Unique molecular species of phosphatidylcholine containing very-long-chain (C_{24} – C_{38}) polyenoic fatty acids in rat brain

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Rat brain has been shown to contain polyenoic very-long-chain fatty acids (VLCFA) belonging to the $n-3$ and $n-6$ series with four, five and six double bonds and even-carbon chain lengths from 24 to 38. These fatty acids are almost exclusively located in unusual molecular species of phosphatidylcholine at the $sn-1$ position of the glycerol backbone, whereas saturated, monoenoic and polyenoic fatty acids with less than 24 carbon atoms are present at the $sn-2$ position. Polyenoic VLCFA phosphatidylcholine in neonatal rat brain is enriched with $n-6$ pentaenoic and $n-3$ hexaenoic VLCFA with up to 36 carbon atoms, whereas the corresponding phospholipid in adult rat brain mainly contains $n-6$ tetraenoic and $n-3$ pentaenoic VLCFA with up to 38 carbon atoms. The total amount of polyenoic VLCFA associated with phosphatidylcholine is highest in the brain of immature animals. Polyenoic VLCFA phosphatidylcholine appears to be predominantly confined to nervous tissue in rats, and it is envisaged that this phospholipid is of physiological significance.

INTRODUCTION

Polyenoic fatty acids with carbon chain lengths greater than 22 (very-long-chain fatty acids, VLCFA) have been reported to be present in the retina of several vertebrate species (Avelaño, 1987; Avelaño & Sprecher, 1987), rat testis (Bridges & Coniglio, 1970; Grogan, 1984), mammalian spermatozoa (Poulos *et al.*, 1986a, 1987) and human brain (Poulos *et al.*, 1986b, 1988; Sharp *et al.*, 1987; Poulos, 1989) and to be synthesized by mouse spermatocytes and spermatids (Grogan & Huth, 1983), human endothelial cells (Rosenthal & Hill, 1984) and human skin fibroblasts (Street *et al.*, 1989) in culture.

In bovine retina, the polyenoic VLCFA belong to the $n-3$ and $n-6$ series, with four, five and six double bonds and carbon chain lengths up to 36. These fatty acids are concentrated in unusual dipolyunsaturated molecular species of phosphatidylcholine and are attached to the $sn-1$ position of the glycerol backbone, whereas docosahexaenoic acid ($C_{22:6}$) is mainly esterified at the $sn-2$ position (Avelaño, 1987, 1988; Avelaño & Sprecher, 1987). Polyenoic VLCFA also occur in retinal phosphatidylcholine of the rabbit, rat, chicken, toad and cod (Avelaño, 1987). The polyenoic VLCFA in ram spermatozoa are $n-3$ and $n-6$ tetra-, penta- and hexa-enoic fatty acids, with up to 34 carbon atoms, and are mainly present in novel molecular species of sphingomyelin (Poulos *et al.*, 1986a, 1987). Sharp *et al.* (1987) and Poulos *et al.* (1988) observed that polyenoic VLCFA in the brain of humans with Zellweger's syndrome (a rare inherited disorder characterized ultrastructurally by a deficiency of tissue peroxisomes) are predominantly $n-6$ penta- and hexa-enoic fatty acids with carbon chain lengths up to 38 and are mostly located in cholesterol esters and molecular species of phosphatidylcholine. In

contrast with cholesterol esters, phosphatidylcholine contained the ultra-long-chain polyenoic fatty acids (i.e. 32–38 carbon atoms). The polyenoic VLCFA are restricted to the $sn-1$ position of phosphatidylcholine, whereas the $sn-2$ position is enriched in saturated, monoenoic and polyenoic fatty acids with less than 24 carbon atoms. Polyenoic VLCFA are present in normal neonatal-human brain in lower amounts, and the fatty acid composition differs in that tetra- and penta-enoic fatty acids predominate.

In the present study we conducted an analysis of the polyenoic VLCFA in rat brain. We were curious to know whether the polyenoic VLCFA in this tissue were esterified in a specific lipid molecule and whether their composition and mass varied with age.

EXPERIMENTAL

Animals

Male Porton rats (*Rattus norvegicus*) used in the present study were housed in wire-mesh cages. Neonatal animals were obtained from females of known gestation time. They were given access to a lactating dam for up to 21 days. Weaned animals were provided with a pelleted rat diet (Charlicks, Adelaide, South Australia, Australia) and water *ad libitum*.

Extraction and analysis of lipids from tissues

Rats (aged 1, 16 and 64 days) were killed by decapitation, and the brain, liver, kidneys, lungs, spleen, heart and testes were rapidly excised. Lipids were extracted from whole brain and portions of other tissues (1–4 g) as described by Folch *et al.* (1957). The total lipid extract was applied to a column (2 cm diam.) of silicic

Abbreviations used: VLCFA, very-long-chain fatty acids; FAB, fast-atom bombardment; CAMIKES, collision-activation mass-analysed ion-kinetic-energy spectroscopy; i.d., internal diameter.

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acid (25 mg of lipid/g of silicic acid), and the neutral lipids, glycolipids and phospholipids were eluted with 10 column vol. of chloroform, 40 column vol. of acetone and 10 column vol. of methanol respectively (Christie, 1982). The phospholipid fraction was resolved into non-acidic and acidic components by applying it to a column (12 cm $\times$ 2 cm diam.) of DEAE-cellulose DE 52 (acetate form). Non-acidic phospholipids were eluted with 10 column vol. of chloroform/methanol (1:1, v/v), and acidic phospholipids were eluted with 10 column vol. of chloroform/methanol (1:1, v/v) made 0.05 M with respect to ammonium acetate, to which was added 20 ml of aq. 28% (w/w) NH_3 /litre (Christie, 1982). The neutral lipid and phospholipid fractions were applied to 20 cm $\times$ 20 cm silica-gel-60 t.l.c. plates (E. Merck, Darmstadt, Germany) with a loading of 20 mg of lipid/plate. The chromatograms were developed in hexane/diethyl ether/acetic acid (40:10:1, by vol.) for neutral lipids and chloroform/methanol/water (35:15:2, by vol.) for phospholipids. The lipid zones were located under u.v. light after spraying the plates with 0.2% (w/v) dichlorofluorescein in 95% (v/v) ethanol and were eluted from the silica gel with chloroform/methanol/acetic acid/water (50:39:1:10, by vol.) as described by Arvidson (1968). The resulting extracts were partitioned with aq. 1 M- NH_3 to remove the dye and then washed with methanol/water (1:1, v/v). Identification of the various lipids was based on a comparison of their t.l.c. mobility with that of authentic standards. In the case of molecular species of phosphatidylcholine, additional evidence of identity was obtained by collision-activation mass-analysed ion-kinetic-energy spectroscopy (CAMIKES) of ions produced by fast-atom bombardment (FAB) as described by Easton *et al.* (1988). For these studies mass spectra were measured on a Vacuum Generator ZAB 2HF mass spectrometer operating in the positive-ion FAB mode.

Portions of the lipid samples (0.01–1 mg in 2 ml of chloroform) were evaporated to dryness under N_2 and transesterified with 5 ml of 0.27 M- H_2SO_4 in methanol at 75 °C for 4 h; the resulting fatty acid methyl esters were extracted with 1 ml of water and 2 ml of hexane. In some cases 10 nmol of melissic acid ($\text{C}_{30:0}$) was added as a reference standard. G.l.c. of the fatty acid methyl esters was performed on a Perkin-Elmer Sigma 2B gas chromatograph equipped with a 12 m $\times$ 0.33 mm (i.d.) fused-silica BP-1 column of 1 μm phase thickness (Scientific Glass Engineering Pty. Ltd., Ringwood, Vic., Australia). The average linear velocity of the H_2 carrier gas was 45 cm/s, and the injector and detector temperatures were 250 and 350 °C respectively. The column was initially maintained at 165 °C for 0.5 min, after which the temperature was increased by 4 °C/min to 325 °C and held for 7 min. The peak areas of the various fatty acid methyl esters were determined with a Hewlett-Packard 3390A integrator. The absolute amount of each polyenoic VLCFA recovered after esterification was determined by comparison of the peak area of a particular fatty acid methyl ester relative to that of the known amount of added standard. It should be emphasized that the values obtained are an approximation, since polyenoic VLCFA methyl ester standards were not available to determine their detector response.

For more definitive identification of the fatty acid methyl esters a combined g.l.c.–m.s. technique was employed (Poulos *et al.*, 1986b; Fellenberg *et al.*, 1987).

G.l.c.–m.s. was performed on a JEOL JMS-DX 303 mass spectrometer operating in electron-impact mode and coupled with a Hewlett-Packard 5890 gas chromatograph and a JEOL JMA-DA 5000 data system. Chromatographic separation was achieved with a Scientific Glass Engineering fused-silica BP-1 column (12 m $\times$ 0.22 mm i.d.; 0.25 μm phase thickness) directly inserted into the mass-spectrometer source. The temperature program used was 160–320 °C at 4 °C/min and maintained at 320 °C for 10 min. The injector and ion-source temperatures were 270 and 250 °C respectively, and He gas (1 ml/min) was the carrier. The ionization voltage was 70 eV.

Phospholipase A_2 hydrolysis of molecular species of phosphatidylcholine was carried out by using a commercial preparation of the enzyme (from bee venom). The lipid sample (approx. 50 nmol) was shaken vigorously at room temperature (20–22 °C) for 45 min with 500 ml of water-saturated diethyl ether and 200 μl of 25 mM-Tris/HCl buffer, pH 8.3, containing 5 mM- CaCl_2 and 0.7 unit of enzyme. At the end of the incubation period, 4 ml of chloroform/methanol (2:1, v/v) was added and the mixture evaporated to dryness under N_2 at 40 °C. The residue was dissolved in a small volume of chloroform/methanol (2:1, v/v) and applied as a 3 cm band to a silica-gel-60 t.l.c. plate, which was developed in chloroform/methanol/water (35:15:2, by vol.). The reaction products (1-acyl-*sn*-glycero-3-phosphocholine and non-esterified fatty acid) were eluted, transesterified, and the resulting methyl esters were subjected to g.l.c. as described above. The specificity of the enzyme for the *sn*-2 position was confirmed by using 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine standard as a substrate. Under the incubation conditions described, the products were 1-stearoyl-*sn*-glycero-3-phosphocholine and arachidonic acid, demonstrating that there had been negligible cleavage of the *sn*-1 position by the enzyme.

Chemicals

These were obtained as follows: silicic acid (325 mesh), 2',7'-dichlorofluorescein, phospholipase A_2 (freeze-dried, salt-free powder from bee venom), β -arachidonoyl- γ -stearoyl L- α -phosphatidylcholine from Sigma Chemical Co., St. Louis, MO, U.S.A.; DEAE-cellulose DE 52 from Whatman Bio-Systems Ltd., Maidstone, Kent, U.K.; and melissic acid ($\text{C}_{30:0}$) from Alltech Associates Pty. Ltd., Summer Hill, N.S.W., Australia. All other chemicals were of reagent grade, and solvents were distilled before use.

RESULTS AND DISCUSSION

The polyenoic VLCFA detected in rat brain were predominantly of the *n*–3 and *n*–6 series, with four, five and six double bonds and even-carbon chain lengths up to 38. It should be noted that the polyenoic VLCFA represented a minor component of the total brain fatty acids (less than 1%). Fractionation of rat brain lipids with a combination of silicic acid and DEAE-cellulose DE 52 column chromatography and preparative t.l.c. indicated that the polyenoic VLCFA were mainly present in a non-acidic phospholipid, with trace amounts in cholesterol esters, triacylglycerol, phosphatidic acid and phosphatidylethanolamine. The non-acidic phospholipid migrated with a slightly greater R_f on t.l.c. than the main

phosphatidylcholine zone, which suggested that it was phosphatidylcholine enriched in polyenoic VLCFA (designated 'polyenoic VLCFA phosphatidylcholine'), similar to that described for human brain and bovine retina (Avelaño, 1987; Sharp *et al.*, 1987; Poulos *et al.*, 1988). The spectrum obtained by FAB m.s. analysis of the non-acidic phospholipid contained a strong signal corresponding to an ion at m/z 184. The ion with m/z 184 afforded a CAMIKES mass spectrum containing ions at m/z 60, 86, 125 and 166, which is consistent with the structure of phosphocholine. This provided strong supporting evidence that the lipid was molecular species of phosphatidylcholine.

Phospholipase A₂ hydrolysis of the rat brain polyenoic VLCFA phosphatidylcholine produced two products: a non-esterified fatty acid and a lipid migrating with a slightly greater mobility on t.l.c. than the product formed by hydrolysis of brain phosphatidylcholine containing fatty acids with carbon chain lengths of less than 24, i.e. 1-acyl-*sn*-glycero-3-phosphocholine. Polyenoic VLCFA were found only in the liberated 1-acyl-*sn*-glycero-3-phosphocholine, which indicated that these fatty acids were found to the *sn*-1 position of the glycerol moiety. The major non-esterified fatty acid released from the *sn*-2 position were palmitic acid (C_{16:0}), palmitoleic acid (C_{16:1}), stearic acid (C_{18:0}), oleic acid (C_{18:1}), eicosaenoic acid (C_{20:1}), eicosatrienoic acid (C_{20:3}), arachidonic acid (C_{20:4}), docosatetraenoic acid (C_{22:4}) and docosa-hexaenoic acid (C_{22:6}). The almost exclusive location of polyenoic VLCFA in phosphatidylcholine in rat brain is similar to human brain (Sharp *et al.*, 1987; Poulos *et al.*, 1988) and retina of vertebrates (including rat) (Avelaño, 1987, 1988; Avelaño & Sprecher, 1987). This is in contrast with ram spermatozoa (Poulos *et al.*, 1987) and ram and rat testis (B. S. Robinson, D. W. Johnson &

A. Poulos, unpublished work), in which polyenoic VLCFA are mainly found in sphingomyelin. It was important to establish whether polyenoic VLCFA phosphatidylcholine exists in other rat tissues besides the brain and retina. This phospholipid was undetectable in the liver, kidneys, lungs, spleen and heart of rats. Phosphatidylcholine species containing polyenoic VLCFA of the *n*-6 series with four and five double bonds and odd- and even-carbon chain lengths up to 32 were found to be a very minor component of the rat testis. Thus it seems that polyenoic VLCFA phosphatidylcholine is essentially restricted to neural tissue.

The polyenoic VLCFA composition of molecular species of phosphatidylcholine in rat brain varied with age, as shown in Fig. 1. Polyenoic VLCFA phosphatidylcholine in 1-day-old-rat brain was enriched with *n*-6 pentaenoic and *n*-3 hexaenoic VLCFA with up to 36 carbon atoms, whereas this phospholipid in 64-day-old-rat brain mainly contained *n*-6 tetraenoic and *n*-3 pentaenoic VLCFA with up to 38 carbon atoms. Polyenoic VLCFA phosphatidylcholine in the brain of 16-day-old animals contained *n*-6 tetraenoic and pentaenoic VLCFA and *n*-3 pentaenoic and hexaenoic VLCFA with up to 38 carbon atoms as major components. It is noteworthy that *n*-6 pentaenoic VLCFA were completely absent from phosphatidylcholine in adult rat brain. Hexaenoic VLCFA belonging to the *n*-6 series were only present in the brain phosphatidylcholine of neonatal rats. The polyenoic VLCFA composition of phosphatidylcholine molecular species in the brain of 1-day-old rats (Fig. 1) is similar to that previously reported for the brain of humans aged 4 days to 6 weeks (Sharp *et al.*, 1987; Poulos *et al.*, 1988; Poulos, 1989). Table 1 indicates that the total amount of

Fig. 1. Developmental changes in the polyenoic VLCFA composition of phosphatidylcholine molecular species in rat brain

Polyenoic VLCFA phosphatidylcholine was isolated from the brain of 1-, 16- and 64-day-old rats, and fatty acid methyl esters were subjected to g.l.c. as described in the Experimental section. The chromatograms displayed depict only the VLCFA. Their identity was determined by g.l.c.-m.s. For convenience the original labelling of the peaks has been retained; '24:5 *n*-6', e.g., means 'C_{24:5, n-6}'.

Table 1. Developmental changes in the amount of polyenoic VLCFA associated with phosphatidylcholine molecular species in rat brain

Polyenoic VLCFA phosphatidylcholine was isolated from the brain of 1-, 16- and 64-day-old rats and polyenoic VLCFA methyl esters were subjected to quantitative g.l.c. as described in the Experimental section. Each value represents the amount of polyenoic VLCFA recovered from polyenoic VLCFA phosphatidylcholine and is the mean $\pm$ range of duplicate determinations. Key to superscripts: ^a contains corresponding $n-3$ pentaenoic VLCFA; ^b contains corresponding $n-3$ hexaenoic VLCFA.

Fatty acid (carbon atoms: double bonds, series)	Age (days)...	Amount of polyenoic VLCFA (nmol/g wet wt.)		
		1	16	64
24:4, $n-6$		5.35 $\pm$ 0.10 ^a	16.77 $\pm$ 0.07 ^a	3.44 $\pm$ 0.03 ^a
26:4, $n-6$		0.07 $\pm$ 0.02	0.62 $\pm$ 0.01	0.05 $\pm$ 0.01
28:4, $n-6$		N.D. [†]	0.08 $\pm$ 0	N.D.
32:4, $n-6$		0.03 $\pm$ 0	0.22 $\pm$ 0.01	0.06 $\pm$ 0.01 ^a
34:4, $n-6$		0.72 $\pm$ 0.04	0.97 $\pm$ 0.07 ^a	0.20 $\pm$ 0.02 ^a
36:4, $n-6$		0.12 $\pm$ 0.03	2.02 $\pm$ 0.18 ^a	0.64 $\pm$ 0.06 ^a
38:4, $n-6$		N.D.	0.05 $\pm$ 0.02 ^a	0.05 $\pm$ 0.03 ^a
24:5, $n-6$		16.24 $\pm$ 0.11 ^b	8.82 $\pm$ 0.07 ^b	N.D.
26:5, $n-6$		0.48 $\pm$ 0.01 ^b	0.25 $\pm$ 0.02 ^b	N.D.
28:5, $n-6$		0.12 $\pm$ 0.01 ^b	0.08 $\pm$ 0 ^b	N.D.
30:5, $n-6$		0.04 $\pm$ 0.01 ^b	N.D.	N.D.
32:5, $n-6$		0.60 $\pm$ 0.01 ^b	0.37 $\pm$ 0.02 ^b	N.D.
34:5, $n-6$		1.20 $\pm$ 0.30 ^b	0.67 $\pm$ 0.02 ^b	N.D.
36:5, $n-6$		0.41 $\pm$ 0.03 ^b	1.12 $\pm$ 0.03 ^b	N.D.
38:5, $n-6$		N.D.	0.02 $\pm$ 0.01 ^b	N.D.
26:6, $n-6$		0.02 $\pm$ 0	N.D.	N.D.
32:6, $n-6$		0.01 $\pm$ 0	N.D.	N.D.
34:6, $n-6$		0.03 $\pm$ 0.01	N.D.	N.D.
36:6, $n-6$		0.02 $\pm$ 0.01	N.D.	N.D.
24:6, $n-3$		*	*	2.69 $\pm$ 0.31
26:6, $n-3$		*	*	N.D.
28:6, $n-3$		*	*	N.D.
30:6, $n-3$		*	N.D.	N.D.
32:6, $n-3$		*	*	0.06 $\pm$ 0.01
34:6, $n-3$		*	*	0.05 $\pm$ 0.01
36:6, $n-3$		*	*	0.08 $\pm$ 0.01
38:6, $n-3$		N.D.	*	0.01 $\pm$ 0
Total ...		25.46	32.06	7.33

* Included in the amount of corresponding $n-6$ pentaenoic VLCFA.

† N.D., not detectable.

polyenoic VLCFA associated with phosphatidylcholine was substantially higher in the brain of 1- and 16-day-old rats than in 64-day-old animals. This was mainly due to lower quantities of $n-6$ pentaenoic and $n-3$ hexaenoic VLCFA in the adult. The level of $n-6$ and $n-3$ polyenoic VLCFA with 24 carbon atoms was higher than those with longer carbon chain lengths in brain phosphatidylcholine at all ages. The content of polyenoic VLCFA with 28–30 carbon atoms in phosphatidylcholine species in rat brain was relatively low. The values presented in Table 1 are comparable with those published for human brain by Sharp *et al.* (1987). An adult rat brain was dissected into left and right cerebral hemispheres, brain stem and cerebellum, and the polyenoic VLCFA phosphatidylcholine was analysed. The composition and amount of polyenoic VLCFA in phosphatidylcholine were similar in each section, inferring an even distribution of the phospholipid throughout the entire brain (P. Sharp, B. S. Robinson & A. Poulos, unpublished work).

The polyenoic VLCFA in rat brain are likely to be synthesized by chain elongation and desaturation of shorter-chain $n-3$ and $n-6$ precursors in a manner similar to that reported for bovine retina (Rotstein &

Aveldaño, 1988), rat testicular cells (Grogan, 1984) and human endocytes (Rosenthal & Hill, 1984). Indeed, preliminary experiments have indicated that 28–36-carbon tetraenoic VLCFA and 26–28-carbon pentaenoic VLCFA are labelled from [1-¹⁴C]hexacosatetraenoic acid (C_{26:4, $n-6$}) in the brain of neonatal rats (B. S. Robinson, D. W. Johnson & A. Poulos, unpublished work). The presence of polyenoic VLCFA at the *sn*-1 position of molecular species of phosphatidylcholine in brain is intriguing, because this position is usually occupied by saturated fatty acids (Christie, 1982) and thus suggests the existence of enzymological specificity. The polyenoic VLCFA may be introduced into the phosphatidylcholine molecule via the CDP-choline pathway, methylation of phosphatidylethanolamine, acylation of lysophosphatidylcholine or a base-exchange reaction (Pelech & Vance, 1984). Lipid precursors containing polyenoic VLCFA were not detected in rat brain, presumably owing to rapid turnover, and hence the exact stage at which polyenoic VLCFA are incorporated into phosphatidylcholine is unclear. It is interesting that the $n-6$ pentaenoic VLCFA and $n-6$ and $n-3$ hexaenoic VLCFA content of molecular species of phosphatidyl-

choline dramatically decreased in rat brain with maturation. In adult rat brain these pentaenoic and hexaenoic VLCFA may be synthesized and/or incorporated into phosphatidylcholine at a lower rate than in neonatal rat brain. Conversely, these pentaenoic and hexaenoic VLCFA may be oxidized and/or released from phosphatidylcholine at a higher rate in the brain of the mature animals. Rotstein *et al.* (1987) reported that the levels of $n-3$ very-long-chain hexaenoic fatty acids and their precursor docosahexaenoic acid ($C_{22:6,n-3}$) associated with dipolyunsaturated phosphatidylcholine species in the rat retina decrease with age and this is thought to have a detrimental effect on vision. In this case, there appears to be a decrease in the synthesis of docosahexaenoic acid ($C_{22:6,n-3}$) from docosapentaenoic acid ($C_{22:5,n-3}$) in older rat retina, with a secondary effect on the hexaenoic VLCFA.

The physiological role of polyenoic VLCFA phosphatidylcholine in brain is unknown. Aveldano (1987, 1988) speculated that the polyenoic VLCFA phosphatidylcholine in retina may be needed to make photoreceptor membrane proteins (predominantly rhodopsin) assume certain functional conformations, and hence play a crucial role in the visual process. It is conceivable that the corresponding phospholipid species in brain is also required for the correct orientation of intrinsic membrane proteins. Alternatively, the brain phosphatidylcholine containing polyenoic VLCFA and/or its polyenoic VLCFA breakdown products (non-esterified fatty acids, lysophosphatidylcholine and diacylglycerol) may be metabolically active compounds and important in cell regulation. The function of polyenoic VLCFA phosphatidylcholine may be more significant at a particular stage of brain development. Clearly, further research is necessary to elucidate fully the properties of this unique brain lipid.

We are grateful to Ms. Peta L. Knapman for typing the manuscript. We thank Dr. Christopher Easton of the Department of Organic Chemistry, University of Adelaide, South Australia, for the identification of molecular species of phosphatidylcholine by m.s. B. S. R. was supported by a grant

from the Adelaide Medical Centre for Women and Children Research Trust, and D.W.J. was funded by a grant from the National Health and Medical Research Council of Australia.

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Received 21 August 1989/25 October 1989; accepted 9 November 1989