Biosynthesis of anandamide and *N*-palmitoylethanolamine by sequential actions of phospholipase A_2 and lysophospholipase D

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Anandamide (an endocannabinoid) and other bioactive long-chain NAEs (*N*-acylethanolamines) are formed by direct release from *N*-acyl-PE (*N*-acyl-phosphatidylethanolamine) by a PLD (phospholipase D). However, the possible presence of a two-step pathway from *N*-acyl-PE has also been suggested previously, which comprises (1) the hydrolysis of *N*-acyl-PE to *N*-acyl-lysoPE by PLA_1/PLA_2 enzyme(s) and (2) the release of NAEs from *N*-acyl-lysoPE by lysoPLD (lysophospholipase D) enzyme(s). In the present study we report for the first time the characterization of enzymes responsible for this pathway. The PLA_1/PLA_2 activity for *N*-palmitoyl-PE was found in various rat tissues, with the highest activity in the stomach. This stomach enzyme was identified as group IB $sPLA_2$ (secretory PLA_2), and its product was determined as *N*-acyl-1-acyl-lysoPE. Recombinant group IB, IIA and V of $sPLA_2$s were also active with *N*-palmitoyl-PE, whereas group X $sPLA_2$ and cytosolic $PLA_2\alpha$ were inactive. In addition, we found wide distribution of lysoPLD activity generating *N*-palmitoylethanolamine from *N*-palmitoyl-lysoPE in rat tissues, with higher activities in the brain and testis. Based on several lines of enzymological evidence, the lysoPLD enzyme could be distinct from the known *N*-acyl-PE-hydrolysing PLD. $sPLA_2$-IB dose dependently enhanced the production of *N*-palmitoylethanolamine from *N*-palmitoyl-PE in the brain homogenate showing the lysoPLD activity. *N*-Arachidonoyl-PE and *N*-arachidonoyl-lysoPE as anandamide precursors were also good substrates of $sPLA_2$-IB and the lysoPLD respectively. These results suggest that the sequential actions of PLA_2 and lysoPLD may constitute another biosynthetic pathway for NAEs, including anandamide.

Key words: *N*-acylethanolamine (NAE), *N*-acylphosphatidylethanolamine, anandamide, endocannabinoid, fatty acid, phospholipase D (PLD).

INTRODUCTION

Ethanolamides of long-chain fatty acids, collectively referred to as NAEs (*N*-acylethanolamines), are present in various mammalian tissues [1–5]. NAEs were previously reported to have anti-inflammatory and membrane-stabilizing actions, and noted to markedly increase in degenerating tissues and cells [6–9]. Later, *N*-arachidonoylethanolamine (anandamide) was identified as an endogenous ligand of the cannabinoid receptors [10] and vanilloid receptor [11], and shown to exert various cannabimimetic activities [12]. In contrast, NAEs with a saturated or monounsaturated fatty acid did not activate cannabinoid receptors, but they were shown to possess various biological activities. For example, *N*-palmitoylethanolamine was reported to be anti-inflammatory and anti-nociceptive [13–15], *N*-oleoylethanolamine to be anorexic via peroxisome-proliferator-activated receptor-α [16,17], and *N*-stearoylethanolamine to be pro-apoptotic [18].

It is generally accepted that NAE is principally biosynthesized in animal tissues from PE (phosphatidylethanolamine) by two sequential enzyme reactions [1–5,19]. The first reaction is transfer of an acyl-group from the *sn*-1 position of glycerophospholipid to PE by an acyltransferase, resulting in the generation of *N*-acyl-PE. Subsequently, NAE is released from *N*-acyl-PE by a phosphodiesterase of PLD (phospholipase D), which we recently cloned [20].

In addition to this direct release of NAE by PLD, Natarajan et al. [21] suggested previously an alternative biosynthetic pathway containing two-step reactions from *N*-acyl-PE to NAE. In this pathway, shown in Scheme 1, *N*-acyl-PE is first hydrolysed to *N*-acyl-lysoPE and a non-esterified fatty acid by an enzyme having PLA_1 or PLA_2 activity, and NAE is then released from *N*-acyl-lysoPE by a lysoPLD (lysophospholipase D)-like enzyme. The PLA_1- or PLA_2-catalysed reaction of this pathway was suggested by the previous observation that *N*-acyl-lysoPE was detected as a catabolite from radiolabelled *N*-acyl-PE in the homogenates of rat heart, rat brain and dog brain [21–23]. It is also noted that commercially available venom and pancreatic PLA_2s have been used to prepare radiolabelled *N*-acyl-lysoPE from radiolabelled *N*-acyl-PE [21,22,24]. The occurrence of a lysoPLD-like enzyme producing NAE was suggested by the finding that radiolabelled *N*-acyl-lysoPE was hydrolysed to NAE by the microsomal preparations of rat heart and dog brain [21,22]. However, enzymes responsible for this alternative pathway in the animal tissues have been hardly characterized, and the precise chemical structure of *N*-acyl-lysoPE has not been determined. It also remained unknown whether *N*-acyl-lysoPE-hydrolysing lysoPLD was identical to the PLD directly hydrolysing *N*-acyl-PE.

In the present study, in order to clarify the physiological significance of this NAE biosynthetic pathway via *N*-acyl-lysoPE,

Abbreviations used: LPA, lysophosphatidic acid; lysoPLD; lysophospholipase D; MAFP, methyl arachidonyl fluorophosphonate; NAE, *N*-acylethanolamine; PE, phosphatidylethanolamine; PL, phospholipase; $cPLA_2\alpha$, cytosolic $PLA_2\alpha$; $sPLA_2$, secretory PLA_2.

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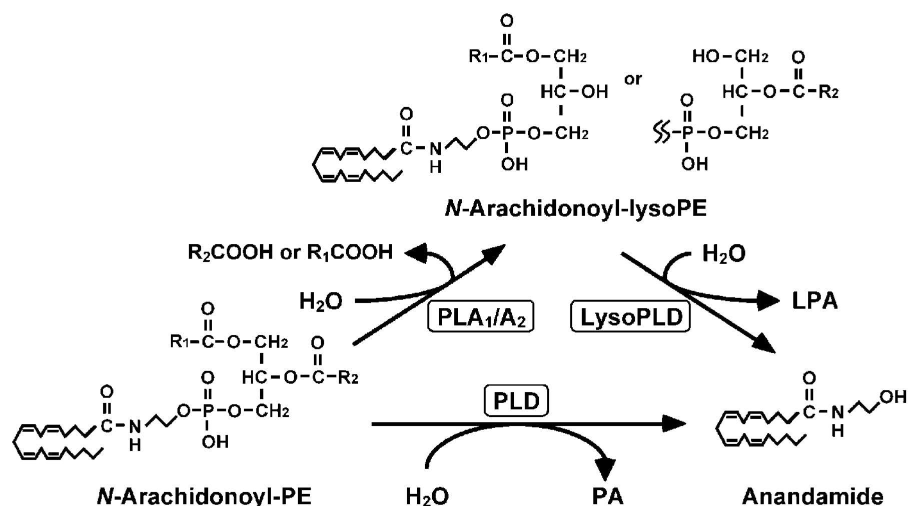

Scheme 1 Diagram of the biosynthetic pathways of anandamide

The PLA_1/PLA_2-lysoPLD pathway and the confirmed PLD pathway are shown. PA, phosphatidic acid.

we focused on the related enzymes. The results suggest the involvement of several, but not all, PLA_2 isoenzymes in the synthesis of NAEs, including anandamide. Moreover, we propose the existence of lysoPLD distinct from the known PLD acting on *N*-acyl-PE.

EXPERIMENTAL

Materials

[1-^{14}C]Palmitic acid, 1-palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphoethanolamine (2-[^{14}C]linoleoyl-PE) and 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine (2-[^{14}C]-arachidonoyl-PE) were purchased from PerkinElmer Life Science (Boston, MA, U.S.A.); [1-^{14}C]arachidonic acid, Macro-Prep High Q, HiTrap SP HP, and Hybond-P from Amersham Biosciences (Piscataway, NJ, U.S.A.); arachidonic acid from Nu-Chek-Prep (Elysian, MN, U.S.A.); palmitic acid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine from Sigma (St. Louis, MO, U.S.A.); 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine from Avanti Polar Lipids (Alabaster, AL, U.S.A.); MAFP (methyl arachidonyl fluorophosphonate) from Cayman Chemical (Ann Arbor, MI, U.S.A.); 3(2)-*t*-butyl-4-hydroxyanisole from Wako Pure Chemical (Osaka, Japan); Triton X-100 from Nacalai Tesque (Kyoto, Japan); *n*-octyl β-D-glucoside from Dojindo (Kumamoto, Japan); Coomassie Brilliant Blue R-250 from ICN (Aurora, OH, U.S.A.); protein assay dye reagent concentrate from Bio-Rad (Hercules, CA, U.S.A.); Microcon YM-10 from Millipore (Bedford, MA, U.S.A.); precoated silica gel 60 F_{254} aluminum sheets for TLC (20 cm × 20 cm, 0.2 mm thickness) from Merck (Darmstadt, Germany); pCR3.1, pRc/CMV, RPMI medium 1640, LipofectAMINE Plus, and fetal calf serum from Invitrogen (Carlsbad, CA, U.S.A.); pBK-CMV from Stratagene (La Jolla, CA, U.S.A.); HEK-293 cells from Health Science Research Resources Bank (Osaka, Japan). 1,2-Dioleoyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]palmitoyl)ethanolamine (*N*-[^{14}C]palmitoyl-PE) and 1,2-dioleoyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]arachidonoyl)ethanolamine (*N*-[^{14}C]arachidonoyl-PE) were prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and either [1-^{14}C]palmitic acid or [1-^{14}C]-arachidonic acid respectively, according to the method of Schmid et al. [22]. 1-Palmitoyl-2-[1-^{14}C]-linoleoyl-*sn*-glycero-3-phospho(*N*-palmitoyl)ethanolamine (*N*-palmitoyl-2-[^{14}C]linoleoyl-PE) was also prepared by the same method using 2-[^{14}C]-linoleoyl-PE and palmitic acid. 1-Oleoyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]palmitoyl)ethanolamine (*N*-[^{14}C]palmitoyl-lysoPE) and 1-oleoyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]arachidonoyl)ethanolamine (*N*-[^{14}C]arachidonoyl-lysoPE) were enzymically prepared from *N*-[^{14}C]palmitoyl-PE or *N*-[^{14}C]arachidonoyl-PE respectively, using rat stomach $sPLA_2$-IB (secretory PLA_2-IB) purified as described below. The products were purified by TLC with a mixture of chloroform/methanol/28 % ammonium hydroxide (40:10:1, by vol.) or chloroform/methanol/acetic acid (9:1:1, by vol.).

Preparation of enzymes

Male Wistar rats (250–500 g, Charles River Japan) were anesthetized by diethyl ether and killed by cervical dislocation. Various organs were removed, cut into small pieces and then homogenized in 5 times the volume (v/w) of 20 mM ice-cold Tris/HCl (pH 7.4) containing 0.32 M sucrose with a Polytron homogenizer. The homogenates were centrifuged at 800 ***g*** for 15 min and the resultant supernatant was used in the experiments shown in Figures 1, 4 and 6. For the experiments shown in Figure 5, the 800 ***g*** supernatants of rat heart and brain were further centrifuged at 105 000 ***g*** for 50 min, and the obtained pellets (particulate fractions) were suspended in 20 mM Tris/HCl (pH 7.4). The particulate fractions were then treated with 1 % (w/v) octyl glucoside, and the supernatant obtained by further centrifugation at 105 000 ***g*** for 50 min was used as solubilized proteins. Protein concentration was determined by the method of Bradford [25] with BSA as a standard.

Purification of the stomach PLA_2

Rat stomach was homogenized in 9 times the volume (v/w) of 20 mM ice-cold Tris/HCl (pH 7.4) containing 0.32 M sucrose, and its particulate fraction was prepared as described above. The particulate fraction suspended in 20 mM Tris/HCl (pH 7.4) was subjected to one cycle of freezing and thawing, solubilized

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by 1 % (w/v) octyl glucoside, and centrifuged at 105 000 **g** for 50 min. The resultant supernatant was heated at 70 °C for 10 min, and the developed precipitate was removed by centrifugation at 105 000 **g** for 50 min. The supernatant (4.5 mg of protein) was 10-fold diluted with water and loaded on to a Macro-Prep High Q anion-exchange column (bed volume, 3 ml) pre-equilibrated with 20 mM Tris/HCl (pH 7.4). The pass-through fractions containing the enzyme were then loaded on to a HiTrap SP HP cation-exchange column (bed volume, 5 ml) pre-equilibrated with 20 mM Tris/HCl (pH 7.4) containing 0.1 % (w/v) octyl glucoside (buffer A). After washing the column with 15 ml of buffer A, the enzyme was eluted with 5 ml of buffer A containing 50 mM NaCl. Prepared purified enzyme was then stored at − 80 °C until use.

Protein sequencing

The purified stomach enzyme was concentrated by a centrifugal filter device (Microcon YM-10), subjected to SDS/PAGE (12.5 % gel), and electrotransferred on to a hydrophobic PVDF membrane (Hybond-P). The band stained with Coomassie Brilliant Blue R-250 was excised, and its N-terminal sequence was determined with a Procise Model 492 protein sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Expression of recombinant PLA_2s

HEK-293 cells were cultured in RPMI medium 1640 containing 10 % fetal calf serum. As described previously, the cDNAs for rat $sPLA_2$-IB [26] and human $sPLA_2$-X [27] were subcloned into pCR3.1, the cDNAs for human $sPLA_2$-IIA [28] and human $sPLA_2$-V [26] into pRc/CMV, and mouse $cPLA_2\alpha$ (cytosolic $PLA_2\alpha$) cDNA into pBK-CMV [28,29]. Transfection was performed according to the manufacturer's instruction: 8–16 μg of plasmid was mixed with 32 μl of LipofectAMINE Plus™ in 800 μl of serum-free RPMI medium 1640 for 30 min and then added to cells that had attained approx. 70 % confluence in a 10 cm dish containing 8 ml of serum-free RPMI medium 1640. After incubation for 4–6 h, the medium was removed, and the transfected cells were further cultured in the presence of serum for 66–68 h. Since $sPLA_2$-IB and $sPLA_2$-X were secreted outside the cells, the culture supernatants were used as the enzyme preparations. For $cPLA_2\alpha$, the cells were harvested with the aid of trypsin, washed twice, suspended in 10 mM Tris/HCl (pH 7.4) and 150 mM NaCl, and sonicated three times each for 3 s. The resultant cell lysate was used as the enzyme preparation. For $sPLA_2$-IIA and $sPLA_2$-V, after the culture medium was removed, the cells were incubated for 15 min at 37 °C with 5 ml of the culture medium containing 1 M NaCl. This procedure allowed the cell surface-associated $sPLA_2$s to be recovered in the medium [28], which was used for the enzyme assay.

Enzyme assay

For the PLA_2 assay with native rat enzymes, the enzymes were incubated with 100 μM or 200 μM ^{14}C-labelled substrate (1000 cpm/nmol, dissolved in 5 μl of ethanol) in 100 μl of 50 mM Tris/acetic acid (pH 8.0) containing 10 mM $CaCl_2$ at 37 °C for 5 min (for the purified stomach enzyme and the homogenates of stomach, pancreas, and small intestine) or 30 min (for the homogenates of other tissues). For the PLA_2 assay with recombinant PLA_2s expressed in HEK-293 cells, the enzymes prepared as described above were incubated at 37 °C for 5 min ($sPLA_2$-IB) or 30 min (other PLA_2s) with 100 μM ^{14}C-labelled substrate (1000 cpm/nmol, dissolved in 5 μl of ethanol) in 100 μl of the following buffers for each PLA_2 isoenzyme: $sPLA_2$-IB, 50 mM Tris/acetic acid (pH 8.0) containing 10 mM $CaCl_2$; $sPLA_2$-IIA, 50 mM Tris/HCl (pH 9.0) containing 10 mM $CaCl_2$; $sPLA_2$-V, 50 mM Tris/acetic acid (pH 6.0) containing 10 mM $CaCl_2$; $sPLA_2$-X, 50 mM Tris/HCl (pH 7.4) containing 10 mM $CaCl_2$; and $cPLA_2\alpha$, 100 mM Tris/HCl (pH 9.0) containing 4 mM $CaCl_2$ [30–33]. The reaction was terminated by the addition of 0.3 ml of a mixture of chloroform/methanol (2:1, v/v) containing 5 mM 3(2)-*t*-butyl-4-hydroxyanisole. In the assays to detect the radiolabelled non-esterified fatty acids produced, 25 μl of 1 M citric acid was included in the mixture to facilitate the extraction of the radiolabelled non-esterified fatty acid into the organic phase. After centrifugation, 100 μl of the organic phase was spotted on a silica gel thin-layer plate (10 cm height) and subjected to TLC at 4 °C for 25 min with a mixture of chloroform/methanol/28 % ammonium hydroxide (40:10:1, by vol.) to detect *N*-[^{14}C]palmitoyl-lysoPE and *N*-[^{14}C]arachidonoyl-lysoPE produced or with chloroform/methanol/acetic acid (9:1:1, by vol.) to detect radiolabelled non-esterified fatty acids produced. Distribution of radioactivity on the plate was quantified by a BAS1500 bioimaging analyser (Fujix, Tokyo, Japan).

For the lysoPLD assay, the enzyme was incubated with 25–200 μM *N*-[^{14}C]palmitoyl-lysoPE or *N*-[^{14}C]arachidonoyl-lysoPE (1000 cpm/nmol, dissolved in 5 μl of ethanol) in 100 μl of 50 mM Tris/HCl (pH 8.0) at 37 °C for 30 min. The reaction was terminated by the addition of 0.3 ml of a mixture of chloroform/methanol (2:1, v/v) containing 5 mM 3(2)-*t*-butyl-4-hydroxyanisole. TLC was performed with a mixture of chloroform/methanol/28 % ammonium hydroxide (40:10:1, by vol.) at 4 °C for 25 min. All the enzyme assays were performed in triplicate.

RESULTS

Tissue distribution of *N*-acyl-PE-hydrolysing PLA_1/PLA_2 activity

We first examined the *N*-acyl-PE-hydrolysing PLA_1/PLA_2 activity with the homogenate of rat brain. The homogenate was allowed to react with *N*-[^{14}C]palmitoyl-PE in the presence of 10 mM $CaCl_2$, and the products were separated from the remaining substrate by TLC. The results revealed that the brain had PLA_1/PLA_2 activity for converting *N*-[^{14}C]palmitoyl-PE to *N*-[^{14}C]palmitoyl-lysoPE, in addition to the PLD activity hydrolysing the same substrate to *N*-[^{14}C]palmitoylethanolamine, in agreement with a previous report [24]. These reactions did not occur with the heat-treated homogenate. By this method, we examined the tissue distribution of the PLA_1/PLA_2 activity for *N*-[^{14}C]palmitoyl-PE in rat. The results showed a wide distribution of the enzyme activity (Figure 1). Among them, by far the highest activity was observed in stomach with a specific activity of 49.1 ± 3.5 nmol/min per mg of protein (means ± S.D., $n = 3$) at 37 °C, followed by pancreas (7.8 ± 0.2) and small intestine (1.4 ± 0.2). The other tested tissues showed lower activities (0.02–0.17 nmol/min per mg of protein).

Purification, identification and substrate specificity of stomach *N*-acyl-PE-hydrolysing PLA_1/PLA_2

For the purpose of the identification of the stomach PLA_1/PLA_2, we attempted to purify the enzyme. After the solubilization of the enzyme from the particulate fraction by 1 % octyl glucoside, the protein sample was subjected sequentially to heat treatment at 70 °C for 10 min and two chromatographic steps using Macro-Prep High Q and HiTrap SP HP. Through this procedure the specific PLA_1/PLA_2 activity with *N*-[^{14}C]palmitoyl-PE was increased 472-fold from 71 nmol/min per mg of protein

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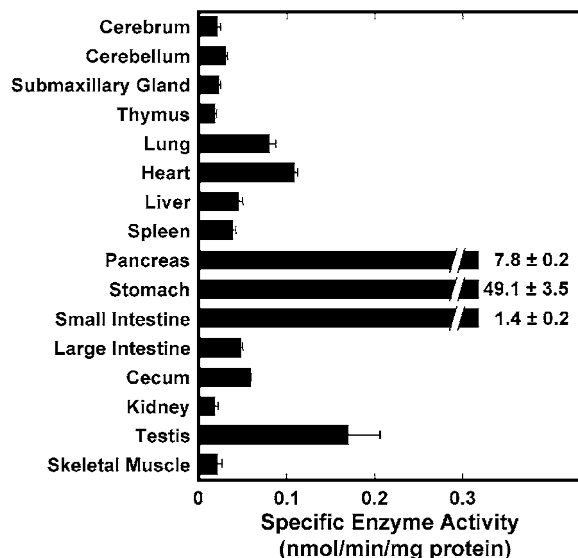

Figure 1 Distribution of the *N*-palmitoyl-PE-hydrolysing PLA_1/PLA_2 activity in rat tissues

The homogenates of the indicated rat organs (4 μg of protein for stomach, 25 μg of protein for pancreas, or 100 μg of protein for other organs) were allowed to react with 100 μM *N*-[^{14}C]palmitoyl-PE. The results are expressed as the means $\pm$ S.D. ($n = 3$).

Table 1 Purification of the *N*-palmitoyl-PE-hydrolysing PLA_1/PLA_2 enzyme from rat stomach

The *N*-palmitoyl-PE-hydrolysing PLA_1/PLA_2 was purified from rat stomach as described in the Experimental section.

Purification step	Total protein (mg)	Total activity (μmol/min)†	Specific activity (μmol/min per mg of protein)†	Yield (%)	Purification (fold)
Solubilized proteins*	21	1.5	0.071	100	1
Heat treatment	4.5	0.86	0.19	57	2.7
Macro-Prep High Q	0.38	2.9	7.7	193	108
HiTrap SP HP	0.032	1.1	34	70	472

* Proteins were solubilized by 1 % octyl glucoside from 105 000 ***g*** pellet of rat stomach homogenates.

† The enzyme assays were performed by the incubation with 100 μM *N*-[^{14}C]palmitoyl-PE in the presence of 10 mM $CaCl_2$ at 37 °C for 5 min.

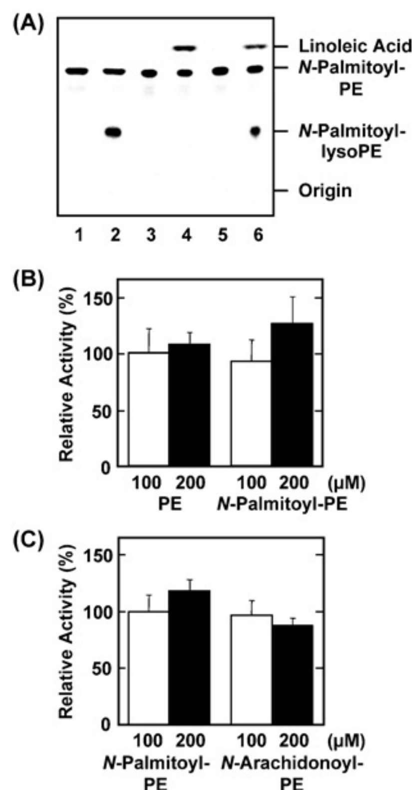

Figure 2 Reactivity of the purified stomach $sPLA_2$-IB with *N*-acyl-PE

(**A**) Identification of the product from *N*-palmitoyl-PE by the purified $sPLA_2$-IB. The purified $sPLA_2$-IB of rat stomach (4 ng of protein) (lanes 2, 4 and 6) or the protein-free buffer (lanes 1, 3 and 5) was allowed to react with 100 μM of *N*-[^{14}C]palmitoyl-PE (lanes 1 and 2), *N*-palmitoyl-2-[^{14}C]linoleoyl-PE (lanes 3 and 4), or the mixture of both substrates in a ratio of 1:1 (lanes 5 and 6). The products were separated by TLC with a mixture of chloroform/methanol/acetic acid (9:1:1, by vol.). (**B**, **C**) The substrate specificity of the purified stomach $sPLA_2$-IB. The purified stomach $sPLA_2$-IB (6 ng of protein) was allowed to react with 100 μM (open columns) or 200 μM (closed columns) of 2-[^{14}C]linoleoyl-PE (PE) (**B**), *N*-palmitoyl-2-[^{14}C]linoleoyl-PE (*N*-Palmitoyl-PE) (**B**), *N*-[^{14}C]palmitoyl-PE (*N*-Palmitoyl-PE) (**C**), or *N*-[^{14}C]arachidonoyl-PE (*N*-Arachidonoyl-PE) (**C**). The activity with 100 μM 2-[^{14}C]linoleoyl-PE (19 μmol/min per mg of protein) (**B**) or with 100 μM *N*-[^{14}C]palmitoyl-PE (28 μmol/min per mg of protein) (**C**) was expressed as 100 %. The results are expressed as the means $\pm$ S.D. ($n = 3$).

to 34 μmol/min per mg of protein (Table 1). The purified enzyme preparation gave a single protein band around 17 kDa, as analysed by SDS/PAGE. The N-terminal sequence of this 17-kDa protein was determined by Edman degradation as Ala-Val-Trp-Gln-Phe-Arg-Asn-Met-Ile-Lys-Xaa-Thr-Ile-Pro-Gly-Ser-Asp. This sequence was identical to Ala^{23}–Asp^{39} of rat $sPLA_2$-IB (assuming Xaa to be Cys) [34]. Thus we concluded that the purified stomach enzyme hydrolysing *N*-palmitoyl-PE was $sPLA_2$-IB.

In order to determine which acyl group at *sn*-1 or *sn*-2 position of *N*-palmitoyl-PE was removed by the purified $sPLA_2$-IB, we used two types of *N*-palmitoyl-PE in which a different carbon atom was radiolabelled. With *N*-[^{14}C]palmitoyl-PE as the substrate of the purified $sPLA_2$-IB, only *N*-[^{14}C]palmitoyl-lysoPE was detected as the radiolabelled product by TLC (Figure 2A, lane 2). In contrast, the enzyme converted *N*-palmitoyl-2-[^{14}C] linoleoyl-PE exclusively to radiolabelled linoleic acid (Figure 2A, lane 4). Furthermore, both *N*-[^{14}C]palmitoyl-lysoPE and [^{14}C]linoleic acid were produced from a mixture of *N*-[^{14}C]-palmitoyl-PE and *N*-palmitoyl-2-[^{14}C]linoleoyl-PE (Figure 2A, lane 6). If the enzyme cleaves *N*-palmitoyl-2-[^{14}C]linoleoyl-PE at *sn*-1 position, *N*-palmitoyl-2-[^{14}C]linoleoyl-lysoPE should be detected. These results demonstrated that the purified $sPLA_2$-IB exclusively released the acyl group at the *sn*-2 position of *N*-palmitoyl-PE.

Furthermore, the reactivity of the purified stomach $sPLA_2$-IB with *N*-palmitoyl-PE was compared with that of PE, a general substrate of PLA_2 (Figure 2B). The results indicated that *N*-palmitoyl-2-[^{14}C]linoleoyl-PE was as reactive as 2-[^{14}C]linoleoyl-PE both at 100 μM and 200 μM. We also assessed the reactivity with *N*-[^{14}C]arachidonoyl-PE, a precursor of anandamide, which was also found to be an active substrate (Figure 2C).

Hydrolytic activity of various PLA_2 isoenzymes with *N*-palmitoyl-PE

We next examined whether PLA_2 isoenzymes, other than $sPLA_2$-IB, were also able to act as PLA_2 toward *N*-acyl-PE. We expressed a series of representative PLA_2 isoenzymes in HEK-293 cells, and measured their activities with *N*-acyl-PE. We used group IB, IIA,

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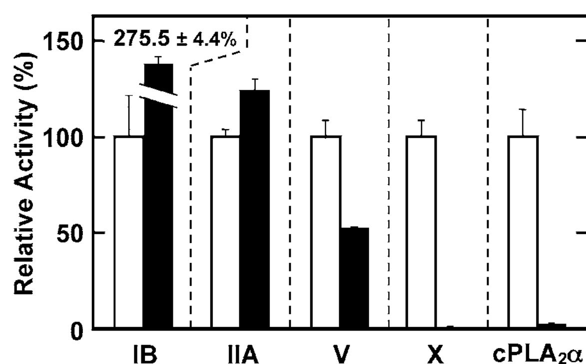

Figure 3 Reactivity of recombinant PLA$_2$s with *N*-[^{14}C]palmitoyl-PE

The recombinant sPLA$_2$-IB, -IIA, -V, -X and cPLA$_2\alpha$ expressed in HEK-293 cells were prepared as described in the Experimental section, and their reactivities with 100 μM 2-[^{14}C]linoleoyl-PE (open columns for sPLA$_2$s), 2-[^{14}C]arachidonoyl-PE (an open column for cPLA$_2\alpha$), *N*-palmitoyl-2-[^{14}C]linoleoyl-PE (closed columns for sPLA$_2$s), or *N*-palmitoyl-2-[^{14}C]arachidonoyl-PE (a closed column for cPLA$_2\alpha$) were measured under appropriate conditions for each PLA$_2$ isoenzyme. The activities of each PLA$_2$ isoenzyme with 2-[^{14}C]linoleoyl-PE or 2-[^{14}C]arachidonoyl-PE were expressed as 100 % as follows: sPLA$_2$-IB, 5.5 nmol/min per 10^6 cells; sPLA$_2$-IIA, 0.37 nmol/min per 10^6 cells; sPLA$_2$-V, 1.6 nmol/min per 10^6 cells; sPLA$_2$-X, 2.0 nmol/min per 10^6 cells; cPLA$_2\alpha$, 39 pmol/min per mg of protein. The results are expressed as the means $\pm$ S.D. ($n = 3$).

V and X of sPLA$_2$s secreted from HEK-293 cells and cPLA$_2\alpha$ in the whole cell lysate. The endogenous PE-hydrolysing activities of mock-transfected or parent HEK-293 cells were less than 5 % of those of the cells transfected with each sPLA$_2$ isoenzyme (results not shown). Only in the case of cPLA$_2\alpha$ was the endogenous PLA$_2$ activity with PE about 15 % of that of the cPLA$_2\alpha$-transfected cells (results not shown). As shown in Figure 3, sPLA$_2$-IB, -IIA, and -V hydrolysed *N*-[^{14}C]palmitoyl-PE at rates of more than 50 % of their corresponding [^{14}C]PE-hydrolysing activities. In contrast, sPLA$_2$-X and cPLA$_2\alpha$ were almost inactive with *N*-[^{14}C]palmitoyl-PE.

Tissue distribution of *N*-acyl-lysoPE-hydrolysing lysoPLD activity

We found the lysoPLD activity releasing *N*-[^{14}C]palmitoyl-ethanolamine from *N*-[^{14}C]palmitoyl-lysoPE in the homogenate of rat brain. This reaction did not occur with the heat-treated homogenate. When the homogenates were subjected to ultra-centrifugation, most of the lysoPLD activity was recovered in the particulate fraction, rather than cytosol. When the particulate fraction was allowed to react with *N*-[^{14}C]palmitoyl-lysoPE in a range of pH 4–11, the optimum pH was found to be around 8. The addition of 2 mM EDTA into the reaction mixture almost completely abolished the enzyme activity, suggesting the requirement for bivalent cations. However, the addition of 10 mM $MgCl_2$ did not stimulate the activity significantly, and 10 mM $CaCl_2$ inhibited the enzyme by 80 %. We therefore carried out the lysoPLD assay in Tris/HCl buffer at pH 8 without adding bivalent cations, and examined the tissue distribution of the lysoPLD activity toward *N*-[^{14}C]palmitoyl-lysoPE in rat. As shown in Figure 4, the enzyme activities were widely distributed among various organs, with higher activities in the brain and testis.

Characterization of brain *N*-acyl-lysoPE-hydrolysing lysoPLD

MAFP, a serine hydrolase inhibitor, is reported to have little inhibitory effect on the heart *N*-acyl-PE-hydrolysing PLD at least up to 1 μM [23,35]. In accordance with the previous result, MAFP had no effect on the brain PLD activity toward *N*-[^{14}C]palmitoyl-PE up to 3 μM (Figure 5A). The brain PLD activity was stimulated about 3-fold by 0.06 % Triton X-100 as was reported previously [22,36], and the Triton X-100-stimulated PLD activity was also insensitive to MAFP (results not shown). In contrast, MAFP dose dependently inhibited the lysoPLD activity of the brain particulate fraction toward *N*-[^{14}C]palmitoyl-lysoPE with an IC_{50} value of about 0.2 μM (Figure 5A).

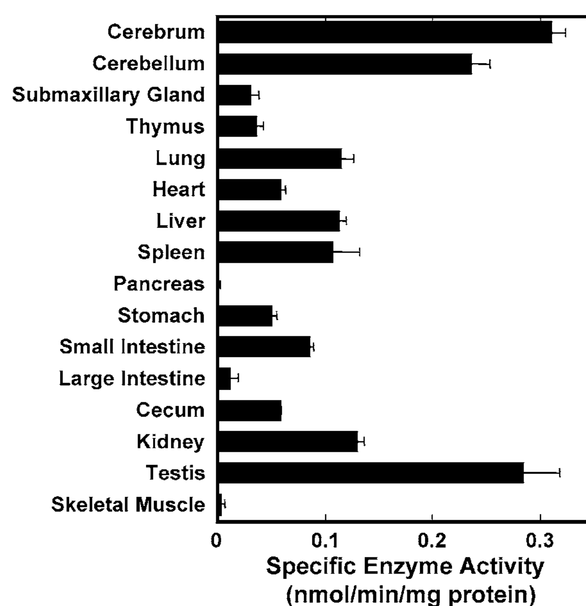

Figure 4 Distribution of the *N*-palmitoyl-lysoPE-hydrolysing lysoPLD activity in rat tissues

The homogenates of the indicated rat organs (100 μg of protein) were allowed to react with 100 μM *N*-[^{14}C]palmitoyl-lysoPE. The results are expressed as the means $\pm$ S.D. ($n = 3$).

N-Acyl-PE-hydrolysing PLD could be solubilized from rat heart microsomes with 1 % octyl glucoside [35,36]. The rat brain PLD was also efficiently solubilized with the same detergent (Figure 5B). However, the *N*-[^{14}C]palmitoyl-lysoPE-hydrolysing lysoPLD was only partially solubilized by the same procedure. These findings provided strong support for the presence of *N*-acyl-lysoPE-hydrolysing lysoPLD enzyme distinct from *N*-acyl-PE-hydrolysing PLD in the rat brain.

We also examined the reactivity of rat brain lysoPLD with *N*-arachidonoyl-lysoPE, a precursor of anandamide. When varying concentrations (25–200 μM) of the substrate were used, the enzyme activity to release [^{14}C]anandamide from *N*-[^{14}C]arachidonoyl-lysoPE increased almost linearly, showing a very high K_m value. The rate was comparable with that to release *N*-[^{14}C]-palmitoylethanolamine from *N*-[^{14}C]palmitoyl-lysoPE at all the substrate concentrations examined (Figure 5C).

Synthesis of *N*-palmitoylethanolamine by the sequential actions of sPLA$_2$-IB and lysoPLD

Finally, we assessed whether *N*-palmitoylethanolamine could actually be synthesized by the combination of PLA$_2$ and lysoPLD. When we mixed increasing amounts of the purified stomach sPLA$_2$-IB with a constant amount of the brain homogenate as a source of lysoPLD, the formation of *N*-[^{14}C]palmitoylethanolamine from *N*-[^{14}C]palmitoyl-PE increased with a concomitant accumulation of *N*-[^{14}C]palmitoyl-lysoPE as an intermediate

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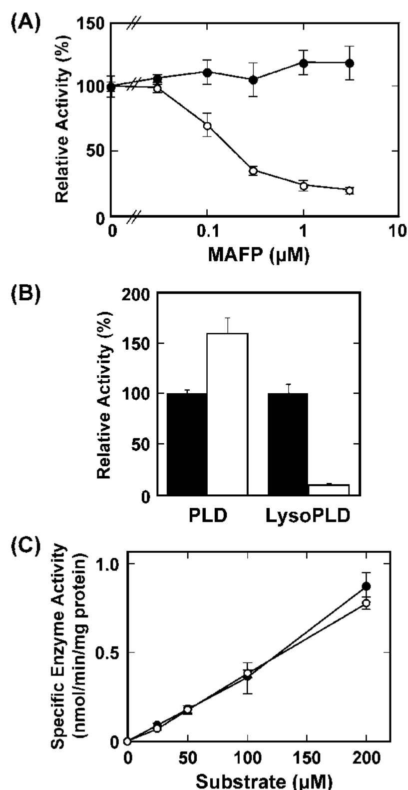

Figure 5 Characterization of *N*-acyl-lysoPE-hydrolysing lysoPLD from rat brain

(**A**) Distinct sensitivity to MAFP between PLD and lysoPLD. The particulate fraction of rat brain was allowed to react with 100 μM of *N*-[^{14}C]palmitoyl-PE (●, PLD) or *N*-[^{14}C]palmitoyl-lysoPE (○, lysoPLD) in the presence of the indicated concentrations of MAFP (dissolved in 5 μl of DMSO). There were no detergents in the reaction mixture. Each activity in the absence of MAFP (PLD, 0.017 nmol/min per mg of protein; lysoPLD, 0.31 nmol/min per mg of protein) was expressed as 100 %. (**B**) Different effects of octyl glucoside on the solubilization of PLD and lysoPLD from the particulate fraction. The particulate fraction of rat brain (closed columns) or proteins solubilized by 1 % octyl glucoside from the particulate fraction (open columns) were allowed to react with 100 μM *N*-[^{14}C]palmitoyl-PE (for PLD assay) or *N*-[^{14}C]palmitoyl-lysoPE (for lysoPLD assay). Final concentrations of octyl glucoside in the reaction mixture were adjusted to 0.13 %. For PLD assay, 0.06 % Triton X-100 was added as an enzyme activator [35]. Each activity in the particulate fractions (PLD, 1.3 nmol/min per g of wet tissue; lysoPLD, 7.7 nmol/min per g of wet tissue) was expressed as 100 %. (**C**) Dependence of the lysoPLD activity on substrate concentrations. The particulate fraction of rat brain (100 μg of protein) was allowed to react with the indicated concentrations of *N*-[^{14}C]palmitoyl-lysoPE (●) or *N*-[^{14}C]arachidonoyl-lysoPE (○). The results are expressed as the means ± S.D. ($n = 3$).

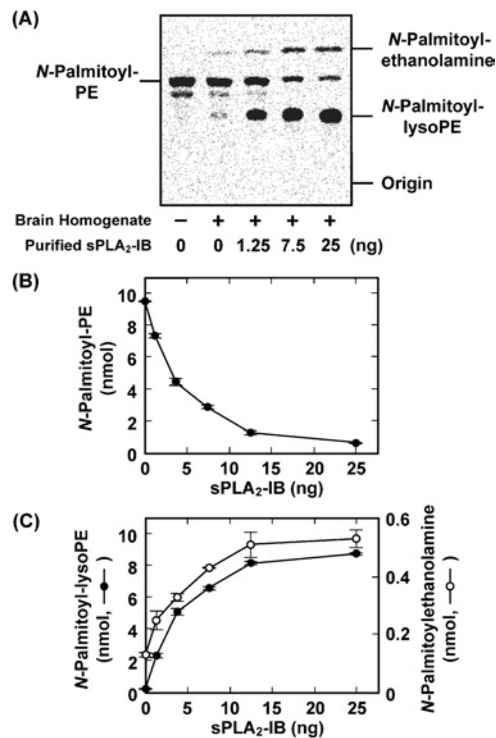

Figure 6 Formation of *N*-palmitoylethanolamine by the sequential action of sPLA$_2$-IB and lysoPLD

The mixtures of an increasing amount (0–25 ng of protein) of the purified stomach sPLA$_2$-IB and a constant amount of the brain homogenate (150 μg of protein) were allowed to react with 100 μM *N*-[^{14}C]palmitoyl-PE (1000 cpm/nmol, dissolved in 5 μl of ethanol). A representative image of the TLC plate is shown in (**A**). The remaining *N*-[^{14}C]palmitoyl-PE (**B**) and the produced *N*-[^{14}C]palmitoyl-lysoPE (**C**, ●) and *N*-[^{14}C]palmitoylethanolamine (**C**, ○) were quantified, and the results are expressed as the means ± S.D. ($n = 3$).

(Figure 6). In the presence of 1 μM MAFP to inhibit lysoPLD, such an increase in the generation of [^{14}C]palmitoylethanolamine was not observed (results not shown).

DISCUSSION

Recent progress in the studies on biological activities of anandamide and other NAEs urges us to investigate the regulatory mechanisms in the biosynthesis of NAEs. It is generally accepted that NAE is directly released from *N*-acyl-PE by PLD [1–5,19]. We recently cloned this PLD which is structurally and catalytically distinguishable from the known PLDs [20]. In addition, an alternative pathway via *N*-acyl-lysoPE has been suggested earlier [21] (Scheme 1). However, the enzymes involved have not been characterized. The present study focused on the enzymes responsible for this biosynthetic pathway of NAE from *N*-acyl-PE to *N*-acyl-lysoPE. We clarified that several PLA$_2$ isoenzymes can generate *N*-acyl-lysoPE from *N*-acyl-PE, and that lysoPLD releasing NAEs from *N*-acyl-lysoPE is catalytically distinct from the *N*-acyl-PE-hydrolysing PLD. The tissue distribution studies revealed the wide distributions of both of these two enzyme activities. The substrate specificity experiments showed that the precursors of anandamide are also the substrates of both the enzymes. Notably, in the brain particulate fraction, the specific activity of lysoPLD (0.31 nmol/min per mg of protein) was far greater than that of PLD (0.017 nmol/min per mg of protein) (Figure 5A). In general, *N*-acyl-PE exists in a much higher amount than NAE in animal tissues [2]. Hence, upon the activation of sPLA$_2$, NAEs including anandamide could be generated by this pathway. We showed that NAE was actually formed from *N*-acyl-PE by the combination of sPLA$_2$-IB and lysoPLD (Figure 6). However, further investigation will be required to clarify how much this pathway contributes to the *in vivo* formation of NAEs.

Since the activity of *N*-palmitoyl-PE-hydrolysing PLA$_1$/PLA$_2$ was by far the highest in stomach among the tested organs, we purified the enzyme to apparent homogeneity from stomach and identified it to be sPLA$_2$-IB, that is the pancreatic-type PLA$_2$. The identification was further confirmed by the finding that recombinant sPLA$_2$-IB was also active with *N*-palmitoyl-PE. The high content of sPLA$_2$-IB in rat stomach is in good agreement with the previous observation that sPLA$_2$-IB is abundantly expressed in rat gastric mucosa [37]. Therefore, it seemed that the extremely high *N*-palmitoyl-PE-hydrolysing activity in stomach is mostly derived from sPLA$_2$-IB. It is likely that *N*-acyl-PE in the diet is

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first digested mainly by sPLA$_2$-IB in the gastrointestinal tract. The enzyme activity of stomach sPLA$_2$-IB toward both PE and *N*-acyl-PE were similar between 100 μM and 200 μM of the substrate concentrations (Figures 2B and 2C), indicating that the standard enzyme assay with 100 μM of the substrate was performed near the saturation levels of the substrates.

We also demonstrated that the purified stomach sPLA$_2$-IB cleaved *N*-palmitoyl-PE exclusively at *sn*-2 position to release a fatty acid. Accordingly, the *N*-palmitoyl-lysoPE produced by sPLA$_2$-IB retains the acyl group at *sn*-1 position. However, we cannot rule out the possibility that PLA$_1$ and other phospholipases hydrolyse *N*-acyl-PE to generate *N*-acyl-2-acyl-lysoPE retaining the acyl group at *sn*-2 position. Furthermore, intramolecular trans-acylation of PE by *N*-acyltransferase may also yield *N*-acyl-2-acyl-lysoPE, although the *sn*-1 position of this molecule would be quickly subjected to reacylation [3].

In mammals, the PLA$_2$ family comprises at least 19 isoenzymes, which are categorized into four families; sPLA$_2$s, cPLA$_2$s, Ca^{2+}-independent PLA$_2$s and platelet-activating factor acetylhydrolases [38–40]. We examined the contribution of PLA$_2$ isoenzymes other than sPLA$_2$-IB to the hydrolysis of *N*-acyl-PE with recombinant enzymes of several representative PLA$_2$s. The results showed that not only sPLA$_2$-IB, but also sPLA$_2$-IIA and sPLA$_2$-V could hydrolyse *N*-palmitoyl-PE at comparable rates to the hydrolysis of PE, one of the most common substrates of PLA$_2$. Thus PLA$_1$/PLA$_2$ activity in different organs (Figure 1) may be attributed to different PLA$_2$ isoenzymes. Since sPLA$_2$s, especially sPLA$_2$-IIA, are up-regulated at various inflamed sites and implicated in the inflammatory processes [39], the pathway via *N*-acyl-lysoPE might be involved in the production of anti-inflammatory NAEs at the inflamed sites. In contrast, sPLA$_2$-X and cPLA$_2\alpha$ hardly hydrolysed *N*-palmitoyl-PE, implying that PLA$_2$ isoenzymes may be functionally classified in terms of the capability of hydrolysing *N*-acyl-PE.

We found a lysoPLD activity hydrolysing *N*-acyl-lysoPE to NAE in various tissues of rat. One of the most interesting findings in the present study is the presence of *N*-acyl-lysoPE-hydrolysing lysoPLD enzyme distinct from the *N*-acyl-PE-hydrolysing PLD enzyme. This proposal is based on the differences in tissue distribution, inhibitory effect of MAFP, and effect of octyl glucoside on the solubilization between these two enzyme activities (Figures 4 and 5). As to the tissue distribution in rat, it was reported previously that the PLD activity was the highest in the heart, followed by the brain and testis [22,35]. In contrast, in the present study we revealed a relatively low lysoPLD activity in the heart, in comparison with the brain and testis (Figure 4). Since recombinant *N*-acyl-PE-hydrolysing PLD of mouse had a low hydrolysing activity toward *N*-acyl-lysoPE [20], the observed lysoPLD activities for *N*-acyl-lysoPE may be partly attributed to the PLD enzyme rather than the lysoPLD enzyme. Indeed, the brain lysoPLD activity for *N*-acyl-lysoPE was not completely inhibited by MAFP, even at the highest concentration tested, and the remaining activity might be derived from PLD which was insensitive to MAFP (Figure 5A). Furthermore, a previous study with the microsome of dog brain reported that Triton X-100 stimulated the hydrolysis of *N*-acyl-PE to NAEs, but inhibited that of *N*-acyl-lysoPE [21]. This observation may provide further support for the presence of a lysoPLD enzyme distinct from the *N*-acyl-PE-hydrolysing PLD enzyme.

Recently, a lysoPLD releasing LPA (lysophosphatidic acid) from lysophosphatidylcholine was purified from plasma and identified to be a soluble form of autotaxin, a member of the ecto-nucleotide pyrophosphatase/phosphodiesterase family [41,42]. Autotaxin is released extracellularly after the intramolecular cleavage and it exerts its enzymic activity outside the cells [43], whereas the brain *N*-acyl-lysoPE-hydrolysing lysoPLD was tightly bound to the membrane and the efficiency of solubilization was poor even in the presence of octyl glucoside (Figure 5B). Further investigations including the protein purification and cDNA cloning are required for its molecular characterization.

It has been a matter of debate whether or not an anandamide-selective biosynthetic pathway exists in animal tissues. The *N*-acyl-PE-hydrolysing PLD was reported not to show selectivity in terms of *N*-acyl species [20,44]. In the present study, *N*-arachidonoyl-PE and *N*-arachidonoyl-lysoPE as precursors of anandamide could be substrates of sPLA$_2$-IB and lysoPLD respectively, but their reaction rates were similar to those for *N*-palmitoyl-PE and *N*-palmitoyl-lysoPE respectively. Since both *N*-palmitoylethanolamine and anandamide were produced at the same rate in this pathway, other NAEs would be then expected to be produced and exert their biological activity as a group (rather than as individual molecular species). Further investigation will be necessary to elucidate the possible presence of the anandamide-selective biosynthetic pathway.

It is noted that the hydrolysis of *N*-acyl-lysoPE by lysoPLD resulted in the production of LPA, in addition to NAEs. LPA is mitogenic and angiogenic, and is implicated in the progression of cancer through its G-protein-coupled receptors [45]. On the other hand, NAEs were shown to have anti-tumour activity [46]. It is interesting that the pathway discussed in the present study simultaneously produces these two classes of bioactive lipids, which have apparently opposite effects on cancer cells.

In summary, we investigated for the first time the PLA$_2$ and lysoPLD enzymes responsible for the possible biosynthetic route of NAEs via *N*-acyl-lysoPE. Not all, but several sPLA$_2$ isoenzymes were found to participate in this pathway, and lysoPLD was suggested to be a distinct enzyme from the *N*-acyl-PE-hydrolysing PLD. The present study gives a further insight into the understanding of the biosynthesis and physiological roles of NAEs.

This work was partially supported by a grant-in-aid from Kagawa Medical University.

REFERENCES

1 Schmid, H. H. O., Schmid, P. C. and Natarajan, V. (1990) *N*-Acylated glycerophospholipids and their derivatives. Prog. Lipid Res. **29**, 1–43

2 Hansen, H. S., Moesgaard, B., Hansen, H. H. and Petersen, G. (2000) *N*-Acylethanolamines and precursor phospholipids – relation to cell injury. Chem. Phys. Lipids **108**, 135–150

3 Schmid, H. H. O. and Berdyshev, E. V. (2002) Cannabinoid receptor-inactive *N*-acylethanolamines and other fatty acid amides: metabolism and function. Prostaglandins Leukot. Essent. Fatty Acids **66**, 363–376

4 Sugiura, T., Kobayashi, Y., Oka, S. and Waku, K. (2002) Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. Prostaglandins Leukot. Essent. Fatty Acids **66**, 173–192

5 Schmid, H. H. O. (2000) Pathways and mechanisms of *N*-acylethanolamine biosynthesis: can anandamide be generated selectively? Chem. Phys. Lipids **108**, 71–87

6 Epps, D. E., Schmid, P. C., Natarajan, V. and Schmid, H. H. O. (1979) *N*-Acylethanolamine accumulation in infarcted myocardium. Biochem. Biophys. Res. Commun. **90**, 628–633

7 Schmid, P. C., Krebsbach, R. J., Perry, S. R., Dettmer, T. M., Maasson, J. L. and Schmid, H. H. O. (1995) Occurrence and postmortem generation of anandamide and other long-chain *N*-acylethanolamines in mammalian brain. FEBS Lett. **375**, 117–120

8 Kondo, S., Sugiura, T., Kodaka, T., Kudo, N., Waku, K. and Tokumura, A. (1998) Accumulation of various *N*-acylethanolamines including *N*-arachidonoylethanolamine (anandamide) in cadmium chloride-administered rat testis. Arch. Biochem. Biophys. **354**, 303–310

9 Hansen, H. S., Lauritzen, L., Strand, A. M., Moesgaard, B. and Frandsen, A. (1995) Glutamate stimulates the formation of *N*-acylphosphatidylethanolamine and *N*-acylethanolamine in cortical neurons in culture. Biochim. Biophys. Acta **1258**, 303–308

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10 Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. and Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science **258**, 1946–1949
11 Di Marzo, V., De Petrocellis, L., Fezza, F., Ligresti, A. and Bisogno, T. (2002) Anandamide receptors. Prostaglandins Leukot. Essent. Fatty Acids **66**, 377–391
12 Di Marzo, V. (1998) 'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance. Biochim. Biophys. Acta **1392**, 153–175
13 Calignano, A., La Rana, G., Giuffrida, A. and Piomelli, D. (1998) Control of pain initiation by endogenous cannabinoids. Nature (London) **394**, 277–281
14 Calignano, A., La Rana, G. and Piomelli, D. (2001) Antinociceptive activity of the endogenous fatty acid amide, palmitylethanolamide. Eur. J. Pharmacol. **419**, 191–198
15 Lambert, D. M., Vandevoorde, S., Jonsson, K. O. and Fowler, C. J. (2002) The palmitoylethanolamide family: a new class of anti-inflammatory agents? Curr. Med. Chem. **9**, 663–674
16 Rodriguez de Fonseca, F., Navarro, M., Gomez, R., Escuredo, L., Nava, F., Fu, J., Murillo-Rodriguez, E., Giuffrida, A., LoVerme, J., Gaetani, S. et al. (2001) An anorexic lipid mediator regulated by feeding. Nature (London) **414**, 209–212
17 Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodriguez De Fonseca, F., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G. and Piomelli, D. (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. Nature (London) **425**, 90–93
18 Maccarrone, M., Pauselli, R., Di Rienzo, M. and Finazzi-Agro, A. (2002) Binding, degradation and apoptotic activity of stearoylethanolamide in rat C6 glioma cells. Biochem. J. **366**, 137–144
19 Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C. and Piomelli, D. (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature (London) **372**, 686–691
20 Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T. and Ueda, N. (2004) Molecular characterization of a phospholipase D generating anandamide and its congeners. J. Biol. Chem. **279**, 5298–5305
21 Natarajan, V., Schmid, P. C., Reddy, P. V. and Schmid, H. H. O. (1984) Catabolism of *N*-acylethanolamine phospholipids by dog brain preparations. J. Neurochem. **42**, 1613–1619
22 Schmid, P. C., Reddy, P. V., Natarajan, V. and Schmid, H. H. O. (1983) Metabolism of *N*-acylethanolamine phospholipids by a mammalian phosphodiesterase of the phospholipase D type. J. Biol. Chem. **258**, 9302–9306
23 Petersen, G. and Hansen, H. S. (1999) *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D lacks the ability to transphosphatidylate. FEBS Lett. **455**, 41–44
24 Petersen, G., Chapman, K. D. and Hansen, H. S. (2000) A rapid phospholipase D assay using zirconium precipitation of anionic substrate phospholipids: application to N-acylethanolamine formation *in vitro*. J. Lipid Res. **41**, 1532–1538
25 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
26 Murakami, M., Koduri, R. S., Enomoto, A., Shimbara, S., Seki, M., Yoshihara, K., Singer, A., Valentin, E., Ghomashchi, F., Lambeau, G., et al. (2001) Distinct arachidonate-releasing functions of mammalian secreted phospholipase A_2s in human embryonic kidney 293 and rat mastocytoma RBL-2H3 cells through heparan sulfate shuttling and external plasma membrane mechanisms. J. Biol. Chem. **276**, 10083–10096
27 Murakami, M., Kambe, T., Shimbara, S., Higashino, K., Hanasaki, K., Arita, H., Horiguchi, M., Arita, M., Arai, H., Inoue, K. and Kudo, I. (1999) Different functional aspects of the group II subfamily (Types IIA and V) and type X secretory phospholipase A_2s in regulating arachidonic acid release and prostaglandin generation. Implications of cyclooxygenase-2 induction and phospholipid scramblase-mediated cellular membrane perturbation. J. Biol. Chem. **274**, 31435–31444
28 Murakami, M., Shimbara, S., Kambe, T., Kuwata, H., Winstead, M. V., Tischfield, J. A. and Kudo, I. (1998) The functions of five distinct mammalian phospholipase A_2s in regulating arachidonic acid release. Type IIA and type V secretory phospholipase A_2s are functionally redundant and act in concert with cytosolic phospholipase A_2. J. Biol. Chem. **273**, 14411–14423
29 Nakatani, Y., Tanioka, T., Sunaga, S., Murakami, M. and Kudo, I. (2000) Identification of a cellular protein that functionally interacts with the C2 domain of cytosolic phospholipase $A_2\alpha$. J. Biol. Chem. **275**, 1161–1168
30 Murakami, M., Nakatani, Y. and Kudo, I. (1996) Type II secretory phospholipase A_2 associated with cell surfaces via C-terminal heparin-binding lysine residues augments stimulus-initiated delayed prostaglandin generation. J. Biol. Chem. **271**, 30041–30051
31 Murakami, M., Kambe, T., Shimbara, S. and Kudo, I. (1999) Functional coupling between various phospholipase A_2s and cyclooxygenases in immediate and delayed prostanoid biosynthetic pathways. J. Biol. Chem. **274**, 3103–3115
32 Ishizaki, J., Suzuki, N., Higashino, K., Yokota, Y., Ono, T., Kawamoto, K., Fujii, N., Arita, H. and Hanasaki, K. (1999) Cloning and characterization of novel mouse and human secretory phospholipase A_2s. J. Biol. Chem. **274**, 24973–24979
33 Atsumi, G., Tajima, M., Hadano, A., Nakatani, Y., Murakami, M. and Kudo, I. (1998) Fas-induced arachidonic acid release is mediated by Ca^{2+}-independent phospholipase A_2 but not cytosolic phospholipase A_2, which undergoes proteolytic inactivation. J. Biol. Chem. **273**, 13870–13877
34 Ohara, O., Tamaki, M., Nakamura, E., Tsuruta, Y., Fujii, Y., Shin, M., Teraoka, H. and Okamoto, M. (1986) Dog and rat pancreatic phospholipases A_2: complete amino acid sequences deduced from complementary DNAs. J. Biochem. (Tokyo) **99**, 733–739
35 Liu, Q., Tonai, T. and Ueda, N. (2002) Activation of *N*-acylethanolamine-releasing phospholipase D by polyamines. Chem. Phys. Lipids **115**, 77–84
36 Ueda, N., Liu, Q. and Yamanaka, K. (2001) Marked activation of the *N*-acylphosphatidylethanolamine-hydrolyzing phosphodiesterase by divalent cations. Biochim. Biophys. Acta **1532**, 121–127
37 Sakata, T., Nakamura, E., Tsuruta, Y., Tamaki, M., Teraoka, H., Tojo, H., Ono, T. and Okamoto, M. (1989) Presence of pancreatic-type phospholipase A_2 mRNA in rat gastric mucosa and lung. Biochim. Biophys. Acta **1007**, 124–126
38 Six, D. A. and Dennis, E. A. (2000) The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim. Biophys. Acta **1488**, 1–19
39 Murakami, M. and Kudo, I. (2002) Phospholipase A_2. J. Biochem. (Tokyo) **131**, 285–292
40 Kudo, I. and Murakami, M. (2002) Phospholipase A_2 enzymes. Prostaglandins Other Lipid Mediat. **68–69**, 3–58
41 Tokumura, A., Majima, E., Kariya, Y., Tominaga, K., Kogure, K., Yasuda, K. and Fukuzawa, K. (2002) Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. J. Biol. Chem. **277**, 39436–39442
42 Umezu-Goto, M., Kishi, Y., Taira, A., Hama, K., Dohmae, N., Takio, K., Yamori, T., Mills, G. B., Inoue, K., Aoki, J. and Arai, H. (2002) Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. J. Cell Biol. **158**, 227–233
43 Stracke, M. L., Clair, T. and Liotta, L. A. (1997) Autotaxin, tumor motility-stimulating exophosphodiesterase. Adv. Enzyme Regul. **37**, 135–144
44 Sugiura, T., Kondo, S., Sukagawa, A., Tonegawa, T., Nakane, S., Yamashita, A., Ishima, Y. and Waku, K. (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. Eur. J. Biochem. **240**, 53–62
45 Mills, G. B. and Moolenaar, W. H. (2003) The emerging role of lysophosphatidic acid in cancer. Nat. Rev. Cancer **3**, 582–591
46 De Petrocellis, L., Melck, D., Bisogno, T. and Di Marzo, V. (2000) Endocannabinoids and fatty acid amides in cancer, inflammation and related disorders. Chem. Phys. Lipids **108**, 191–209

Received 6 January 2004/19 February 2004; accepted 3 March 2004
Published as BJ Immediate Publication 3 March 2004, DOI 10.1042/BJ20040031

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