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Rat Brain Phosphatidyl-*N,N*-Dimethylethanolamine Is Rich in Polyunsaturated Fatty Acids

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Abstract: Phosphatidyl - *N,N* - dimethylethanolamine (PDME), an intermediate in the formation of phosphatidylcholine (PC) by the sequential methylation of phosphatidylethanolamine (PE), was purified from rat brain and its fatty acid (FA) composition compared with those of brain PC and PE. The proportion of polyunsaturated fatty acids (PUFAs) in the PDME (29.8%) was similar to that of PE (27.7%) and much greater than in PC (2.8%). Like the PUFAs of PE, the major PUFAs found in PDME were arachidonic acid (20:4) and docosahexaenoic acid (22:6). An isotopic method was developed to quantify the PDME purified from brain; a tritiated methyl group from CH_3I was transferred to the PDME in the presence of cyclohexylamine to form [^{3}H]PC, and the radioactivity of the PC was then counted. The concentration of rat brain PDME obtained using this method (33.0 ± 1.8 μg/g brain) was very similar to that obtained using quantitative GLC analysis of its FAs (36.9 ± 1.8 μg/g). The FAs in the PE and PC of rat brain synaptosomes were also analyzed; too little PDME was present in synaptosomes to permit similar analysis. The percentage of unsaturated FAs in synaptosomal PE was even higher (43.4 vs. 27.7) than that in PE prepared from whole brain. Since synaptosomes have a very high activity of phosphatidyl-*N*-methyltransferase, the enzyme complex that methylates PE to form PC, this enzyme may serve, in nerve endings, to produce a particular pool of PC, rich in PUFAs, which may have a distinct physiological function. **Key Words:** Fatty acid composition—Phosphatidylethanolamine-*N*-methyltransferase — Phosphatidyl - *N,N* - dimethylethanolamine—Rat brain. **Tacconi M. and Wurtman R. J.** Rat brain phosphatidyl-*N,N*-dimethylethanolamine is rich in polyunsaturated fatty acids. *J. Neurochem.* **45**, 805–809 (1985).

Phosphatidylcholine (PC) in cells can be synthesized by three pathways: by combining CDP-choline and diacylglycerol; by exchanging free choline for a serine in phosphatidylserine (PS) or an ethanolamine in phosphatidylethanolamine (PE) (base-exchange); or by sequentially methylating PE. Of these pathways only the latter actually forms new choline molecules. PE-methylation activity, catalyzed by the enzymes phosphatidylethanolamine-*N*-methyltransferase (PEMT), has been demonstrated in many tissues (Bremer and Greenberg, 1961; Hirata and Axelrod, 1978, 1980) including brain (Blusztajn et al., 1979; Crews et al., 1980). The PE in brain is very rich in polyunsaturated fatty acids (PUFAs), whereas brain PC, probably formed via all three pathways, has a much lower average PUFA content (Skrbic and Cumings, 1970; Albert and Anderson, 1977; Crawford and Wells, 1979). It therefore seemed possible that the brain PC formed via PEMT might contain more PUFAs than the PC provided by CDP-choline or base-exchange pathways, and might fulfill different physiological functions. (For example, a PC rich in PUFAs might modify its microenvironment, either by changing the viscosity of the biomembrane or by being a better substrate for phospholipases, thereby making PUFAs or other degradation products, such as choline, more available for further metabolic processes.) In attempting to characterize the pool of PC formed via PE methylation, we examined the

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Abbreviations used: BHT, butylated hydroxytoluene; FA, fatty acid; PC, phosphatidylcholine; PDME, phosphatidyl-*N,N*-dimethylethanolamine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine-*N*-methyltransferase; PL, phospholipid; PMME, phosphatidyl-*N*-monomethylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid. Fatty acids are abbreviated as number of carbon atoms.

fatty acid (FA) composition of brain phosphatidyl-*N,N*-dimethylethanolamine (PDME), an intermediate in the formation of the PC.

MATERIALS AND METHODS

Male Sprague-Dawley rats (200–300 g, Charles River Laboratories) were housed under a 12-h light-dark schedule (VITA-LITE; Duro-Test, North Bergen, NJ, U.S.A.) and given free access to food (Charles River Laboratories, RMH 3000) and water. The animals were decapitated and their brains quickly removed, weighed, and immediately processed. To prepare synaptosomes, brains were homogenized in 0.32 *M* sucrose and centrifuged under a sucrose gradient as described by Dodd et al. (1981). The protein contents of synaptosomes were determined according to Lowry et al. (1951).

Extraction of phospholipids and separation into classes

Brain tissues (0.5–1 g) were homogenized in chloroform-methanol (2:1) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant and lipids were extracted by the method of Folch et al. (1957) with minor modifications; they were first homogenized in 10 volumes of methanol, then the chloroform (20 volumes) was added. Extracts were stirred for 5 min, after which they were filtered over filter paper and the filters were washed with additional chloroform-methanol (2:1); the mixture was then layered with 0.75% wt/vol aqueous KCl (0.2 ml/ml), nitrogen was layered over it, and it was allowed to stand overnight. The upper phase was discarded and the lower phase, containing phospholipids (PLs), was evaporated to dryness under vacuum. When the lipids in synaptosomes were to be extracted, aliquots corresponding to 200–300 μg protein were resuspended in 200 μl of 0.32 *M* sucrose and extracted with chloroform-methanol (2:1), as described above. To separate PLs from neutral lipids, the extracts were redissolved in chloroform and layered onto a 1 × 25 cm column of activated silicic acid (10 g, Biosil A, 100–200 mesh, Bio-Rad) (Rouser et al., 1976). Chloroform, acetone, and methanol (10 column volumes each) were used to elute lipids; the first two eluates—the neutral lipids and free FAs in chloroform, and the cerebrosides and sulfatides in acetone—were discarded. PLs eluted with methanol were evaporated to dryness under vacuum and redissolved in 2 ml chloroform; aliquots were then spotted onto silica gel plates (Silica gel G, Adsorbosil Plus I, Alltech) and chromatographed using the solvent system chloroform-methanol-ammonia, 70:30:4 by vol.

Purification of PDME

PDME has a R_f of 0.62 in the solvent system previously described; it thus separates well from the major PLs [PE, R_f = 0.47; PC, R_f = 0.33; PS, R_f = 0.07; phosphatidyl-*N*-monomethylethanolamine (PMME), R_f = 0.40] but not from others such as cardiolipin and, possibly, the cerebrosides. To obtain pure PDME in sufficient amounts for FA analysis and quantitation, aliquots of extracts containing total PLs, corresponding to 300–400 mg brain, were run on TLC plates as described above. In each plate a mixture of brain extract, to which PDME standard was added, was always run in parallel and exposed to iodine vapors or rhodamine spray (after protecting the samples) to visualize the TLC region containing PDME. A 1-cm band, corresponding to the R_f of a true PDME standard, was scraped, extracted three times with methanol (2 + 1 + 1 ml) containing 0.01% BHT, evaporated to dryness, spotted on high-performance TLC plates (HETLC-HL Uniplates with preadsorbent, 10 × 10 cm, Analtech, Newark, DE, U.S.A.), and chromatographed in chloroform-methanol-water (70:30:4). A brain sample to which PDME standard had been added was always run in parallel. After visualization as described above, the band corresponding to the PDME standard was scraped and then transferred in glass tubes for further analysis. In one experiment, uniformly labeled [^{14}C]PDME (0.05 μCi/mg) was extracted and chromatographed following the procedure described above; the radioactivity recovered was 55%.

Analysis of FA composition

Silica gels containing PDME (purified by the above procedure), PE, and PC (separated by TLC using chloroform-methanol-ammonia, 70:30:4) were methylated directly by alkaline methanolysis (Glass, 1971). Diheptadecanoyl-PC (20 μg) was added to each sample as internal standard. Each sample was mixed thoroughly with 1 ml saturated NaOH in chloroform-methanol (2:1); 10 min later, 1 ml 1 *M* HCl in saline was added and the sample was mixed and centrifuged. Approximately 300 μl of the lower phase was then aspirated, evaporated to dryness, and resuspended in 20 μl chloroform, 1–2 μl of which were injected in a gas chromatography apparatus Packard 430, equipped with a FID detector and an electronic integrator. The chromatographic column (6 feet long and 2 mm internal diameter) contained SP 2330 as liquid phase; the carrier gas was nitrogen (22 ml/min). The initial temperature was kept at 160°C for 4 min, and then was increased to 220°C at a rate of 2°C/min; the injector and detector temperatures were both 275°C. The FA methylesters were identified by comparing their retention factors with those of standard solutions run under identical conditions. Measurements of peak areas were made with the automatic integrator attached to the gas chromatograph. FA contents were expressed as percentages of total FAs. Total FAs in PDME were quantitated by comparison with the internal standard (diheptadecanoyl-PC) and corrected for recoveries.

Methylation of PDME to PC

To affirm the authenticity of the FAs obtained through the above procedure, an additional assay was developed involving the transfer of a tritiated methyl group from CH_3I to the PDME, yielding tritiated PC (Stoffel, 1975). Aliquots of 200–300 mg of brain were extracted and chromatographed as described above. Silica gel containing PDME was scraped and then extracted three times with methanol; the extracts were evaporated to dryness. To the dry extracts 2 μl of cyclohexylamine (50 μmol), 24 μl [^{3}H]CH_3I (25 μmol, 0.1 μCi/μmol), and then methanol to a final volume of 500 μl were added. The tubes were flushed with nitrogen and then tightly capped and stored at room temperature in the dark for about 20 h, after which 2 ml of diethylether were added. The mixture was then washed in sequence with 5% wt/vol sodium metabisulfite, 1 *M* HCl, and water (1.5 ml each). The washings were pooled and further extracted with 2 ml chloroform, and added quantitatively to the ether phase. The ether-

chloroform mixture was then evaporated to dryness and the extracts were redissolved in small aliquots of chloroform-methanol (1:1) and spotted onto TLC plates (Adsorbosil, Plus I, Alltech) using a mixture containing chloroform-methanol-ammonia, 70:30:4. The areas corresponding to PC (indicated by addition of true PC standard) were scraped into scintillation vials, to which 1 ml methanol and 10 ml Betafluor were added, and their radioactivities counted using a liquid scintillation counter. Two sets of standard curves containing known concentrations of PDME were run in parallel: external standard of 5, 10, 20, and 40 μg PDME, dissolved in chloroform (which were pipetted into the reaction tubes and directly methylated), and internal standards of 10, 20, 40, and 60 μg of PDME, in duplicate, which were separated and extracted using the same two TLC systems utilized for brain extracts. Recoveries calculated by comparing the two sets of standards were 50%.

Phosphorus analysis of brain PLs was performed according to the method of Svanborg and Svennerholm (1961).

To prevent oxidation, samples were stored under nitrogen and all the glassware used was flushed with nitrogen before use.

Materials

Chloroform and methanol of reagent grade were redistilled before use. PDME from egg was obtained from Gibco Laboratories, NY, U.S.A. PE (dipalmitoyl) and PC (dipalmitoyl and diheptadecanoyl) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). [^{3}H]CH_3I (100 μCi/μmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Other chemicals were of reagent grade.

RESULTS

The percent FA composition of the PDME in rat brain was analyzed and compared to the FA composition of brain PE and PC in five separate experiments (Table 1a). The major FAs in brain PDME were oleic (33.6%), stearic (20.4%), palmitic (11.1%), arachidonic (10.6%), and docosahexaenoic acid (12.8%). This FA pattern was very similar to that of brain PE (which contained 10.4% arachidonate and 12.1% docosahexaenoate), but differed markedly from that of brain PC (which contained almost 50% of palmitic acid and only 2.8% of PUFAs).

The FA compositions of synaptosomal PE and PC were also measured (Table 1b). (We obtained too little PDME from synaptosomes to permit measurement of its FAs.) The levels of docosahexaenoic and arachidonic acids (as percents of total FAs) in synaptosomal PE and PC were both considerably greater than their levels in PE and PC from total brain. The enrichment in synaptosomal PUFAs was particularly evident when the polyunsaturated versus monounsaturated ratio was calculated: this was threefold greater in synaptosomal PE, twofold greater in synaptosomal PC.

The amount of PDME obtained from rat brain, quantitated by GLC analysis of its FAs, was 36.9 μg/g fresh tissue (Table 2). To ascertain whether the material isolated and analyzed for FA composition contained only PDME, we also measured brain PDME using an isotopic method based on the transfer of a methyl group from tritiated CH_3I to PDME to form tritiated PC (as described in Materials and Methods). This experiment affirmed that at least 90% of the FAs recovered by GLC analysis of supposed PDME were in fact related to PDME. PDME accounted for 0.1% of total PL: its levels were only 0.3–0.4% of those of PC (270 μg/mg PL) or PE (360 μg/mg PL), respectively.

TABLE 1a. *Percent fatty acid composition of rat brain PE, PC, and PDME*

Carbon no.	FAs [as % total (mean ± SEM)]		
	PE	PDME	PC
14:0	tr	tr	tr
16:0	9.1 ± 0.8	11.1 ± 1.7	46.6 ± 1.7
16:1	0.7 ± 0.1	2.9 ± 1.1	0.8 ± 0.2
18:0	27.2 ± 2.0	20.4 ± 3.0	17.1 ± 3.1
18:1	29.1 ± 1.5	33.6 ± 3.3	30.9 ± 1.4
18:2	0.5 ± 0.1	2.9 ± 1.1	0.3 ± 0.1
20:0	0.2 ± 0.0	0.1 ± 0.01	ND
20:1	6.2 ± 0.5	1.9 ± 0.7	1.7 ± 0.4
20:3–22:0	0.5 ± 0.1	1.3 ± 0.2	0.1 ± 0.00
20:4	10.4 ± 0.8	10.6 ± 1.7	1.6 ± 0.2
20:5–22:2	0.5 ± 0.2	0.5 ± 0.08	<0.1
22:4	3.7 ± 0.6	1.7 ± 0.1	<0.1
22:6	12.1 ± 3.5	12.8 ± 1.7	0.7 ± 0.1
Total monounsaturated	36.0	38.0	33.4
Total polyunsaturated	27.7	29.8	2.8
Poly/mono	0.78	0.77	0.08

TABLE 1b. *Fatty acid composition of PE and PC in rat brain synaptosomes*

Carbon no.	FAs [as % total (mean ± SEM)] PE	PC
14:0	tr	tr
16:0	8.9 ± 1.9	51.0 ± 2.3
16:1	0.7 ± 0.2	1.1 ± 0.2
18:0	30.8 ± 1.1	16.2 ± 2.1
18:1	14.8 ± 0.8	26.3 ± 1.4
18:2	0.8 ± 0.1	0.4 ± 0.01
20:0	<0.1	<0.1
20:1	1.3 ± 0.2	0.7 ± 0.2
20:3–22:0	0.3 ± 0.03	ND
20:4	14.9 ± 0.3[a]	2.4 ± 0.3[a]
20:5–22:2	0.6 ± 0.2	ND
22:4	4.4 ± 0.2	ND
22:6	22.5 ± 1.2[a]	1.8 ± 0.5[a]
Total monounsaturated	16.8	28.1
Total polyunsaturated	43.4	4.6
Poly/mono	2.58	0.16

ND, not detected.

[a] $p < 0.01$. This was greater than its percentage in whole brain PC (ANOVA: two-tailed t test). Plasmologen PE was not separated.

DISCUSSION

These studies show that the rat's brain contains only small quantities of PDME, equivalent to approximately 0.1% of total brain PLs, or 0.3–0.4% of the amount of PE and PC, and that the FA composition of the PDME is very similar to that of PE and different from that of PC. The relatively low levels of PDME in brain are compatible with either of two interpretations, i.e., either that the PEMT pathway is a relatively minor source of brain PC (compared with the contributions of the CDP-choline and base-exchange pathways), or that the addition of the third methyl group to the PDME is a very rapid process, such that very little of the intermediate accumulates. The first interpretation is probably more likely: the CDP-choline pathway (and, probably to a lesser extent, base-exchange) is considered to provide most of the PC in the brain (Ansell, 1973; Orlando et al., 1977), and as little as 1% may be derived via the PEMT activity (Percy et al., 1982). Moreover, when synaptosomes are incubated with labeled *S*-adenosylmethionine and exogenous PMME the main product of the reaction obtained after 30 min is PDME and not PC (Blusztajn et al., 1979), suggesting that at least in vitro, the addition of the third methyl group is not a fast process. The major difference between the FA compositions of brain PDME and PC also could be explained similarly by hypothesizing that a relatively small amount of PC containing large proportions of PUFAs (such as arachidonic and docosahexaenoic acids) derives from the PEMT pathway, and that this small pool is diluted with much larger amounts of PC coming from other pathways, and containing mostly saturated FAs.

TABLE 2. *Concentration of PDME in rat brain*

	Method of analysis FAME-GLC	CH_3I methylation
μg/g brain ± SEM	36.9 ± 1.83	33.0 ± 1.82
μg/mg PL ± SEM	1.22 ± 0.06	1.09 ± 0.06

Five separate experiments were performed for each analysis, each using approximately 300 mg brain. Fatty acid methyl ester GLC (FAME-GLC) was performed on PDME purified from brain, as described in Materials and Methods, by methylation of its fatty acids in the presence of diheptadecanoyl-PC as an internal standard. To a similar amount of purified PDME a tritiated methyl group from CH_3I was transferred to form labeled PC, the radioactivity of which was then counted and quantitated by comparison with a PDME standard curve processed in the same way. Corrections for possible losses during the extraction procedure were obtained by calculating the recovery of uniformly labeled [^{14}C]PDME added to brain samples at the beginning of the extraction.

Our data on the FA composition of rat brain PDME are consistent with observations on PLs in other tissues; PC produced by hepatic PEMT was found to be particularly rich in arachidonic acid (LeKim et al., 1973; Trewhella and Collins, 1973); in rabbit leukocytes the arachidonic acid released by the addition of chemoattractants was found to be derived mainly from PC produced via PEMT (Hirata et al., 1979). Moreover, Hitzemann (1982) found that both PEMT activity and the percent of arachidonoyl-PC were high in brains of 14-day-old rats.

Since the overall PUFA content in brain PC is much smaller than in other tissues [in the liver, for example, it is 40% (Getz et al., 1961) compared with only 5–6% in brain], the pool derived from PEMT, although small, may be particularly important for membrane functions [especially at the nerve endings, where PEMT activity is highest (Blusztajn et al., 1979)]. The PEMT pathway might be even more important in cholinergic nerve endings, where the PC it forms may be used as a source of choline for acetylcholine synthesis (Maire and Wurtman, 1984), especially when neuronal firing is increased or the supply of choline is limited (Maire and Wurtman, 1985).

In conclusion, our findings show that only small amounts of PDME are present in rat brain (33.6 μg/g brain, representing only 0.1% of total PLs) and that the FA composition of this PDME is similar to that of PE and very different from that of the total pool of its product, PC. Since most of the brain PC probably derives from the CDP-choline pathway, the pool of PC deriving from PEMT, which is enriched in PUFAs, may have particular functions.

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