Molecular composition of the phosphatidylcholines produced by the phospholipid methylation pathway in rat brain *in vivo*

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We examined the formation *in vivo* of molecular subspecies of brain phosphatidylcholine (PC) via the phospholipid-methylation pathway. [^3H]Methionine was infused into a lateral cerebral ventricle, and ^3H -labelled PC was isolated from brains of rats 0.1–18 h after the infusions. Three major subspecies of this PC, differing in their fatty acid compositions, were separated on silver-impregnated t.l.c. plates, and the proportions of radioactivities in these three PC fractions were determined. The results indicate that newly-formed PC synthesized by methylation of phosphatidylethanolamine at 0.1 h after [^3H]methionine contains a significantly higher proportion of polyunsaturated subspecies (i.e. those with six or four double bonds) than does PC obtained later times after injection of [^3H]methionine. This change in the composition of ^3H -labelled brain PC occurs gradually and is not due to an influx of radioactive PC from the periphery. Our data suggest that polyunsaturated PC (hexaenes and tetraenes) produced in the brain by methylation of phosphatidylethanolamine turns over faster than does that containing more-saturated fatty acids.

INTRODUCTION

Phosphatidylcholine (PC) is produced in the brain by three biochemical pathways. Two of these, the CDP-choline cycle (Kennedy & Weiss, 1956) and the base exchange pathway (Kanfer, 1972), form PC by utilizing pre-existing free choline. The third pathway, that involving phospholipid methylation (Bremer & Greenberg, 1960) which makes PC by three sequential methylations of phosphatidylethanolamine (PE), is the only known pathway capable of generating choline moieties *de novo*. Brain PC has been shown to be produced via PE methylation both *in vivo* (Chida & Arakawa, 1971; Morgans & Abdel-Latif, 1974; Zawad & Brown, 1985) and *in vitro* by various subcellular fractions (Blusztajn *et al.*, 1979; Crews *et al.*, 1980); this pathway has also been identified in neuronal and glial cell cultures (Strittmatter *et al.*, 1979; Dainous *et al.*, 1982; Yavin, 1985).

Although it has been estimated that only about 1% of the total brain PC originates by the phospholipid-methylation pathway (Percy *et al.*, 1982; Lakher *et al.*, 1986), the PC generated by this pathway in synaptosomal preparations *in vitro* is particularly enriched in polyunsaturated fatty acids (especially docosahexaenoic, 22:6, and arachidonic, 20:4) compared with 'bulk' synaptosomal PC (Tacconi & Wurtman, 1985b) or with the PC produced in synaptosomes by the two other biosynthetic pathways (Lakher *et al.*, 1986). The possibilities were considered that newly synthesized PC formed via methylation of PE either maintains its original fatty acid composition but is diluted with more-saturated PCs formed by the other pathways, or that this PC, once synthesized, is subjected to a deacylation–reacylation process, which renders it more saturated (Tacconi & Wurtman, 1985b).

We have measured the proportions of the particular molecular subspecies of isotopically labelled PC present in rat brain at various intervals (0.1–18 h) after injection of [^3H]methionine into a lateral cerebral ventricle. PC was extracted from the brains, isolated by t.l.c. and separated by argentation chromatography into three groups of molecular subspecies which differed in their degree of unsaturation. The possible contribution of peripheral radioactive compounds to the accumulation of [^3H]PC was assessed by comparing the levels of radioactive PC in brain halves ipsilateral and contralateral to the [^3H]methionine infusions.

MATERIALS AND METHODS

Materials

L-[methyl- ^3H]Methionine (sp. radioactivity 5–15 Ci/mmol) and L-[methyl- ^{14}C]methionine (sp. radioactivity 40–55 mCi/mmol) were purchased from New England Nuclear. Phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.); choline chloride was from Sigma; monomethylethanolamine, dimethylethanolamine and 1,6-diphenylhexatriene were from Aldrich; 2,6-di-*t*-butyl-*p*-cresol was from Eastman Kodak; AgNO₃ was from Mallinckrodt. These and all other reagents were of analytical or highest available grade.

Intracerebroventricular infusions of radiolabelled methionine

Male Sprague–Dawley rats (Charles River Laboratories) weighing 250–300 g received L-[methyl- ^3H]methionine or L-[methyl- ^{14}C]methionine (25 μCi in 2 μl of saline) by injection into a lateral cerebral ventricle via a plastic cannula guide implanted 3–5 days previously

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyltrimethylethanolamine.

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Table 1. Accumulation of [³H]PC in the left and right brain-halves in rats receiving [³H]methionine intraperitoneally or intracerebroventricularly

Rats (four to six per group) were killed at the intervals indicated after receiving [³H]methionine (25 μ Ci) into the right cerebral ventricle or intraperitoneally. Both halves of the animals' brains were assayed separately for [³H]PC. Data given represent the proportions (means $\pm$ S.E.M.) of [³H]PC present in the right half of the brain.

Type of injection	Time after [³ H]methionine injection (h)	Cannula in the right side	Cannula in the left side	[³ H]PC in the right half of rat brain (%)
Intraperitoneal	20.0	Yes	No	55 $\pm$ 2
Intraventricular	0.1	Yes	Yes	93 $\pm$ 6
into the right ventricle	6.0	Yes	Yes	90 $\pm$ 5
	20.0	Yes	Yes	83 $\pm$ 5

(Russel & Macri, 1978). Radiolabelled methionine was infused unilaterally over a period of 4.5 min using an infusion pump (Harvard Apparatus, Mills, MA, U.S.A.). In some of the experiments plastic cannulas were implanted bilaterally to ensure that the presence of the cannula on one side of the brain does not cause a preferential influx of radioactive material to the vicinity of cannula. In control experiments, rats with unilaterally implanted plastic cannula guides received [³H]methionine (25 μ Ci in 0.2 ml of saline) intraperitoneally. Rats were killed by 3.5 s of focused microwave irradiation of the head (BN-K2 Microwave Oven, 1.2 kW; General Medical Engineering, Peabody, MA, U.S.A.) at various intervals after injection.

Extraction and purification of isotopically labelled PC

Brain samples were homogenized in 6 ml of a chloroform/methanol (2:1, v/v) mixture (Folch *et al.*, 1959), containing 2,6-di-*t*-butyl-*p*-cresol as an anti-oxidant (1 mg/10 ml), in a Teflon/glass homogenizer. Water (1.2 ml) was added to the extracts; samples were vortex-mixed for 2 min and the phases were separated by centrifugation at 10000 *g* for 15 min. The upper phase was used for determination of *S*-adenosylmethionine and *S*-adenosyl[³H]methionine according to Gharib *et al.* (1982). The lower (chloroform) phase was taken to dryness under N₂, redissolved in 0.2 ml of chloroform/methanol (1:1, v/v) and applied to preparative t.l.c. plates (Silica Gel G Taper Plates; Analtech, Newark, DE, U.S.A.). Chloroform extracts obtained from 0.5 g of brain tissue were spread over 6 cm of a t.l.c. plate along with standards of PC, PMME and PDME. The plates were developed in a system of chloroform/methanol/propan-2-ol/0.25% KCl/triethylamine (30:9:25:6:18, by vol.). Phospholipids were visualized under u.v. light after spraying the plates with 0.1% 1,6-diphenylhexatriene in light petroleum, and then were extracted from the plates with 3 $\times$ 2.5 ml of methanol; the methanol was removed under N₂, and the PMME and PDME fractions were taken for measurement of radioactivity. The PC fractions were redissolved in 0.15 ml of chloroform/methanol (1:1, v/v). Aliquots of these extracts were taken for counting of radioactivity, and the rest was applied to t.l.c. plates containing AgNO₃ to separate molecular subspecies of PC (Arvidson, 1965). Radioactivities in phospholipids were expressed as percentages of total brain radioactivities to diminish the effects of between-sample variations (Gaitonde & Richter, 1956).

To verify the identity of the radioactivity associated with [³H]PC bands in the above t.l.c. system, aliquots of extracts containing [³H]PC were taken to dryness and subjected to acid hydrolysis with 0.3 ml of 6 M-HCl for 1 h at 90 °C. The HCl was removed under a vacuum, and the extracts were rechromatographed onto t.l.c. plates (Whatman LK6D) in the system acetonitrile/ethanol/acetic acid/8.3% (w/v) NaCl (48:8:1:24, by vol.). A choline standard was run along with the samples and visualized with I₂ vapour. Silica gel areas on the t.l.c. plates that corresponded to the choline standard were extracted with 1 ml of 50% (v/v) ethanol in 0.2 M-HCl, and the radioactivities in the extracts were measured. PC recovery was monitored by the addition of [¹⁴C]PC to unlabelled brain extracts.

Separation of molecular subspecies of PC

Molecular subspecies of PC were resolved according to their degree of unsaturation by a t.l.c. method similar to that described by Arvidson (1965). Plates (Silica Gel HL; Analtech) were dipped into a saturated solution of AgNO₃ in methanol for 10–15 min and then dried for 1 h at 80 °C. Methanol (h.p.l.c. grade) was dried with anhydrous Na₂SO₄, filtered and distilled immediately before use. PC extracts obtained from 0.5 g of brain tissue were spread over 20 cm of a t.l.c. plate. The chromatographic system contained chloroform/methanol/water (50:34:7, by vol.). Phospholipids were visualized under u.v. light by spraying with 1,6-diphenylhexatriene as above.

To identify the positions of endogenous subspecies of PC on the plates the following PC standards were cochromatographed with brain PC extracts: 1-palmitoyl-2-stearoyl-PC (16:0, 18:0), 1-palmitoyl-2-oleoyl-PC (16:0, 18:1), dioleoyl-PC (18:1, 18:1), 1-palmitoyl-2-arachidonoyl-PC (16:0, 20:4), 1-stearoyl-2-arachidonoyl-PC (18:0, 20:4), dilinoleoyl-PC (18:2, 18:2), dilinolenoyl-PC (18:3, 18:3) and diarachidonoyl-PC (20:4, 20:4) (all from Avanti Polar Lipids). We have previously evaluated the fatty acid compositions of the endogenous synaptosomal PC comprising the three major PC fractions on argentation t.l.c. plates and demonstrated that the positions of exogenous PC standards may be utilized to identify the positions of endogenous PC subspecies with the same number of double bonds (Tacconi & Wurtman, 1985b). Silica gel areas corresponding to individual subspecies of PC were scraped into tubes and extracted twice with 3 ml of

chloroform/methanol/acetic acid/water (50:39:1:10, by vol.) (Arvidson, 1965).

PC recoveries were examined by applying different unlabelled molecular species of PC onto silver-impregnated plates, extracting them with 2 × 3 ml of the same extracting mixture, and measuring their phosphorus contents by the method of Svanborg & Svennerholm (1961).

PC extracts were dried under a vacuum, and redissolved in 5 ml of Hydrofluor (National Diagnostics, Highland Park, NJ, U.S.A.). Silver ions were precipitated with 0.2 ml of 2 M-HCl, and AgCl was sedimented by centrifugation (1000 *g* for 20 min). Radioactivities were measured in clear supernatant fluids after addition of 10 ml of Hydrofluor, by using a liquid-scintillation spectrometer (Beckman LS 7500).

RESULTS

Estimation of peripheral contribution to the accumulation of radioactive PC in rat brain

Total amounts of radioactivity in brains of rats receiving [³H]methionine intracerebrally averaged $14.0 \pm 9.6\%$ (mean $\pm$ S.D.) of the amount that had been infused. This suggests that most of the injected radioactivity is lost from the brain, and enters the general circulation. To determine whether ³H-labelled compounds from the periphery contributed to the accumulation of the [³H]PC in rat brains after intracranial injections of [³H]methionine, the levels of [³H]PC were measured in the two sides of brain, ipsilateral and contralateral to the injections (Table 1). At all times studied, most (83–93%) of the [³H]PC was found within the brain half ipsilateral to the [³H]methionine injections. Since plastic cannula guides were placed symmetrically into the right and left sides of the brain, the asymmetric distribution of [³H]PC is not due to a lesion produced by implantation.

In supplementary studies we also found that levels of [³H]PC within the right and left halves of the brain were essentially equal in rats receiving [³H]methionine intraperitoneally despite the implantation of plastic cannula guides on the right sides of their brains (Table 1).

Accumulation of radiolabelled PC in rat brain

The intracerebroventricular injection of radioactive methionine led to a gradual accumulation of radiolabelled PC in rat brain (Fig. 1a). This increase was concomitant with an exponential decrease in the levels of S-adenosyl[³H]methionine that had been synthesized in brain tissue from the administered [³H]methionine (Fig. 1a).

[³H]PC represented $0.21 \pm 0.04\%$ and $1.59 \pm 0.21\%$ of the total brain radioactivity at 0.5 h and 18 h, respectively, after intracerebral [³H]methionine. In contrast, the percentages of radioactivity in [³H]PMME and [³H]PDME, the two intermediates in the phospholipid methylation pathway, reached peaks 1.5 h after intracerebral [³H]methionine, reaching $0.14 \pm 0.02\%$ ([³H]PMME) and $0.16 \pm 0.01\%$ ([³H]PDME) of total brain radioactivity, and decreased thereafter (Figs. 1b and 1c).

In control experiments brains from rats killed at 0.5 h and 18 h after receiving [³H]methionine intracerebroventricularly were homogenized in 0.4 M-HClO₄. Samples were centrifuged, and the resulting pellets were extracted

Fig. 1. Accumulations of S-adenosyl[³H]methionine, [³H]PC, [³H]PMME and [³H]PDME in brains of rats receiving intracerebroventricular [³H]methionine

Rats (four to six per group) were killed at intervals after receiving [³H]methionine (25 μ Ci) intracerebroventricularly. Brains were assayed for total radioactivity, S-adenosyl[³H]methionine ([³H]AdoMet), [³H]PC, [³H]PMME and [³H]PDME as described in the Materials and methods section. Radioactivities in AdoMet, PC, PMME and PDME are expressed as percentages of total brain radioactivity. Values given are means $\pm$ S.E.M.

with chloroform. Most of the [³H]methionine retained by the brain tissue was apparently incorporated into proteins, inasmuch as the proportion of radioactive material that was insoluble in both HClO₄ and chloroform continued to rise, from $37.9 \pm 0.5\%$ of total brain ³H 0.5 h after [³H]methionine administration to $77.8 \pm 1.5\%$ at 18 h. In contrast, chloroform-soluble radioactive materials represented only $0.7 \pm 0.1\%$ and $3.2 \pm 0.2\%$ of total brain ³H at 0.5 h and 18 h after [³H]methionine. The proportion of radioactive material that was soluble in HClO₄ (e.g. unchanged [³H]methionine) decreased from $61.5 \pm 2.2\%$ of total brain ³H 0.5 h after [³H]methionine administration to $19.0 \pm 1.2\%$ at 18 h.

Separation of the molecular subspecies of PC

Argentation chromatography allowed us to separate brain PC into three major bands. These represented three major fractions of brain PC that could be distinguished according to the degree of unsaturation in their fatty acids. The lower band (*R_F* 0.15–0.18) cochromatographed with a hexaenoic PC standard, dilinolenoyl-PC (18:3, 18:3), i.e. with six double bonds in its fatty acids. The middle band (*R_F* 0.30–0.35) corresponded to tetraenoic PC standards: 1-palmitoyl-2-arachidonoyl-PC (16:0,

Table 2. Molecular species of [³H]PC formed in brains of rats receiving [³H]methionine intracerebroventricularly

Rats (three to five per group) were killed at the intervals indicated after receiving [³H]methionine into the right lateral cerebral ventricle. PC was isolated by t.l.c. from the right brain halves, its molecular species were separated by argentation t.l.c., silica gel areas corresponding to the three major fractions of PC were scraped from the plates and extracted, and aliquots of these extracts were used for determinations of radioactivity and phosphorus, as described in the Materials and methods section. Synthetic PC species were used as standards. Values given are means $\pm$ S.E.M.

Time after [³ H]methionine (h)	[³ H]PC species (% of total [³ H]PC)		
	Saturates + monoenes + dienes	Tetraenes	Hexaenes
0.1	30 $\pm$ 4	29 $\pm$ 6	41 $\pm$ 8
6.0	57 $\pm$ 3	22 $\pm$ 3	20 $\pm$ 1
18.0	64 $\pm$ 2	22 $\pm$ 1	14 $\pm$ 2
Phosphorus in unlabelled PC (% of total PC)			
	78 $\pm$ 1	13 $\pm$ 1	9 $\pm$ 1

Fig. 2. Proportions of polyunsaturated and more-saturated subspecies of radiolabelled PC in brains of rats receiving radioactive methionine intracerebroventricularly

Rats (three to five per group) were killed at various intervals after receiving [³H]- or [¹⁴C]-methionine (25 μ Ci) into the right lateral cerebral ventricle. Radioactive PC was isolated by t.l.c. from right brain halves; its molecular subspecies were separated by argentation t.l.c. as described in the Materials and methods section. Appropriate synthetic PC species were used as standards. The three types of symbols (circle, square and triangle) represent data (means $\pm$ S.E.M.) from three independent experiments. Open symbols refer to the polyunsaturated molecular subspecies of radiolabelled PC (hexaenes + tetraenes); filled symbols refer to the more-saturated subspecies (fully saturated + monoenes + dienes). Vertical bars indicate S.E.M. values. Note the time-related changes in the proportions of the polyunsaturated to the more-saturated subspecies of radiolabelled PC.

20:4), 1-stearoyl-2-arachidonoyl-PC (18:0, 20:4) and dilinoleoyl-PC (18:2, 18:2). The upper band (R_F 0.45–0.50) comigrated with saturated molecular species of PC, as well as with mono- and di-enes: 1-palmitoyl-2-stearoyl-PC (16:0, 18:0), 1-palmitoyl-2-oleoyl-PC (16:0, 18:1) and dioleoyl-PC (18:1, 18:1). These bands, correspondingly, contained $9.4 \pm 0.5\%$, $12 \pm 0.7\%$ and $77.9 \pm 0.6\%$ of the total brain PC as assayed by phosphorus (Table 2). Total PC concentration in the brain tissue was found to be $14.6 \pm 1.0 \mu\text{mol/g}$. PC recoveries (determined as described in the Materials and methods section) from the silver-impregnated plates were found to be 75–80% for all molecular species of PC that were examined. We have also determined that 70–75% of the radioactivity in the t.l.c.-isolated PC fraction from rat brain comigrated with a choline standard on another t.l.c. plate after it had been subjected to acid hydrolysis in 6 M-HCl (results not shown).

Distribution of radioactivity among molecular subspecies of the radiolabelled PC

The proportions of radioactivities accumulated in the three major fractions of PC varied depending on the time after injection of radiolabelled methionine (Fig. 2). Thus, 0.1 h after the injections most of the radioactivity was found in the two polyunsaturated fractions of PC: hexaenes ($41 \pm 8\%$) and tetraenes ($29 \pm 6\%$). In contrast, brain PC analysed 18 h later had most of its radioactivity ($64 \pm 2\%$) in more saturated molecular subspecies of PC. Similar results were obtained in three independent experiments using either [³H]- or [¹⁴C]-methionine (combined results are presented in Fig. 2).

DISCUSSION

Several previous reports suggested that the phospholipid methylation pathway in the liver forms species of PC containing high proportions of arachidonic acid (Glenn & Austin, 1971; LeKim *et al.*, 1973; Threwhella & Collins, 1973). We have also previously shown that methylation of PE in rat brain synaptosomes generates a pool of PC unusually rich in polyunsaturated fatty acids (docosahexaenoic and arachidonic) compared with the 'bulk' pool of synaptosomal PC. At the same time, the molecular composition of this newly synthesized PC resembles that of its precursor, synaptosomal PE (Tacconi & Wurtman, 1985a,b). Once formed, a molecule of PC may remain unchanged, simply mixing with the 'bulk' pool of PC; alternatively, its fatty acid composition may be modified by an enzymatic deacylation–reacylation cycle catalysed by phospholipase A₂ and acyltransferases (Bell & Coleman, 1980). Finally, PC may be enzymically degraded to its chemical components such as choline and phosphatidic acid or phosphorylcholine and diacylglycerol. These compounds, in turn, may be reutilized for the synthesis *de novo* of PC via the CDP-choline cycle (choline or phosphorylcholine) or in the base-exchange pathway (choline).

We now have demonstrated that the molecular composition of the isotopically labelled PC formed in rat brain *in vivo* from radioactive methionine gradually changes with time (Fig. 2). Most of the radiolabelled PC (about 65–70%) isolated from rat brains 0.1 h after the injection of labelled methionine comigrated with the standards of PC containing hexaenoic or tetraenoic fatty acids. The proportions of radioactivity in these highly

polyunsaturated molecular subspecies of PC decreased with time, such that 18 h later most of the radioactivity (62–66%) was found in the fraction containing fully saturated PC and PC with one or two double bonds (Fig. 2). The distribution of the isotopically labelled PC among the three major fractions on the argentation t.l.c. plates 18 h after rats received radioactive methionine tended to approach that of the unlabelled brain PC, as assayed by phosphorus content.

Since most of the [^3H]PC synthesized in rat brain from [^3H]methionine that has been injected into a lateral cerebral ventricle is found on the side of injection (Table 1), the observed time-dependent changes in the molecular composition of radiolabelled PC are not due to a dilution of the brain PC with that formed on the periphery.

There may be three plausible explanations of our observations. First, radiolabelled PC formed by phospholipid methylation could have been degraded to its chemical constituents, e.g. choline, and then resynthesized by the CDP-choline cycle or by the base-exchange pathway. This mechanism would imply that PC made via methylation of PE would have a short half-life; that intermediates of this process, e.g. radioactive free choline or phosphorylcholine, could be found in the brain after administering radioactive methionine; and that PC generated via the two other synthetic pathways would contain much smaller proportions of the polyunsaturated fatty acids compared with PC formed by the transmethylation pathway. Indeed, concomitant with the formation of [^3H]PC, production of free [^3H]choline was observed in human neuroblastoma cell line (LA-N-2) when the cells were cultured in the presence of [^3H]methionine (Blusztajn *et al.*, 1986). On the other hand, arguing against this hypothesis, it was shown that the [^3H]PC that is newly formed by rat brain synaptosomes via any of the three pathways contain much larger proportions of polyunsaturated fatty acids than does 'bulk' synaptosomal PC (Holbrook *et al.*, 1985).

Second, the fatty acid composition of the radiolabelled PC could be changed by deacylation–reacylation reactions which have been shown to operate in the brain *in vitro* (Webster & Alpern, 1964). Although certain polyunsaturated phospholipid pools, e.g. phosphatidic acid, are thought to be predominantly formed via a deacylation–reacylation cycle rather than through their synthesis *de novo*, reports on the specificity of different fatty acids *in vivo* and *in vitro* are inconsistent (Bell & Coleman, 1980). Our experiments seem to suggest that the polyunsaturated fatty acids (especially those with six double bonds) in the newly synthesized PC can be replaced by more-saturated fatty acids. The fate of unlabelled polyunsaturated fatty acids that will thus be liberated from PC could not be traced in our experiments. It is possible that some of these molecules are 'channelled' to other phospholipids, just as [^{14}C]docosahexaenoic acid that was injected into the rat eye was first incorporated into phosphatidic acid but 60 min later was found mostly in PE (Bazan & Bazan, 1985). This type of mechanism would allow the creation of differences in the fatty acid compositions of various phospholipids, as are known to exist between PC and PE.

Third, it is possible that polyunsaturated fatty acids of isotopically labelled PC underwent enzymic saturation while still a part of the phospholipid. This hypothesis would imply, in agreement with our observations, that

the half-lives of the polyunsaturated fatty acids in PC are much shorter than that of the whole PC molecule (Fig. 2). It appears, however, that experimental data to support or disprove this mechanism are not currently existent.

In summary, we have shown that the [^3H]PC newly synthesized via methylation of PE in rat brain is unusually rich in polyunsaturated fatty acids, specifically those with six and four double bonds. This PC pool, once formed, tends gradually to become more saturated, approaching the composition of unlabelled brain PC.

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