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Enzymatic Synthesis of Anandamide, an Endogenous Cannabinoid Receptor Ligand, through N-Acylphosphatidylethanolamine Pathway in Testis: Involvement of Ca^{2+}-Dependent Transacylase and Phosphodiesterase Activities

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Rat testis was shown to contain significant amounts of both N-acylethanolamine, including N-arachidonoylethanolamine (anandamide), and N-acylphosphatidylethanolamine (N-acylPE), including N-arachidonoylPE. The fatty acid profiles of the N-acyl moieties of the two classes resembled each other. We confirmed that testis microsomes contain a phosphodiesterase activity catalyzing the release of anandamide from N-arachidonoylPE. They also contain an enzyme activity catalyzing the transfer of arachidonic acid from the 1-position of diacylphospholipids to PE to form N-arachidonoylPE. These results suggest that the N-acylPE pathway is important in the synthesis of anandamide in this tissue.

Anandamide (N-arachidonoylethanolamine) is an endogenous cannabinoid receptor ligand (1). It has various cannabimimetic activities in vitro and in vivo; for example, it inhibits forskolin-induced accumulation of intracellular cyclic AMP, it inhibits N-type Ca^{2+} channels in several types of cells in vitro, and it induces antinociception, hypothermia, hypomotility and catalepsy when administered to experimental animals (1–5). However, the available information on the metabolism of anandamide is limited. In particular, it is necessary to clarify the biosynthetic route of anandamide in order to better understand the metabolism and the physiological roles of anandamide in mammalian tissues.

Several investigators have already reported that anandamide can be synthesized from free arachidonic acid and ethanolamine (6–8). Nonetheless, the physiological significance of this pathway is uncertain because of the high Km values for the substrates; the reaction may be attributed to the reversed action of amidase (9). Furthermore, we recently found that the fatty acid composition of N-acylethanolamines in brain does not reflect that of free fatty acids present in the same tissue, even though no apparent fatty acid specificity of the enzyme activity itself (T. Sugiura, unpublished data).

In this study, we investigated whether there is a case for another synthetic pathway of anandamide in rat testis and testis microsomes, involving formation of N-arachidonoylPE from diarachidonoylphosphatidylcholine (PC)[2] and PE, and the hydrolysis of N-arachidonoylPE to release N-arachidonoylethanolamine, anandamide.

MATERIALS AND METHODS

Materials. [^{3}H] Arachidonic acid (100 Ci/mmol) and [^{3}H]oleic acid (5 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA). Unlabeled fatty acids and snake venom (*Naja naja atra*) were obtained from Sigma (St. Louis, MO). DioleoylPE, phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), dicyclohexylcarbodiimide and

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[2] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; fatty acids are designated in terms of number of carbon atoms:number of double bonds, e.g., 18:1 for oleic acid.

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1-anthroyl cyanide were from Wako Pure Chem. Ind. (Osaka, Japan). Various types of N-acylethanolamines were prepared according to the method of Devane et al. (1).

Preparation of di[^{3}H]arachidonoylPC, 1-arachidonoyl-2-[^{3}H]arachidonoylPC and N-[^{3}H]arachidonoylPE. [^{3}H]Arachidonic acid (10 μmol, 5 μCi/ μmol) was converted to fatty acid anhydride by mixing with 10 mg of dicyclohexylcarbodiimide in 0.25 ml of chloroform (without ethanol) (10). Then, 10 mg of glycero-3-phosphocholine (GPC) (Sigma, St. Louis, MO) and 4 mg of dimethylaminopyridine were added to the tube, and the mixture was stirred overnight. 1-Arachidonoyl-2-[^{3}H]arachidonoylPC was prepared from 1-arachidonoylGPC and [^{3}H]arachidonic anhydride as described elsewhere (10). The resultant di[^{3}H]arachidonoylPC or 1-arachidonoyl-2-[^{3}H]arachidonoylPC was purified by TLC developed with chloroform:methanol:water (65:25:4, v/v). To prepare N-[^{3}H]arachidonoylPE, [^{3}H]arachidonic acid (10 μmol, 5 μCi/μmol) was first converted to fatty acyl chloride by treatment with oxalyl chloride. Then, [^{3}H]arachidonoyl chloride was mixed with dioleoylPE in chloroform containing a small amount of pyridine. The resultant N-[^{3}H]arachidonoylPE was purified by TLC developed with chloroform:methanol:water (75:25:4, v/v) (solvent system A). Purification of N-acylethanolamines and N-acylPE: Testes were obtained from Wistar male rats (350-400 g body weight). Total lipids were extracted by the method of Bligh and Dyer (11). A portion of total lipids (equivalent to 10 g wet tissue), with 2 nmol of N-heptadecanoyl(17:0)ethanolamine or N-17:OPE added as an internal standard, was first fractionated by TLC developed with chloroform:methanol: NH_4OH (80:20:2, v/v) (solvent system B). The area corresponding to N-acylethanolamine or N-acylPE was scraped off the TLC plates and lipids were extracted by the method of Bligh and Dyer. N-Acylethanolamine was further purified by two-dimensional TLC developed first with petroleum ether:diethyl ether:acetone:acetic acid (30:40:20:1, v/v) (solvent system C) and then with the organic layer of an ethyl acetate:petroleum ether:acetic acid:water (100:50:20:100, v/v) mixture (6). N-acylPE was purified by two-dimensional TLC developed first with solvent system C and then with solvent system A.

HPLC analysis of N-acylethanolamine and N-acylethanolamine moiety of N-acylPE. Purified N-acylethanolamine was converted to the 1-anthroyl derivative by treatment with 1-anthroyl cyanide and quinuclidine dissolved in acetone at 45°C for 75 min. The 1-anthroyl derivative of N-acylethanolamine was separated by TLC developed with petroleum ether:diethyl ether:acetic acid (40:60:1, v/v). N-acylPE was hydrolyzed by phospholipase D (*Streptomyces chromofuscus*, Boehringer Mannheim GmbH, FRG). The resultant N-acylethanolamine was purified by TLC developed with solvent system C and then converted to the 1-anthroyl derivative as described above. The 1-anthroyl derivative of N-acylethanolamine was analyzed in a HPLC system equipped with a fluorescence detector (excitation 370 nm; emission 470 nm), using a CAPCELL PAK C18 SG column (Shiseido, Tokyo, Japan) (4.6 mm × 250 mm × 2) and acetonitrile:isopropanol:water (80:3:17, v/v) as the mobile phase.

Analysis of fatty acids esterified at the 1-position of PC and PE. PC and PE were purified by two-dimensional TLC (12), then hydrolyzed with snake venom phospholipase A_2 (12), which was boiled for 10 min to inactivate lysophospholipases prior to use. The resultant lysophospholipids were separated by TLC and transmethylated with 0.5 M methanolic sodium methoxide (12). Fatty acid methyl esters were analyzed by GLC.

Degradation of N-[^{3}H]arachidonoylPE by testis microsomes. Microsomes were prepared as described elsewhere (10). Microsomes (250 μg protein) were incubated with N-[^{3}H]arachidonoylPE (5 nmol) in 250 μl of 20 mM Hepes buffer (pH 7.4) containing 2 mM DTT, 2 mM $CaCl_2$ or EGTA, 2 mM Triton X-100 and 1 mM PMSF at 37°C for 20 min (13). After the incubation, total lipids were extracted and fractionated by two-dimensional TLC developed first with solvent system C and then with solvent system B. The radioactivities in N-acylethanolamine and N-acylPE fractions were estimated.

Formation of N-[^{3}H]arachidonoylPE from di[^{3}H]arachidonoylPC and PE. Microsomes (1 mg protein) were incubated with di[^{3}H]arachidonoylPC (30 nmol, 5 μ Ci/ μ mol) and dioleoylPE (250 nmol) in 1.25 ml of 20 mM Hepes buffer (pH 7.4) containing 2 mM DTT and 2 mM $CaCl_2$ or EGTA at 37°C for 3 h. After the incubation, total lipids were extracted and fractionated by TLC developed first with solvent system C and then with solvent system B. Purified N-acylPE was then hydrolyzed by phospholipase D (*Streptomyces chromofuscus*). The resultant N-acylethanolamine was purified by TLC developed with solvent system C. The radioactivity in this fraction was estimated.

RESULTS AND DISCUSSION

First, we examined whether rat testis contains N-acylethanolamines including N-arachidonoylethanolamine, anandamide. As shown in Table 1, rat testis contains various types of N-acylethanolamines. The predominant species was N-16:0, accounting for 60% of the total. The rank order of the major species was as follows: N-16:0 > N-18:0 > N-20:4(n-6) = N-18:2(n-6) = N-18:1(n-9) > N-18:1(n-7). Then, we examined the level of N-acylPE and the fatty acid profile of its N-acyl moiety (Table 1). Rat testis contains a relatively high level of N-acylPE (2525 pmol/g wet tissue), which is about 20 times higher than the level of N-acylethanolamine present in the same tissue. Notably, the fatty acid composition of the N-acyl moiety of N-acylPE resembles that of N-acylethanolamine. This observation strongly suggests a metabolic relationship between N-

TABLE 1
Fatty Acid Composition of N-acyl Moieties of N-acylethanolamine and N-acylPE in Rat Testis

	N-acylethanolamine		N-acylPE	
Fatty acyl moieties	pmol/g tissue	(%)	pmol/g tissue	(%)
16:0	73.9 ± 7.5	60.4	1631.9 ± 207.5	64.6
16:1(n–7)	1.1 ± 0.3	0.9	8.8 ± 1.1	0.3
18:0	21.4 ± 1.7	17.5	330.4 ± 60.1	13.1
18:1(n−7)	4.0 ± 0.3	3.3	70.4 ± 12.2	2.8
18:1(n−9)	5.5 ± 0.9	4.5	69.2 ± 14.7	2.7
18:2(n−6)	5.6 ± 1.8	4.6	68.1 ± 19.4	2.7
20:4(n−6)	6.0 ± 1.4	4.9	274.3 ± 136.8	10.9
Others	4.8 ± 1.1	3.9	72.0 ± 46.0	2.9
Total	122.3 ± 7.5	100	2525.1 ± 412.2	100

The data are the means ± SD of four determinations.

acylethanolamine and N-acylPE. So, we investigated whether N-arachidonoylethanolamine, anandamide, can be synthesized enzymatically from the corresponding N-arachidonoylPE.

As shown in Fig. 1, rat testis microsomes contain an enzyme activity catalyzing the release of N-arachidonoylethanolamine from N-arachidonoylPE. The enzyme reaction proceeded in the presence of EGTA. The addition of $CaCl_2$ instead of EGTA did not markedly affect the enzyme activity. No appreciable activity was observed with boiled microsomes. These results indicate that N-arachidonoylPE occurring in rat testis (Table 1) might be a stored precursor form of N-arachidonoylethanolamine, anandamide.

The question then arises, how is N-arachidonoylPE supplied? To answer this question, we examined the ability of several compounds to act as arachidonoyl donors in the formation of N-arachidonoylPE. No appreciable amount of radioactivity was found in the N-acylPE fraction when microsomes were incubated with free [^{3}H]arachidonic acid and PE even in the presence of CoA and ATP-Mg^{2+} (data not shown). On the other hand, we found that testis microsomes contain an enzyme activity catalyzing the gradual transfer of [^{3}H]arachidonic acid from di[^{3}H]arachidonoylPC to PE to form N-[^{3}H]arachidonoylPE (Fig. 2). The presence of Ca^{2+} is essential for the enzyme activity, though the enzyme reaction proceeded in the absence of other cofactors such as CoA and ATP-Mg^{2+}. We also found that di[^{3}H]oleoylPC acted as an acyl donor in the formation of N-[^{3}H]oleoylPE, similarly to the case of di[^{3}H]arachidonoylPC (data not shown). However, no detectable N-[^{3}H]arachidonoylPE was formed when 1-arachidonoyl-2-

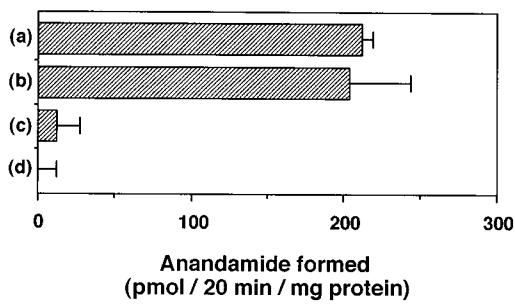

FIG. 1. Enzymatic release of N-[^{3}H] arachidonoylethanolamine (anandamide) from N-[^{3}H]arachidonoylPE by rat testis microsomes. Microsomes ((a) and (b)) or boiled (100°C 5 min) microsomes ((c) and (d)) were incubated with N-[^{3}H]arachidonoylPE in the presence of 2 mM $CaCl_2$ ((a) and (c)) or 2 mM EGTA ((b) and (d)) at 37°C for 20 min as described in Materials and Methods. The data are the means ± SD of four determinations.

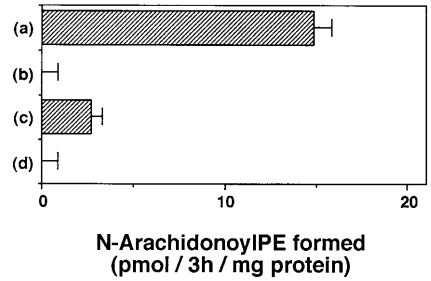

FIG. 2. Enzymatic formation of N-[^{3}H]arachidonoylPE from di[^{3}H]arachidonoylPC and PE by rat testis microsomes. Microsomes ((a) and (b)) or boiled microsomes ((c) and (d)) were incubated with di[^{3}H]arachidonoylPC and PE in the presence of 2 mM $CaCl_2$ ((a) and (c)) or 2 mM EGTA ((b) and (d)) at 37°C for 3 h as described in Materials and Methods. The data are the means ± SD of three determinations.

[^{3}H]arachidonoylPC was employed as an acyl donor (data not shown), suggesting that [^{3}H]arachidonic acid was transferred from the 1-position of di[^{3}H]arachidonoylPC to PE.

Next, we examined the levels of arachidonic acid at the 1-position of testicular phospholipids. We found that significant amounts of arachidonic acid are esterified at the 1-position of PC and PE (Table 2). This is consistent with the earlier observation by Blank et al. (14) that rat testis contains diarachidonoyl species of PC and PE.

The results of the present study clearly indicate that anandamide can be synthesized enzymatically from N-arachidonoylPE, which is actually present in testis, and that testis microsomes can synthesize N-arachidonoylPE from PE and arachidonic acid at the 1-position of diacyl phospholipids such as diarachidonoylPC. To our knowledge, this is the first report showing the occurrence of anandamide in testis and presenting direct evidence for the presence of both the enzyme activities and the substrates implicated in the synthesis of anandamide via the N-acylPE pathway in this tissue.

Previously, Schmid and colleagues (15-18) reported that ischemic dog heart and brain contain significant amounts of both N-acyl(16:0, 18:0, 18:1, 18:2)ethanolamine and N-acyl(16:0, 18:0, 18:1, 18:2)PE. They showed that these tissues contain phospholipase D activity, which cleaves N-acylPE to release N-acylethanolamine (13). Further, they demonstrated that microsomal fractions obtained from these tissues contain an enzyme activity catalyzing the transfer of fatty acids esterified at the 1-position of diacylphospholipids to PE to form N-acylPE in the presence of Ca^{2+} (17,18). Nevertheless, they did not detect arachidonic acid-containing species of N-acylethanolamine or N-acylPE. Also, these enzyme activities were not examined using arachidonic acid-containing species as substrates. Recently, Piomelli and colleagues (19) demonstrated that

TABLE 2
Fatty Acid Composition at the 1-Position of PC and PE in Rat Testis

Fatty acyl moieties	PC	PE
16:0	72.2 ± 1.8	50.8 ± 1.6
18:0	13.9 ± 1.0	24.4 ± 2.1
18:1(n−7) + 18:1(n−9)	6.8 ± 0.2	12.8 ± 0.7
18:2(n−6)	1.5 ± 0.1	1.6 ± 0.4
20:4(n−6)	3.4 ± 0.4	6.8 ± 1.8
22:4(n−6)	0.2 ± 0.0	0.9 ± 0.4
22:5(n−6)	1.5 ± 0.7	2.0 ± 0.5
Others	0.5 ± 0.1	0.7 ± 0.1
Total	100	100

The data are the means ± SD of six determinations.

anandamide can be synthesized from N-arachidonoylPE through the action of a phosphodiesterase in cultured neuronal cells. However, it remained to be established which enzyme activity and substrates are implicated in the formation of N-arachidonoylPE. In the present study, we showed that Ca^{2+}-dependent transacylase and arachidonic acid at the 1-position of diacyl phospholipids are involved in the synthesis of N-arachidonoylPE, as in the case of saturated, monoenoic or dienoic fatty acid-containing species. Our finding of relatively similar fatty acid profiles in N-acylethanolamines, N-acylPE and the 1-position of diacylPC or PE strongly suggests that the N-acylPE pathway is important in the synthesis of anandamide as well as other N-acylethanolamines in this type of tissue.

Finally, our finding that anandamide is present in rat testis is particularly noteworthy for the following reasons: firstly, Schuel et al. (20) reported that anandamide reduces sperm fertilizing capacity in sea urchin by inhibiting the acrosome reaction, and secondly, several investigators have already demonstrated that cannabinoid receptor (CB1) is present in human testis (21) and mouse uterus (22). These observations strongly suggest that anandamide plays important physiological roles in the reproductive system, as in the nervous system.

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