Differential incorporation of docosahexaenoic and arachidonic acids in frog retinal pigment epithelium

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Abstract Vertebrate retinas contain a high level of docosahexaenoic acid (DHA, 22:6n-3) and a relatively low level of arachidonic acid (AA, 20:4n-6). Although it is not known how DHA is selectively concentrated in the retina, the retinal pigment epithelium (RPE) is intimately involved in this process as these cells phagocytize shed rod outer segments (containing DHA) and take up DHA from the circulation. To explore the role of the RPE in the DHA enrichment in the retina, native frog RPE cells (RPE-eyecups) were incubated with [^{3}H]DHA or [^{3}H]AA for short times, or pulsed for up to 1 h with the two labeled fatty acids plus labeled glycerol simultaneously, followed by a chase for up to 7 h. The incorporation of label into various lipids was monitored by thin-layer or high performance liquid chromatography. In short-time incubations, a greater amount of DHA was incorporated into neutral lipids (primarily triglycerides, TG) compared with AA, while similar amounts of both fatty acids were found in phospholipids. On a relative basis, DHA was esterified mainly into TG, with AA into phospholipids. The uptake of each fatty acid correlated linearly with concentration (30-120 nM) and the incorporation pattern was distinct. Interestingly, the uptake and incorporation of labeled DHA or AA were not affected by the presence of unlabeled AA or DHA, respectively. In a pulse-chase experiment, RPE cells were active in the de novo synthesis of glycerolipids, particularly TG, which achieved a specific activity as high as diglycerides and ten times higher than the major phospholipids. The incorporation of DHA into most glycerolipids occurred to a large extent during de novo glycerolipid synthesis, whereas AA was incorporated mainly by deacylation-reacylation reactions. Our results suggest different metabolic pathways for handling DHA and AA in frog RPE. The preferential incorporation of DHA into TG suggests that TG might play an important role in the selective enrichment of DHA in the retina and in the recycling of photoreceptor DHA between the RPE and the retina. —**Chen, H., and R. E. Anderson.** Differential incorporation of docosahexaenoic and arachidonic acids in frog retinal pigment epithelium. *J. Lipid Res.* 1993. **34:** 1943-1955.

Supplementary key words glycerol • lipids • phagocytosis • retina • triglycerides

Docosahexaenoic (DHA, 22:6n-3) and arachidonic (AA, 20:4n-6) acids are the major polyunsaturated fatty acids (PUFAs) of the n-3 and n-6 families of essential fatty acids that are found in animals. While AA is broadly distributed in all biological membranes, DHA is specifically localized in excitable membranes of neural tissues (1, 2). Rod photoreceptor outer segments of vertebrate retinas are highly enriched in DHA which makes up about half of the total fatty acids in phospholipids; AA is a minor component, representing 3-5% of the fatty acids (3). The level of DHA in the retina is among the highest reported for any membrane. Over the last several years, evidence has accumulated that DHA is essential for maintaining the normal structure and function of the retina (4-14).

The rod outer segments consist of an orderly stack of several hundred discs surrounded by a plasma membrane. These disc membranes are constantly being renewed (15, 16). Newly formed discs are added at the base of the outer segments and older membranes are slowly displaced apically until they reach the tip of the outer segments, where discs are shed as a single package and phagocytized by the adjacent retinal pigment epithelium (RPE). The complete renewal of rod outer segment membranes takes about 9-10 days for mammals and 40-60 days for amphibians (17). Because large amounts of outer segment membranes are turned over each day, it was thought that a dietary deprivation of n-3 essential fatty acids would led to a rapid depletion of DHA from these membranes. However, rats and monkeys raised on n-3-deficient diets showed a slow decrease in the DHA content of their rod outer segments or retinas (6, 7, 18-26). Compared with non-neural tissues, the retina displays a stronger ability to conserve this PUFA (18, 22, 23). The turnover of rod outer segment DHA is much slower

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Abbreviations: PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; AA, arachidonic acid; RPE, retinal pigment epithelium; TLC, thin-layer chromatography; RBP, Ringer's-bicarbonate-pyruvate buffer; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; TG, triglycerides; DG, diglycerides; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; FFA, free fatty acids.

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than the renewal rate of these membranes during n-3 deficiency as well as n-3 dietary supplementation (27). Thus, the retina has a remarkable intrinsic capacity to conserve this long-chain PUFA.

Evidence thus far indicates that rod outer segment DHA is conserved through recycling between the retina and the RPE (26-31). Rod outer segment tips phagocytized by the RPE contain large amounts of DHA that is rapidly processed and efficiently shuttled back to the retina (28, 29). This DHA recycling provides a sustained DHA flow to the retina for the synthesis of outer segment lipids, independent of dietary intake of n-3 fatty acids. Yet, the question of how DHA of rod outer segment membranes is metabolized in the RPE is not known. As the RPE takes up and enriches both DHA and AA from the circulation (32, 33), how is DHA selectively transported to the retina? In the present study, we gave free DHA and AA to the RPE and compared the incorporation of these two PUFAs into RPE lipids. Our results indicate that DHA is metabolized differently from AA in the RPE. The preferential incorporation of DHA into RPE neutral lipids may promote the selective recycling of this fatty acid from the RPE to the retina.

EXPERIMENTAL PROCEDURES

Materials

Adult *Rana pipiens* (medium size) were purchased from J. M. Hazen (Alburg, VT). Crickets were obtained from Fluker's Cricket Farm (Baton Rouge, LA). [4,5-^{3}H]DHA (23.0 Ci/mmol) and [5,6,8,9,11,12,14,15-^{3}H]AA (79.9 Ci/mmol) were from New England Nuclear (Boston, MA). [2-^{3}H]glycerol (1 Ci/mmol) was from Amersham (Arlington Heights, IL). All organic solvents and unlabeled glycerol were HPLC grade and obtained from Fisher (Pittsburgh, PA). KG silica 60 thin-layer chromatographic (TLC) plates (10 × 20 cm, 250 μm) were from EM Science (Gibbstown, NJ). Essential fatty acid-free bovine serum albumin (BSA) was from Sigma (St. Louis, MO). Unlabeled DHA and AA were from Nu-Chek Prep (Elysian, MN).

Animals

Frogs were maintained at room temperature (22°C) in a plastic cage with constant running water; they were fed crickets once per week. The animals were regulated to a diurnal cycle (10-h dark and 14-h light) of ceiling fluorescent illumination. The frogs used in this study were kept in this environment for less than 2 months. The treatment of frogs was in accordance with the guidelines of the Association for Research in Vision and Ophthalmology. The experimental protocol was reviewed and approved by the Animal Protocol Review Committee of Baylor College of Medicine (Houston, TX).

BSA-fatty acid complex

[^{3}H]DHA or [^{3}H]AA of appropriate specific radioactivity was complexed with delipidated BSA as following. Aliquots of tritium-labeled fatty acids (and unlabeled ones where specified) in ethanol solution were transferred to screw-capped tubes and the solvent was evaporated under a N_2 stream. Aliquots of BSA in 50 mM $NaHCO_3$ were added subsequently, so that BSA to fatty acid molar ratio was 1:2. The fatty acid-protein mixture was sonicated for 5-10 min at room temperature, after which the mixture appeared clear.

Preparation of RPE-eyecups

Frogs, dark-adapted overnight, were killed by decapitation and pithed immediately under dim red light in the early morning prior to normal light onset. Eyes were enucleated and eyecups were prepared by removal of the anterior segment, lens, and vitreous humor. After placing in Ca^{2+}-free Ringer's buffer (34) for 10-20 min at room temperature, retinas were gently removed from the RPE. The resultant RPE-eyecups were washed at least three times with chilled Ringer's-bicarbonate-pyruvate buffer (RBP, pH 7.4) containing 120 mM NaCl, 5.1 mM KCl, 2.75 mM $CaCl_2$, 1.25 mM $MgSO_4$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, 18 mM pyruvate, and 5 μg/ml each of penicillin and streptomycin, and collected in a glass flask containing 5 ml ice-cold RBP until all dissections were completed. The RBP was gassed for 1 h with O_2/CO_2 (95:5) prior to use. All of the above operations and subsequent incubations were conducted under dim red light.

RPE-eyecup incubation

RPE-eyecups were resuspended in 3 ml fresh RBP and preincubated for 30 min at 24°C with mild shaking under a constant stream of humidified O_2/CO_2. Incubations were started by adding 100 μl of 50 mM $NaHCO_3$ (pH 8.7) containing known amounts of [^{3}H]glycerol, [^{3}H]DHA, and/or [^{3}H]AA (conjugated with BSA at a molar ratio of 2:1). After incubating for the indicated time periods under the same conditions as preincubation, RPE-eyecups were washed three times with 10 ml chilled RBP and RPE cells were subsequently isolated. For the pulse-chase experiment, the washed eyecups were transferred to 3 ml fresh RBP containing known amounts of unlabeled glycerol, unlabeled DHA, and unlabeled AA conjugated with BSA, and incubated further for up to 7 h. At the end of the incubation periods, RPE-eyecups were washed with chilled RBP and RPE cells were prepared by gentle agitation with a small camel-hair brush. The recovered RPE cells were subsequently purified on ficoll gradients (34) and extracted immediately two times with chloroform-methanol (35). The preparation time for RPE cells was about 40 min during which the cells were kept at 0°C in Ca^{2+}-free Ringer's.

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Lipid separation and saponification

The extracted lipids were resolved into phospholipids, triglycerides, free fatty acids, and diglycerides by one-dimensional TLC using a solvent system hexane–diethyl ether–glacial acetic acid 70:35:1 (v/v/v). Lipid classes were localized by brief iodine staining and scraped for radioactivity counting. Aliquots of lipid extracts were taken for organic phosphorus (36) and total radioactivity determinations. When individual phospholipid class separation was desired, the extracted lipids were resolved by two-dimensional, three-step TLC (37) with the modifications: hexane–diethyl ether 140:35 (v/v) for the second-step separation and chloroform–methanol–acetone–acetic acid–water 80:26:30:24:8 (by volume) for the third step. After visualizing with 2,7-dichlorofluorescein and UV light, the silica gels containing individual lipid classes were scraped, suspended in 1 ml of 2.0% KOH (w/v) in ethanol, and saponified at 100°C for 30 min. A known amount of heptadecanoic acid (17:0, internal standard) was added prior to the saponification. After cooling to room temperature, 1 ml water and 100 μl concentrated HCl were added to the saponification mixture and the released free fatty acids were extracted three times with 2 ml hexane. The remaining aqueous phase was dissolved in 15 ml Flo-Scint A (Packard Instrument, Meriden, CT) and the radioactivity of [^{3}H]glycerol was measured in a Tri-Carb liquid scintillation counter (Packard).

HPLC analysis of fatty acids

The extracted free fatty acids from individual lipid classes including the internal standard were phenacyl-derivatized and analyzed on a Supelco LC-18 column (25 cm × 4.6 mm, I.D., 5 μm particle size) with a linear gradient of acetonitrile–water 80:20 to 92:8 (v/v) for 50 min at a flow rate of 2 ml/min (38). The phenacyl esters were monitored at 242 nm and peak areas were integrated using Spectra-Physics 4270 integrator (San Jose, CA) and post-run processed with the WINner/286 program. The eluted fractions corresponding to the identity of DHA and AA standards were collected separately in scintillation vials, mixed with Flo-Scint A, and counted for radioactivity. HPLC peaks were confirmed by gas–liquid chromatographic analysis of collected effluents (38). The molar absorptivity (peak areas/nmol) of individual phenacyl esters was determined (38) and used for quantitation of fatty acids separated by HPLC.

RESULTS

DHA and AA levels in frog RPE

The lipids of RPE cells are composed primarily of phospholipids (80%), triglycerides (10.5%), and free fatty acids (7.7%) (**Table 1**). The fatty acid composition of individual lipid classes determined by HPLC analysis (data not shown) is similar to that obtained by gas–liquid chromatographic analyses (32), and establishes the HPLC method as a satisfactory procedure for fatty acid determination. The level of DHA and AA (mol% of total fatty acids) was similar in free fatty acids (FFA), phosphatidic acid (PA), diglycerides (DG), lysophosphatidylethanolamine (L-PE), and lysophosphatidylcholine (L-PC) (Table 1). However, triglycerides (TG), phosphatidylcholine (PC), and phosphatidylserine (PS) had more DHA than AA, whereas phosphatidylethanolamine (PE) and phosphatidylinositol (PI) had about 2- and 35-fold more AA than DHA, respectively.

TABLE 1. Lipid composition and relative content of DHA and AA in individual lipids of frog RPE cells

Lipid	Class	DHA	AA
FFA	7.7 ± 1.9	7.8 ± 1.5	8.5 ± 1.2
PA	0.8 ± 0.1	8.9 ± 1.3	10.7 ± 1.9
DG	2.5 ± 0.4	7.6 ± 1.6	8.7 ± 2.1
TG	10.5 ± 1.4	17.8 ± 3.5	7.9 ± 2.8
PI	6.2 ± 0.5	1.1 ± 0.4	38.8 ± 1.3
L-PE	6.2 ± 1.4	24.4 ± 3.3	22.3 ± 5.0
L-PC	4.0 ± 1.1	19.8 ± 3.5	16.1 ± 2.2
PE	19.5 ± 2.0	11.2 ± 2.5	28.4 ± 1.9
PC	37.9 ± 2.2	14.7 ± 1.1	11.6 ± 2.0
PS	4.6 ± 0.9	20.7 ± 3.6	10.6 ± 1.2

Lipids were isolated from frog RPE cells and resolved into classes by two-dimensional, 3-step TLC. Individual lipid classes were saponified and the resulting fatty acids were converted to phenacyl esters and quantitated by HPLC. Lipid class data (mol%) were calculated from the total fatty acids of each class divided by a factor of 1 (for FFA, L-PE, and L-PC), 2 (for PA, DG, PE, PC, PI, and PS), or 3 (for TG), and normalized for the ten classes. Data on DHA and AA are the mole percentages of these two PUFAs in total fatty acids of each lipid class. Values are means ± SD of ten determinations. Abbreviations: FFA, free fatty acids; PA, phosphatidic acid; DG, diglycerides; TG, triglycerides; PI, phosphatidylinositol; L-PE, lyso-phosphatidylethanolamine; L-PC, lyso-phosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine.

Time-course of uptake and incorporation of DHA and AA

DHA and AA were rapidly taken up by frog RPE cells during the 1 h incubation at the fatty acid concentration of 60 nM (**Fig. 1**). The uptake of DHA was greater than AA, and appeared to be more sustained at the later time points (Fig. 1A). The partition of DHA and AA between phospholipids and neutral lipids was markedly different: over half of the DHA was incorporated into TG and 40% into phospholipids (Fig. 1B), whereas the reverse was observed for AA (Fig. 1C). While similar amounts (nmol/mg Pi) of both fatty acids were incorporated into phospholipids, the incorporation of DHA into TG was 2 times greater than that of AA (Figs. 1D and 1E). In addition, FFA and DG had about 2 and 3 times more DHA compared with AA, respectively. Thus, DHA and AA have different patterns of incorporation into RPE lipids.

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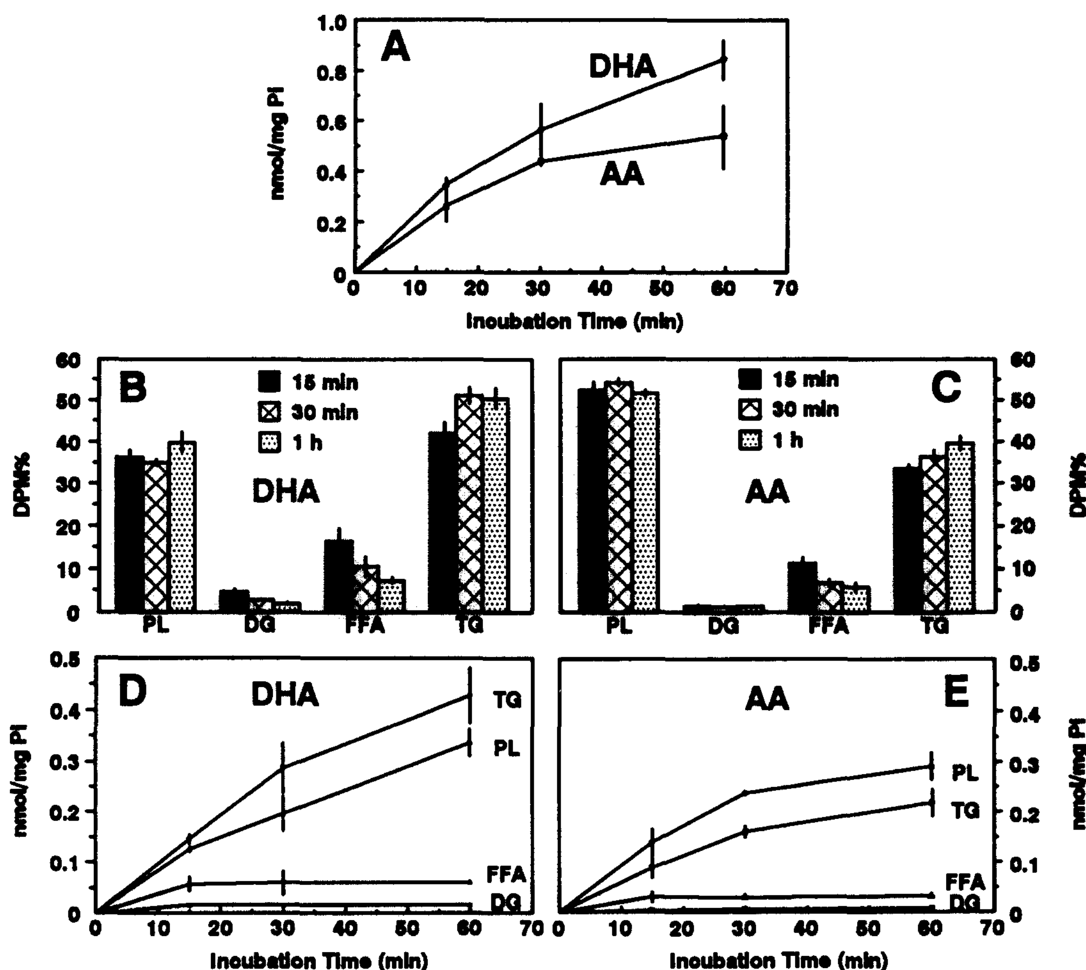

Fig. 1. Uptake and incorporation of [^{3}H]DHA and [^{3}H]AA into frog RPE cells at fatty acid concentration of 60 nM. Freshly prepared RPE-eyecups were incubated continuously with labeled DHA or labeled AA for up to 1 h. At the indicated times, RPE cells were isolated for lipid extraction. Aliquots of the extracts were taken for total radioactivity and lipid phosphorus determinations. The remaining lipids were resolved into classes by one-dimensional TLC and individual lipids were scraped for radioactivity counting. Values are means ± SD of three independent determinations of one incubation in which 20 frog eyecups were used. Panel A, the time-course of uptake (nmol/mg lipid phosphorus) of DHA and AA into total RPE lipids. Panel B, the relative percentage of DHA label in phospholipids (PL), diglycerides (DG), free fatty acids (FFA), and triglycerides (TG). Panel C, the relative percentage of AA label in the four lipid classes. Panel D, the time-course of incorporation (nmol/mg lipid phosphorus) of DHA into the four lipid classes. Panel E, the time-course of incorporation (nmol/mg lipid phosphorus) of AA into the four lipid classes.

Concentration-dependent uptake and incorporation of DHA and AA

In short-time (15 min) incubations, a linear increase in uptake was observed for both DHA and AA between 30 and 120 nM, with the slightly greater uptake rate for DHA than for AA (**Fig. 2A**). The profile of incorporation was unchanged for both fatty acids within the 4-fold concentration range: the highest labeling was in TG (>40%) for DHA and phospholipids (>50%) for AA (Figs. 2B and 2C). When the amount (nmol/mg Pi/15 min) of label in each lipid class was determined, all neutral lipids (TG, FFA, and DG) had 2–3 times more DHA, while phospholipids had slightly less DHA, than AA (Figs. 2D and 2E). These results once again indicate differences in the incorporation of DHA and AA into RPE cellular lipids.

Interaction of DHA and AA in uptake and incorporation

Fig. 3 shows the effects of unlabeled DHA or AA on the uptake and incorporation of labeled AA or DHA, respectively, into RPE cells. When unlabeled AA (60 and 600 nM) was coincubated with 60 nM labeled DHA, the amount of DHA taken up by RPE cells was identical to the control where AA was absent in the incubation (Fig. 3A). Likewise, unlabeled DHA had no observable effect on the uptake of labeled AA (Fig. 3B). Furthermore, for both DHA and AA, the relative distribution of radioactivity (DPM%) among phospholipids, DG, FFA, and TG was not significantly altered by the presence of the unlabeled fatty acids, except a slightly lower percentage of radioactivity in FFA when the concentration of unlabeled

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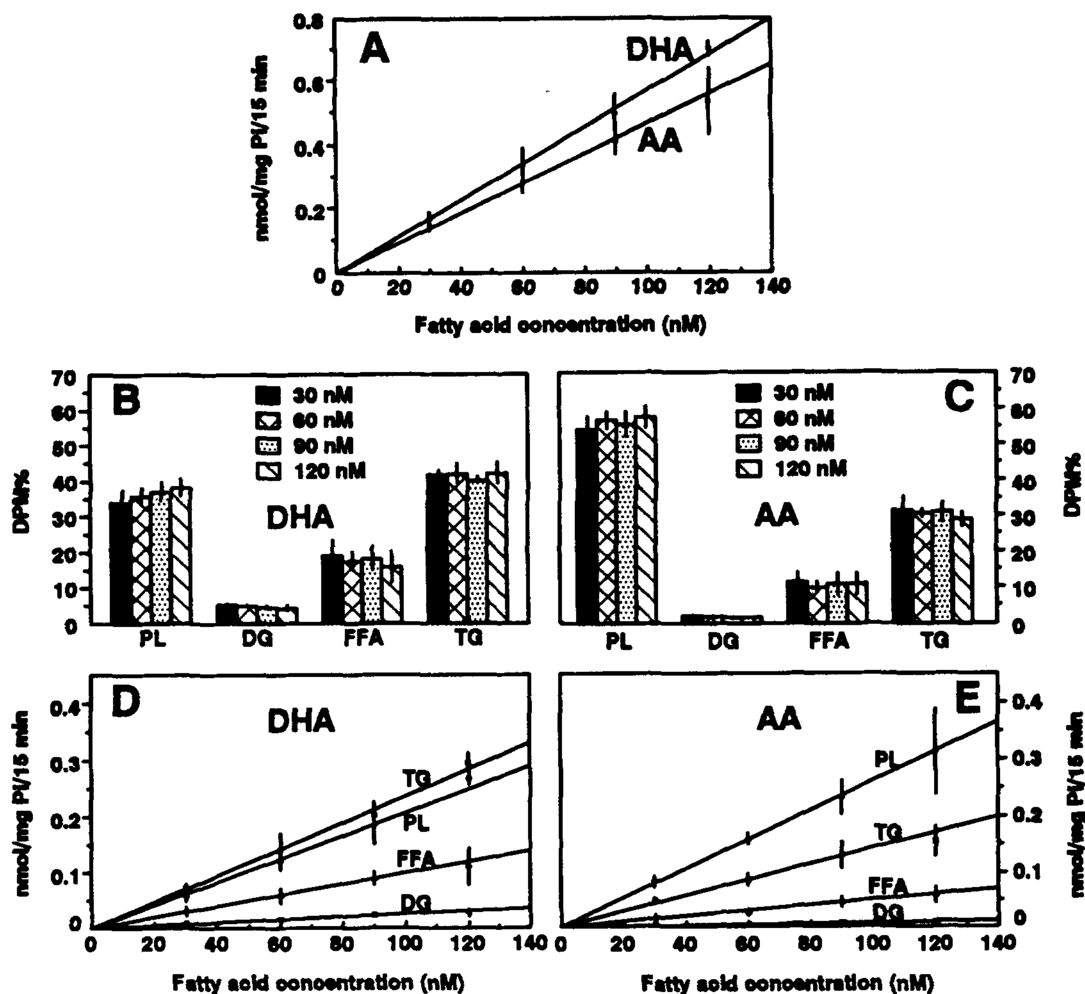

Fig. 2. Concentration-dependent uptake and incorporation of [^{3}H]DHA and [^{3}H]AA into frog RPE cells at the range of 30–120 nM. Freshly prepared RPE-eyecups were incubated with labeled DHA or labeled AA for 15 min. At the end of the incubation period, RPE cells were isolated for lipid extraction. Aliquots of the extracts were taken for total radioactivity and lipid phosphorus determinations. The remaining lipids were resolved into classes by one-dimensional TLC and individual lipids were scraped for radioactivity counting. Values are means ± SD of three independent determinations of one incubation in which 20 frog eyecups were used. Panel A, the concentration-dependent uptake (nmol/mg lipid phosphorus/15 min) of DHA and AA into total RPE lipids. Panel B, the relative percentage of DHA label in PL, DG, FFA, and TG. Panel C, the relative percentage of AA label in the four lipid classes. Panel D, the concentration-dependent incorporation (nmol/mg lipid phosphorus/15 min) of DHA into the four lipid classes. Panel E, the concentration-dependent incorporation (nmol/mg lipid phosphorus/15 min) of AA into the four lipid classes.

fatty acid was 10 times higher than the labeled acid (Figs. 3C and 3D). Thus, there appears to be no interaction between DHA and AA for uptake and incorporation into RPE lipids under our experimental conditions.

Glycerol labeling of frog RPE lipids

To study the de novo synthesis and turnover of glycerolipids, RPE cells were labeled with [2-^{3}H]glycerol for up to 1 h, or pulsed for 1 h followed by a chase for up to 7 h. The glycerol radioactivity of each glycerolipid was determined by counting the aqueous phase of the saponification mixture. As this radioactivity could be partially lost and/or contaminated by the label from the fatty acids, duplicate controls were carried out in which [^{3}H]glycerol was excluded from the incubation and RPE cells were labeled only with [^{3}H]DHA and [^{3}H]AA for 1 h. The aqueous and lipid phases of each saponified glycerolipid were counted separately and the ratio (aqueous/lipid) of radioactivity for each glycerolipid was calculated and found to be low (approximately 5%). This value was used for correcting the [^{3}H]glycerol radioactivity obtained directly from the measurement of the aqueous labeling. The mass of each glycerolipid was determined by HPLC quantitation of fatty acid phenacyl esters. The recovery of internal standard (17:0) in almost all cases was over 95%. The specific activity (dpm/nmol) of [^{3}H]glycerol for each glycerolipid was obtained by dividing the corrected radioactivity by the calculated original mass.

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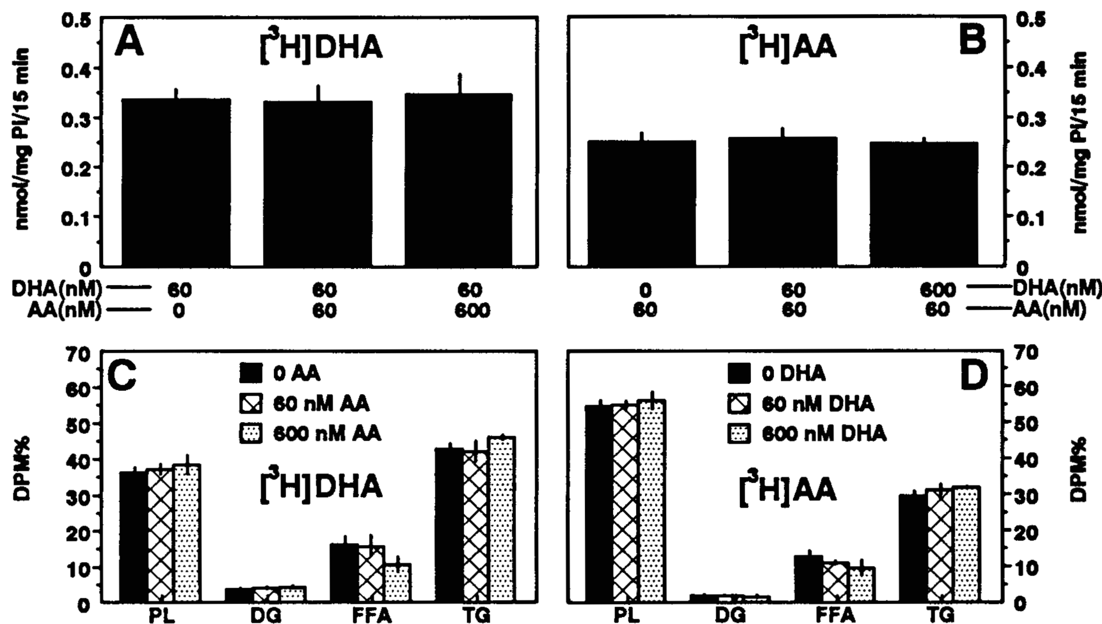

Fig. 3. Interaction between DHA and AA in uptake and incorporation into frog RPE cells. Freshly prepared RPE-eyecups were incubated with 60 nM labeled DHA or 60 nM labeled AA in the presence of 0, 60, and 600 nM unlabeled AA (for [3H]DHA labeling) or DHA (for [3H]AA labeling) for 15 min. At the end of the incubation period, RPE cells were isolated for lipid extraction. Aliquots of the extracts were taken for total radioactivity and lipid phosphorus determinations. The remaining lipids were resolved into classes by one-dimensional TLC and individual lipids were scraped for radioactivity counting. Values are means ± SD of three independent determinations of one incubation in which 20 frog eyecups were used. Panel A, the effects of unlabeled AA on [3H]DHA uptake (nmol/mg lipid phosphorus/15 min) into total RPE lipids. Panel B, the effects of unlabeled DHA on [3H]AA uptake (nmol/mg lipid phosphorus/15 min) into total RPE lipids. Panel C, the relative percentage of DHA label in PL, DG, FFA, and TG at the three concentrations of AA. Panel D, the relative percentage of AA label in the four lipid classes at the three concentrations of DHA.

The [3H]glycerol was rapidly incorporated into all of the glycerolipids (**Fig. 4A**). The highest specific activity during the pulse was attained by PA, followed by neutral glycerolipids (DG and TG); PE, PC, and PI had a similar value which was about one order of magnitude lower than PA, DG, and TG. The [3H]glycerol specific activities of glycerolipids are compatible with the well-established metabolic linkage of PA to DG and then to TG or phospholipids (PE, PC, and PS), and reflect these precursor-product relationships. As anticipated, the specific activities of [3H]glycerol steadily increased in all glycerolipids during the 1 h of continuous pulse and declined during the chase period. Within the cold chase, PA disclosed an exceedingly rapid turnover. Both DG and TG turned over more slowly than PA and reached the same specific activity which was the highest among all glycerolipids in this period. Interestingly, the specific activity of [3H]glycerol was increasing in TG during the initial 1 h of the chase, while that in PA was dramatically falling, demonstrating a rapid flux of glycerol label from PA through DG to TG.

The relative incorporation of [3H]glycerol into various glycerolipids during the 8-h incubation is depicted in Fig. 4B. Initially, 75% of the label was evenly distributed among PA, TG, and PC. With time, there was a rapid and remarkable reduction of relative labeling in PA and a concomitant increase in TG, with the most dramatic change occurring during the first hour of the chase. The percentage of labeling in other glycerolipids fluctuated, but the changes were much smaller than those in PA and TG. By the end of the incubation, the predominant portion of glycerol label (>50%) was present in TG, in proportions much greater than the relative TG mass (10% of the total lipids). PC, DG, and PE contained 20%, 11%, and 10% of the label, respectively, whereas PI and PS had less than 5%.

DHA labeling of frog RPE lipids

The specific activity (dpm/nmol) of [3H]DHA in each glycerolipid was calculated by dividing the radioactivity by the mass of DHA, both of which were determined by reverse-phase HPLC. The time-course of specific activities of DHA in various glycerolipids is illustrated in **Fig. 5A.** The highest specific activity was in PA during the first 30-min incubation, followed by PI, DG, and TG, which had similar values; PE and PC had much lower values, while PS was the lowest. The specific activity of [3H]DHA in PI increased rapidly, and its value was greater than its metabolic precursor (PA) by the end of the 1-h pulse and remained higher throughout the chase period. It is noteworthy that the specific activity of [3H]glycerol for PI was much lower than PA, DG, and TG

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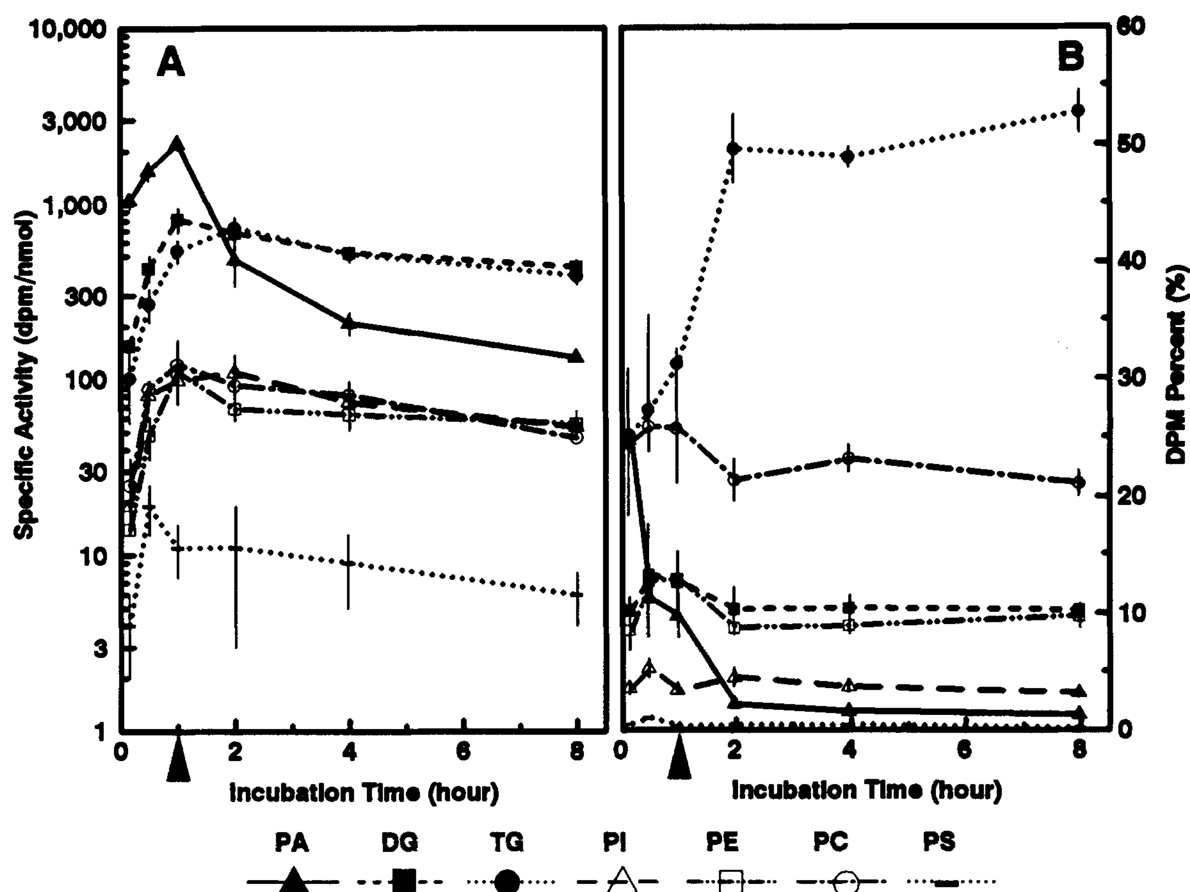

Fig. 4. Labeling of RPE glycerolipids with [^{3}H]glycerol as a function of incubation time. Freshly prepared RPE-eyecups were incubated with 50 μCi [^{3}H]glycerol (16.7 μM) and labeled fatty acids for up to 1 h, or pulsed for 1 h followed by a cold chase for up to 7 h in the presence of 167 μM unlabeled glycerol. At the indicated times, RPE cells were prepared and extracted lipids were resolved into classes by two-dimensional, 3-step TLC, and saponified with 2% KOH. The [^{3}H]glycerol radioactivity of each glycerolipid was determined by counting the aqueous phase of the saponification mixture and correcting for contamination by the labeled fatty acids. The correction factor was estimated by comparing the radioactivity of the aqueous phase to that of the lipid phase of individual saponified glycerolipids from the RPE cells which were incubated only with labeled DHA and AA for 1 h. The mass of each glycerolipid was determined by HPLC analysis of fatty acid phenacyl esters using 17:0 as an internal standard. Values are means ± SD of three independent incubations, except for the 10-min time point (n = 2). Twenty eyecups were used for each incubation. Panel A contains the specific activities and panel B illustrates the relative distribution of the label.

during the 8-h incubation period (Fig. 4A). As observed with the [^{3}H]glycerol labeling, the specific activity of [^{3}H]DHA in PA declined rapidly during the chase, especially within the first 2 h; the specific activities in DG and TG declined more slowly than PA and reached the same value. Interestingly, PE and PC had specific activities of [^{3}H]DHA not significantly different from each other during the 8-h incubation.

When the specific activity profiles of [^{3}H]glycerol and [^{3}H]DHA were compared (Figs. 4A and 5A), a striking similarity was found in all glycerolipids, except for PI. These data suggest that the incorporation and turnover of [^{3}H]DHA in most RPE glycerolipids follow the de novo pathway, whereas deacylation-reacylation reactions contribute to a great extent to the incorporation of [^{3}H]DHA into PI.

The relative distribution of [^{3}H]DHA radioactivity among glycerolipids of frog RPE cells is presented in Fig. 5B. A greater percentage of DHA labeling was incorporated into TG than into any other glycerolipids. A rapid and dramatic increase in the proportion of labeling was observed in TG within the initial 30 min of the pulse, when the proportion of labeling was rapidly declining in FFA (data not shown) and to a lesser extent, in PA. The percentage of DHA labeling in PE and PC appeared to increase during the 1-h pulse, yet the total change was much smaller than that found in TG. Relatively, little DHA label was incorporated into DG, PI, or PS throughout the incubation. By the end of the 8-h incubation, TG had 55% DHA labeling, while phospholipids (PC, PE, PI, and PS) had a total of 32%.

AA labeling of frog RPE lipids

In contrast to [^{3}H]DHA, [^{3}H]AA exhibited a different labeling pattern (**Fig. 6A**). Except at 10 min when they shared the same specific activity with PA, TG had the

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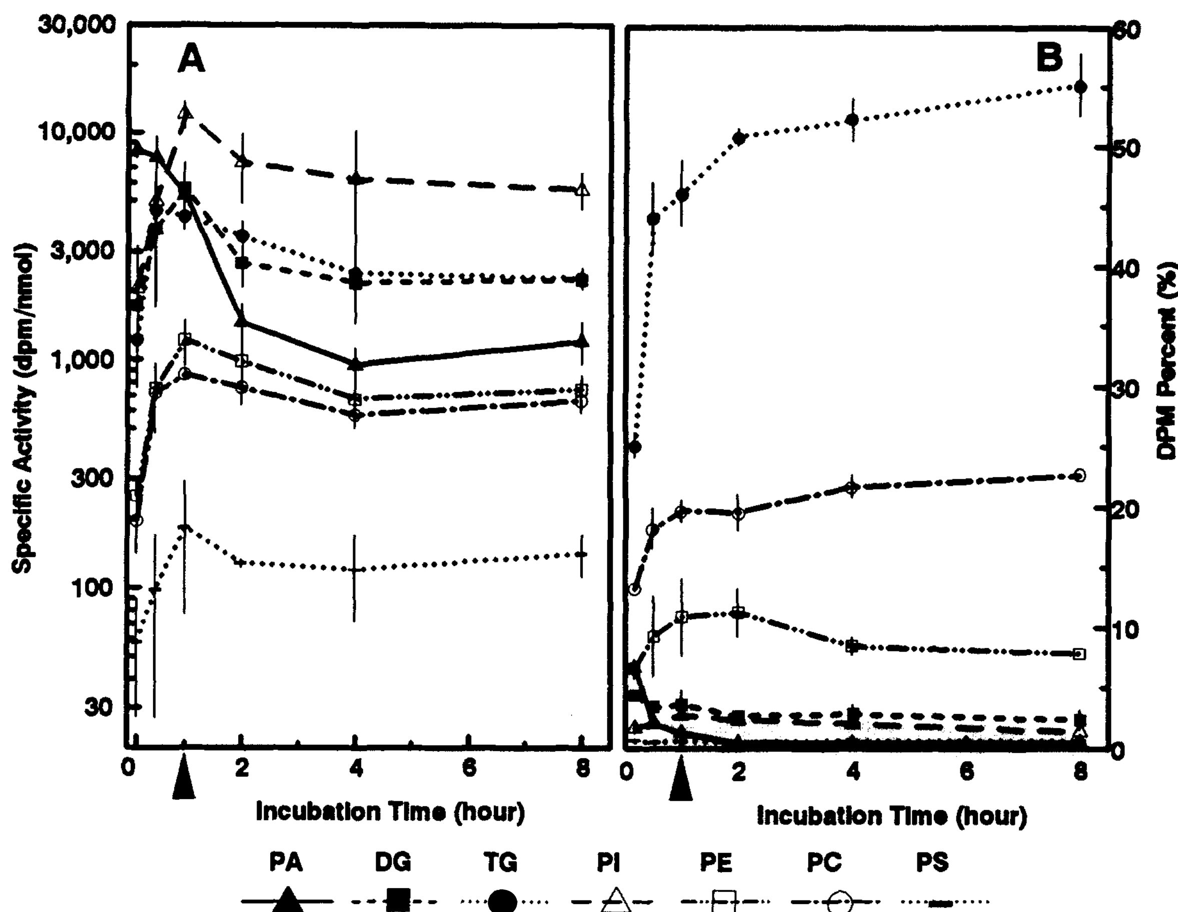

Fig. 5. Labeling of RPE glycerolipids with [³H]DHA as a function of incubation time. RPE-eyecups were freshly prepared and incubated with 6 μCi [³H]DHA (83 nM) and other labeled compounds for up to 1 h, or pulsed for 1 h followed by a chase period for up to 7 h in the presence of 16.7 μM unlabeled DHA. The lipids of RPE cells isolated after the incubations were separated by two-dimensional, 3-step TLC and saponified with 2% KOH. The released fatty acids of each glycerolipid, after phenacyl derivatization, were separated and quantitated by reverse-phase HPLC as described under Experimental Procedures. The specific activity of [³H]DHA was determined by dividing the radioactivity by the mass of DHA. Thus, the specific activities refer to [³H]DHA dpm/nmol DHA. Results are expressed as means ± SD of three independent incubations, except for the 10-min time point (n = 2). Each incubation contained 20 frog eyecups. Panel A contains the specific activities and panel B illustrates the relative distribution of the label.

highest specific activity of [³H]AA at all time points, which was much higher than their de novo precursors (PA and DG). Similarly, DG had a specific activity of [³H]AA higher than PA after the initial 10 min of the pulse. All of the measured phospholipids (PI, PE, PC, PS) showed similar values which were only slightly lower than PA.

The relative distribution of [³H]AA radioactivity among glycerolipids was also markedly different from that of [³H]DHA (Fig. 6B). A much smaller percentage of AA labeling was in PA (<2%) at 10 min, compared with the DHA labeling (7%). Although a relatively large percentage of labeled AA was incorporated into TG by the end of the incubation, this level (38%) was lower than [³H]DHA (55%). However, the relative AA labeling in PI (10-15%) was much greater than [³H]DHA (2%). The proportions of AA labeling in PC, PE, PI, and PS by 8 h were 26%, 16%, 10%, and 2% (total 54%), respectively, which was greater than that of DHA labeling in these phospholipids (total 32%).

Comparison of de novo synthesis and deacylation-reacylation reactions

To evaluate the contribution of de novo synthesis and deacylation-reacylation reactions in the incorporation of DHA and AA into RPE glycerolipids, the ratios of specific activity of labeled fatty acids to labeled glycerol were calculated (**Table 2**). These ratios represent amounts of labeled fatty acids incorporated through de novo synthesis and/or deacylation-reacylation reactions relative to that of the labeled glycerol incorporated by de novo synthesis. Ratios higher than that of PA are indicative of transacylation reactions in the incorporation of fatty acids into glycerolipids. When the ratios of [³H]DHA to [³H]glycerol were compared, all glycerolipids showed

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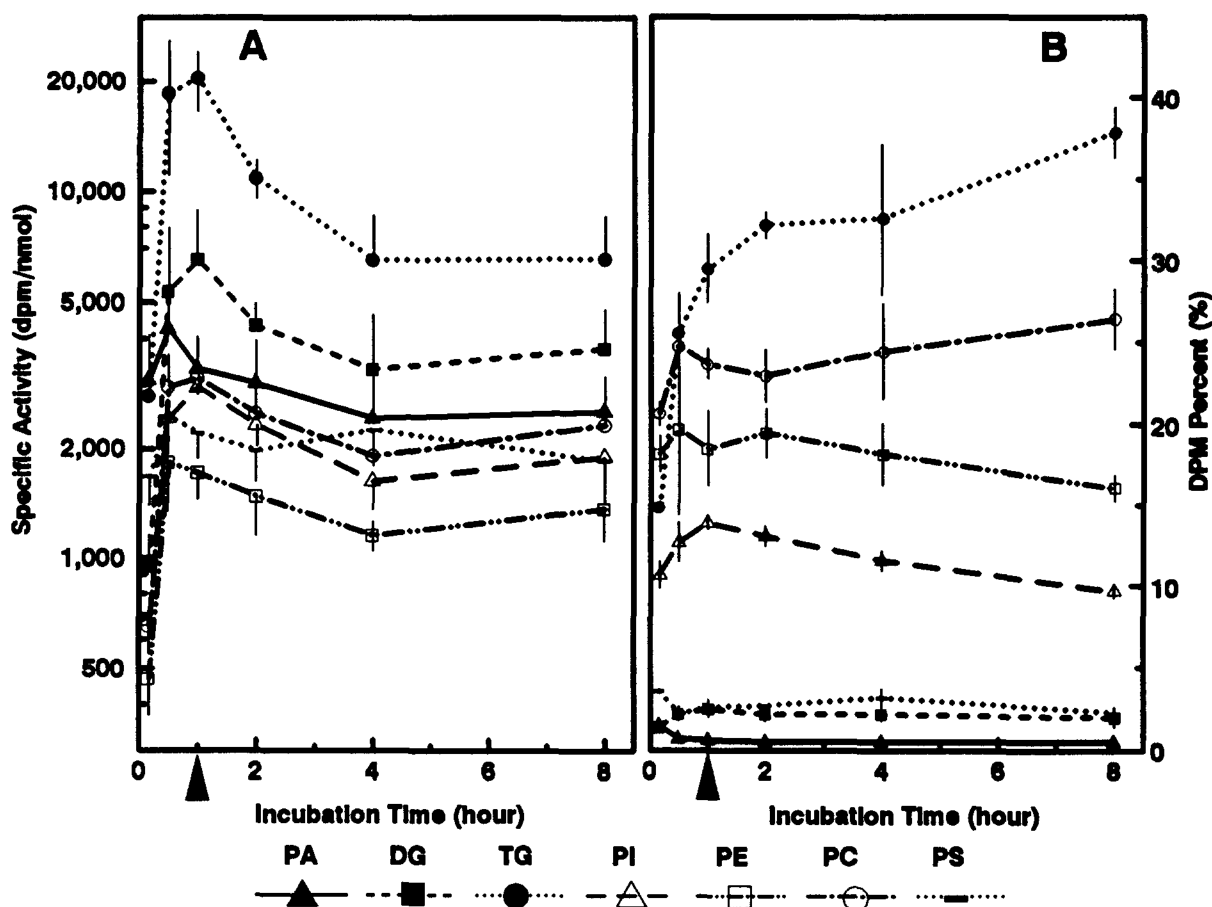

Fig. 6. Labeling of RPE glycerolipids with [^{3}H]AA as a function of incubation time. RPE-eyecups were freshly prepared and incubated with 7 μCi [^{3}H]AA (30 nM) and other labeled compounds for up to 1 h, or pulsed for 1 h followed by a chase for up to 7 h in the presence of 4.2 μM unlabeled AA. The lipids of RPE cells prepared at the end of the incubation periods were separated by two-dimensional, 3-step TLC and saponified with 2% KOH. The released fatty acids of each glycerolipid, after phenacyl derivatization, were separated and quantitated by reverse-phase HPLC as described under Experimental Procedures. The specific activity of [^{3}H]AA was determined by dividing the radioactivity by the mass of AA. Thus, the specific activities refer to [^{3}H]AA dpm/nmol AA. Results are expressed as means ± SD of three independent incubations, except for the 10 min time point (n = 2). Each incubation contained 20 frog eyecups. Panel A contains the specific activities and panel B illustrates the relative distribution of the label.

similar ratios, except for PI which had a much higher value. These data confirmed the incorporation of DHA into glycerolipids (except PI) by a de novo pathway. When the ratios of [^{3}H]AA to [^{3}H]glycerol were compared, higher values appeared in TG, PI, PE, PC, and PS than in PA. Therefore, the acylation of AA into these glycerolipids is, to a great extent, through deacylation–reacylation reactions.

Comparison of DHA and AA incorporation into RPE glycerolipids

Fig. 7 illustrates the dpm ratios of DHA/AA for various lipids from RPE cells incubated for up to 8 h. This ratio was relatively constant in the FFA pool. However, PA had a ratio strikingly higher than FFA and other lipids during the pulse; DG was lower than PA, but higher than the remaining lipids at 10 min. These data support the notion that DHA is more favorably used for de novo synthesis compared to AA. The dpm ratio of DHA/AA was much higher in TG (around 0.7) than in phospholipids (PE, PC, PS, and PI; average value 0.2) and remained the highest during the chase, confirming the preferential incorporation of DHA into RPE TG.

DISCUSSION

Docosahexaenoic acid is the major end product of the n−3 essential fatty acid family and is present in very large amounts in the retina, especially in rod photoreceptor disc membranes (1–3). Unlike DHA, arachidonic acid, the major end product of elongation and desaturation of n−6 PUFAs, is present in low levels in the retina, although it is widely distributed in relatively large amounts in many other tissues (2). The role of the RPE in the selective enrichment of DHA in the retina has been explored

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TABLE 2. Ratios of specific activity of labeled fatty acids to labeled glycerol in frog RPE glycerolipids

A: Ratio of specific activity of [^{3}H]DHA (dpm/nmol) to [^{3}H]glycerol (dpm/nmol)

Lipid	10 min	30 min	1 h	1 + 1 h	1 + 3 h	1 + 7 h
PA	8 ± 1	5 ± 1	2 ± 0	3 ± 1	5 ± 0	9 ± 2
DG	11 ± 1	9 ± 1	7 ± 1	4 ± 1	4 ± 1	5 ± 1
TG	12 ± 1	17 ± 1	8 ± 1	5 ± 1	5 ± 0	6 ± 0
PI	108 ± 4	61 ± 5	123 ± 18	68 ± 11	84 ± 11	103 ± 14
PE	18 ± 4	16 ± 3	11 ± 2	14 ± 3	11 ± 0	13 ± 1
PC	8 ± 1	8 ± 3	7 ± 3	8 ± 1	7 ± 0	14 ± 1
PS	10 ± 3	5 ± 3	16 ± 2	12 ± 4	13 ± 5	23 ± 8

B: Ratio of specific activity of [^{3}H]AA (dpm/nmol) to [^{3}H]glycerol (dpm/nmol)

Lipid	10 min	30 min	1 h	1 + 1 h	1 + 3 h	1 + 7 h
PA	3 ± 1	3 ± 1	2 ± 0	6 ± 1	11 ± 2	9 ± 2
DG	6 ± 1	9 ± 1	8 ± 2	8 ± 1	8 ± 2	9 ± 3
TG	28 ± 2	51 ± 4	38 ± 4	21 ± 2	12 ± 1	19 ± 3
PI	35 ± 2	26 ± 2	30 ± 3	31 ± 3	23 ± 4	36 ± 5
PE	35 ± 5	42 ± 6	16 ± 2	22 ± 3	18 ± 3	24 ± 0
PC	26 ± 7	30 ± 8	26 ± 6	31 ± 8	24 ± 3	45 ± 5
PS	419 ± 15	125 ± 17	206 ± 18	180 ± 25	250 ± 17	309 ± 18

Frog RPE-eyecups were freshly prepared and incubated for 10 min, 30 min, and 1 h with [^{3}H]glycerol (16.7 μM), [^{3}H]DHA (83 nM), and [^{3}H]AA (30 nM) together, or pulsed for 1 h and then chased for 1 h, 3 h, and 7 h. At the indicated times, RPE cells were isolated and lipids were extracted and resolved into classes by two-dimensional, 3-step TLC. Individual lipids were saponified with 2% KOH and the resulting free fatty acids, after phenacyl derivatization, were analyzed by HPLC as described under Experimental Procedures. The specific activities of [^{3}H]glycerol, [^{3}H]DHA, and [^{3}H]AA in glycerolipids were determined independently (see Figs. 4A, 5A, and 6A), and the ratios of specific activity of labeled fatty acids to labeled glycerol were calculated. Results are means ± SD of three independent incubations, except for the 10-min time point (n = 2). Each incubation contained 20 frog eyecups.

in the present study by comparing the metabolism of DHA and AA in this cell. We have found two major differences. First, there is a quantitative difference in the incorporation of these two PUFAs into RPE cellular lipids. This is especially evident when the incorporation is expressed as an absolute amount (Figs. 1D, 1E, 2D, and 2E). DHA was more favorably used for the synthesis of neutral lipids (DG and TG) than AA when the concentration of these two fatty acids in the incubation medium was the same. Second, there is a qualitative difference in the incorporation mechanism employed by the RPE. DHA absorbed by RPE cells is incorporated primarily through de novo synthesis, whereas AA is esterified by acyl exchange reactions. This conclusion was reached by comparing the specific activity of DHA or AA with glycerol (Figs. 4-6 and Table 2) and by the DHA/AA ratios in PA and DG (Fig. 7).

RPE cells are engaged in the phagocytosis and degradation of shed photoreceptor outer segment membranes (15, 16). These membranes contain a large amount of DHA (28, 29) which is recycled by the RPE back to the retina for the regeneration of new photoreceptor membranes. The processing of phagocytized outer segment membranes must be rapid as photoreceptor shedding occurs every day. The efficient recycling of DHA from the RPE to the retina constitutes the major mechanism for DHA conservation in the retina (26-31). Nevertheless, the detailed aspects of DHA metabolism in the RPE and its subsequent trafficking through the interphotoreceptor matrix have not yet been defined.

Our findings provide some insight into these issues. According to our results, it appears that DHA, released from phagocytized photoreceptor outer segment membranes by RPE endogenous lysosomes, is rapidly incorporated into PA, most of which is destined for TG through the de novo synthetic pathway. AA, a minor component of rod outer segment membranes, is incorporated primarily into phospholipids and to a relatively lesser extent into TG, mainly by exchanging with the fatty acids already present in these lipid fractions. Because of the active metabolism of TG (Fig. 4 and ref. 39), we expect that other fatty acids would also be incorporated into RPE TG. As a result, a large accumulation of TG as cytoplasmic lipid droplets would occur after photoreceptor shedding, which should correlate with the degradation of phagocytized outer segment membranes. This prediction assumes that free fatty acids are released during the phagolysosomal degradation. Baker et al. (40) have reported that the number of oil droplets (TG as the major component) in rat RPE is dramatically increased after rod photoreceptor shedding and the change is strongly correlated with the clearance of large phagosomes. Similarly, autoradiographic studies of Gordon, Rodriguez de Turco, and Bazan (41) following labeled DHA injection showed that the radioactivity in oil

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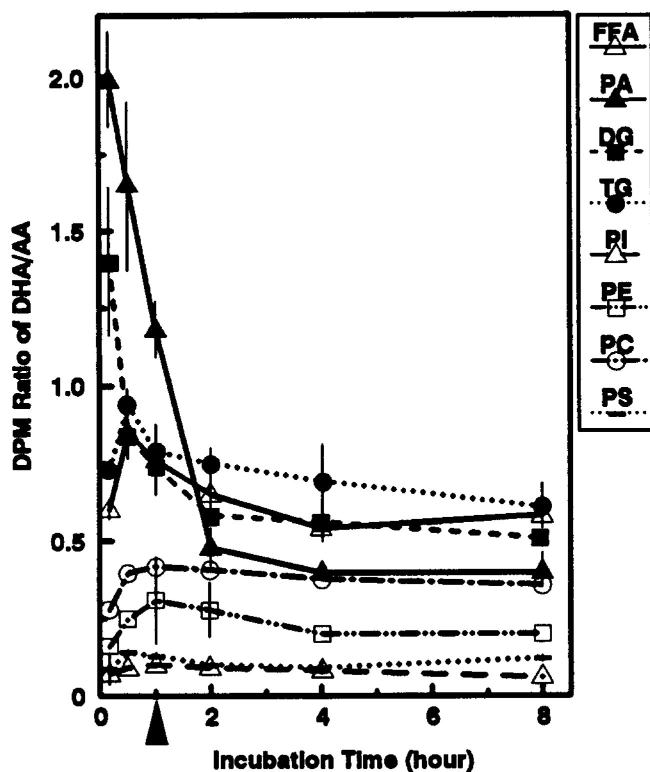

Fig. 7. Ratios (dpm) of DHA/AA in RPE lipids as a function of incubation time. RPE-eyecups were incubated with 83 nM [³H]DHA and 30 nM [³H]AA for up to 1 h, or pulsed for 1 h followed by a cold chase for up to 7 h in the presence of 16.7 μM unlabeled DHA and 4.2 μM unlabeled AA. The lipids of RPE cells were isolated and separated by two-dimensional, 3-step TLC, and saponified with 2% KOH. The released fatty acids, after phenacyl derivatization, were separated and quantitated by reverse-phase HPLC. Fractions of DHA and AA eluted from the HPLC column, were collected separately and counted for radioactivity. Results are expressed as means ± SD of three independent incubations, except for the 10-min time point (n = 2). Each incubation contained 20 frog eyecups.

droplets of frog RPE increased following rod outer segment shedding. By incubating frog RPE cells with [³H]DHA- and [³H]AA-labeled rod outer segment membranes for 2 h, followed by a chase for up to 8 h, we found that, although DHA and AA did not label rod outer segment TG, the percentage of DHA and AA labeling in RPE TG dramatically increased with incubation time (42). These observations agree with our biochemical data and support the physiological relevance of our present findings.

The storage of rod outer segment DHA in TG as oil droplets, as discussed above, supports the significance of the de novo synthetic pathway for the DHA incorporation into RPE lipids. The synthesis proceeding de novo, in contrast to deacylation-reacylation, results in a net increase in RPE cellular lipids, which can accommodate the large amounts of fatty acids (primarily DHA) occurring during the phagolysosomal degradation of shed photoreceptor membranes. TG droplets are relatively inert, variable in size and number, and metabolically labile, and serve as a fatty acid reservoir in numerous cells (43). Thus, RPE cells can store DHA derived from shed rod outer segments in TG that are made by de novo synthesis. Although AA appears also to be transferred from the rod outer segment membranes to RPE TG, the incorporation is limited and appears to be mediated through a different mechanism (Figs. 1 and 6).

Due to the lack of storage of fatty acids (esterified into TG), the retina requires a constant DHA supply (3). The transient storage of DHA derived from the digestion of daily shed photoreceptor membranes by RPE cells as TG droplets conserves this important retinal component from oxidation or loss into the circulation. This allows the RPE cells to gradually release DHA and to maintain a ready supply for photoreceptor membrane synthesis. TG DHA can be shipped out of RPE cells readily, as cellular TG are generally synthesized and degraded at a faster rate than phospholipids (43-48). A rapid disappearance of TG droplets after the removal of exogenous fatty acids has been demonstrated in fibroblasts (48). Oil droplets of rat RPE are also metabolized rapidly in response to rod outer segment shedding (40).

How is DHA maintained at a high level in the retina? Our present results indicate that RPE cells can selectively enrich DHA over AA. When these two fatty acids are presented at the same concentration to RPE cells, DHA is more favorably used for the synthesis of neutral lipids (Figs. 1, 2, and 7). Consistently, TG of frog RPE are more enriched in DHA than in AA in the steady state (Table 1 and ref. 32). This enrichment is small (2-3-fold) relative to the difference found in rod outer segments (10-fold). However, the extent of DHA enrichment by RPE cells increases with incubation time (Fig. 1). In addition, it should be kept in mind that RPE cells phagocytize daily a large amount of membranes (rod outer segments) that are already highly enriched in DHA (28, 29). Thus, even a small selectivity towards the DHA enrichment by the RPE can profoundly facilitate the maintenance of the unique photoreceptor fatty acid profile.

The recycling of DHA between the RPE and the retina appears to be the major route for DHA conservation in the retina (26-31). Stinson, Wiegand, and Anderson (27) followed the radioactivity of [³H]DHA injected intravitreally and observed a 19-day half-life of turnover in rod outer segment phospholipids from rats fed linseed oil (53% 18:3n-3, 20% 18:2n-6), but no apparent DHA turnover in rats fed safflower oil (83% 18:2n-6 and <1% 18:3n-3). As the turnover time for rat rod outer segment membranes is known to be 9-10 days (17, 26), the slow or no apparent turnover of DHA radioactivity in rat rod outer segments suggest that this PUFA is recycled by the RPE after the daily shedding under n-3 deficiency as well as n-3 enrichment conditions. Of interest, these results also suggest that the amounts of DHA arriving from the

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circulation to the retina, which dilute the pool of labeled DHA in the retina, are surprisingly small even during n-3 supplementation. It is not known at present whether AA derived from rod outer segment membranes is also recycled by the RPE. However, we observed that rod outer segment AA was also incorporated into RPE TG, suggesting that this PUFA is also conserved by the RPE.

In summary, we have found that DHA and AA, the major components of two essential fatty acid families, are metabolized through different mechanisms in the RPE. This distinct metabolic behavior relates directly to the daily processing of PUFAs derived from shed photoreceptor membranes and may be partly responsible for the conservation of DHA by the RPE and for the selective enrichment of DHA in the retina.

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