Eur. J. Biochem. *240*, 53−62 (1996)

Transacylase-mediated and phosphodiesterase-mediated synthesis of *N*-arachidonoylethanolamine, an endogenous cannabinoid-receptor ligand, in rat brain microsomes

Comparison with synthesis from free arachidonic acid and ethanolamine

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(Received 22 April 1996) − EJB 96 0590/5

The levels of *N*-arachidonoylethanolamine (anandamide), an endogenous cannabinoid-receptor ligand, and a relevant molecule, *N*-arachidonoylphosphatidylethanolamine (*N*-arachidonoylPtdEtn), in rat brain were investigated using a newly developed sensitive analytical method. We found that rat brain contains small but significant amounts of these two types of *N*-arachidonoyl lipids (4.3 pmol/g tissue and 50.2 pmol/g tissue, respectively). Then, we investigated how *N*-arachidonoylethanolamine (anandamide) is produced in the brain. We found that anandamide can be formed enzymatically via two separate synthetic pathways in the brain: enzymatic condensation of free arachidonic acid and ethanolamine; and formation of *N*-arachidonoylPtdEtn from PtdEtn and arachidonic acid esterified at the 1-position of phosphatidyl-choline (PtdCho), and subsequent release of anandamide from *N*-arachidonoylPtdEtn through the action of a phosphodiesterase. We confirmed that rat brain contains both the enzyme activities and lipid substrates involved in these reactions. Several lines of evidence strongly suggest that the second pathway, rather than the first one, meets the requirements and conditions for the synthesis of various species of *N*-acylethanolamine including anandamide in the brain.

Keywords: anandamide (*N*-arachidonoylethanolamine); *N*-arachidonoylphosphatidylethanolamine; cannabinoid; transacylase; phosphodiesterase.

Anandamide (*N*-arachidonoylethanolamine) is a novel type of icosanoid that was described as the first endogenous cannabinoid-receptor ligand in mammals [1]. It binds to cannabinoid receptors with high affinity [1−6], exhibits potent pharmacological activity *in vitro* or *in vivo* comparable to those of other psycotropic cannabinoids [1, 2, 7−10] and is assumed to be one of the important endogenous modulators of several synaptic functions [11, 12]. Due to its possible physiological importance and possible therapeutic applications, much attention is being paid to this bioactive lipid molecule. However, little is known of the mechanism underlying the biosynthesis, the tissue levels or the metabolic regulation of anandamide and related molecules in mammalian tissues and cells.

Several investigators have already reported that anandamide can be synthesized enzymatically from free arachidonic acid and ethanolamine using a brain homogenate [13], microsomes [14, 15], the P_2 fraction [16] and cytosol [14]. However, the physiological significance of this pathway has not yet been established because of the high K_m values for substrates [14−16]. The reaction may be attributed to the reverse action of amidohydrolase [15], yet information on this enzyme activity remains limited. However, Di Marzo et al. [17], recently suggested the possibility that anandamide can be synthesized from *N*-arachidonoylphosphatidylethanolamine (*N*-arachidonoylPtdEtn) through the action of a phosphodiesterase. However, the details of this enzyme pathway remain quite obscure. For example, little information is available concerning the tissue level of *N*-arachidonoylPtdEtn. Furthermore, the mechanism underlying the formation of *N*-arachidonoylPtdEtn has not yet been elucidated. It will be very important to clarify the mechanism and the regulation of the biosynthesis of anandamide in order to better understand the physiological roles of this bioactive lipid molecule.

In the present study, we investigated the enzyme activities involved in the synthesis of anandamide using rat brain microsomes. We found that the *N*-arachidonoylPtdEtn pathway, rather than synthesis from free arachidonic acid and ethanolamine, accounts well for the synthesis of various types of *N*-acylethanollamine including anandamide present in this tissue.

MATERIALS AND METHODS

Materials. [^{3}H]Arachidonic acid (100 Ci/mmol), [^{14}C]arachidonic acid (57 mCi/mmol), [^{14}C]palmitic acid (57 mCi/mmol), [^{14}C]stearic acid (58 mCi/mmol) [^{14}C]linoleic acid (53 mCi/mmol), and 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (57 mCi/mmol) were purchased from Dupont-New England Nuclear. [^{3}H]Palmitic acid (52 Ci/mmol), [^{3}H]oleic acid (5 Ci/mmol) and [^{14}C]oleic acid (54 mCi/mmol) were purchased from Amersham. Unlabeled fatty acids, *sn*-glyc-

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Abbreviations. $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

Enzymes. Phospholipase A_2 (EC 3.1.1.4); phospholipase D (EC 3.1.4.4).

ero-3-phosphocholine, essentially fatty-acid-free BSA and phospholipase A_2 (*Naja naja*) were obtained from Sigma. Dioleoylglycerophosphoethanolamine, phenylmethylsulfonyl fluoride ($PhMeSO_2F$), dithiothreitol, dicyclohexylcarbodiimide and 1-anthroyl cyanide were from Wako Pure Chem. Ind. 9-Anthroyldiazomethane was from Funakoshi Co., Ltd. Ethanolamine hydrochloride and dimethylaminopyridine were from Aldrich Chem. Co. Quinuclidine was from Molecular Probes, Inc. Ethanol-free chloroform (stabilized with 2-methyl-2-butene) and ethanolamine (2-aminoethanol) were obtained from Tokyo Kasei Co., Ltd. Phospholipase D from *Streptomyces chromofuscus* and that from cabbage were obtained from Boehringer Mannheim GmbH. Docosapentaenoic acid (*n*-6) was prepared as follows. Rat testis total lipids were treated with sodium methoxide, then the resultant fatty acid methyl esters were separated by TLC and further fractionated by 20% $AgNO_3$-impregnated TLC. The 22:5(*n*-6)-enriched fraction (pentaenoic species) was extracted, subjected to alkaline hydrolysis and purified by TLC. Wistar rats (male, 330−430 g body mass) were obtained from Sankyo Lab. Service.

Preparation of radiolabeled phospholipids. First, radiolabeled fatty acids {([^{3}H]20:4 (arachidonic), [^{3}H]16:0 (palmitic), [^{14}C]18:0 (stearic) and [^{3}H]18:1 (oleic)} were diluted with unlabeled fatty acids to 5 mCi/mmol. The radiolabeled fatty acids (3 mg) were then mixed with 10 mg of dicyclohexylcarbodiimide dissolved in 0.25 ml of chloroform (without ethanol) at room temperature for 2 h to yield [^{3}H]/[^{14}C]fatty acid anhydride [18]. Then, 10 mg of glycero-3-phosphocholine and 4 mg of dimethylaminopyridine were added to the tube [19, 20]. The mixture was then stirred in the tube, sealed with N_2 gas, at room temperature for 24 h. The resultant di-[^{3}H]/[^{14}C]acylglycerophosphocholine was purified by TLC with chloroform/methanol/water (65:25:4, by vol.). To prepare di-[^{3}H]arachidonoylglycerophosphoethanolamine, di-[^{3}H]arachidonoylglycerophosphocholine was treated with phospholipase D (cabbage) in the presence of ethanolamine (20%, mass/vol). The resultant di[^{3}H]-arachidonoylglycerophosphoethanolamine was purified with the TLC system described above. 1-Arachidonoyl-2-[^{3}H]arachidonoylglycerophosphocholine was prepared as follows. Chemically synthesized diarachidonoylglycerophosphocholine was subjected to phospholipase A_2 hydrolysis. The resultant 1-arachidonoylglycerophosphocholine was purified by TLC with chloroform/methanol/water (65:25:4, by vol.), then mixed with dimethylaminopyridine and [^{3}H]arachidonoyl anhydride (5 mCi/mmol) prepared as described above, and dissolved in 0.25 ml chloroform containing 0.5% acetic anhydride [20]. After standing for 24 h, the resultant 1-arachidonoyl-2-[^{3}H]arachidonoylglycerophosphocholine was purified by TLC with the solvent system described above. To prepare *N*-[^{14}C]acylPtdEtn, first, [^{14}C]fatty acids (10 mCi/mmol) were converted to fatty acyl chlorides by treatment with oxalyl chloride. The resultant [^{14}C]fatty acyl chlorides were mixed with dioleoylglycerophosphoethanolamine dissolved in chloroform containing 5% pyridine. After standing for 60 min, *N*-[^{14}C]acylPtdEtn was purified by TLC with chloroform/methanol/water (75:25:4, by vol.).

HPLC analysis of 9-anthroyldiazomethane-derivatized free fatty acids by HPLC. Fatty acids were derivatized with 9-anthroyldiazomethane according to the method of Nakagawa and Waku [21]. 9-Anthroyldiazomethane-derivatized individual fatty acids were purified by TLC with petroleum ether/diethyl ether/acetic acid (90:10:1, by vol.; R_f = 0.35−0.42: the R_f values varied somewhat, depending on the degree of unsaturation and the chain length), and separated from each other with a HPLC system (Shimadzu LC-6A) equipped with a reverse-phase column (CAPCELL PAK C18 SG, 5 μm, 4.6 mm × 250 mm × 2, Shiseido and a fluorescence detector (excitation at 365 nm; emission at 412 nm). Acetonitrile/isopropanol/water (90:4:6, by vol.) was used as the mobile phase. The flow rate was 1.2 ml/min. Analysis of free fatty acids in rat brain was carried out as follows. Brains were removed from decapitated rats, washed with ice-cold saline and then homogenized in chloroform/ethanol/water (1:2:0.7, by vol.) with a Waring blender. The post-decapitation period was usually 15−20 min. Total lipids were extracted by the method of Bligh and Dyer [22]. A portion of total lipids (usually equivalent to 0.05 g wet tissue), with 2 nmol of 17:0 as an internal standard, was separated by two-dimensional TLC developed first with petroleum ether/diethyl ether/acetone/acetic acid (30:40:20:1, by vol.) and then with chloroform/methanol/NH_4OH (80:20:2, by vol.). Lipid spots were visualized under ultraviolet light (365 nm) after spraying with primuline. The free fatty acid fraction (R_f = 0.80−0.84 for the first dimension and 0.09−0.13 for the second dimension) was scraped from a TLC plate and extracted from the silica gel by the method of Bligh and Dyer. Free fatty acids were derivatized with 9-anthroyldiazomethane and analyzed as described above.

HPLC analysis of 1-anthroyl derivatives of *N*-acylethanolamines by HPLC. Various types of standard *N*-acylethanolamine including *N*-17:0 ethanolamine were prepared from various types of fatty acyl chloride and ethanolamine by the method of Devane et al. [1]. *N*-Acylethanolamine was converted to 1-anthroyl derivatives by the modified method of Goto et al. [23]. Briefly, *N*-acylethanolamine (2 nmol) was treated with 1-anthroyl cyanide (1 mg/ml) in 0.2 ml acetone containing 0.08% quinuclidine at 45 °C for 75 min. The reaction was stopped by the addition of 50 μl methanol. The 1-anthroyl derivative of *N*-acylethanolamine was purified by TLC with petroleum ether/diethyl ether/acetic acid (40:60:1, by vol.; R_f = 0.11−0.16). The purified 1-anthroyl derivative of *N*-acylethanolamine was analyzed with an HPLC system equipped with a reverse-phase column (Shiseido CAPCELL PAK C18 SG, 4.6 mm × 250 mm × 2) and a fluorescence detector (excitation at 370 nm; emission at 470 nm).

Acetonitrile/isopropanol/water (80:3:17, by vol.) was used as the mobile phase. The flow rate was 1.4 ml/min. The derivatization with 1-anthroyl cyanide occurred linearly at least up to 100 nmol of *N*-acylethanolamine for both *N*-heptadecanoyl (17:0) species and *N*-arachidonoyl (20:4) species (data not shown). The detection limit for the 1-anthroyl derivative of *N*-acylethanolamine with this system was around 0.3 pmol. To examine *N*-acylethanolamines in the brain, total lipids were extracted from decapitated rat brains. Butylhydroxytoluene (final, 0.05%) was added to avoid lipid peroxidation. *N*-Acylethanolamine was purified as follows. A portion of the total lipids (usually equivalent to 2 g wet tissue), with 2 nmol of *N*-heptadecanoyl ethanolamine added as an internal standard, was first fractionated by TLC with chloroform/methanol/NH_4OH (80:20:2, by vol.; in a tank sealed with N_2 gas. The area corresponding to standard *N*-acylethanolamine (a mixture of several saturated and unsaturated species was used because of the different R_f values of saturated and unsaturated species: R_f = 0.77−0.79) was scraped off a TLC plate and extracted from the silica gel by the method of Bligh and Dyer. The extraction was conducted in the presence of butylhydroxytoluene (0.001%) in a N_2-gas-sealed tube. *N*-Acylethanolamine was further purified by TLC with petroleum ether/diethyl ether/acetone/acetic acid (30:40:20:1, by vol.; R_f = 0.25−0.27), and by subsequent two-dimensional TLC developed first with petroleum ether/diethyl ether/aceton/acetic acid (30:40:20:1, by vol.) and then with an organic layer of ethyl acetate/petroleum ether/acetic acid/water (100:50:20:100, by vol.; R_f = 0.31−0.34). A stream of N_2 gas was used to remove solvents from the TLC plates. Purified *N*-acylethanolam-

ine was converted to its 1-anthroyl derivative and then analyzed as described above.

HPLC analysis of 1-anthroyl derivatives of the *N*-acyl moiety of *N*-acylPtdEtn. Various types of *N*-acylPtdEtn including *N*-17:0 PtdEtn were prepared from various types of fatty acyl chloride and dioleoylglycerophosphoethanolamine as described above. To examine the *N*-acyl moiety of *N*-acylPtdEtn in rat brain, a portion of the total lipids (equivalent to 2 g wet tissue), with 2 nmol of *N*-17:0 PtdEtn added as an internal standard, was first fractionated by TLC with chloroform/methanol/NH_4OH (80:20:2, by vol.). The area corresponding to standard *N*-acylPtdEtn (a mixture of N-saturated and N-unsaturated species, $R_f = 0.64-0.68$) was scraped off a TLC plate and extracted from the silica gel by the method of Bligh and Dyer. Similar care was taken to avoid lipid peroxidation throughout the analytical procedures as in the case of the analysis of *N*-acylethanolamine. *N*-AcylPtdEtn was further purified by two-dimensional TLC developed first with petroleum ether/diethyl ether/acetone/acetic acid (30:40:20:1, by vol.; $R_f = 0.01$) and then with chloroform/methanol/water (75:25:4, by vol.; $R_f = 0.56-0.60$). The purified *N*-acylPtdEtn was then subjected to phospholipase D (*Streptomyces chromofuscus*) treatment. Briefly, 1 U of phospholipase D, 1 ml diethyl ether, 1 ml 2 mM sodium acetate, pH 8.0, and *N*-acylPtdEtn were mixed and stirred at room temperature for 2 h. We confirmed that more than 95% of the radioactivity of *N*-[^{14}C]18:1 PtdEtn (100 nmol) was recovered in the *N*-acylethanolamine fraction (data not shown). The resultant *N*-acylethanolamine was extracted and purified by TLC with petroleum ether/diethyl ether/acetone/acetic acid (30:40:20:1, by vol.) *N*-Acylethanolamine was analyzed with a HPLC system after conversion to its 1-anthroyl derivative as described above.

Enzymatic formation of *N*-acylethanolamine from radiolabeled fatty acids and ethanolamine. Rat brain microsomes were prepared as described earlier [20]. The protein content was estimated according to the method of Lowry et al. [24]. The standard assay conditions were as follows. Brain microsomes (250 μg protein) were incubated with ethanolamine hydrochloride (2.5 μmol) and various types of free [^{14}C]fatty acids (2.5 nmol) dissolved in 0.2% BSA (final concentration of BSA, 0.02%) in 250 μl 20 mM Hepes, pH 8.0, containing 2 mM dithiothreitol and 2 mM EGTA at 37°C for 20 min. The incubation was stopped by adding chloroform/methanol (1:2, by vol.). The total lipids were extracted by the method of Bligh and Dyer. Lipids were fractionated by two-dimensional TLC developed first with petroleum ether/diethyl ether/acetone/acetic acid (30:40:20:1, by vol.) and then with chloroform/methanol/NH_4OH (80:20:2, by vol.). The spots of *N*-acylethanolamine and free fatty acids were scraped off a TLC plate, and then the radioactivity was estimated.

Enzymatic release of *N*-acylethanolamine from radiolabeled *N*-acylPtdEtn. Rat brain microsomes (250 μg protein) were incubated with various types of *N*-[^{14}C]acylPtdEtn (2.5 nmol) in 250 μl 20 mM Hepes, pH 7.4, containing 2 mM dithiothreitol, 2 mM $CaCl_2$ or EGTA, 2 mM Triton X-100 and 1 mM $PhMeSO_2F$ at 37°C for 20 min according to the method of Schmid et al. [25], with slight modifications. The incubation was stopped by adding chloroform/methanol (1:2, by vol.), and then the total lipids were extracted by the method of Bligh and Dyer. Lipids were fractionated by two-dimensional TLC developed first with petroleum ether/diethyl ether/acetone/acetic acid (30:40:20:1, by vol.) and then with chloroform/methanol/NH_4OH (80:20:2, by vol.). The radioactivity in *N*-acylethanolamine and *N*-acylPtdEtn fractions was estimated.

Enzymatic formation of *N*-acylPtdEtn from radiolabeled phospholipids. The standard assay conditions were as follows. Rat brain microsomes (100 μg protein) were incubated with various types of radiolabeled diacyl phospholipids (5 nmol), lysophospholipids (5 nmol) or free fatty acids (5 nmol) [each 5 mCi/mmol of fatty acyl moiety, except in the case of 1-Pam-2-[^{14}C]arachidonoylGro*P*Cho (Pam, palmitoyl; Gro, glycerol) (57 mCi/mmol)] and dioleoyl glycerophosphoethanolamine (250 nmol) in 250 μl 20 mM Hepes, pH 7.4, containing 2 mM dithiothreitol and 2 mM $CaCl_2$ at 37°C for 2 h. The incubation was stopped by adding chloroform/methanol (1:2, by vol.), and then total lipids were extracted by the method of Bligh and Dyer. The lipids were fractionated by two-dimensional TLC developed first with petroleum ether/diethyl ether/acetone/acetic acid (30:40:20:1, by vol.) and then with chloroform/methanol/NH_4OH (80:20:2, by vol.). The *N*-acylPtdEtn fraction was further purified by TLC with chloroform/methanol/water (75:25:4, by vol.). The radioactivity in the *N*-acylPtdEtn fraction was estimated. In some cases, the purified *N*-acylPtdEtn was further hydrolyzed with phospholipase D (*S. chromofuscus*). The resultant *N*-acylethanolamine was purified by TLC with petroleum ether/diethyl ether/acetone/acetic acid (30:40:20:1, by vol.). The radioactivity recovered in the *N*-acylethanolamine fraction was estimated.

Analysis of fatty acids at the 1-position of diacyl phospholipids. Total lipids were obtained from rat whole brains, rat brain microsomes and rat brain synaptosomes prepared as described earlier [26] by the method of Bligh and Dyer. PtdCho and PtdEtn were separated by two-dimensional TLC developed first with chloroform/methanol/NH_4OH (65:35:5, by vol.) and then with chloroform/acetone/methanol/acetic acid/water (5:2:1:1.3:0.5, by vol.) [27]. Then, PtdCho and PtdEtn were hydrolyzed with phospholipase A_2 (*N. naja*), which was boiled (100°C for 5 min) prior to use to abolish lysophospholipase activity, in a mixture of 1 ml diethyl ether and 1 ml 0.1 M Tris/HCl, pH 7.4, containing 2 mM $CaCl_2$, as described previously [27]. The mixture without phospholipase A_2 was run as a control. The resultant lysoPtdCho ($R_f = 0.06-0.08$) and lysoPtdEtn ($R_f = 0.20-0.22$) were purified by TLC with chloroform/methanol/water (65:25:4, by vol.). After extraction from the silica gel, the fatty acyl moieties of lysoPtdCho and lysoPtdEtn were converted to fatty acid methyl esters, using 0.5 M methanolic sodium methoxide, and then analyzed by GLC. 17:0 methyl ester was used as an internal standard.

RESULTS

First, we developed a method for the separation and quantification of various molecular species of *N*-acylethanolamine by HPLC after their conversion to 1-anthroyl derivatives. As shown in Fig. 1, the 1-anthroyl derivatives of 18 species of standard *N*-acylethanolamine were separated well from each other on a chromatogram. We then examined the level and fatty acid composition of the *N*-acyl moiety of *N*-acylethanolamine obtained from rat brain using *N*-17:0 ethanolamine as an internal standard. The results of four separate experiments are summarized in Table 1. Predominant fatty acyl moieties of *N*-acylethanolamine were 16:0 and 18:0 accounting for 50.6% and 19.4, respectively. Considerable portions (12.2% and 12.6%) were also accounted for by 18:1(*n*-9)-containing and 18:1(*n*-7)-containing species, respectively. However, the levels of other species were found to be generally very low. For example, the amount of the arachidonoyl species (anandamide) was 4.3 pmol/g wet tissue, accounting for 0.7% of the total. It is apparent, therefore, that the *N*-acyl moiety of *N*-acylethanolamine present in rat brain consists mainly of saturated and monoenoic species.

Next we examined the enzyme activity involved in the synthesis of anandamide from free arachidonic acid and ethanol-

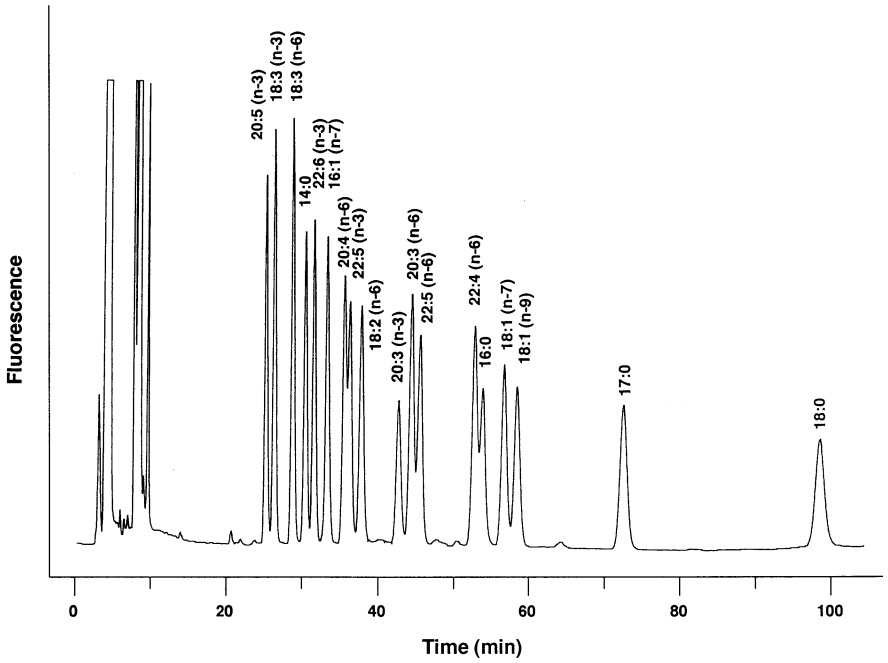

Fig. 1. Separation of 1-anthroyl derivatives of various types of *N*-acylethanolamine by reverse-phase HPLC. *N*-Acylethanolamine was converted to 1-anthroyl derivatives and then analyzed by reverse-phase HPLC as described in Materials and Methods.

Table 1. Fatty acid composition of *N*-acylethanolamine and *N*-acyl moiety of *N*-acylPtdEtn obtained from rat brain. *N*-Acylethanolamine and *N*-acylPtdEtn were obtained from rat brains and analyzed as described in Materials and Methods. *N*-17:0 ethanolamine or *N*-17:0 PtdEtn was used as an internal standard. The data are the means ± SD of four determinations.

Acyl moiety	Composition of			
	N-Acylethanolamine		*N*-AcylPtdEtn	
	pmol/g wet mass	%	pmol/g wet mass	%
14:0	6.3 ± 1.4	1.1 ± 0.2	54.6 ± 14.7	0.5 ± 0.1
16:0	302.5 ± 33.3	50.6 ± 5.6	8 407.9 ± 2051.2	69.6 ± 17.0
16:1 (*n*-7)	5.5 ± 1.4	0.9 ± 0.2	154.9 ± 43.1	1.3 ± 0.4
18:0	115.6 ± 27.7	19.4 ± 4.6	1 475.6 ± 399.3	12.2 ± 3.3
18:1 (*n*-7)	75.2 ± 13.3	12.6 ± 2.2	974.4 ± 301.0	8.1 ± 2.5
18:1 (*n*-9)	73.1 ± 44.1	12.2 ± 7.4	823.5 ± 318.4	6.8 ± 2.6
18:2 (*n*-6)	4.6 ± 1.4	0.8 ± 0.2	93.3 ± 38.0	0.8 ± 0.3
20:4 (*n*-6)	4.3 ± 1.1	0.7 ± 0.2	50.2 ± 27.8	0.4 ± 0.2
22:6 (*n*-3)	6.1 ± 0.6	1.0 ± 0.1	25.8 ± 12.8	0.2 ± 0.1
Others	4.1 ± 2.4	0.7 ± 0.4	11.6 ± 10.0	0.1 ± 0.1
Total	597.3 ± 104.4	100	12 071.8 ± 3143.4	100

amine. As shown in Fig. 2, rat brain microsomes contain an enzyme activity that catalyzes the formation of anandamide from free arachidonic acid and ethanolamine. The reaction increased with time (Fig. 2 A), with increasing amounts of microsomal protein (Fig. 2 B) and with increasing concentrations of the substrates (Fig. 2 D and E). The optimal pH was around 8.0−9.0 (Fig. 2 C). No appreciable activity was observed when boiled microsomes were employed. The enzyme reaction followed the typical Michaelis-Menten equation (data not shown): the apparent K_m value for ethanolamine (assayed in the presence of 100 μM free arachidonic acid) was 135 mM and that for arachidonic acid (assayed in the presence of 150 mM ethanolamine) was 153 μM.

The fatty acid specificity of the enzyme reaction was studied next. As shown in Fig. 3, various types of fatty acids acted as acyl donors in the formation of *N*-acylethanolamine. Differences in potency appear not to be so prominent among these fatty acids, though 20:4, 18:0 and 18:2 were incorporated at slightly slower rates than 16:0 and 18:1. This clearly indicates that the enzyme reaction is not specific to certain species of fatty acids such as arachidonic acid. This observation prompted us to examine the free-fatty-acid pool in the brain.

Table 2 shows the level and profile of free fatty acids present in decapitated rat brains. We confirmed that large amounts of free fatty acids are present in decapitation-induced ischemic rat brains. The predominant species were 16:0, 18:0 and 20:4, followed by 18:1 [(*n*-9) and (*n*-7)]. Assuming that 80% of the tissue is water, the tissue levels of the above fatty acids were calculated roughly to be 82 μM, 150 μM, 123 μM and 33 μM, respectively. These concentrations of free fatty acids appear to be enough for them to serve as acyl donors in the enzymatic formation of *N*-acylethanolamine, providing that a sufficient amount of ethanolamine is present. However, the profile of free fatty acids present in the brain (Table 2) appears to be consider-

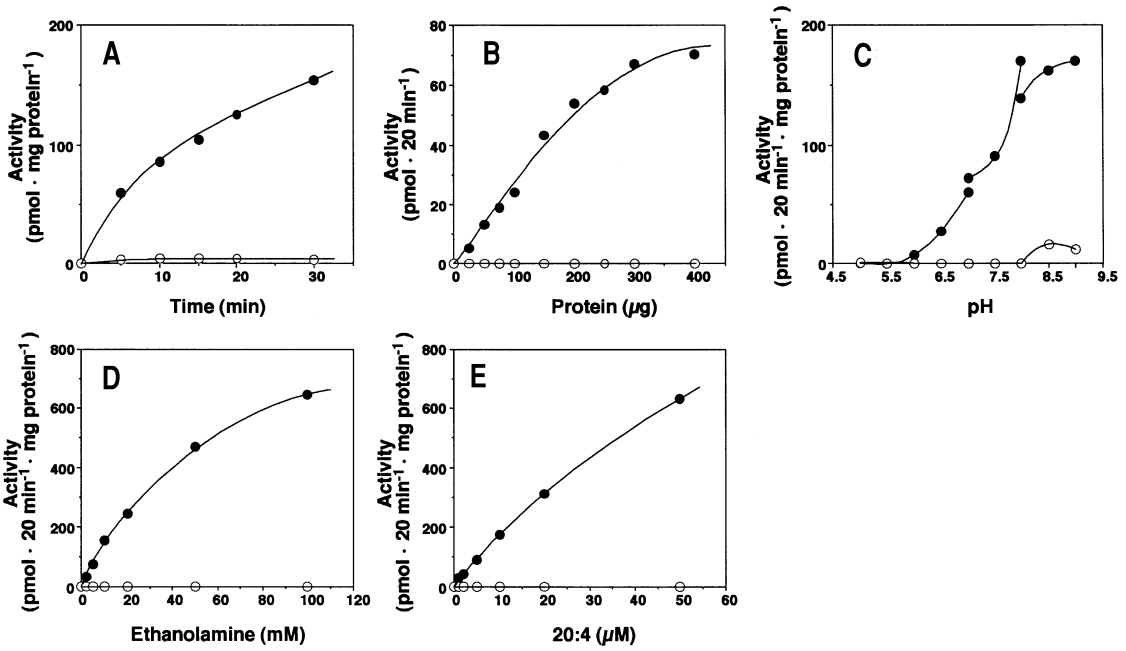

Fig. 2. Kinetics of the enzymatic formation of anandamide from free 20:4 and ethanolamine. Rat brain microsomes (250 μg protein) were incubated with free [^{14}C]20:4 (2.5 nmol) and ethanolamine (2.5 μmol) in 250 μl of 20 mM Hepes, pH 8.0, containing 2 mM dithiothreitol and 2 mM EGTA at 37 °C for 20 min, as described in Materials and Methods. Total lipids were extracted and fractionated by two-dimensional TLC. The radioactivity in the *N*-acylethanolamine fraction was estimated. The values are the means of three determinations. (●) microsomes; (○) boiled (100 °C for 5 min) microsomes. pH 5.0−7.0, 20 mM Mes buffer; pH 7.0−8.0, 20 mM Hepes buffer; pH 8.0−9.0, 100 mM borate buffer.

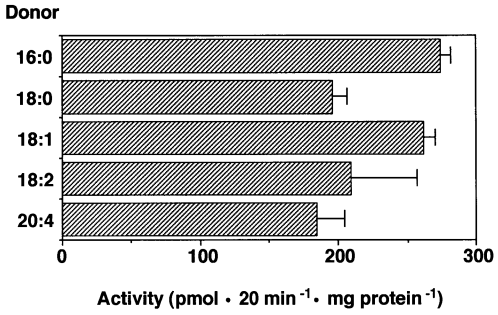

Fig. 3. Enzymatic formation of various types of *N*-acylethanolamine from free fatty acids and ethanolamine. Rat brain microsomes (250 μg protein) were incubated with free [^{14}C]fatty acids (2.5 nmol) and ethanolamine (2.5 μmol) at 37 °C for 20 min as in Fig. 2. Total lipids were extracted and fractionated by two-dimensional TLC. The radioactivity in the *N*-acylethanolamine fraction was estimated. The values are the means ± SD of three determinations.

Table 2. Levels and profile of free fatty acids in rat brain. Fatty acids were obtained from decapitated rat brains as described in Materials and Methods. 17:0 was used as an internal standard. The data are the means ± SD of four determinations.

Fatty acid	Free fatty acid content	
	nmol/g wet mass	%
14:0 + 16:1 (*n*-7)	9.4 ± 9.9	2.8 ± 3.0
16:0	65.2 ± 9.4	19.5 ± 2.8
18:0	120.2 ± 13.5	36.0 ± 4.0
18:1 (*n*-7) + (*n*-9)	26.2 ± 6.0	7.8 ± 1.8
18:2 (*n*-6)	1.5 ± 1.8	0.4 ± 0.5
20:4 (*n*-6)	98.1 ± 17.9	29.4 ± 5.4
22:6 (*n*-3)	11.6 ± 3.1	3.5 ± 0.9
Others	2.0 ± 0.7	0.6 ± 0.2
Total	334.2 ± 41.7	100

ably different from that of the *N*-acyl moiety of *N*-acylethanolamine present in the same tissue (Table 1), although the enzyme involved in the synthesis of *N*-acylethanolamine from free fatty acids and ethanolamine did not exhibit prominent fatty acid specificity (Fig. 3). So, we examined the enzyme activities and substrate availabilities for a possible alternative synthetic pathway for *N*-acylethanolamine, which involves the formation and hydrolysis of *N*-acylPtdEtn.

The fatty acid composition of the *N*-acyl moiety of *N*-acylPtdEtn is shown in Table 1. The predominant fatty acids of the *N*-acyl moiety were 16:0 (69.6%) and 18:0 (12.2%). 18:1(*n*-9) and 18:1 (*n*-7) also accounted for considerable portions of the *N*-acyl moiety: 6.8% and 8.1%, respectively. However, the proportions of other fatty acids such as 20:4 were very low. Such a fatty acid profile resemble that of *N*-acylethanolamine rather than that of free fatty acids (Table 2), especially in terms of the level of 20:4. Because a small but significant amount of *N*-arachidonoylPtdEtn (50.2 pmol/g wet tissue) was found to be present, we examined whether or not anandamide can be enzymatically formed from *N*-arachidonoylPtdEtn. As shown in Fig. 4, rat brain microsomes contain a phosphodiesterase activity that catalyzes the release of various types of *N*-acylethanolamine, including anandamide, from the respective parent *N*-acylPtdEtn molecules, including *N*-arachidonoylPtdEtn. The enzyme activity was observed even in the presence of EGTA, although the activity was augmented in the presence of 2 mM $CaCl_2$. We confirmed that boiled microsomes did not exhibit any apparent enzyme activity (data not shown). These observations strongly suggest that *N*-arachidonoylPtdEtn, actually shown to be present in rat brain, is a potential precursor form of anandamide.

Then, we studied how *N*-arachidonoylPtdEtn is produced in the brain. We found that rat brain microsomes contain a transacylase activity that catalyzes the gradual transfer of [^{3}H]arachi-

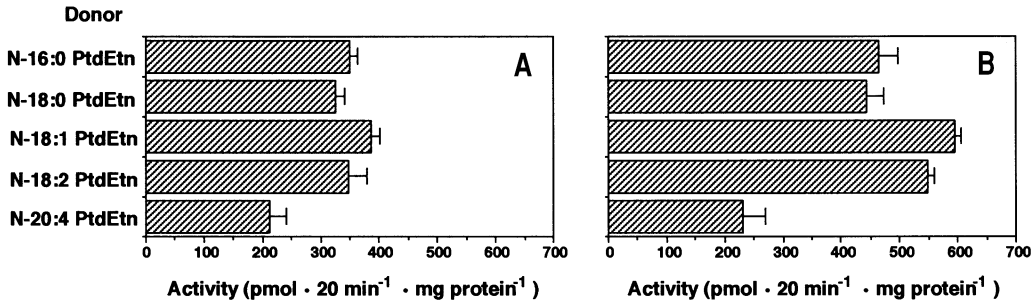

Fig. 4. Enzymatic release of *N*-acylethanolanine from radiolabeled *N*-acylPtdEtn. Rat brain microsomes (250 μg protein) were incubated with various types of *N*-[^{14}C]acylPtdEtn (2.5 nmol) in 250 μl 20 mM Hepes, pH 7.4, containing 2 mM dithiothreitol, 2 mM EGTA (A) or $CaCl_2$ (B), 2 mM Triton X-100 and 1 mM $PhMeSO_2F$ at 37 °C for 20 min, as described in Materials and Methods. Total lipids were extracted and fractionated by two-dimensional TLC. The radioactivity in the *N*-acylethanolamine fraction was estimated. The values are the means ± SD of three determinations.

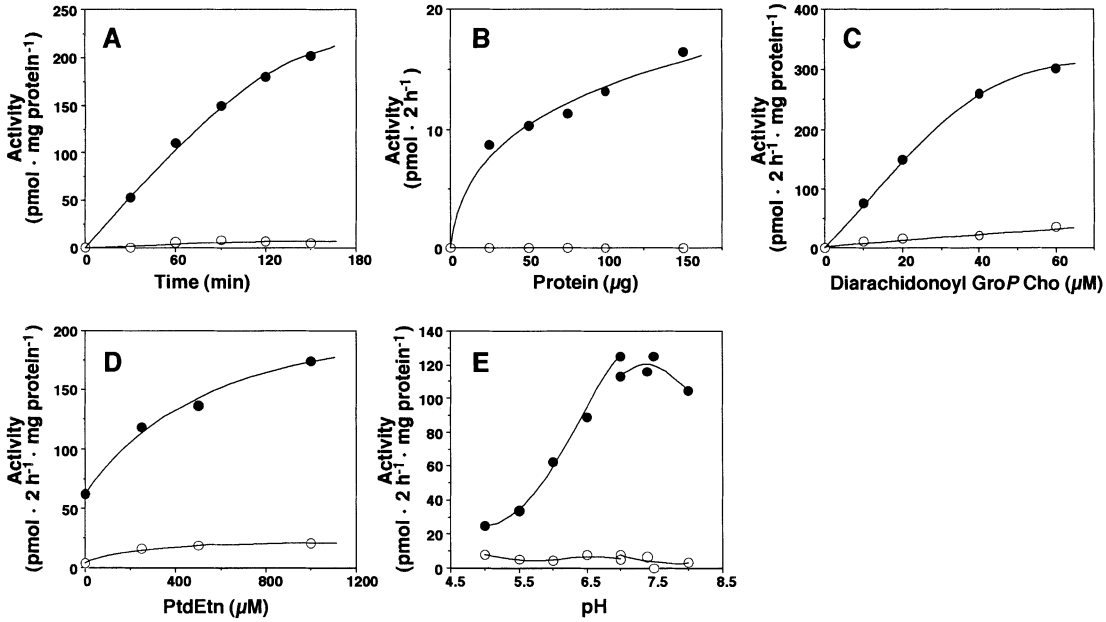

Fig. 5. Kinetics of the enzymatic formation of *N*-[^{3}H]arachidonoylPtdEtn from di-[^{3}H]arachidonoylGro*P*Cho and brain microsomes. Rat brain microsomes (100 μg protein) were incubated with di-[^{3}H]arachidonoylGro*P*Cho (5 nmol) and Ole_2Gro*P*Etn (250 nmol) in 250 μl 20 mM Hepes, pH 7.4, containing 2 mM dithiothreitol and 2 mM $CaCl_2$ at 37 °C for 2 h. Total lipids were extracted and fractionated by two-dimensional TLC. *N*-Acylethanolamine was further purified by TLC as described in Materials and Methods. The radioactivity in the *N*-acylPtdEtn fraction was estimated. The values are the means of three to four determinations. (●) microsomes; (○) boiled microsomes. pH 5.0−7.0, 20 mM Mes buffer; pH 7.0−8.0, 20 mM Hepes buffer.

donic acid from di-[^{3}H]arachidonoylGro*P*Cho to PtdEtn to form *N*-[^{3}H]arachidonoylPtdEtn. The enzyme reaction increased with time (Fig. 5A), with microsomal protein (Fig. 5B) and with increasing concentrations of the substrate, di-[^{3}H]arachidonoylGro*P*Cho (Fig. 5C). The enzyme reaction was also augmented in the presence of exogenously added Ole_2Gro*P*Etn (Ole, oleoyl; Fig. 5D) though the reaction occurred to some extent in the absence of exogenous Ole_2Gro*P*Etn, suggesting that endogenous microsomal PtdEtn also acts as an acceptor. The optimal pH was around 7.0−7.5 (Fig. 5E). Free [^{3}H]arachidonic acid did not react with the NH_2 group of PtdEtn, at least under the present assay conditions (data not shown). Furthermore, apparent activity was not observed when boiled microsomes were employed. These results indicate that the transfer of [^{3}H]arachidonic acid from di-[^{3}H]arachidonoylGro*P*Cho to PtdEtn is an enzymatic transacylation reaction. We also found that the presence of Ca^{2+} is required for the enzyme activity (data not shown); the addition of EGTA instead of Ca^{2+} abolished the enzyme activity. In order to confirm that [^{3}H]arachidonic acid was linked to the NH_2 group of PtdEtn, we examined the product generated on mild alkaline treatment of [^{3}H]-labeled *N*-acylPtdEtn by TLC. The radioactivity was mainly recovered in the glycerophospho(*N*-acyl)ethanolamine fraction (Fig. 6), indicating that [^{3}H]arachidonic acid reacted with the NH_2 moiety of PtdEtn.

Then, we investigated the donor specificity of the enzyme reaction (Fig. 7). Noticeably, 1-arachidonoyl-2-[^{3}H]arachidonoylGro*P*Cho was shown not to act as an acyl donor, indicating that [^{3}H]arachidonic acid was not transferred from the 2-position but from the 1-position of di-[^{3}H]arachidonoylGro*P*Cho to the NH_2 group of PtdEtn. We also confirmed that 1-Pam-2-[^{14}C]arachidonoylGro*P*Cho does not serve as an acyl donor in the formation of *N*-[^{3}H]arachidonoylPtdEtn (data not shown). The structure of diacyl phospholipid appears to be crucially important; no appreciable amount of [^{3}H]arachidonic acid was transferred from 1-[^{3}H]arachidonoylGro*P*Cho. No appreciable transfer was also observed when free [^{3}H]arachidonic acid was added as an acyl donor. However, we found that various types of diacyl Gro*P*Cho in addition to di-[^{3}H]arachidonoylGro*P*Cho

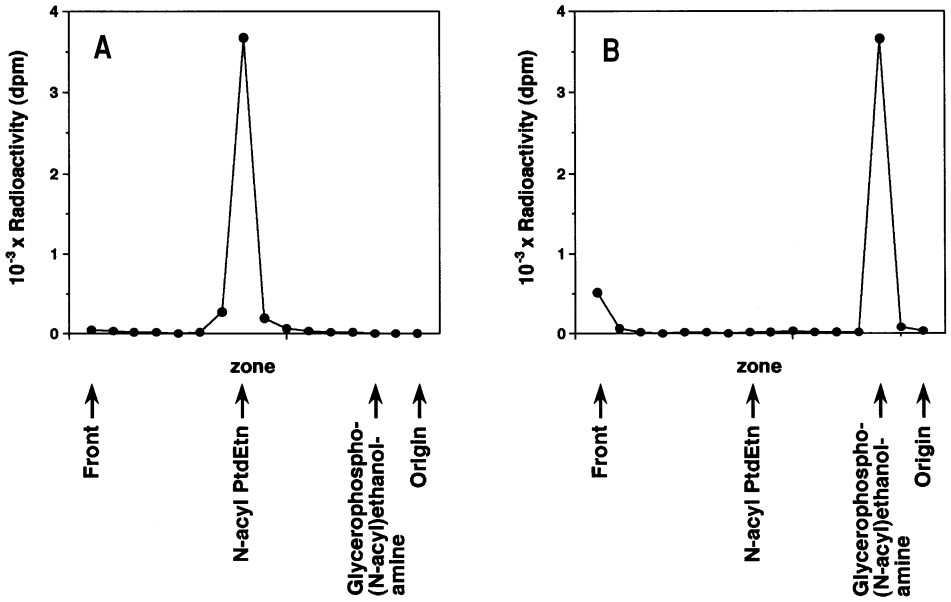

Fig. 6. Effect of mild alkaline treatment of *N*-[^{3}H]arachidonoylPtdEtn synthesized enzymatically from di-[^{3}H]arachidonoylGro*P*Cho and PtdEtn by brain microsomes. Rat brain microsomes were incubated with di-[^{3}H]arachidonoylGro*P*Cho and PtdEtn in Hepes buffer (pH 7.4) containing 2 mM dithiothreitol and 2 mM $CaCl_2$ at 37 °C for 2 h. The resultant *N*-[^{3}H]arachidonoylPtdEtn was purified by two-dimensional TLC and subsequent TLC as described in Materials and Methods. *N*-[^{3}H]arachidonoylPtdEtn was then treated with 1 ml 0.2 M NaOH (90% methanol) for 20 min. In the control, 90% methanol without NaOH was added to *N*-[^{3}H]arachidonoylPtdEtn. After the addition of 2 ml 0.2 M HCl, the reaction products were extracted by the method of Bligh and Dyer, and then fractionated by TLC with chloroform/methanol/water (75:25:4, by vol.). The TLC plate was scraped at 1-cm intervals for liquid-scintillation counting. (A) Without alkaline treatment; (B) with alkaline treatment.

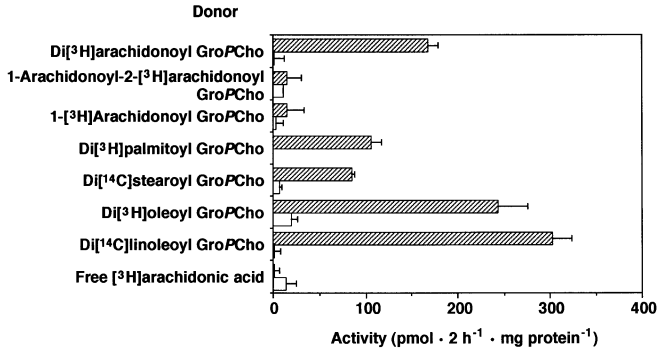

Fig. 7. Enzymatic formation of *N*-acylPtdEtn from radiolabeled lipid donors and PtdEtn by brain microsomes. Rat brain microsomes (250 μg protein) were incubated with radiolabeled phospholipids (5 nmol), lysophospholipids (5 nmol), or free fatty acids (5 nmol) in 250 μl 20 mM Hepes, pH 7.4, containing 2 mM dithiothreitol and 2 mM $CaCl_2$ at 37°C for 2 h. Total lipids were extracted and fractionated by TLC. *N*-AcylPtdEtn was further purified by TLC as described under Materials and Methods. The radioactivity in the *N*-acylPtdEtn fraction was estimated. The values are the means ± SD of three determinations. Hatched bars, microsomes; open bars, boiled microsomes.

Table 3. Fatty acid distribution at the 1-position of PtdCho and PtdEtn of rat whole brain. PtdCho and PtdEtn were obtained from rat whole brain total lipids and subjected to phospholipase A_2 hydrolysis. The resultant lysoPtdCho and lysoPtdEtn were purified and transmethylated. Fatty acid methyl esters were analyzed by GLC. The incubation without phospholipase A_2 was run as a control to correct the data. 17:0 methyl ester was used as an internal standard. The data are the means ± SD of six determinations. n.d., not detected.

Fatty acid	Composition of	
	PtdCho	PtdEtn
	%	
16:0	47.6 ± 1.7	14.4 ± 2.0
18:0	33.2 ± 1.5	64.7 ± 2.5
18:1 (*n*-7) + (*n*-9)	14.6 ± 1.0	20.7 ± 1.6
18:2 (*n*-6)	0.4 ± 0.2	n. d.
20:4 (*n*-6)	0.3 ± 0.1	n. d.
22:6 (*n*-3)	1.2 ± 0.2	n. d.
Others	2.7 ± 0.6	0.2 ± 0.3
Total	100	100

act as acyl donors in the formation of *N*-acylPtdEtn. In contrast to the case of PtdCho, however, the formation of *N*-[^{3}H]arachidonoylPtdEtn was not apparent when di-[^{3}H]arachidonoylGro-*P*Etn was added as an acyl donor, at least under the present experimental conditions (data not shown).

Finally, we examined the fatty acid distribution at the 1-position of PtdCho and PtdEtn obtained from rat whole brain, microsomes and synaptosomes. As shown in Table 3, we confirmed that a small amount of arachidonic acid was present at the 1-position of PtdCho of rat brain. Similar results were observed for PtdCho obtained from microsomes and synaptosomes (data not shown), though PtdCho of these fractions contain slightly higher amounts of palmitic acid and lower amounts of stearic acid, compared with that of whole brain. These observations support the hypothesis that at least a part of *N*-arachidonoyl-PtdEtn, shown to be present in brain, is formed from PtdEtn and arachidonic acid esterified at the 1-position of diacyl phospholipids, especially PtdCho, through a transacylation pathway.

DISCUSSION

There is increasing evidence that anandamide exhibits potent cannabimimetic activity *in vitro* and *in vivo*. It is rather surprising, however, that little information is so far available concern-

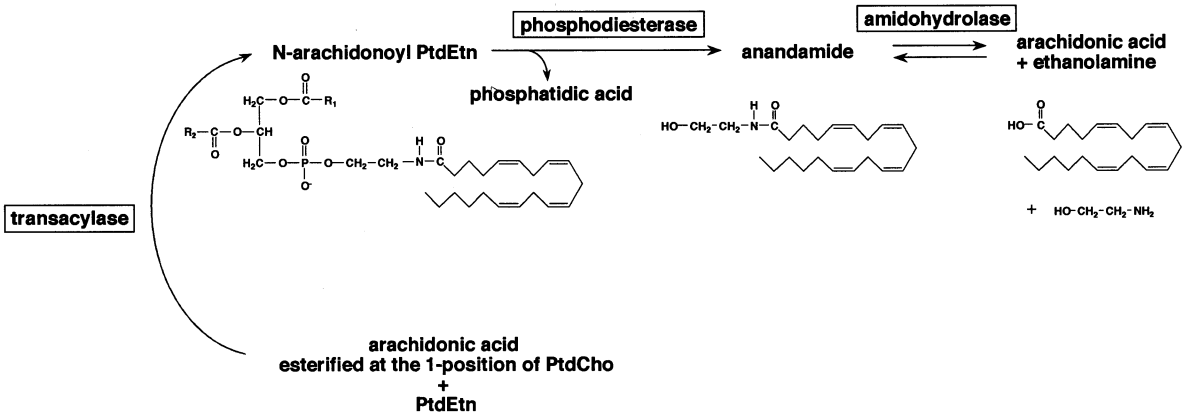

Fig. 8. Possible synthetic pathways for *N*-arachidonoylethanolamine (anandamide).

ing the exact tissue level of anandamide. It will be of great value, therefore, to examine in detail the fatty acid composition of *N*-acylethanolamine in various mammalian tissues, especially in terms of the proportion of arachidonoyl species. In this study, we investigated the fatty acid composition of *N*-acylethanolamine obtained from decapitated rat brains using a newly developed sensitive analytical method. We found that decapitated rat brains actually contain a small amount of the *N*-arachidonoyl species of *N*-acylethanolamine (Table 1). Devane et al. [1] reported that 0.6 mg of anandamide was obtained from 4.5 kg porcine brain. This level is about 90-times higher than that obtained from rat brains in this study. The exact reason for the difference is not clear. It may be due to the different experimental conditions such as the animal species used and the procedures employed. Very recently, Schmid and co-workers [28] reported that small amounts of the *N*-arachidonoyl species (about 1% of the total *N*-acylethanolamine) were present in pig and cow brains. Further studies are needed to accumulate information on the levels of anandamide in various mammalian tissues under physiological and pathophysiological conditions.

Elucidation of the mechanism underlying the biosynthesis of anandamide should also be very important for a better understanding of this novel type of bioactive lipid. However, it is not yet fully understood how anandamide is synthesized in mammalian tissues. Here we demonstrated that anandamide can be formed via two separate synthetic pathways in the brain (Fig. 8). One is formation from free arachidonic acid and ethanolamine, and the other involves the formation of *N*-arachidonoylPtdEtn through a transacylation reaction and subsequent release of anandamide by means of a phosphodiesterase activity.

Previously, several investigators demonstrated that anandamide can be formed from free arachidonic acid and ethanolamine by rat brain homogenates [13], rabbit brain microsomes and cytosol [14], bovine brain P_2 fraction [16] and porcine brain microsomes [15]. The results as to the kinetics of the formation of anandamide from free arachidonic acid and ethanolamine in the present study with rat brain microsomes are in general agreement with those observed in these previous studies. The apparent K_m values for arachidonic acid and ethanolamine observed in this study and in others [14−16] were both substantially high, suggesting that the increased availabilities of these substrates are important in the induction of the synthesis of anandamide via this pathway. We (Fig. 3) and Ueda et al. [15] confirmed that the enzyme activity itself does not exhibit apparent fatty acid specificity. However, Kruszka and Gross [14], and Devane and Axelrod [16] reported that arachidonic acid is a preferred substrate compared with other fatty acids such as palmitic acid. The difference may be due to the different enzyme sources and the different assay conditions. In any case, it is obvious that various types of fatty acids are capable, more or less, of serving as substrates in the formation of *N*-acylethanolamine in this system. Udenfriend and co-workers [29, 30] have already demonstrated the synthesis of *N*-acylethanolamine from various types of free fatty acids and ethanolamine by rat liver microsomes and several rat tissue homogenates in the 1960s, though they did not refer to arachidonic acid.

Decapitation-induced ischemic rat brains contain a large amount of free fatty acids including arachidonic acid (Table 2), as has also been demonstrated by others [31]. The tissue levels of free fatty acids, including arachidonic acid, shown in this study appear to be high enough for the synthesis of *N*-acylethanolamine via this pathway. Assuming that *N*-acylethanolamine is produced mainly via this pathway, the profile of free fatty acids would affect the fatty acid composition of *N*-acylethanolamine, because the enzyme activity itself is not specific to certain species of fatty acids. The fatty acid profile of *N*-acylethanolamine (Table 1) was, however, shown to be considerably different from the profile of free fatty acids present in the same tissue (Table 2): the level of arachidonic acid in the former fraction was very low.

There are several possible explanations for the very low proportion of *N*-arachidonoyl species among various *N*-acylethanolamine species. For instance, the degradation of *N*-arachidonoyl species in living tissues is very rapid compared with in other fatty acyl species. Desarnaud et al. [32], and Ueda et al. [15] demonstrated that the degradation of *N*-arachidonoyl species is rapid compared with other fatty acyl species, especially *N*-palmitoyl species, using brain microsomes. This may account, at least in part, for the low proportion of N-arachidonoyl species. However, the most probable explanation appears to be that a large part, if not all, of *N*-acylethanolamine is produced from *N*-acylPtdEtn, rather than from free fatty acids and ethanolamine, as has been indicated by Schmid and co-workers [33−36]. Several investigators have demonstrated the synthesis of *N*-acylethanolamine from free fatty acids and high concentrations of ethanolamine by a degradation enzyme (amidohydrolase) acting in reverse [15, 36]. However, the tissue levels of ethanolamine are known to be low (3.14 μmol/g tissue for rat brain [37], 3.4 μmol/g tissue for cat brain [38] and 0.388 μmol/g tissue for mouse liver [39]); it seems unlikely that amidohydrolase synthesizes anandamide from free arachidonic acid and ethanolamine in living tissues unless there are some special metabolic pools for these substrates.

The other synthetic pathway for *N*-acylethanolamine is formation from *N*-acylPtdEtn through a phospholipase D−type reaction, which was proposed by Schmid and co-workers [25, 33−

36, 40] for saturated, monoenoic and dienoic species of *N*-acylethanolamine. Since little is known concerning the case of *N*-arachidonoyl species, we investigated the possibility that the synthesis of anandamide occurs via this pathway as in the case of other *N*-acylethanolamine species. While this study was in progress, Di Marzo et al. [17] also suggested that anandamide can be synthesized through this pathway. However, the details of the mechanism underlying the formation of anandamide through this pathway remain quite obscure. In particular, precise information on pre-existing *N*-acylPtdEtn, especially on *N*-arachidonoyl species, is not yet available. Here we showed that decapitated rat brains contain a small but significant amount of *N*-arachidonoylPtdEtn (Table 1). We further demonstrated that rat brain microsomes contain an enzyme activity that catalyzes the release of anandamide from *N*-arachidonoylPtdEtn (Fig. 4). These observations constitute clear evidence that anandamide can be synthesized from pre-existing *N*-arachidonoylPtdEtn by means of a phosphodiesterase activity in this tissue. The reason why the presence of *N*-arachidonoyl species of both *N*-acylethanolamine and *N*-acylPtdEtn was overlooked in previous studies may be that the amounts of *N*-arachidonoyl species in these fractions are too small for GLC analysis. In this study, we developed a sensitive analytical method utilizing 1-anthroyl derivatives and reverse-phase HPLC, which makes the detection of 0.3 pmol of *N*-acylethanolamine possible. As for the fluorometric analysis of *N*-acylethanolamine, Koga et al. [41] also reported a procedure employing a fluorogenic reagent, 4-(*N*-chloroformylmethyl-*N*-methyl) amino-7-*N*,*N*′-dimethylaminosulfonyl-2,1,3-benzoxadiazole and reverse-phase HPLC.

Schmid and co-workers [35, 36, 42−44] reported that fatty acids esterified at the 1-position of diacyl phospholipids are transferred to the NH_2 moiety of PtdEtn to form *N*-acylPtdEtn. Here we showed that this is the case for arachidonic acid (Figs 5−7). A portion of di-[^{3}H]arachidonoylGro*P*Cho was also hydrolyzed by phospholipase A_2 and/or A_1 activities (1 nmol · $2h^{-1}$ · 0.1 mg $protein^{-1}$) during the incubation with brain microsomes (data not shown). However, we confirmed that neither free arachidonic acid nor arachidonic acid esterified at the 2-position was transferred to the NH_2 moiety of PtdEtn. Furthermore, [^{3}H]Arachidonate-containing lysoPtdCho failed to act as an acyl donor (Fig. 7). A very small amount of [^{3}H]arachidonic acid was sometimes incorporated into the *N*-acylPtdEtn fraction in the presence of ATP · Mg^{2+} and CoA (data not shown). However, the radioactivity was not recovered in the *N*-acylethanolamine fraction after the treatment of *N*-acylPtdEtn with phospholipase D, indicating that [^{3}H]arachidonic acid was not incorporated into the NH_2 moiety of PtdEtn even in this case (data not shown). Overall, it appears that the very low proportion of arachidonic acid at the 1-position of diacyl phospholipids in this tissue (Table 3) is responsible, in a large part, for the very low proportion of *N*-arachidonoyl species of *N*-acylPtdEtn (Table 1). Thus, the level of arachidonic acid at the 1-position of diacylphospholipids, especially PtdCho, is an important factor determining the tissue level of *N*-arachidonoylPtdEtn, which, in turn, would influence the level of anandamide in the same tissue.

As for *N*-acylPtdEtn, it has already been reported that this rather unusual phospholipid is present mainly in degenerating mammalian tissues, such as ischemic tissues, and is not usually detected in high amounts in normal tissues [36]. The mechanism underlying the induction of the biosynthesis of *N*-acylPtdEtn is not yet fully understood. The entry of Ca^{2+} into cells is regarded as an important signal triggering the formation of *N*-acylPtdEtn; the transacylase catalyzing the transfer of fatty acids esterified at the 1-position of diacyl phospholipids to the NH_2 moiety of PtdEtn is a Ca^{2+}-dependent enzyme [35, 36, 42−44]. Di Marzo et al. [17], and Hansen et al. [45] reported that the formation of either *N*-acylPtdEtn or *N*-acylethanolamine in [^{3}H]ethanolamine-labeled neuronal cells was augmented when the cells were stimulated with ionomycin, A23187 or glutamate, all of which are known to cause Ca^{2+} influx. Nonetheless, not much is known concerning the case of the *N*-arachidonoyl species as yet, because most of the radioactivity was incorporated into other species such as the *N*-palmitoyl and *N*-stearoyl species [17, 45]. Further studies are necessary to clarify the regulation of the biosynthesis of *N*-arachidonoylPtdEtn as well as anandamide in stimulated tissues and cells.

Recently, we [26], and Mechoulam et al. [46] found that 2-arachidonoylglycerol, possibly generated through increased inositol phospholipid metabolism, exhibits binding affinity toward cannabinoid receptor(s). Although its binding affinity was considerably lower than that of anandamide [26], its amount was approximately 800-times higher than that of anandamide shown in the present study ([26] and Table 1). Noticeably, different from the case of 2-arachidonoylglycerol, we failed to detect any selective or preferential synthetic pathways for the *N*-arachidonoyl species of *N*-acylPtdEtn and *N*-acylethanolamine, compared with other species. Thus, the relative physiological importance or role allotment of these cannabinoid-receptor-ligand candidates remains to be determined.

In conclusion, we demonstrated that anandamide can be formed through two separate synthetic pathways in the brain: formation from free arachidonic acid and ethanolamine; and formation via the *N*-acylPtdEtn pathway. We found that the second pathway explains well the fatty acid composition of *N*-acylPtdEtn actually present in tissues. Considering the finding that the level of anandamide in the brain is very low, however, it is apparent that detailed studies are still required for a full understanding of the physiological significance of anandamide in the brain.

We wish to thank Takayuki Miyazaki and Taku Muto for their technical assistance. This study was supported in part by a Grant-in Aid from the Ministry of Education, Science and Culture of Japan (07672387).

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