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Phosphatidylcholine produced in rat synaptosomes by *N*-methylation is enriched in polyunsaturated fatty acids

(brain/phospholipids/membranes/phosphatidylethanolamine *N*-methyltransferase)

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ABSTRACT **Rat brain synaptosomes contain an enzyme, phosphatidylethanolamine *N*-methyltransferase (EC 2.1.1.17), that catalyzes the methylation of phosphatidylethanolamine to form its mono-, di-, and trimethyl (phosphatidylcholine) derivatives. Synaptosomal phosphatidylethanolamine is much richer in polyunsaturated fatty acids (43.4%) than is synaptosomal phosphatidylcholine (4.6%). It thus seemed possible that the phosphatidylcholine derived via the *N*-methylation of phosphatidylethanolamine might also be especially enriched in polyunsaturated fatty acids. To test this hypothesis, we examined the incorporation of [^{3}H]methyl groups into various molecular species of phosphatidylcholine, by incubating rat synaptosomes for 10, 30, or 90 min in a medium containing *S*-adenosyl[*methyl*-^{3}H]methionine. Phosphatidylcholine was extracted and separated from other lipids by TLC, after which its molecular species were isolated by argentation TLC (which distinguishes among the phospholipid molecules by the degree of unsaturation of their fatty acid moieties.) We found that approximately 65% of the [^{3}H]methyl incorporated into phosphatidylcholine during the incubation period was present in the fraction associated with pentaene or hexaene fatty acids; an additional 30% was present in the tetraene fraction, while the remaining phosphatidylcholine radioactivity was distributed between the dienes and monoenes. Similar distributions were observed among synaptosomes incubated for 10 or 30 min; however, after 90 min the phosphatidyl[^{3}H]choline contained proportionately less of the tetraenes. These observations indicate that neuronal phosphatidylcholine molecules formed via *N*-methylation are especially richer in polyunsaturated fatty acids, and they raise the possibility that these molecules constitute a distinct pool with particular physiologic functions.**

Three synthetic pathways can produce phosphatidylcholine (PtdCho) molecules in cells: the Kennedy pathway, which combines CDP-choline with 1,2-diacylglycerol; the base-exchange pathway, which exchanges free choline with a serine or ethanolamine in phosphatidylserine or phosphatidylethanolamine (PtdEtn); and the sequential *N*-methylation of PtdEtn, forming its mono-, di-, and trimethyl (PtdCho) derivatives. The latter is the only pathway capable of producing new choline moieties in the brain, which otherwise depends on the circulation for its choline supply (1, 2). This pathway, catalyzed by phosphatidylethanolamine *N*-methyltransferase (PtdEtnMeTase; EC 2.1.1.17), has been demonstrated in brain (3, 4), its activity being highest in synaptosomes (5).

The fatty acid composition of synaptosomal PtdEtn, which is the precursor for the PtdCho that is formed via methylation (and *a* precursor for that formed via the base exchange pathway), differs markedly from that of PtdCho, containing 43.4% polyunsaturated fatty acids as opposed to 4.6% in PtdCho. Two hypotheses have been proposed (6) to explain this difference: i.e., that the PtdCho formed by methylation (or base exchange) maintains the fatty acid composition of the precursor PtdEtn but is diluted with much larger amounts of PtdCho formed via the CDP-choline pathway; or that this PtdCho, once formed, is deacylated and then reacylated, changing its fatty acid composition to that associated with the bulk of the PtdCho. We found that brain phosphatidyl-*N*,*N*-dimethylethanolamine (PtdMe$_2$Etn), an intermediate in the synthesis of PtdCho (via PtdEtnMeTase), which makes up only 0.1% of total phospholipids, is, like PtdEtn, very rich in polyunsaturated fatty acids: 10.6% of its total fatty acids are arachidonic acid and 12.8% are docosahexaenoic acid, while only 11% are palmitic acid (6) (all percentages are on a molar basis). This suggests that the fatty acid composition of the PtdEtn tends to be preserved during at least the first two steps in its *N*-methylation. We now describe the incorporation of [^{3}H]methyl groups into the various molecular species of PtdCho that are formed (by methylation) when rat synaptosomes are incubated with *S*-adenosyl[*methyl*-^{3}H]methionine (Ado[^{3}H]Met).

MATERIALS AND METHODS

Preparation of Synaptosomes. Male Sprague–Dawley rats (200–300 g, Charles River Breeding Laboratories) were housed under a 12-hr light–dark schedule (Vita-Lite, Duro-Test, North Bergen, NJ) and had free access to food (Charles River Breeding Laboratories, RMH 3000) and water. The animals were decapitated, and their brains were quickly removed and homogenized in 10 vol of 0.32 M sucrose, using a Potter–Elvehjem homogenizer. Synaptosomes were then prepared from the homogenate by centrifugation under a sucrose gradient as described by Dodd *et al.* (7). Synaptosomal proteins were measured by the method of Lowry *et al.* (8).

Measurement of PtdEtnMeTase Activity. PtdEtnMeTase activity in synaptosomes was assayed by the method described by Leprohon *et al.* (9). Aliquots of synaptosomes (representing 0.2–0.3 mg of proteins) were resuspended in 0.32 M sucrose to which 50 mM Tris·HCl buffer (containing 5 mM $MgCl_2$, 0.2 mM EDTA, and 0.1 mM ATP) was then added to a final volume of 120 μl per tube. Reactions were started by the addition of 10 μM (2.5 μCi) of Ado[^{3}H]Met (22 μCi/mmol, Amersham; 1 Ci = 37 GBq). Samples were incubated for 10, 30, or 90 min at 37°C. Reactions were stopped by addition of 3 ml of chloroform/methanol/HCl,

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Abbreviations: AdoMet, *S*-adenosylmethionine; PtdCho, phosphatidylcholine; Lyso-PtdCho, lysophosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdEtnMeTase, phosphatidylethanolamine *N*-methyltransferase; PtdMeEtn, phosphatidylmonomethylethanolamine; PtdMe$_2$Etn, phosphatidyl-*N*,*N*-dimethylethanolamine.

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100:50:1 (vol/vol). The extracts were washed twice with 2 ml of 0.75% KCl in 50% (vol/vol) methanol, to remove water-soluble radioactive materials. The chloroform phases were then taken to dryness in a Savant Speed Vac lyophilizing system; the dry extracts containing phospholipids were redissolved in 50 μl of chloroform/methanol, 1:1 (vol/vol), and spotted on silica gel plates (silica gel G, Absorbosil Plus-1, Applied Science Laboratories, State College, PA) and developed in chloroform/propionic acid/1-propanol/water, 2:2:3:1 (vol/vol). Lysophosphatidylcholine (Lyso-PtdCho), PtdCho, PtdEtn, and PtdMeEtn standards (Sigma) were run in parallel. Compounds were visualized with iodine, and the regions of the silica gels corresponding to the added standards were scraped into scintillation vials; the phospholipids were then eluted with 1 ml of methanol to which 10 ml of Hydrofluor (National Diagnostics, Somerville, NJ) had been added, and their radioactivities were measured.

Separation of PtdCho in Molecular Species by Argentation Chromatography. Synaptosomes containing approximately 1 mg of protein were incubated in Tris buffer for 10, 30, or 90 min in the presence of Ado[^{3}H]Met; phospholipids were then extracted and separated into fractions, as described above. The silica gel corresponding to PtdCho was scraped and then extracted with 1, 1, and 0.5 ml of methanol, and the combined extracts were evaporated to dryness. The extracts were then redissolved in 100 μl of chloroform/methanol, 1:1 (vol/vol), and spotted on $AgNO_3$-impregnated plates (10). High-performance TLC plates (HETLC-HL, 10 × 10 cm, Analtech, Newark, DE) were dipped into a saturated solution of $AgNO_3$ in methanol and left in the dark for 10–15 min. (To avoid deposition of crystals on the plates, the saturated $AgNO_3$ solution was filtered immediately before each use.) The plates were then gently removed from the $AgNO_3$ solution, dried with hot air for 5 min, and then activated at 120°C for 30 min. Each sample was spotted on the plate and allowed to migrate up to 7 cm in chloroform/methanol/water, 50:34:7.5 (vol/vol). A mixture containing 1,2-dipalmitoyl, 1-palmitoyl, 2-oleoyl, 1,2-dioleoyl, 1-palmitoyl, 2-arachidonoyl, and 1,2-dilinolenoyl PtdCho (Avanti Biochemicals) was run in parallel as standards. Fractions corresponding to the reference standards were scraped and transferred into scintillation vials; the phospholipids were extracted with 1 ml of methanol to which 10 ml of Hydrofluor had been added and their radioactivities were measured. Analyses of the phosphorus contents in the various phospholipids were performed on PtdCho fractions (11).

Determination of Fatty Acid Composition of PtdCho Species. To measure the relative abundance of particular fatty acids in the various molecular species of PtdCho prepared as described above, their fatty acids were analyzed by gas chromatography. Aliquots of synaptosomes containing 1.2–1.4 mg of protein were incubated as described above, in the presence of 10 μM AdoMet. PtdCho was purified by TLC, extracted with methanol, and spotted on $AgNO_3$-impregnated plates as described above. After development, each plate was divided into fractions 0.5 cm high, and these were scraped into glass tubes and directly methylated by alkaline methanolysis (12). Twenty micrograms of diheptadecanoyl PtdCho was added to each sample as an internal standard; then 1 ml of saturated NaOH in chloroform/methanol, 2:1 (vol/vol), was added, after which the samples were mixed thoroughly. After 10 min, 1 ml of 1 M HCl in saline was added and samples were mixed and centrifuged. Approximately 300 μl of the lower phase was aspirated (with a 500-μl syringe) and then evaporated to dryness and resuspended in 20 μl of chloroform, 1-2 μl of which was injected into a gas chromatographic apparatus (Hewlett–Packard 5880, equipped with a flame ionization detector and an electronic integrator). A fused capillary column (SP233, 30 m long × 0.25 mm internal diameter; Supelco, Bellefonte, PA) was used; the carrier gas was helium; its flow rate was 1 ml/min, the split ratio was 1:30. The initial column temperature was 190°C; after 10 min, it was increased to 210°C at the rate of 2°C/min. Injector and detector temperatures were both 250°C.

The fatty acid methyl esters were identified by comparing their retention factors with those of standard fatty acid methyl esters, chromatographed under indentical conditions. Measurements of peak areas were made with an automatic integrator attached to the gas chromatographic apparatus. Fatty acid contents were expressed as mol percents of total fatty acids.

Triplicate samples were used for each incubation time in each experiment, and experiments were repeated three or more times.

RESULTS

The methylation of endogenous substate by PtdEtnMeTase was studied by incubating synaptosomes with 10 μM Ado[^{3}H]Met for 10, 30, or 90 min at 37°C (Table 1). Ptd[^{3}H]MeEtn was the major reaction product at all times, followed by $PtdMe_2$[^{3}H]Etn and Ptd[^{3}H]Cho. Lyso-Ptd[^{3}H]Cho was also measurable in amounts equal to 20–30% those of total Ptd[^{3}H]Cho. (Since the lipids were extracted under acidic conditions, part of the radioactivity in the Lyso-PtdCho fractions may actually have represented hydrolyzed Ptd[^{3}H]Cho plasmalogen.)

To measure the ^{3}H labeling of various PtdCho species formed via PtdEtnMeTase, PtdCho molecules were further fractionated, according to the number of double bonds present in their fatty acids, by argentation chromatography. Seventy to 80% of the total radioactivity spotted on plates were recovered in fractions corresponding to PtdCho standards (whose fatty acids contained zero, one, two, four, or six double bonds) (Table 2). The fraction corresponding to the pentaenes plus hexaenes, containing 4–6% of total phosphorus, incorporated largest amounts of ^{3}H (almost half of the radioactivities); the fraction corresponding mainly to the arachidonate PtdCho (8–10% of total phosphorus) contained about 25% of the radioactivity; no radioactivity was detected in the saturated fraction and only 2–2.5% in the monoene and diene fractions. Specific activities were calculated (fmol of ^{3}H incorporated in each fraction per nmol of phosphorus). The radioactivity incorporated after 30 min of incubation into the pentaenes plus hexaenes fraction was 199 ± 10 fmol/nmol, and that in the tetraenes was 66 ± 10

Table 1. [^{3}H]Methyl incorporation into phospholipids by synaptosomal PtdEtnMeTase

Time of incubation, min	PtdEtnMeTase activity, pmol of [^{3}H]methyl incorporated per mg of protein			
	PtdMeEtn	$PtdMe_2$Etn	PtdCho	Lyso-PtdCho
10	1.72 + 0.33	0.77 + 0.13	1.05 + 0.15	0.20 + 0.01
30	3.86 + 0.77	2.89 + 0.31	1.81 + 0.26	0.61 + 0.13
90	4.14 + 0.52	3.29 + 0.63	2.58 + 0.10	0.53 + 0.01

Rat synaptosomes were incubated with 10 μM Ado[^{3}H]Met for 10, 30, or 90 min. Data represent mean ± SEM in triplicate experiments.

Table 2. [^{3}H]Methyl incorporation into various molecular species of PtdCho by synaptosomal PtdEtnMeTase

Time of incubation, min	Fatty acids in PtdCho species					
	Saturated	Monoenes	Dienes	Tetraenes	Pentaenes + hexaenes	Total*
	[^{3}H]Methyl incorporated, fmol/mg of protein					
10	ND	17 ± 5	18 ± 4	184 ± 38	452 ± 46	899 ± 24
30	ND	18 ± 2	26 ± 6	471 ± 68	846 ± 44	1660 ± 94
90	ND	52 ± 1	38 ± 10	441 ± 27	1720 ± 50	3010 ± 161
	P_i content,† nmol/mg of protein					
30	10.3 ± 2.9	47.2 ± 2.3	1.6 ± 2.3	7.2 ± 0.3	4.3 ± 0.2	70.5 ± 5.0

Data represent mean ± SEM of triplicate experiments. ND, not detected.

*Total fmol of [^{3}H]methyl spotted on each plate per mg of protein. Approximately 70–80% of total radioactivity could be identified.

†Phosphorus content in individual PtdCho fractions 30 min after incubation. "Total" is the sum for all fractions. When the incubation was prolonged to 90 min, the amounts of saturated and monoenoic PtdCho were slightly increased (17.5 ± 1.2 and 59.5 ± 3.4 nmol/mg of protein, respectively), while the polyene fractions remained basically unchanged.

fmol/nmol; these were 500 and 160 times higher, respectively, than the radioactivities incorporated into the monoene fractions (0.4 ± 0.02), which represented the major molecular species of PtdCho. The production of labeled tetraenes and pentaenes plus hexaenes doubled (452 ± 46 to 845 ± 38, and 184 ± 38 to 471 ± 68 fmol/mg of protein, respectively) when the incubation time was prolonged from 10 to 30 min. However, when incubations were longer (90 min) the labeling on the pentaenes plus hexaenes continued to increase (to 1721 ± 50 fmol/mg of protein) but no further increase was observed in the tetraenes (441 ± 27 fmol/mg).

To verify the relative abundance of unlabeled fatty acids of each fraction, gas chromatographic analysis was performed on samples that had been incubated for various intervals (Table 3). (Incubation had no effect on the proportions of various unlabeled fatty acids present in each fraction, so data were pooled.) Seventy percent of the unlabeled fatty acids in PtdCho were found in association with the fraction containing mostly monoenes, 16% were in the saturated fraction, and only 2%, 8.2%, and 4.6%, respectively, were in the fractions corresponding to dienes, tetraenes, and pentaenes plus hexaenes.

DISCUSSION

These data show that the synaptosomal PtdCho molecules formed by the *N*-methylation of endogenous substrate are considerably richer in polyunsaturated fatty acids than is the bulk of unlabeled PtdCho, which is largely formed via the CDP-choline cycle. The Ptd[^{3}H]Cho contained no detectable saturated fatty acids and only 1–2% of its fatty acids were monoenes and dienes; in contrast, the unlabeled PtdCho contained 16% saturated fatty acids and 70% monoenes. These results are consistent with other studies suggesting that N-methylation produces a PtdCho enriched in arachidonic acid in tissues other than brain (13, 14) and confirm our previous findings (6) that the brain $PtdMe_2Etn$ (an intermediate in the sequential methylation of PtdEtn) also contains high amounts of polyunsaturated fatty acids (27% of the total fatty acids), resembling closely the fatty acid distribution of brain PtdEtn. Since less than 5% of the total fatty acids in synaptosomal PtdCho are polyunsaturated, it seems likely that the PtdCho derived via PtdEtnMeTase constitutes, at steady state, only a small pool and is heavily diluted by more saturated PtdCho species formed from preexisting choline via the CDP-choline pathway (which is thought to produce PtdCho species rich in monoenes and saturated fatty acids) (15, 16). On the other hand, it is also possible that much of the PtdCho newly formed by methylation is rapidly deacylated by phospholipase A_2 to Lyso-PtdCho and then reacylated with more saturated fatty acids.

In support of this latter possibility, we found no increase in the tetraene content of synaptosomal Ptd[^{3}H]Cho between 30 and 90 min of incubation, but during this period other fatty

Table 3. Fatty acid composition of various molecular species of unlabeled PtdCho in rat synaptosomes

Carbon atoms: double bonds	Fatty acids in PtdCho species				
	Saturated	Monoenes	Dienes	Tetraenes	Pentaenes + hexaenes
	mol %				
14:0	0.1 ± 0.00	0.9 ± 0.2	0.8 ± 0.06	0.8 ± 0.0	0.6 ± 0.05
16:0	82.8 ± 0.5	40.5 ± 2.1	23.1 ± 1.1	28.4 ± 3.6	22.2 ± 1.7
16:1	0.7 ± 0.2	2.9 ± 0.1	1.6 ± 0.2	0.8 ± 0.2	1.4 ± 0.5
18:0	7.5 ± 1.0	12.6 ± 0.8	27.1 ± 3.0	26.8 ± 0.9	25.8 ± 2.2
18:2	—	0.2 ± 0.0	35.9 ± 4.0	—	—
20:0	0.2 ± 0.0	0.5 ± 0.0	—	—	—
20:1	—	1.9 ± 0.08	—	—	—
20:4	—	0.5 ± 0.06	1.5 ± 0.7	32.7 ± 3.2	2.3 ± 0.3
22:4	—	—	—	3.9 ± 0.8	—
22:5	—	—	—	—	3.4 ± 0.6
22:6	—	—	—	—	38.5 ± 3.0
	nmol/mg of protein				
Total fatty acids	27.0 ± 4.7	118.0 ± 7.5	3.4 ± 1.2	14.0 ± 1.7	7.7 ± 1.5

Results are the mean ± SEM of five determinations. Data indicate the percent of total fatty acids represented by each fatty acid in separated PtdCho species. (Some contamination occurred: for example, the saturated species contained 8.6% monoenes.)

acid species in the phospholipid continued to increase. This indicates either that formation of [^{3}H]arachidonoyl PtdCho stops after short incubation periods or, more likely, that such incorporation continues, but the arachidonic acid in PtdCho is hydrolyzed faster than other fatty acids. Arachidonoyl PtdCho in synaptosomes is known to be 2–3 times more active as a substrate for phospholipase A_2 than are other PtdCho species (17). If *N*-methylation of PtdEtn *in vivo* also yields a PtdCho rich in arachidonate that turns over rapidly, this pool may, by liberating arachidonic acid and Lyso-PtdCho, have specific physical roles. Arachidonic acid is the precursor of prostaglandins, which may modulate neurotransmitter release (18, 19); Lyso-PtdCho is a strong detergent and may also directly modify membrane viscosity or be further degraded [by lysophospholipases, which seem particularly active in synaptosomes (20)] to yield glycerophosphocholine and choline. We have previously shown that when synaptosomes are incubated in the presence of Ado[^{3}H]Met, free [^{3}H]choline also appears in the medium in amounts that account for as much as 20% of the radioactivity present as [^{3}H]PtdCho (5). These data suggest an important role for *N*-methylation in supplying choline at cholinergic nerve endings, where the choline is needed to sustain acetycholine synthesis, especially when the neurons are firing frequently and when choline is in short supply (21, 22).

In conclusion, we show that synaptosomal PtdCho formed via *N*-methylation differs from the bulk of the PtdCho by containing mostly hexaenoic and tetraenoic fatty acids, and the latter probably are hydrolyzed rapidly to yield fatty acids and other degradation products that may have important physiological roles.

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