Biosynthesis of anandamide and N-palmitoylethanolamine by sequential actions of phospholipase A2 and lysophospholipase D

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Anandamide (an endocannabinoid) and other bioactive long-chain NAEs (N-acyl-PE) 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 been suggested previously, which comprises (1) the hydrolysis of N-acyl-PE to N-acyl-lysoPE by PLA2/PLA1 enzyme(s) and (2) the release of NAEs from N-acyl-

INTRODUCTION

Ethanolamides of long-chain fatty acids, collectively referred to as NAEs (N-acyl-PE), are present in various mammalian tissues [1–5]. NAEs were previously reported to have anti-inflammatory and membrane-stabilizing activities, and are known to markedly increase in degenerating tissues and cells [6–9]. Later, N-arachidonoyl ethanolamine (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 were shown to possess various biological activities. For example, N-palmitoylethanolamine was reported to be anti-inflammatory and anti-nociceptive [13–15], N-oleoyl ethanolamine to be anorectic via peroxisome-proliferator-activated receptor-α [16,17], and N-stearoyl ethanolamine to be pro-apoptotic [18].

It is generally accepted that NAEs is principally biosynthesized in animal tissues from PE (phosphatidylethanolamine) by two sequential enzyme reactions [1–5,19]. The first reaction is the 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 PLA1 or PLA2 activity, and NAE is then released from N-acyl-lysoPE by a lysoPLD (lysophospholipase D)-like enzyme. The PLA1- or PLA2-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 homogenate of rat heart, rat brain and dog brain [21–23]. It is also noted that commercially available venom and pancreatic PLA1s 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 largely 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-lysoPE.

In the present study, in order to clarify the physiological significance of this NAE biosynthetic pathway via N-acyl-lysoPE, 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. sPLA1-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 sPLA1-IB and the lysoPLD respectively. These results suggest that the sequential actions of PLA1 and lysoPLD may constitute another biosynthetic pathway for NAEs, including anandamide.

Key words: N-acyl-ethanolamine (NAE), N-acylphosphatidylethanolamine, anandamide, endocannabinoid, fatty acid, phospholipase D (PLD).
we focused on the related enzymes. The results suggest the involvement of several, but not all, PLA2 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]CPalmitic acid, 1-palmitoyl-2-[1-14C]linoleoyl-sn-glycero-3-phosphoethanolamine (2-[14C]linoleoyl-PE) and 1-palmitoyl-2-[1-14C]arachidonoyl-sn-glycero-3-phosphoethanolamine (2-[14C]arachidonoyl-PE) were purchased from PerkinElmer Life Science (Boston, MA, U.S.A.); [1-14C]arachidonic acid, Macroprep 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-lino- leoyl-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 F254 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-[1-13C]palmitoyl)ethanolamine (N-[13C]palmitoyl-PE) and 1,2-dioleoyl-sn-glycero-3-phospho(N-[1-14C]arachidonoyl)ethanolamine (N-[14C]arachidonoyl-PE) were prepared from 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine and either [1-14C]palmitic acid or [1,14C]-arachidonic acid respectively, according to the method of Schmid et al. [22]. 1-Palmitoyl-2-[1,13C]-linoleoyl-sn-glycero-3-phospho(N-palmitoyl)ethanolamine (N-palmitoyl-2-[13C]linoleoyl-PE) was also prepared by the same method using 2-[14C]linoleoyl-PE and palmitic acid. 1-Oleoyl-sn-glycero-3-phospho(N-[1-13C]palmitoyl)ethanolamine (N-[13C]palmitoyl-lysoPE) and 1-oleoyl-sn-glycero-3-phospho(N-[1-14C]arachidonoyl)ethanolamine (N-[14C]arachidonoyl-lysoPE) were enzymically prepared from N-[14C]palmitoyl-PE or N-[1-14C]arachidonoyl-PE respectively, using rat stomach sPLA2-IB (secretory PLA2-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 20mM 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 105000 g for 50 min, and the obtained pellets (particulate fractions) were suspended in 20mM 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 105000 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 PLA2**

Rat stomach was homogenized in 9 times the volume (v/w) of 20mM 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 20mM Tris/HCl (pH 7.4) was subjected to one cycle of freezing and thawing, solubilized...
by 1% (w/v) octyl glucoside, and centrifuged at 105 000g for 50 min. The resultant supernatant was heated at 70°C for 10 min, and the developed precipitate was removed by centrifugation at 105 000g 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 PLA2s**

HEK-293 cells were cultured in RPMI medium 1640 containing 10% fetal calf serum. As described previously, the cDNAs for rat sPLA2-IB [26] and human sPLA2-X [27] were subcloned into pCR3.1, the cDNAs for human sPLA2-IIA [28] and human sPLA2-V [26] into pRC/CMV, and mouse cPLA2α (cytosolic PLA2α) 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 PlusTM 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 sPLA2-IB and sPLA2-V were secreted outside the cells, the culture supernatants were used as the enzyme preparations. For cPLA2α, 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 sPLA2-IIA and sPLA2-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 sPLA2s to be recovered in the medium [28], which was used for the enzyme assay.

**Enzyme assay**

For the PLA2 assay with native rat enzymes, the enzymes were incubated with 100 µM or 200 µM 14C-labelled substrate (1000 cpm/nmol, dissolved in 5 µl of ethanol) in 100 µl of 50 mM Tris/acetate acid (pH 8.0) containing 10 mM CaCl2, 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 PLA2 assay with recombinant PLA2s expressed in HEK-293 cells, the enzymes prepared as described above were incubated at 37°C for 5 min (sPLA2-IB) or 30 min (other PLA2s) with 100 µM 14C-labelled substrate (1000 cpm/nmol, dissolved in 5 µl of ethanol) in 100 µl of the following buffers for each PLA2 isoenzyme: sPLA2-IB, 50 mM Tris/acetate acid (pH 8.0) containing 10 mM CaCl2; sPLA2-IIA, 50 mM Tris/HCl (pH 9.0) containing 10 mM CaCl2; sPLA2-V, 50 mM Tris/acetate acid (pH 6.0) containing 10 mM CaCl2; sPLA2-X, 50 mM Tris/HCl (pH 7.4) containing 10 mM CaCl2; and cPLA2α, 100 mM Tris/HCl (pH 9.0) containing 4 mM CaCl2 [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-[14C]palmitoyl-lysoPE and N-[14C]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 analyzer (Fujix, Tokyo, Japan).

For the lysoPLD assay, the enzyme was incubated with 25–200 µM N-[14C]palmitoyl-lysoPE or N-[14C]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 PLA2/PLA2 activity**

We first examined the N-acyl-PE-hydrolysing PLA2/PLA2 activity with the homogenate of rat brain. The homogenate was allowed to react with N-[14C]palmitoyl-PE in the presence of 10 mM CaCl2, and the products were separated from the remaining substrate by TLC. The results revealed that the brain had PLA2/PLA2 activity for converting N-[14C]palmitoyl-PE to N-[14C]palmitoyl-lysoPE, in addition to the PLD activity hydrolysing the same substrate to N-[14C]palmitoylthanolamine, 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 PLA2/PLA2 activity for N-[14C]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 PLA2/PLA2**

For the purpose of the identification of the stomach PLA2/PLA2, 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 PLA2 activity for N-[14C]palmitoyl-PE was increased 472-fold from 71 nmol/min per mg of protein.
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-[14C]palmitoyl-PE. The results are expressed as the means ± S.D. (n = 3).

**Table 1** Purification of the N-palmitoyl-PE-hydrolysing PLA1/PLA2 enzyme from rat stomach

The N-palmitoyl-PE-hydrolysing PLA1/PLA2 was purified from rat stomach as described in the Experimental section.

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

* 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-[14C]palmitoyl-PE in the presence of 10 mM CaCl2 at 37 °C for 5 min.

**Figure 1** Distribution of the N-palmitoyl-PE-hydrolysing PLA1/PLA2 activity in rat tissues

The N-terminal sequence of this 17-kDa protein preparation gave a single protein band around 17 kDa, as analysed by SDS/PAGE. The N-terminal sequence of this 17-kDa protein preparation was identified as Ala-Val-Trp-Gln-

**Figure 2** Reactivity of the purified stomach sPLA2-IB with N-acyl-PE

(A) Identification of the product from N-palmitoyl-PE by the purified sPLA2-IB. The purified sPLA2-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-[14C]palmitoyl-PE (lanes 1 and 2), N-palmitoyl-2-[14C]linoleoyl-PE (lanes 3 and 4), or the mixture of both substrates at 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, vol.). (B, C) The substrate specificity of the purified stomach sPLA2-IB. The purified stomach sPLA2-IB (6 ng of protein) was allowed to react with 100 µM (open columns) or 200 µM (closed columns) of 2-[14C]linoleoyl-PE (PE) (B), N-palmitoyl-2-[14C]linoleoyl-PE (N-Palmitoyl-PE) (B), N-[14C]palmitoyl-PE (N-Palmitoyl-PE) (C), or N-[14C]arachidonoyl-PE (N-Arachidonoyl-PE) (C). The activity with 100 µM 2-[14C]linoleoyl-PE (19 µmol/min per mg of protein) (B) or with 100 µM N-[14C]palmitoyl-PE (28 µmol/min per mg of protein) (C) was expressed as 100%. The results are expressed as the means ± S.D. (n = 3).

**Hydrolytic activity of various PLA2 isoenzymes with N-palmitoyl-PE**

We next examined whether PLA2 isoenzymes, other than sPLA2-IB, were also able to act as PLA2 toward N-acyl-PE. We expressed a series of representative PLA2 isoenzymes in HEK-293 cells, and measured their activities with N-acyl-PE. We used group IB, IIA,
genous PLA2 activity with PE about 15
per 10⁶ cells; sPLA2-IIA, 0.37 nmol/min per 10⁶ cells; sPLA2-V, 1.6 nmol/min per 10⁶ cells;
cPLA2
2-[14C]arachidonoyl-PE (a closed column for cPLA2
PE or 2-[14C]arachidonoyl-PE were expressed as 100 % as follows: sPLA2-IB, 5.5 nmol/min
sPLA2-X, 2.0 nmol/min per 10⁶ cells; cPLA2
of more than 50
sPLA2-IB, -IIA, and -V hydrolysed
transfected cells (results not shown). As shown in Figure 3,
conditions for each PLA2 isoenzyme. The activities of each PLA2 isoenzyme with 2-[14C]linoleoyl-
expressed as the means
± S.D. (n = 3).

Tissue distribution of N-acyl-lysoPE-hydrolysing lysoPLD activity
We found the lysoPLD activity releasing N-[14C]palmitoyl-ethanolamine from N-[14C]palmitoyl-lysoPE in the homogenate of rat brain. This reaction did not occur with the heat-treated homogenate. When the homogenates were subjected to ultracentrifugation, most of the lysoPLD activity was recovered in the particulate fraction, rather than cytosol. When the particulate fraction was allowed to react with N-[14C]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, 10 mM MgCl₂ did not stimulate the activity significantly, and 10 mM CaCl₂ 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-[14C]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-[14C]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-[14C]palmitoyl-lysoPE with an IC₅₀ value of about 0.2 μM (Figure 5A).

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-[14C]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 activity 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 [14C]anandamide from N-[14C]arachidonoyl-lysoPE increased almost linearly, showing a very high Kₘ value. The rate was comparable with that to release N-[14C]-palmitoylthanolamine from N-[14C]palmitoyl-lysoPE at all the substrate concentrations examined (Figure 5C).

Synthesis of N-palmitoylthanolamine by the sequential actions of sPLA₂-IB and lysoPLD
Finally, we assessed whether N-palmitoylthanolamine could actually be synthesized by the combination of PLA₂ and lysoPLD. When we mixed increasing amounts of the purified stomach sPLA₂-IB with a constant amount of the brain homogenate as a source of lysoPLD, the formation of N-[14C]palmitoylthanolamine from N-[14C]palmitoyl-PE increased with a concomitant accumulation of N-[14C]palmitoyl-lysoPE as an intermediate

Figure 3 Reactivity of recombinant PLA2s with N-[14C]palmitoyl-PE
The recombinant sPLA₂-IB, -IIA, -V, -X and cPLA₂α expressed in HEK-293 cells were prepared as described in the Experimental section, and their activities with 100 μM 2-[14C]linoleoyl-PE (open columns for sPLA₂s), 2-[14C]arachidonoyl-PE (an open column for cPLA₂α), N-palmitoyl-2-[14C]linoleoyl-PE (closed columns for sPLA₂s), or N-palmitoyl-2-[14C]arachidonoyl-PE (a closed column for cPLA₂α) were measured under appropriate conditions for each PLA2 isoenzyme. The activities of each PLA2 isoenzyme with 2-[14C]linoleoyl-PE or 2-[14C]arachidonoyl-PE were expressed as 100 % as follows: sPLA₂-IB, 5.5 nmol/min per 10⁶ cells; sPLA₂-IIA, 0.37 nmol/min per 10⁶ cells; sPLA₂-V, 1.6 nmol/min per 10⁶ cells; sPLA₂-X, 2.0 nmol/min per 10⁶ cells; cPLA₂α, 39 pmol/min per mg of protein. The results are expressed as the means ± S.D. (n = 3).

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-[14C]palmitoyl-lysoPE. The results are expressed as the means ± S.D. (n = 3).
lysoPE (\(\text{N}^-\text{acyl-lysoPE}\)) allowed to react with 100 \(\mu\text{M}\) of \(\text{N}\-[14\text{C}]\text{palmitoyl-PE}\) (\(\bullet\), PLD) or \(\text{N}\-[14\text{C}]\text{palmitoyl-lysoPE}\) (\(\circ\), lysoPLD) in the presence of the indicated concentrations of MAFP (dissolved in 5 \(\mu\text{l}\) 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 \(\mu\text{M}\) \(\text{N}\-[14\text{C}]\text{palmitoyl-PE}\) (for PLD assay) or \(\text{N}\-[14\text{C}]\text{palmitoyl-lysoPE}\) (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 \(\mu\text{g}\) of protein) was allowed to react with the indicated concentrations of \(\text{N}\-[14\text{C}]\text{palmitoyl-lysoPE}\) (\(\bullet\)) or \(\text{N}\-[14\text{C}]\text{arachidonoyl-lysoPE}\) (\(\circ\)). The results are expressed as the means ± S.D. (n = 3).

The mixtures of an increasing amount (0–25 ng of protein) of the purified stomach sPLA2-IB and a constant amount of the brain homogenate (150 \(\mu\text{g}\) of protein) were allowed to react with 100 \(\mu\text{M}\) \(\text{N}\-[14\text{C}]\text{palmitoyl-PE}\) (1000 cpm/nmol, dissolved in 5 \(\mu\text{l}\) of ethanol). A representative image of the TLC plate is shown in (A). The remaining \(\text{N}\-[14\text{C}]\text{palmitoyl-PE}\) (B) and the produced \(\text{N}\-[14\text{C}]\text{palmitoyl-lysoPE}\) (\(\bullet\)) and \(\text{N}\-[14\text{C}]\text{palmitoyl-lysoPE}\) (\(\circ\)) were quantified, and the results are expressed as the means ± S.D. (n = 3).

Figure 6 Formation of \(\text{N}\)-palmitoyl-ethanolamine by the sequential action of sPLA2-IB and lysoPLD

Dissection

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 \(\text{N}\)-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 \(\text{N}\)-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 \(\text{N}\)-acyl-PE to \(\text{N}\)-acyl-lysoPE. We clarified that several PLA2 isoenzymes can generate \(\text{N}\)-acyl-lysoPE from \(\text{N}\)-acyl-PE, and that lysoPLD releasing NAEs from \(\text{N}\)-acyl-lysoPE is catalytically distinct from the \(\text{N}\)-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, \(\text{N}\)-acyl-PE exists in a much higher amount than NAE in animal tissues [2]. Hence, upon the activation of sPLA2, NAEs including anandamide could be generated by this pathway. We showed that NAE was actually formed from \(\text{N}\)-acyl-PE by the combination of sPLA2-IB and lysoPLD (Figure 6). However, further investigation will be required to clarify how much this pathway contributes to the \textit{in vivo} formation of NAEs.

Since the activity of \(\text{N}\)-palmitoyl-PE-hydrolysing PLA2/PLA2 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 sPLA2-IB, that is the pancreatic-type PLA2. The identification was further confirmed by the finding that recombinant sPLA2-IB was also active with \(\text{N}\)-palmitoyl-PE. The high content of sPLA2-IB in rat stomach is in good agreement with the previous observation that sPLA2-IB is abundantly expressed in rat gastric mucosa [37]. Therefore, it seemed that the extremely high \(\text{N}\)-palmitoyl-PE-hydrolysing activity in stomach is mostly derived from sPLA2-IB. It is likely that \(\text{N}\)-acyl-PE in the diet is

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first digested mainly by sPLA₂-IB in the gastrointestinal tract. The enzyme activity of stomach sPLA₂-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 sPLA₂-IB cleaved N-palmitoyl-PE exclusively at sn-2 position to release a fatty acid. Accordingly, the N-palmitoyl-lysoPE produced by sPLA₂-IB retains the acyl group at sn-1 position. However, we cannot rule out the possibility that PL₁, and other phospholipases hydrolyse N-acyl-PE to generate N-acyl-2-acyl-lysoPE retaining the acyl group at sn-2 position. Furthermore, intramolecular transacylation 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 PLA₂ family comprises at least 19 isoenzymes, which are categorized into four families; sPLA₂S, cPLA₂S, Ca²⁺-independent PLA₂S and platelet-activating factor acetylhydrolases [38–40]. We examined the contribution of PLA₂ isoenzymes other than sPLA₂-IB to the hydrolysis of N-acyl-PE with recombinant enzymes of several representative PLA₂S. The results showed that not only sPLA₂-IB, but also sPLA₂-IIA and sPLA₂-V could hydrolyse N-palmitoyl-PE at comparable rates to the hydrolysis of PE, one of the most common substrates of PLA₂. Thus PLA₂/PLₐ₂ activity in different organs (Figure 1) may be attributed to different PLA₂ isoenzymes. Since sPLA₂S, especially sPLA₂-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, sPLA₂-X and cPLA₂-α hardly hydrolysed N-palmitoyl-PE, implying that PLA₂ 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 (lyosphosphatidic acid) from lyosphosphatidylcholine was purified from plasma and identified to be a soluble form of autotaxin, a member of the ectonucleotide 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 sPLA₂-IB and lysoPLD respectively, but their reaction rates were similar to those for N-palmitoyl-PE and N-palmitoyl-lysoPE respectively. Since both N-palmitoylthanolamine 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 PLA₂ and lysoPLD enzymes responsible for the possible biosynthetic route of NAES via N-acyl-lysoPE. Not all, but several sPLA₂ 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.

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