1 (without AcOH) and a reaction time of 4 days,20 and the desired product was obtained in approximately 7% yield. When the molar ratio of atRAL to EA reached 1:4 and no AcOH was added, the reaction condition produced pdA2E at a yield of 5% after 3 days (run 5), and increased the yield up to 11% by

Theoretical calculations were implemented using a Gaussian 03 program package (Gaussian, Inc., Pittsburgh, PA, USA).25 Equilibrium geometries of pdA2E and isopdA2E were optimized using the density functional theory (DFT) method at the B3LYP/6-31G (d) level. Density functional theory optimized structures with some relevant molecular orbital were shown by GaussView (Version 5.09) software (Semichem, Inc., Shawnee Mission, KS, USA) to yield higher-quality images of these structures determined. Density functional theory optimized structures were also used for the calculations of UV-visible spectra in acetonitrile by using the BHandHLYP functional at the 6-31G (d) level. Excitation energies and oscillator strengths of excited states in the DFT-optimized geometries of pdA2E and isopdA2E were calculated, and presented in Supplementary Tables S1 and S2, respectively.

Eye Extracts and HPLC Analysis

Bovine RPE/choroids (3 eyes per sample), bovine neural retinas (6 eyes per sample), and murine eyecups (16 eyes per sample) were individually homogenized in a tissue grinder with 50% methanolic chloroform and water as described previously.17 It should be noted that retinae were washed twice after removal from the eyes. For chromatographic separation of compounds, the dC18 column was used with a gradient mobile phase consisting of acetonitrile and water in the presence of 0.1% TFA (75%-90% acetonitrile [0-30 minutes], 90%-100% acetonitrile [30-40 minutes], and 100% acetonitrile [40-100 minutes], with a flow rate of 0.5 mL/min; monitored at 490 nm). As a control, the second half of the sample was directly injected into HPLC.
prolonging the reaction time from 3 to 7 days (run 6). The use of a reactant molar ratio of 1:6 and one equivalent of AcOH as an additive in the biomimetic synthesis led to significant growth in terms of the pdA2E formation after only 3 days (Fig. 2), affording it in approximately 15% yield (17.4 mg, run 7). Detection by reverse-phase HPLC revealed several minor pdA2E isoforms having a molecular ion signal at m/z 658.5 in the pdA2E samples isolated from silica gel chromatography (runs 4 and 7, Table); the contents of these isomers were, respectively, approximately 36%, 26%, 78%, and 45% (Table), as estimated by integrating peak areas. The pdA2E and its major isomer iso pdA2E in pure form were obtained via further HPLC purification with the yields of 5.8% and 2.5%, respectively.

Tissue Constituents and Biomimetic Reaction Mixtures

One-half of a methanolic chloroform extract derived from three bovine RPE/choroids was examined by reverse-phase HPLC, and the eluents were monitored for absorbance at 490 nm. With a total running time of 50 minutes, peaks in the HPLC profile (Fig. 3A) were readily assigned to previously identified lipofuscin constituents, including A2E (Fig. 1A),15 isoA2E (Fig. 1B),16 isoA2E,17 and pdA2E.20 In addition to these fluorophores, a previously unrecognized peak, attributable to a less polar pigment, exhibited an Rf of approximately 45.7 minutes and UV-visible absorbance maxima (λmax) at 492 and 342 nm (Fig. 3A), similar to that of pdA2E (λmax, 492 and 342 nm), but different in shape and relative intensity. We then determined whether the compound with 492/340-nm absorbance maxima in bovine RPE/choroids was generated in reaction mixtures of atRAL and ethanolamine (runs 4–7, Table). Using reverse-phase HPLC, the elution profiles revealed a peak with considerable peak height and (kmax, 492 and 340 nm (Fig. 2), which also eluted with the same Rf as the 492/340-nm peak in extract of bovine RPE/choroids. To further corroborate the consistency of pdA2E and iso pdA2E in bovine RPE/choroids with synthesized compounds, the HPLC chromatogram of bovine RPE/choroid extract (Fig. 3A) was compared with that of a mixture of the second half of bovine RPE/choroid extract and moderate quantities of synthetic pdA2E and iso pdA2E. As expected, peak heights of pdA2E and iso pdA2E were significantly raised (Fig. 3B), and quantification by measuring chromatographic peak areas (mAU) were measured, and calculated using Empower 3 software. Values are expressed as a peak area ratio of pdA2E/iso pdA2E to isoA2E in two portions of bovine RPE/choroid extracts. mAU, milliabsorbance units.
Retinal-Derived Fluorophores pdA2E and isopdA2E

Identification of isopdA2E

A major obstacle to structural elucidation of isopdA2E is to obtain sufficient samples in pure form for nuclear magnetic resonance (NMR) experiments, because the minuscule amount of materials is present in extracts of bovine RPE/choroids. In vitro biomimetic synthesis and the subsequent two-step purification solved this problem and thus the synthetic compound was studied by extensive NMR and MS, and structurally identified. The data manifested that it was a cis (Z)-isomer of pdA2E at the C13C14 double bond. The isopdA2E is a reddish solid that has a molecular formula of C15H10NO as determined by high-resolution ES-MS at m/z 658.4983 (theoretical 658.4982, [M]+). The UV-visible absorbance spectrum of isopdA2E in methanol contained two peaks, with \( \lambda_{\text{max}} \) at 492 nm (6200 M\(^{-1}\) cm\(^{-1}\)) and 340 nm (64000 M\(^{-1}\) cm\(^{-1}\)). The \(^1\)H-NMR spectrum of isopdA2E (Fig. 4B) was very similar, but not identical to that of pdA2E (Fig. 4A). The 125-MHz \(^{13}\)C-NMR spectrum of isopdA2E in CD\(_2\)OD, with the aid of distortionless enhancement by polarization transfer 135, heteronuclear singular quantum correlation, and heteronuclear multiple bond correlation spectroscopic techniques, resolved 47 carbon resonances that came from 10 methyls, 8 methylenes, 17 methines, and 12 quaternary carbons. Homonuclear shift correlation spectroscopy (\(^1\)H–\(^1\)H COSY) permitted the complete identification of methyl and olefinic proton resonances. Importantly, nuclear Overhauser enhancement spectroscopy (NOEY) revealed a significant cross-peak between H\(_a\) (7.81 ppm, d, \( J = 1.9 \) Hz) and H\(_b\) (6.55 ppm, d, \( J = 14.8 \) Hz) in isopdA2E, suggestive of a \( E \)-\( Z \) geometry (Fig. 4C), and, however, the signal was not detected in the NOESY spectrum of pdA2E.20 All other double bonds in two polyenic side chains of isopdA2E were identified as being in \( E \) configurations based on large coupling constants of vicinal olefinic protons (\( J_{1,A} = 16.7 \) Hz, \( J_{11,12} = 16.0 \) Hz, \( J_{2,3} = 15.0 \) Hz, \( J_{11,12} = 15.1 \) Hz) and NOESY correlations (1-CH\(_3\)/8-H, 8-H/10-H, 10-H/12-H, 7-H/9-CH\(_3\), 9-CH\(_3\)/11-H, 11-H/13-CH\(_3\), 13-CH\(_3\)/14-H, 1-CH\(_3\)/8-H, 8-H/10-H, 12-H/14-H, and 16-H/18-H). All proton and carbon chemical shifts in the \(^1\)H- and \(^{13}\)C-NMR spectra were carefully assigned, and presented in Figure 4C and the Materials and Methods section, respectively.

Evaluation of UV-Visible Absorbance Spectra and Electronic Transition Assignments

Density functional theory computed UV-visible absorbance spectra of pdA2E and isopdA2E in acetonitrile using the BHandHLYP functional across the 6-31G (d) basis set. To some extent, simulated spectra of pdA2E (\( \lambda_{\text{max}} \) 536 and 525 nm; Fig. 5A) and isopdA2E (\( \lambda_{\text{max}} \) 339 and 524 nm; Fig. 5C) agree with experimentally determined UV-visible absorbance spectra of pdA2E (\( \lambda_{\text{max}} \) 342 and 492 nm) and isopdA2E (\( \lambda_{\text{max}} \) 340 and 492 nm), although the relatively high \( \lambda_{\text{max}} \) of these two compounds was red-shifted by approximately 30 nm. In the case of isopdA2E, there are mainly two allowed states different in energy in the UV-visible region, and each excited state is characterized through a large electronic transition probability. As depicted in Figure 5D, the first absorbance band (524 nm) is typically of high intensity and is associated with an excitation from the highest occupied molecular orbital (HOMO) (180) to the lowest unoccupied molecular orbital (LUMO) (181), indicative of a charge transfer from the long arm to the pyridinium ring and the short arm. The second absorbance band (359 nm) with comparatively lower intensity involves an excitation from the HOMO-1 (179) to the LUMO+1 (182) and is related to a charge transfer from the short arm to the pyridinium ring and the long arm. Furthermore, an additional minor absorbance band at 415 nm also was detected and is due to an excitation from the HOMO-1 (179) to the LUMO (181) and a charge transfer from the short arm to the pyridinium ring and the long arm. The data clearly supported the conclusion that the 492-nm absorbance was assigned to the long arm of isopdA2E and the 340-nm absorbance to the short arm (Fig. 1D). As expected, these electronic transitions also were observed when DFT-optimized structure of pdA2E was theoretically evaluated for its absorbance maxima (Fig. 5B), thereby confirming that the 492-nm absorbance was directed to the long arm and the 342-nm absorbance to the short arm (Fig. 1C). The N-Cg-Cg1-Cg1-H dihedral angle (\( \phi \)) in DFT-optimized structures of pdA2E and isopdA2E was calculated as \( -43.1^\circ \) (Fig. 1B) and \( +46.2^\circ \) (Fig. 1F), respectively, suggesting that the torsion in spatial structures of these two molecules, at least in part, is due to the mutual repulsion of relevant electrons in this region. Further analysis of their spatial structures also corroborated that a correlation was observed between H\(_a\) and H\(_b\) in isopdA2E rather than pdA2E, consistent with the NOEY data (Fig. 4C).

Photoinduced Interconversion Between pdA2E and isopdA2E

Solutions of these two fluorophores in water (200 \( \mu \)M) containing 2\% DMSO were irradiated by the 490-nm blue-green light, after which the resulting samples were analyzed by HPLC (Fig. 6). Subjecting the pdA2E solution (Fig. 6A) to light for 10 minutes gave rise to two pdA2E isomers called iso1 and iso2, both of which exhibited an \( m/z \) peak at 658.5, consistent with our previous report.20 Importantly, iso2, with \( \lambda_{\text{max}} \) at 340 and 492 nm, was determined to be identical with isopdA2E.
(Fig. 6B), as judged by HPLC co-injection (Fig. 6C), UV-visible absorbance spectra (Figs. 6B, 6D), and ESI-MS (Fig. 3C). The same methods were used to confirm that exposure of the iso-pdA2E solution to light yielded pdA2E and iso1 (Figs. 6D–F). The data are indicative of the ability of light to mediate facile interconversion between pdA2E and iso-pdA2E.

**Phospholipase D-Catalyzed Hydrolysis**

To provide further insight into tissue localization and biosynthetic pathway of pdA2E, we obtained bovine eyes and dissected neural retinas. In one-half of extracts from six bovine neural retinas, we readily observed A2E, isoA2E, and N-retinylidene phosphatidylethanolamine (NRPE) by HPLC.
However, a chromatographic peak attributable to pdA2E was not visible in the HPLC profile while monitoring at 490 nm (Fig. 7A). To determine whether pdA2-PE (Fig. 7D), like A2-PE, is formed in neural retina and serves as the substrate for lysosomal enzymes, we incubated the second half of neural retina extracts with phospholipase D (Fig. 7B). In addition to A2E, isoA2E and an additional unknown peak (*) that exhibited $k_{\text{max}}$ at 336 and 450 nm, a minor peak was assigned to pdA2E on the basis of absorbance spectra and by co-injection with synthetic pdA2E (Fig. 7B). It is not surprising that the NRPE peak disappeared because phospholipase D digested this adduct to give NRE (N-retinylidene ethanolamine) and phospholipids (Fig. 7C); the latter compounds do not elute from a dC18 column. Taken together, these findings corroborate the fact that pdA2-PE is likely generated in bovine neural retinas and functions as an immediate pdA2E precursor, with pdA2E being released from pdA2-PE via enzymatic hydrolysis (Fig. 7D).

**DISCUSSION**

We have demonstrated that atRAL reacts with excess ethanolamine at a molar ratio of 1:1 in the absence of AcOH (run 4, Table) to generate pdA2E, in contrast to the A2E synthesis under the optimal reaction condition, which, however, does not produce pdA2E (run 1, Table). Biomimetic synthesis under different reaction conditions was performed to ascertain whether the atRAL/ethanolamine molar ratio or AcOH affected the production of pdA2E. The Table reflects that the pdA2E preparation is very sensitive to the molar ratio of atRAL to ethanolamine in the reaction mixtures, reflecting that excess ethanolamine may function as a catalyst to aid the formation of pdA2E. By contrast, the addition of AcOH is clearly conducive to the synthesis of pdA2E (run 7, Table), although the acid additive is not essential for its formation (run 3, Table).

Previous studies reveal that A2E (Fig. 1A) exhibits $k_{\text{max}}$ at 340 and 440 nm; the 340-nm absorbance is attributable to a conjugation system having a pyridinium ring and four double bonds on the short arm, and the 440-nm absorbance arises from a conjugation system having the pyridinium ring and five double bonds on the long arm. The difference in the structure of pdA2E compared with A2E is that an additional pentadiene moiety is incorporated into the long arm of pdA2E. With DFT calculations, we confirmed electronic transition assignments on two arms of pdA2E (Fig. 1C). Like the long arm of A2E, the short arm of pdA2E also provides for a conjugation system of a pyridinium ring and five double bonds; yet, it is surprising that the short arm of pdA2E does not retain the 440-nm absorbance of the long arm of A2E, but exhibits $k_{\text{max}}$ at 342 nm that is almost consistent with $k_{\text{max}}$ of the short arm of A2E (340 nm). In contrast to the long arm of A2E, the conjugation system present within the long arm of pdA2E extends and includes six double bonds and a pyridinium ring, to which the 492-nm absorbance is attributed. A possible explanation for the phenomena is that the pyridinium ring head modulates absorbance distribution for two arms with the conjugation system on each arm of A2E being extended, and the longer double-bond conjugation systems on the long arm of pdA2E versus A2E would lead to the red-shifting of $k_{\text{max}}$. The same correlation between $k_{\text{max}}$ and conjugation systems also is observed in isoA2E (Fig. 1B) and isopdA2E (Fig. 1D).

We here compared fluorescence efficiency of several RPE lipofuscin pigments, including pdA2E and isopdA2E, at a concentration of 50 μM (Fig. 8). At an excitation of 488 nm, the wavelength ($\lambda_{\text{ex}}$) generally used for fundus autofluorescence imaging, the fluorescence emission efficiency followed the decreased order: A2E > isoA2E > isoA2E > pdA2E >
isoA2E > atRAL dimer (Fig. 8A). Yet, under the 430-nm excitation, the emission fluorescence exhibited by iisoA2E was of greater efficiency than A2E and isoA2E, and fluorescence intensity of atRAL dimer significantly increased and was higher than that of pdA2E and isoA2E; the fluorescence efficiency of these compounds in decreasing order was iisoA2E > A2E > isoA2E > atRAL dimer > pdA2E > isoA2E (Fig. 8B).

In bovine neural retinas, we observed A2E and isoA2E, as well as NRPE, a PE-atRAL Schiff base adduct (Fig. 7C), that has been previously identified in isolated bovine rod outer segments.25,26 The NRPE is probably a ligand for ABCA4,27–29 the rod photoreceptor-specific ATP-binding cassette transporter that is mutated in recessive Stargardt disease.30 In recent time we proposed a pdA2E biosynthetic cascade20 that is initiated by the formation of a Schiff base between the membrane phospholipid PE and atRAL to create NRPE. This adduct would then undergo a [1,6]-proton tautomerization to generate a phosphatidyl analogue of enamine (PAE). It is speculated that PAE will then form an additional Schiff base with a second molecule of atRAL to give an all-trans-iminium salt. After 6π-aza-electrocyclization to produce a phosphatidyl dihydropyridinium bisretinoid molecule, dihydropyridinium A2-PE, there are two possibly ongoing paths: one is to eliminate two hydrogens to form A2-PE, a phosphatidyl pyridinium bisretinoid that has been identified as an immediate precursor of A2E,21 and another is to further react with a third molecule of atRAL in a Michael-type addition to generate a dihydropyridinium A2-PE-atRAL adduct. Subsequent ring opening and elimination would give rise to dihydropyridinium pdA2-PE, which after facile autoxidation and a two-hydrogen loss would yield pdA2-PE; the latter is cleaved by hydrolysis of the phospholipids to release pdA2E. Considering that dihydropyridinium A2-PE was an unstable intermediate,31 together with a phospholipase D-based assay that has shown the presence of pdA2-PE, a precursor of pdA2E, in neural retinas (Fig. 7), we here reported a revised and expanded pathway for the formation of pdA2E and isoA2E (Fig. 9). Specifically, the third atRAL molecule was reacted with A2-PE rather than dihydropyridinium A2-PE to generate an A2-PE-atRAL adduct via the Michael-type addition, followed by ring opening and

**Figure 8.** Comparison among fluorescence emission spectra of A2E, isoA2E, iisoA2E, pdA2E, isoA2E, and atRAL dimer. The final concentration of these compounds in methanol was 50 μM and tested by a Hitachi F-4500 fluorescence spectrophotometer with the excitation wavelength (λex) at 430 (A) or 488 nm (B).

**Figure 9.** Proposed biosynthetic pathway by which pdA2E forms via pdA2-PE in the retina.
elimination of the crotonaldehyde and vinyl β-ionone ring moieties to produce pdA2-PE. Finally, cleavage of the phosphodiester bond in pdA2-PE, possibly by phospholipase D, yields pdA2E. PdA2E and iso pdA2E can be readily interconverted by light. We previously revealed that pdA2E was readily detected in the RPE/choroid of a single human eye as well as of a single bovine eye. However, in the present work, although we confirmed the presence of a low amount of iso pdA2E in 1.5 bovine RPE/choroids (Fig. 3), it was not detectable in extract of one human RPE/choroid, and was not observed after treatment of hydrophobic extracts from bovine neural retinas with phospholipase D (Fig. 7), probably due to inadequate sensitivity of iso pdA2E for the HPLC detection. Another possible explanation for the phenomena is that the in vivo iso pdA2E level depends on light-induced interconversion of pdA2E and iso pdA2E because we observed that the extent for conversion of pdA2E to iso pdA2E by light was much less in comparison with that for light-mediated conversion of iso pdA2E to pdA2E (Figs. 6B, 6E). Using extracts of 16 eyecups from Abca4/Rd8−/− mice (6–10 months of age), we detected pdA2E at a low level and an absence of iso pdA2E, suggesting that the knockout of the Abca4/Rd8 genes in mice does not favor the formation of pdA2E and iso pdA2E in the retina. One question that we also cannot ignore is that in healthy retinas, the levels of pdA2E and iso pdA2E are found to be much lower compared with that of A2E and isoA2E (Fig. 3), indicative of their relatively weak physiological significance. Nevertheless, given the quantity of pdA2E and iso pdA2E is unknown in diseased retinas and other gene knockout mouse models, a direct statement that these two pigments may not affect RPE cannot be made at the current stage.

A hallmark of aging retina is RPE lipofuscin buildup. An overload of lipofuscin in the RPE has been implicated with retinal degeneration and blindness in patients with AMD. Several investigations of potential treatments for AMD include approaches that would reduce the bisretinoid formation,32 several enzymatically degrade A2E33 and remove retinal-derived adducts from the RPE.34 Our study gives a more complete understanding of components and biosynthesis of adverse retinylipofuscin.

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