Molecular Species Composition of Phosphatidylcholines during the Development of the Avian Embryo Brain

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ABSTRACT

A comparative approach has been used to investigate the molecular species composition of phosphatidylcholine (PC) and its age variation throughout several developmental stages of chick and duck embryo brains. The brain PC consist of 15 major molecular species which do not undergo appreciable variation in their relative abundance either during embryonic development or between equivalent stages of maturation in the 2 avian species. In fact, a highly invariable molecular architecture of PC is shown in the developing organ. Molecular species containing saturated or monounsaturated fatty acids were dominant in all stages of development of the avian embryo brain. Among these molecular species, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine accounted for 75-80% of the total PC.

INTRODUCTION

The known influence of phospholipid molecular species on the physical properties of biological membranes and the activity of membrane proteins (1-3) encourages studies on the precise structure of the major phospholipid classes present in order to correlate certain membrane functions with specific lipid compositions. Additionally, systematic studies of developing living systems, which undergo drastic changes in their functional capacities, could be of interest in determining the possible associated lipid compositional changes (4).

Changes in the diet were found to alter the molecular composition of the phosphoglycerides with limited effects on the overall physical properties of the membrane (5,6). In addition, similarities in the molecular species composition of phosphatidylcholine (PC) from homologous tissues of different mammals have been described (7). One is led to believe by these observations that there exists a certain "tissue specificity" in the molecular structure of some major phospholipids (8) which appears to be important in maintaining the functional activity of the tissue.

We describe here the detailed molecular composition of PC from the avian embryonic brain, seeking variation either resulting from development or from differences in avian species.

MATERIALS AND METHODS

Egg incubation, tissue homogenization and lipid extraction were performed as previously described (9). A variable number of embryo brains (50-150 individuals) were pooled according to age.

Isolation of the PC Samples

PCs were obtained directly from the total lipid extract by thin layer chromatography (TLC) on 1.0 mm layers of Silica Gel G (E. Merck A.G., Darmstat, G.F.R.) using chloroform/methanol/water (65:25:4, v/v/v) as the solvent system. Aliquots containing up to 25 mg of total lipids were applied as narrow bands to 20 x 20 cm plates. Bands of PC were detected by spraying the margins of the plates with the Dittmer reagent (10), then scraped off the plates and extracted by successive treatments with 10 vol of chloroform/methanol (2:1, 1:1, 1:2 and 1:9, v/v) and finally methanol. PC isolated by this method appeared as a single spot in 2-dimensional TLC using chloroform/methanol/water (65:25:4, v/v/v) and *n*-butanol/acetic acid/water (60:20:20, v/v/v) as solvent systems.

Conversion of PC into 1,2-Diacyl-3-Acetyl-*sn*-Glycerol

Twenty-five units of phospholipase C (EC 3.1.4.3) from *Clostridium welchii* (Sigma Chemical Co., St. Louis, MO) were dissolved into 8.0 ml of 0.05 M Tris buffer, pH 7.2, and 10.0 ml of 5 mM $CaCl_2$ and washed twice with ethyl-ether to remove lipid contaminants. To this solution were added PC (35 mg) dissolved in freshly distilled ethyl-ether (5.0 ml). The mixture was shaken under N_2 at 29 C for 2 hr, then it was extracted 5 times with ethyl-ether. Combined extracts were evaporated to dryness and weighed. An aliquot containing up to 1 mg of dry extract was analyzed by TLC (0.5 mm thick) using *n*-hexane/ethyl-ether/acetic

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acid (50:50:1, v/v/v) as the solvent system. The 1,2-diacyl-*sn*-glycerol spot was visualized by spraying the plate with a 0.1% Rhodamine-6-G methanol solution or by exposing it to iodine vapor.

The remaining dry extract was acetylated with a 24-fold molar excess of acetic anhydride and 1 vol of anhydrous pyridine at 40 C for 4 min. The reaction was stopped by adding 3.0 ml of anhydrous methanol. Diacyl-glycerol acetates were extracted 5 times with ethyl-ether and the combined extracts were washed with 2 N HCl (2 vol), 2% $NaHCO_3$ (2 vol) and distilled water (3 vol). The organic phase was dried over anhydrous sodium sulfate, concentrated in a rotary evaporator and purified by TLC on 0.5 mm layers of Silica Gel G, using hexane/ethyl-ether/acetic acid (50:50:1, v/v/v) as the solvent system. Bands of diacyl-glycerol acetates were detected with Rhodamine-6-G solution and the 1,2-diacyl,3-acetyl-*sn*-glycerols were scraped off the plate and eluted from the adsorbent with several volumes of chloroform. Initial PC and 1,2-diacyl,3-acetyl-*sn*-glycerols did not show significant differences in their fatty acid compositions (data not shown).

Subfractionation of 1,2-Diacyl,3-Acetyl-*sn*-Glycerol

1,2-Diacyl,3-acetyl-*sn*-glycerols were resolved by argentation ($AgNO_3$-silica gel) TLC on the basis of total number of double bonds per molecule.

Two plates (200 x 200 x 0.5 mm) were prepared as described by Renkonen (11), except that the plates were dried at 120 C for 35 min. The plates were developed using benzene/chloroform/methanol (90:10:1, v/v/v), followed by benzene/chloroform/methanol (90:10:2.5, v/v/v) in order to obtain better resolution of the most highly unsaturated fractions. After development, the plates were sprayed with a 0.05% methanol/water (1:1, v/v) solution of 2',7'-dichlorofluorescein and the different subfractions were located under ultraviolet (UV) light. Those subfractions corresponding to the various degrees of unsaturation were recovered separately by repeated extraction of the gel scrapings with ether. Silver nitrate was eliminated by washing the extracts with 0.1% NaCl solution. The organic extracts were evaporated to dryness in a rotary evaporator after addition of a small amount of methanol to remove the last traces of water. The ratio of the subfractions obtained was determined by gas liquid chromatography (GLC) by adding methyl pentadecanoate (as in internal standard) to the methyl ester derivatives.

Positional Distribution of Fatty Acids in 1,2-Diacyl,3-Acetyl-*sn*-Glycerol Subfractions

The positional distribution of the fatty acids in the 1,2-diacyl,3-acetyl-*sn*-glycerols was determined by hydrolysis with pancreatic lipase (EC 3.1.1.3) from Calbiochem (Los Angeles, CA).

Two to 3 mg of 1,2-diacyl,3-acetyl-*sn*-glycerol acetates were suspended in 1.0 ml of 1 M Tris buffer pH 8.0, 0.25 ml of 2.2% sodium deoxycholate and 0.1 ml of 0.1% $CaCl_2$. The mixture was sonicated for 15 sec in a MSE ultrasonic disintegrator, then 5-6 mg of pancreatic lipase were added and the mixture was incubated for 1 min at 40 C. The reaction was stopped by adding 6 N HCl (3 ml). Free fatty acids and 2-acyl-*sn*-glycerols were recovered by repeated ether extractions and separated by TLC (0.5 mm Silica Gel G layers) with *n*-hexane/ether/acetic acid (50:50:1, v/v/v) as solvent system. Bands were identified using standards, scraped off the plate and directly converted to methyl esters.

Gas Liquid Chromatography

Preparation of the methyl esters and analysis of the fatty acids by GLC were done as described previously (9).

Analytical data represent an average of 2-4 different samples expressed as molar percentages of total fatty acids. Standard deviation for data in Tables I and II and in Figure 1 was no more than 1.9% and 1.0%, respectively, for molar relative abundances higher and lower than 10%.

RESULTS AND DISCUSSION

Fractionation by argentation-TLC of 1,2-diacyl,3-acetyl-*sn*-glycerols derived from PC of chick (13th, 17th and 21st incubation days) and duck embryo brains (17th, 22nd and 28th incubation days) provides (a) the relative abundance (Table I) and fatty acid composition of each fraction, and (b) the positional distribution of acyl groups (GLC data is available from the authors upon request). From these complementary results, the experimental molecular species composition was estimated (Table II).

Five different groups of 1,2-diacyl,3-acetyl-*sn*-glycerol derivatives (Table I) with different degrees of unsaturation were obtained from all the developmental stages considered either in chick or duck embryo brains, i.e., disaturated, mono-, di-, tetra-, and hexaenoic molecules. The relative abundances of all these 1,2-diacyl, 3-acetyl-*sn*-glycerol subfractions remain fairly constant both throughout development and

TABLE I

Molar Relative Abundance of Different Fraction of 1,2-Diacyl,3-Acetyl-*sn*-glycerols Derived from Avian Embryo Brain PC

Molecular classes[a]	Chick (incubation days)			Duck (incubation days)		
	13	12	21	17	22	28
Disaturated	32.1[b]	34.7	29.4	29.4	37.1	35.2
Monoenoic	44.4	46.4	47.7	49.2	46.6	49.0
Dienoic	8.7	8.0	6.6	4.8	4.5	4.0
Tetraenoic	7.9	6.0	7.3	10.6	6.8	7.0
Hexaenoic	6.9	5.0	8.9	5.9	4.9	4.7

[a]Subfractionation of 1,2-diacyl,3-acetyl-*sn*-glycerols is achieved by argentation-TLC on the basis of unsaturation degree (see Methods).

[b]Molar relative abundance of different 1,2-diacyl,3-acetyl-*sn*-glycerol fractions within the whole population from each developmental stage.

TABLE II

Experimental Molecular Species Composition of PC from Developing Avian Embryo Brain[a]

Molecular classes[b]	Chick (incubation days)			Duck (incubation days)		
	13	17	21	17	22	28
16:0/16:0	25.6	30.6	26.8	26.8	32.2	29.1
18:0/16:0	2.6	3.2	1.5	2.6	2.6	4.4
16:1/16:0	3.6	1.8	1.0	1.0	2.0	1.4
18:1/16:0	11.2	13.7	12.6	15.3	19.2	15.4
16:0/18:1[c] (+16:0/16:1)	24.8	27.6	28.9	27.9	22.4	27.3
18:0/16:1 (+18:0/18:1)	2.4	3.4	4.4	4.0	3.8	6.5
16:0/18:2	3.0	2.6	2.2	1.2	1.6	1.2
18:1/18:1 (+18:1/16:1)	2.4	2.6	3.2	3.0	2.6	2.8
16:0/20:4	3.8	3.9	4.0	6.0	3.6	3.9
18:0/20:4	2.0	1.8	2.4	5.4	2.6	2.8
16:0/22:6	3.9	3.9	5.2	3.1	3.4	2.8
18:0/22:6	1.2	1.8	2.2	1.4	1.2	1.2
Total accounted (Σ)[d]	86.50	96.90	94.40	97.70	97.20	98.80

[a]Molecular species with molar relative abundances higher than 1% are represented.

[b]*sn*-1 position/*sn*-2 position.

[c]*Molecular species in brackets indicate minor constituents in not-fully-resolved mixtures.*

[d]Molar percentage of total PC population accounted by the analytical procedure.

between the 2 avian species considered. Nevertheless, the previously reported increase in the absolute amount of PC during the development of the brain tissue (9) provides a steady developmental increase of all molecular species. An overall 1.6 ± 0.4-fold increase can be calculated for each group within the developmental period considered (Fig. 1).

The percentage distribution of individual PC molecular species is shown in Table II. Fifteen major ($\geq$ 2% of total PC population) molecular species are found which do not undergo significant variation in relative abundance either during the course of developmental studies or between the 2 avian species considered. This maintenance of PC composition greatly contrasts with the changes previously described in chick embryo liver (4) and must be partially attributed to the maintenance of the specificity of PC synthesis by the developing brain (Gonzalez-Ros, in preparation).

PC populations are composed of molecules with selective fatty acid distributions, i.e., unsaturated fatty acids are attached primarily to the *sn*-2 position, whereas saturated acyl groups bind mainly to the *sn*-1 glycerol hydroxyl group, as described elsewhere for animal phosphoglycerides (3).

The most abundant molecular species of PC are monoenoic and disaturated, which account for ca. 50 and 30% of the total PC population, respectively. The most abundant disaturate is 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine which exhibits increased relative levels on the 17th incubation day in the chick embryo (22nd day in duck) in accordance with previously described increased levels of palmitic acid at the same developmental stage (9). Relative abundance of dipalmitoyl-PC from brain is somewhat higher than in the majority of previously described biological systems (7,12), except for lung tissue, where it exists at an even greater amount (3,7,13).

Another type of disaturated PC at lower levels is 1-stearoyl,2-palmitoyl-*sn*-glycero-3-phosphocholine. There was no detection either of 1-palmitoyl,2-stearoyl or 1,2-distearoyl-*sn*-glycero-3-phosphocholine, which is in agreement with the conclusion reached from studies on liposome permeability where these molecules apparently create a much too rigid and impermeable bilayer structure (14-16). In addition, the absence of molecules such as dilauroyl or dimyristoyl-PC is not surprising because of the observed anomalous behavior of these molecular species in membrane model systems (17).

The monoenoic PC molecular species are present in the developing brain at even higher levels than the disaturated ones and the main components are 1-palmitoyl,2-oleoyl, and 1-oleoyl,2-palmitoyl-*sn*-glycero-3-phosphocholine. 1-Oleoyl, 2-palmitoyl-*sn*-glycero-3-phosphocholine probably is uniquely associated with brain tissue since it cannot be detected in other embryonic organs such as liver or lung (4,13), where it was attributed to a positional selectivity shown by the oleoyl group during the biosynthesis of PC (3,18-20). Alternatively, O'Brien and Geison (21) indicated 1,2-dipalmitoyl and 1-palmitoyl,2-oleoyl PC species as the most characteristic in nerve endings and myelin, respectively.

Other possible combinations of monounsaturated (oleic and palmitoleic) and saturated (palmitic and stearic) fatty acids have also been detected by forming monoenoic molecular species, except that stearic acid was never found attached to the *sn*-2 position.

Dienoic PC molecular species represent ca. 7% of the total PC population. They are formed either by having 2 double bonds in the same apolar chain (1-palmitoyl,2-linoleoyl-*sn*-glycero-3-phosphocholine) or by concomitance of 2 monounsaturated apolar chains within the same PC molecule (1,2-dioleoyl-*sn*-glycero-3-phosphocholine). Both of these have been found previously in the brains of higher animals (3,7).

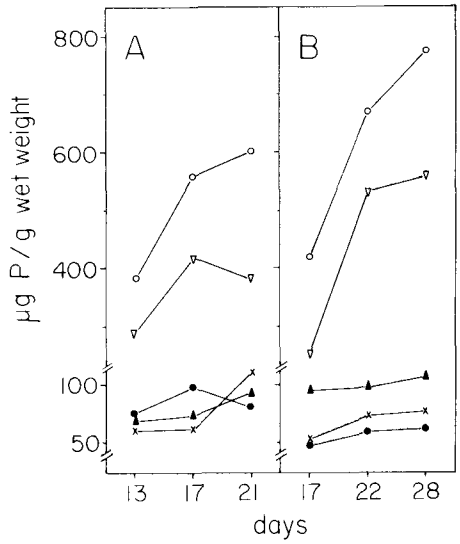

FIG. 1. Developmental changes in the amount of phosphorus contained in molecular species groups of PC with different degrees of unsaturation from chick (A) and duck (B) embryo brain. (▽) disaturated, (○) monoenoic, (●) dienoic, (▲) tetraenoic and (x) hexaenoic molecular species. Phosphorus determinations were performed as previously described (9).

Polyunsaturated PC, composed mainly of tetraenoic and hexaenoic molecular species, have been found with relative abundances between 6-9% in all the developmental stages considered. These are very low values compared to the embryonic chick liver, for example (4). In all cases, preferential combinations of palmitic acid (as different from stearic acid) in the *sn*-1 position with polyunsaturated arachidonic (tetraenoic molecular species) or docosahexenoic (hexaenoic molecular species) acids at the *sn*-2 position are detected. These combinations correspond to polyunsaturated molecular species commonly found in animal tissues, although a predominance of tetraenoic molecular species containing *sn*-1 bound stearic acid, instead of palmitic, also has been reported (3,4,7).

There was no detection of appreciable amounts of dipolyunsaturated (e.g., arachidonic/docosahexenoic, arachidonic/docosahexenoic) molecular species, which agrees with the observed instability of membrane model systems containing high amounts of these phosphoglyceride molecules (15).

Table III shows the molecular composition of PC that would be expected on the basis of a random distribution of the fatty acids present in the initial PC (9). In comparison, experi-

TABLE III

Calculated Molecular Species Composition of PC from the Developing Avian Embryo Brain Assuming Random Distribution of Acyl Groups[a,b]

Molecular classes	Chick (incubation days)			Duck (incubation days)		
	13	17	21	17	22	28
16:0/16:0	29.23	32.97	29.87	26.45	35.29	28.39
18:0/16:0	2.87	2.78	3.66	3.13	3.48	4.63
16:1/16:0	2.76	1.73	1.48	1.87	1.06	0.89
18:1/16:0	13.33	13.39	13.78	14.31	13.66	13.99
16:0/18:1 +16:0/16:1	16.09	15.12	15.26	16.18	14.72	14.88
18:0/16:1 +18:0/18:1	1.57	1.27	1.86	1.91	1.44	2.41
16:0/18:2	1.09	1.30	1.04	0.39	0.30	–
18:1/18:1 +18:1/16:1	7.34	6.14	7.04	8.05	5.70	7.30
16:0/20:4	1.80	2.13	2.07	2.80	2.69	2.74
18:0/20:4	0.17	0.17	0.25	0.33	0.26	0.44
16:0/22:6	1.86	1.90	2.29	1.42	1.77	1.66
18:0/22:6	0.18	0.16	0.28	0.16	0.17	0.27
Total accounted (Σ)	78.29	79.06	78.88	77.00	81.88	79.97

[a]Data on PC fatty acid composition taken from Gonzalez-Ros and Ribera (1979) (9).

[b]Calculation assuming random distribution of acyl groups were done according to Kuksis et al. (1963) (22).

mental values for saturated and monoenoic species containing monounsaturated fatty acids at the *sn*-1 position appear to obey the random distribution, whereas monoenoic species containing monounsaturated fatty acids (mainly oleic acid) at the *sn*-2 position present experimental values deviated positively from the statistical assumption. In the dienoic species fraction, appearance of those pairing saturated and diunsaturated fatty acids apparently is favored whereas the opposite occurs to those containing 2 monounsaturated acyl chains. In addition, it can be observed that experimental values for polyunsaturated species (tetra- and hexaenoic molecules), especially those containing stearic acid, are much higher than those expected from the random distribution assumption. The observed nonrandomness would mean certain acyl groups of PC for the developing brain tend to associate with each other on some basis other than molar concentration. Another possibility is that brain PC represents pooled contributions of molecules from several PC populations, each of which may possess a random distribution for its fatty acids. Whether these results are a reflection of the specificity of endogenous PC synthesis is as yet unknown.

In conclusion, PC from the developing avian brain shows a molecular composition which basically agrees with the trend shown by the brain of higher animals, although it differs from the molecular composition patterns exhibited by other embryonic avian tissues. Monoenoic and disaturated PC species roughly account for 80% of the total PC population where the main components are 1,2-dipalmitoyl, 1-palmitoyl,2-oleoyl and 1-oleoyl,2-palmitoyl-*sn*-glycero-3-phosphocholine. There is a strong maintenance of that pattern both during the embryonic development and between the 2 avian species considered, which may be necessary to keep the functional activity of the embryonic tissue. The mechanism by which this constancy of PC molecular composition is achieved is not understood at this time but may be related to the specificity of endogenous PC synthesis and/or caused by selective transport within the developing encephalon.

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