Molecular Characterization of a Phospholipase D Generating Anandamide and Its Congeners*

Anandamide (N-arachidonoylthanolamine) is known to be an endogenous ligand of cannabinoid and vanilloid receptors. Its congeners (collectively referred to as N-acylethanolamines) also show a variety of biological activities. These compounds are principally formed from their corresponding N-acyl-phosphatidylethanolamines by a phosphodiesterase of the phospholipase D-type in animal tissues. We purified the enzyme from rat heart, and by the use of the sequences of its internal peptides cloned its complementary DNAs from mouse, rat, and human. The deduced amino acid sequences were composed of 393–396 residues, and showed that the enzyme has no homology with the known phospholipase D enzymes but is classified as a member of the zinc metallo-hydrolase family of the β-lactamase fold. As was overexpressed in COS-7 cells, the recombinant enzyme generated anandamide and other N-acylethanolamines from their corresponding N-acyl-phosphatidylethanolamines at comparable rates. In contrast, the enzyme was inactive with phosphatidylcholine and phosphatidylethanolamines at comparable rates. In addition, it was found that the enzyme is strongly suggesting the physiological importance of lipid molecules of this class.

N-Acylethanolamines are ethanolamides of long chain fatty acids that exist in almost all the animal tissues (1–4). Their biological activities have been extensively studied. For example, anandamide (N-arachidonoylthanolamine) acts as a ligand of cannabinoid receptors (5, 6) and vanilloid receptor (7). N-Palmitoyl ethanolamine is known as anti-inflammatory substance (8, 9) and -Palmitoylethanolamine is known as anti-inflammatory substance (8, 9) and

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1 The abbreviations used are: PE, phosphatidylethanolamine; PC, phosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; G1PDH, glyceraldehyde-3-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; N-acetyl-phosphatidylethanolamine; PC, phosphatidylcholine; PLD, phospholipase D; RT-PCR, reverse transcriptase-PCR.

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank/EMBL Data Bank with accession number(s) AB112350, AB112351, and AB112352.

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from their corresponding 1-14C-labeled fatty acids and 1,2-diacyl-PE, and N-palmitoyl-1-palmitoyl-2-[14C]linoleoyl-PE from N-palmitoyl-1-palmitoyl-2-[14C]linoleoyl-PE and palmitic acid according to the method of Schmid et al. (16). N-14C-Acyl-lyso-PEs were prepared from their corresponding N-[14C]Acyl-PEs using pancreatic phospholipase A2, and N-palmitoyl-2-[14C]linoleoyl-phosphatic acid was from N-palmitoyl-2-[14C]linoleoyl-PE using Streptomyces sp. PLD. The products were purified by TLC with a mixture of chloroform, methanol, 28% ammonium hydroxide (90:20:2, v/v).

Enzyme Purification—One hundred and fifty adult Wistar ST rats (SLC, Japan) were anesthetized with diethyl ether and sacrificed by cervical dislocation. Hearts were removed, cut into small pieces, and then homogenized in 5 times the volume (v/v) of 20 mM Tris-HCl (pH 7.4) containing 0.32 M sucrose by a Polytron homogenizer. The homogenates were centrifuged at 800 × g for 5 min, and the resultant pellet was suspended in phosphate-buffered saline (pH 7.4). After freezing and thawing, the sample was centrifuged at 105,000 × g for 55 min. The supernatant was further centrifuged at 105,000 × g for 55 min, and the resultant pellet was suspended in phosphate-buffered saline (pH 7.4). After freezing and thawing, the sample was centrifuged at 105,000 × g for 55 min. The resultant clear supernatant was frozen at −80 °C. After thawing slowly at 4 °C, the sample (50 mg of protein) was loaded on a HitTrap SP HP cation-exchange column (bed volume, 5 ml) pre-equilibrated with 20 mM Tris-HCl (pH 7.4) containing 1% (w/v) CHAPS (buffer A). After washing the column with 20 ml of buffer A, the enzyme was eluted with 28 ml of buffer A containing 50 mM NaCl. Active fractions (14 ml) were diluted 3-fold (v/v) with buffer A and then loaded on a HiTrap Q anion-exchange column (1 ml). After washing the column with 5 ml of 50 mM NaCl and 10 ml of 200 mM NaCl, the enzyme was eluted with 4 ml of 300 mM NaCl. Active fractions were diluted 3-fold with buffer A and loaded on a HiTrap Blue affinity column (1 ml). After washing the column with 5 ml of buffer A and 3 ml of buffer A containing 50 mM potassium phosphate, the enzyme was eluted with 6 ml of 100 mM potassium phosphate. Protein concentration was determined by the method of Bradford (20) with bovine serum albumin as standard. Rat aortic homogenates were centrifuged at −80 °C for 30 min to remove particulate fractions of rat heart that were stored at −80 °C for 1 month. After thawing for 1 week, 4 °C for 24 h, or 25 °C for 3 h, the enzyme activity was lost by 8, 86, 45, or 25%, respectively. The same treatment, the enzyme activity of the octyl glucoside-solubilized proteins was lost by 97, 78, 50, or 28%, respectively.

Microsequencing and Bioinformatics—To isolate internal peptides from the purified rat heart NAPE-PLD, active fractions from the hydroxyapatite column were subjected to SDS-PAGE on a 10% gel. Band A stained with Coomassie (Fig. 1) was excised from the gel and subjected to in-gel digestion using trypsin. The digest mixture was separated by reverse-phase high performance liquid chromatography using a TSKgel ODS-80Ts column (20 × 0.46 cm height) and developed in chloroform–methanol–28% ammonium hydroxide (80:20:2, v/v) at 4 °C for 55 min. The 200-ml fraction was spotted on a silica gel thin layer plate (10 cm height) and developed in chloroform, methanol, 28% ammonium hydroxide (80:20:2, v/v) at 4 °C for 20 min. Distribution of radioactivity on the plate was quantified by a BAS1500 imaging analyzer (Fuji, Tokyo, Japan).

Preparation of Anti-NAPE-PLD Antibodies—Rabbit antisera was raised against a hexahistidine-tagged mouse NAPE-PLD protein. A mouse NAPE-PLD cDNA lacking stop codon was generated by PCR using a forward primer 5′-GGATCCGATGGATGAGTATGAGGACC-3′ and a reverse primer 5′-CTCAGAGGGTATCTCAAAAGCTATT-3′. This cDNA was ligated into a prokaryotic expression vector pTrcHis2B to generate a C-terminally hexahistidine-tagged NAPE-PLD protein. The construct was confirmed by sequencing in both directions and was introduced chemically into competent E. coli TOP10 F’ cells as host. Cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside at an A600 of 0.6–0.7, allowed to grow at 37 °C for 5 h, and pelleted at 8000 × g for 30 min. After freezing and thawing, the cells were resuspended in 50 ml Tris-HCl (pH 7.4) containing 100 mM NaCl (buffer B) and were sonicated on ice 10 times each for 20 s at a high intensity with a 1-min cooling period between each burst. The cell homogenates were then treated with 1% Triton X-100 on ice for 30 min and centrifuged at 10,000 × g for 30 min. The pellet was suspended in buffer B containing 0.5% SDS and centrifuged again at 105,000 × g for 30 min. The resultant clear supernatant was diluted 3-fold with buffer B and was applied onto an nickel-nitrotriacetic acid agarose column (2 ml of bed volume). After washing the column with 20 ml of buffer B containing 10 mM imidazole and 0.05% Triton X-100, the enzyme was eluted with 9 ml of buffer B containing 100 mM imidazole. The enzyme fractions were dialyzed against 50 mM Tris-HCl (pH 7.5) to 30% confluency in a 100-mm dish containing Dulbecco’s modified Eagle’s medium with 10% fetal calf serum in a humidified 5% CO2, 95% air incubator. The cells were then treated with 8 μg of NAPE-PLD-pcDNA3.1(+) and Lipofectamine and cultured at 37 °C for 48 h, with one change of medium at 12 h. The harvested cells were sonicated 3 times each for 3 in 20 mM Tris-HCl (pH 7.4) and used as homogenates. Control COS-7 cells were prepared in the same way, except that the insert-free pcDNA3.1(−) vector was used for transfection. The homogenates were centrifuged at 105,000 × g for 15 min, and the obtained pellet (membrane fraction) was resuspended in 20 mM Tris-HCl (pH 7.4) containing 1% (w/v) octyl glucoside, followed by further centrifugation at 105,000 × g for 30 min. The obtained clear supernatant was referred to as the octyl glucoside-solubilized proteins.

Enzyme Assay—NAPE-PLD was incubated with 100 μM N-[14C]Acyl-PE or N-[14C]Acyl-lyso-PE (10,000 cpm in 5 μl of ethanol) in 100 μl of 50 mM Tris-HCl (pH 7.5) at 37 °C for 10 min. An activator (0.1% Triton X-100, 10 mM MgCl2, or 10 mM CaCl2) was also contained in the reaction mixture. A mixture of chloroform/methanol (2:1, v/v, 0.3 ml) was added to the reaction mixture to terminate the reaction. After centrifugation, 100 μl of the lower layer was spotted on a silica gel thin layer plate (10 cm height) and developed in chloroform, methanol, 28% ammonium hydroxide (80:20:2, v/v) at 4 °C for 20 min. Distribution of radioactivity on the plate was quantified by a BAS1500 imaging analyzer (Fuji, Tokyo, Japan).

Expression of NAPE-PLD in COS-7 Cells—The 1.2-kb NAPE-PLD cDNAs were ligated into the eukaryotic expression vector pcDNA3.1(+). COS-7 cells were grown at 37 °C and 5% CO2 using a 100-mm dish containing Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. The cells were then transfected with 8 μg of NAPE-PLD-pcDNA3.1(+) and Lipofectamine. The cells were allowed to grow at 37 °C for 55 min. The obtained supernatant was further centrifuged at 105,000 × g for 30 min, and the obtained pellet (membrane fraction) was resuspended in 20 mM Tris-HCl (pH 7.4) containing 1% (w/v) octyl glucoside, followed by further centrifugation at 105,000 × g for 30 min. The obtained clear supernatant was diluted 3-fold with buffer B and was applied onto an nickel-nitrotriacetic acid agarose column (2 ml of bed volume). The eluted fractions were dialyzed against 50 mM Tris-HCl (pH 7.5) to 30% confluency in a 100-mm dish containing Dulbecco’s modified Eagle’s medium with 10% fetal calf serum in a humidified 3% CO2, 95% air incubator. The cells were then treated with 8 μg of NAPE-PLD-pcDNA3.1(+) and Lipofectamine and cultured at 37 °C for 48 h, with one change of medium at 12 h. The harvested cells were sonicated 3 times each for 3 in 20 mM Tris-HCl (pH 7.4) and used as homogenates. Control COS-7 cells were prepared in the same way, except that the insert-free pcDNA3.1(−) vector was used for transfection. The homogenates were centrifuged at 105,000 × g for 15 min, and the obtained pellet (membrane fraction) was resuspended in 20 mM Tris-HCl (pH 7.4) containing 1% (w/v) octyl glucoside, followed by further centrifugation at 105,000 × g for 30 min. The obtained clear supernatant was referred to as the octyl glucoside-solubilized proteins.
buffer C at room temperature for 1 h. Finally, NAPE-PLD was visualized using enhanced chemiluminescence and analyzed by a LAS1000plus lumino-imaging analyzer (Fujix, Tokyo, Japan).

Reverse Transcriptase-PCR—Total RNA was isolated from various organs of mice (C57/B16 Cr, Charles River, Japan) with Trizol. Primers used are as follows: for NAPE-PLD, denaturation at 94 °C for 30 s, annealing at 58 °C for 2 min (24, 26, or 28 cycles); for GAPDH, denaturation at 94 °C for 48 s, annealing at 58 °C for 2 min, and extension at 72 °C for 2 min (25 cycles). Active fractions from HiTrap Q, For NAPE-PLD, denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 2 min (24, 26, or 28 cycles); for GAPDH, denaturation at 94 °C for 48 s, annealing at 70 °C for 48 s, and extension at 72 °C for 1 min (25 cycles).

### RESULTS

**Identification and Cloning of NAPE-PLD cDNA**—In earlier studies we solubilized the NAPE-PLD from the 105,000 × g pellet of rat heart homogenates, and partially purified it by ion-exchange column chromatographies (13, 14). We further purified the rat heart enzyme by the use of a combination of four column chromatographies to a specific activity of 406 nmol/min/mg protein at 37 °C (Table I). As analyzed by SDS-PAGE, the final preparation was not completely pure, and several major protein bands were still detected (Fig. 1). However, since we noted that the intensity of Band A with a molecular mass of about 46 kDa changed in agreement with the NAPE-hydrolyzing activity through the hydroxyapatite chromatography, we presumed that Band A was the enzyme protein.

The protein of Band A was digested with trypsin. The resulting peptides were separated by reverse-phase high performance liquid chromatography and were microsequenced. By this procedure, we could determine five peptide sequences. By the use of data base, the three peptide sequences (KLHDEEEIQELQA, LHDEEIQELQAQ, and LLAELEQLK) were found to be highly homologous (AI857635, AL564333, AW293283, BG977438, BQ224588, BU507936, and BX411424) were found to be highly homologous to the mouse cDNA at the protein level. We prepared PCR primers based on these nucleotide sequences, and we cloned rat and human homologues from rat brain and human megakaryoblastic leukemia cells (CMK cells) by RT-PCR (Fig. 2). The amino acid sequences deduced from the cDNAs of three animal species were composed of 396 (mouse and rat) and 393 (human) residues, and their molecular weights were calculated to be 45,816 (mouse), 45,737 (rat) and 45,596 (human). Their amino acid identity was 95.5% (between mouse and rat), 89.1% (between mouse and human), and 90.4% (between rat and human). The sequences showed no homology with those of the reported PLD such as mammalian PLD1 (24) and PLD2 (25), Saccharomyces cerevisiae SPO14PLD1 (26), Strepzymeces antibioticus PLD (27), and glycosylphosphatidylinositol-specific PLD (28).

The zinc metallohydrolase family of the β-lactamase fold is a large superfamily of proteins including a wide variety of hydrolases such as class B β-lactamase, glyoxalase II, arylsulfatase, cytidine monophosphate-N-acetylneuraminic acid hydroxylase, and cAMP phosphodiesterase (22, 23). Proteins belonging to this superfamily are characterized by an HX(E/H)XD(C/R/S)−HX50−70HX15−40/CSDX0−70H motif that is highly conserved among members of this family. This motif is presumed to participate in zinc coordination and hydrolysis reaction (22, 23). As shown in Fig. 3, this motif was completely conserved in the putative NAPE-PLD of the three animal species, suggesting that the catalytic activity of NAPE-PLD is correlated with the zinc content.

**Overexpression and Characterization of NAPE-PLD**—We overexpressed the mouse cDNA in COS-7 cells by the lipofection method. The homogenates of the transfected cells were

### Table I

Purification of NAPE-PLD from rat heart

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Activitya</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>105,000 × g pellet from homogenates</td>
<td>1861</td>
<td>118</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteins solubilized with octyl glucoside</td>
<td>497</td>
<td>397b</td>
<td>0.80c</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Active fractions from HiTrap HP</td>
<td>55</td>
<td>209</td>
<td>3.8</td>
<td>53</td>
<td>4.8</td>
</tr>
<tr>
<td>Active fractions from HiTrap Q</td>
<td>0.73</td>
<td>73</td>
<td>20</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>Active fractions from HiTrap Blue</td>
<td>0.18</td>
<td>48</td>
<td>53</td>
<td>12</td>
<td>67</td>
</tr>
<tr>
<td>Active fractions from hydroxyapatite</td>
<td>0.033</td>
<td>13</td>
<td>406</td>
<td>3.4</td>
<td>508</td>
</tr>
</tbody>
</table>

* Only one unit is defined as the amount of enzyme required to hydrolyze 1 nmol of N-palmitoyl-PE per min at 37 °C.

**Fig. 1.** Purification of rat heart NAPE-PLD as shown by SDS-PAGE. A, the active fractions at each step (0.1–10 μg of protein) were subjected to 10% PAGE in the presence of 0.1% SDS, followed by staining with silver nitrate. Lane 1, the 105,000 × g pellet of the rat heart homogenates; lane 2, octyl glucoside-solubilized proteins; lane 3, HiTrap SP HP; lane 4, HiTrap Q; lane 5, HiTrap Blue; lane 6, Bio-Gel HTP (hydroxyapatite). B, the active fraction from the hydroxyapatite column was 50-fold concentrated and was subjected to SDS-PAGE followed by staining with Coomassie.
allowed to react with N-[^14]C]palmitoyl-PE, and the product was separated by TLC. The result showed the generation of N-[^14]C]palmitoylethanolamine with a specific activity of 19 H11006 1 nmol/min/mg protein at 37°C (Fig. 4, lane 3).

In contrast, the enzyme activity in the homogenates of COS-7 cells transfected with the insert-free vector was very low (0.02 H11006 0.004 nmol/FIG. 2. Deduced amino acid sequences from mouse, rat, and human NAPE-PLD CDNAs. Sequence identity shared by three NAPE-PLD proteins is shown by asterisks. Underlines denote sequences corresponding with the peptide fragments obtained by tryptic digestion of the rat heart enzyme.

Fig. 3. Comparison of NAPE-PLD to representative members of the zinc metallohydrolase family of the β-lactamase fold. Alignment over the highly conserved segments among representative members of the zinc metallohydrolase family of the β-lactamase fold is shown. Highly conserved residues are shown in boldface type and are boxed. GenBankTM accession numbers of Bacillus cereus β-lactamase, human glyoxalase II, Desulfovibrio gigas ROO (rubredoxin oxygen:oxidoreductase), human ENAC1 (aryl sulfatase), E. coli PhnP (phosphonate uptake and biodegradation), mouse CMP-NeuAc hydroxylase (cytidine monophosphate-N-acetylneuraminic acid hydroxylase), and S. cerevisiae PDE1 (cAMP phosphodiesterase) are M19530, NM005326, AF218053, AF308695, AE000482, NM007717, and P22434, respectively.

To test the ability of NAPE-PLD to react with N-[^14]C]palmitoyl-PE, and the product was separated by TLC. The result showed the generation of N-[^14]C]palmitoylethanolamine with a specific activity of 19 ± 1 mmol/min/mg protein at 37°C (Fig. 4, lane 3). In contrast, the enzyme activity in the homogenates of COS-7 cells transfected with the insert-free vector was very low (0.02 ± 0.004 mmol/
min/mg protein), and an enormous amount of the homogenates gave a faint band of the product (Fig. 4, lane 2). The rat and human homologues were also expressed in COS-7 cells by the same method, and the homogenates of the transfected cells exhibited the NAPE-PLD activities.

As analyzed by Western blotting with polyclonal antibody raised against a mouse recombinant NAPE-PLD, the homogenates of COS-7 cells transfected with mouse or rat cDNA revealed an immunoreactive band around 46 kDa (Fig. 5, lanes 3 and 4). In contrast, the homogenates of COS-7 cells transfected with the insert-free vector did not show the immunoreactive protein band (lane 2). The antibody also stained a 46-kDa band with the insert-free vector did not show the immunoreactive band around 46 kDa (Fig. 5, lane 1), authentic N-[14C]palmitoylthanolamine (NPE) was spotted. Positions of authentic compounds are indicated by arrows.

When the cell homogenates were subjected to ultracentrifugation, the enzyme activity was mostly recovered in the membrane fraction (31 ± 1 nmol/min/mg protein) with a much lower activity in the cytosol (4 ± 1 nmol/min/mg protein). Again, the membrane fraction of COS-7 cells transfected with the insert-free vector showed a very low activity (0.05 ± 0.005 nmol/min/mg protein). Its localization in the membrane fraction was in agreement with that of the native enzyme (15). The recombinant mouse enzyme was stimulated 1.7-fold by 0.1% Triton X-100. The enzyme could be solubilized from the membrane with 1% octyl glucoside, and the soluble enzyme was activated 1.9- and 1.8-fold by 10 mM CaCl2 and MgCl2, respectively. NAPE-PLD also hydrolyzed N-stearoyl-PE, and [14C]acyl-PE as substrates (Table II).

**TABLE II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km</th>
<th>Vmax</th>
<th>Vmax/Km</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>μmol/min</td>
<td>nmol/min/mg protein</td>
<td>mg protein</td>
</tr>
<tr>
<td>NPE</td>
<td>3.3 ± 1.7</td>
<td>97.9 ± 11.8</td>
<td>30.9 ± 8.6</td>
</tr>
<tr>
<td>NArPE</td>
<td>2.8 ± 0.5</td>
<td>73.2 ± 3.1</td>
<td>26.1</td>
</tr>
<tr>
<td>NOPE</td>
<td>2.9 ± 0.8</td>
<td>83.4 ± 3.9</td>
<td>28.8</td>
</tr>
<tr>
<td>NSPE</td>
<td>3.4 ± 0.8</td>
<td>101.4 ± 9.0</td>
<td>29.8</td>
</tr>
<tr>
<td>Npalmitoyl-lyso-PE</td>
<td>4.0 ± 1.6</td>
<td>26.7 ± 1.6</td>
<td>6.7</td>
</tr>
<tr>
<td>Lyso-NArPE</td>
<td>4.0 ± 1.6</td>
<td>26.7 ± 1.6</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Moreover, we investigated whether or not PC and PE were also substrates for NAPE-PLD. However, although *Streptomyces* sp. PLD generated phosphatidic acid from PC and PE, even a large amount of the recombinant enzyme could not hydrolyze PC or PE (Fig. 6).

Transphosphatidylation is a unique catalytic property of PLD, which generates phosphatidyl alcohol rather than phosphatidic acid in the presence of primary alcohols such as ethanol and butanol (29). When *Streptomyces* sp. PLD as a positive control was incubated with N-palmitoyl-1-palmitoyl-2-[14C]linooley-PE in the presence of ethanol, [14C]phosphatidylethanol was produced (Fig. 7, lane 5). In contrast, recombinant NAPE-PLD produced [14C]phosphatidic acid exclusively. [14C]Phosphatidylethanol was not detected (Fig. 7, lane 5).

**Organ Distribution of NAPE-PLD**—The organ distribution of the NAPE-PLD activity was examined using microsomes from various murine organs (Fig. 8A). The N-palmitoyl-PE hydrolyzing activity was widely distributed with higher specific activities in brain, kidney, and testis. As shown in Fig. 8, B and C, the distribution of murine NAPE-PLD at mRNA and protein levels was not completely identical but similar to that of the...
enzyme activity. These results suggest that the enzyme identified by us is principally responsible for the NAPE-PLD activity in various organs.

**DISCUSSION**

The major biosynthetic pathway of anandamide and other bioactive N-acylethanolamines is composed of two enzyme reactions: 1) transfer of an acyl group from the sn-1 position of glycerophospholipid to the amino group of PE resulting in the formation of NAPE, and 2) subsequent hydrolysis of NAPE to N-acylethanolamine and phosphatidic acid (1–4). The former reaction is catalyzed by a Ca$^{2+}$-dependent N-acyltransferase and the latter reaction by a phosphodiesterase of the PLD type (NAPE-PLD). However, molecular characterization of enzymes involved in this pathway has not been performed. Here for the first time we report the molecular cloning and expression of NAPE-PLD that specifically generates N-acylethanolamines including anandamide from their corresponding NAPEs. The cDNA cloning revealed that the enzyme belongs to the zinc metallohydrolase family of the **

**Fig. 6. Reactivity of recombinant NAPE-PLD with PC and PE.** The 105,000 × g pellet from the homogenates of COS-7 cells overexpressing mouse NAPE-PLD (25 μg of protein) (lanes 2 and 5), Streptomyces sp. PLD (2 units) (lanes 3 and 6), and buffer alone (lanes 1 and 4) were allowed to react with 100 μM 1-palmitoyl-2-[14C]linoleoyl-PE (2,000 cpm) (lanes 1–3) or 100 μM 1-palmitoyl-2-[14C]linoleoyl-PE (2,000 cpm) (lanes 4–6) in the presence of 0.1% Triton X-100 at 37 °C for 15 min. Positions of authentic compounds are indicated by arrows. PA, phosphatidic acid.

**Fig. 7. Transphosphatidylation activity of recombinant NAPE-PLD.** The 105,000 × g pellet from mouse NAPE-PLD-expressing COS-7 cell homogenates (7 μg of protein) (lanes 2 and 3), Streptomyces spp. PLD (5 units) (lanes 4 and 5), and buffer alone (lane 1) were allowed to react with 100 μM N-palmitoyl-1-palmitoyl-2-[14C]linoleoyl-PE (NPPE) (2,000 cpm) in the presence (lanes 1, 3, and 5) or absence (lanes 2 and 4) of 3% ethanol at 37 °C for 1 h. Triton X-100 (0.1%) was also included. Positions of authentic compounds are indicated by arrows. PA, phosphatidic acid; PET, phosphatidylethanol.

**Fig. 8. Distribution of the NAPE-PLD activity, mRNA, and protein level in mouse organs.** A, the 105,000 × g pellet (30–235 μg of protein) from the homogenates of each mouse organ was allowed to react with 100 μM N-[14C]palmitoyl-PE in the presence of 0.1% Triton X-100 and 1 μM methylarachidonilfluorophosphonate (14) (added to inhibit hydrolysis of the produced N-palmitoylthanolamine by fatty acid amide hydrolase). Mean values ± S.D. are shown (n = 3). B, distribution of the NAPE-PLD and GAPDH mRNA was analyzed by RT-PCR as described under “Experimental Procedures.” C, distribution of the NAPE-PLD protein was analyzed by Western blotting as described under “Experimental Procedures.” The 105,000 × g pellet (50 μg of protein) from the homogenates of each mouse organ were used. Lane R, recombinant mouse PLD used as a positive control.
Mutational studies using the recombinant enzyme will be required to elucidate catalytic mechanisms of NAPE-PLD. Because NAPE-PLD is classified as a phosphodiesterase based on the type of reaction, we should note that Vibrio fischeri and S. cerevisiae cAMP phosphodiesterases, and E. coli phosphodiesterase (Elac) are included in this family (22, 23, 34, 35).

Second, NAPE-PLD was catalytically distinguishable from the known PLD. Previous reports showed that microsomes of dog brain and rat heart had a low PLD activity toward PC and PE in addition to the NAPE-PLD activity (15, 16). However, our present studies revealed that the recombinant NAPE-PLD had no activity with PC or PE (Fig. 6). It should be noted that PC is a common substrate of mammalian PLD1 and PLD2 (29).

We also confirmed that the enzyme does not catalyze transphosphatidylation to generate phosphatidyl alcohol in the presence of primary alcohols (Fig. 7). The lack of transphosphatidylation was in agreement with the previous results (18) with microsomes of rat brain and heart having the NAPE-PLD activity. These catalytic properties of NAPE-PLD indicate that the enzyme is not only structurally but also functionally distinct from PLD members of the HKD/phosphatidyltransferase gene family (29).

Third, our results demonstrated that not only N-arachidonoyl-PE (a precursor of anandamide) but also other NAPEs with different N-acyl groups are good substrates of the recombinant NAPE-PLD (Table II). It has been a matter of debate whether or not an anandamide-specific biosynthetic pathway exists in animal tissues because most of endogenous N-acyl ethanolamines except anandamide do not function as cannabinoid receptor ligands (4). Sugiuira et al. (17) reported previously that rat brain microsomes showed the PLD activity for all of NAPEs with different N-acyl groups (16:0, 18:0, 18:1, 18:2, and 20:4) and that N-arachidonoyl-PE was less active than other NAPEs. Our results with the recombinant enzyme confirm this earlier finding and explain why the composition of naturally occurring N-acyl ethanolamines tends to resemble the N-acyl composition of the precursor NAPE (4).

Some exceptional tissues such as uterus (36) and some tumor tissues (37) in which anandamide is relatively abundant may possess other enzymes or pathways to contribute to the selective generation of anandamide.

Fourth, we detected the N-palmitoyl-PE hydrolyzing activity in almost all the mouse organs with higher specific activities in brain, kidney, and testis (Fig. 8A). Earlier, Schmid et al. (16) examined the organ distribution of NAPE-PLD with rat organ homogenates and reported that the heart exhibited the highest activity, followed by brain, testis, kidney, spleen, liver, and lung. We also showed a similar distribution of the enzyme in rat with the proteins solubilized from rat organ microsomes (14). Petersen et al. (38) reported that among bovine organs the brain exhibited the highest specific activity, followed by kidney, spleen, lung, heart, and liver. From these results, the brain consistently displays a high NAPE-PLD activity over animal species. In contrast, remarkable species difference of the NAPE-PLD activity has been found with the heart (39). Furthermore, we revealed that relative amounts of the mRNA and protein of NAPE-PLD in different organs showed similar patterns to potency of the NAPE-PLD activity (Fig. 8, B and C). The results suggest that the enzyme identified by us is principally responsible for the NAPE-PLD activity in various organs, although we cannot rule out the possibility that isozymes or different biosynthetic pathways also participate in the generation of N-acyl ethanolamines. Future studies using specific inhibitors and gene-disrupted animals of NAPE-PLD will elucidate this question. Age-dependent change of the NAPE hydrolyzing activity was also reported with rat brain (40). Thus, it will be of interest to investigate the regulatory mechanism for the expression levels of mRNA and protein of NAPE-PLD.

Previously, we showed that NAPE-PLD partially purified from rat heart was markedly stimulated with Triton X-100 and divalent cations including Ca2+ and Mg2+ (13). Marked stimulatory effects of Triton X-100, Ca2+, and Mg2+ were also observed with the native enzyme of mouse brain (data not shown). However, the recombinant mouse enzyme was stimulated only 2-fold by these activators. The reason for this difference remains unclarified, and we cannot rule out the possibility that there is a subtle structural difference between the native enzyme and recombinant enzyme. Further examination on the cofactor requirement will be necessary with the purified recombinant enzyme.

Recent studies with gene-disrupted mice of the anandamide-degrading enzyme (fatty acid amide hydrolase (41)) emphasize physiological importance of anandamide as a neuromodulator (42–44). Not only anandamide but also other N-acyl ethanolamines were dramatically accumulated in the gene-disrupted mice (44). However, physiological significance of cannabinoid receptor-inactive N-acyl ethanolamines remains unclarified. Identification of the gene of NAPE-PLD enables us to generate model animals in which NAPE-PLD is knocked out or overexpressed. In addition, the structural analysis of the recombinant NAPE-PLD protein should help us to elucidate the catalytic and regulatory mechanisms of NAPE-PLD in biologic responses and to develop its selective inhibitors. These future studies will contribute to better understanding of the physiological and pathophysiological significance of anandamide and other bioactive N-acyl ethanolamines in mammals.

REFERENCES

A Phospholipase D Generating Anandamide and Its Congeners

Molecular Characterization of a Phospholipase D Generating Anandamide and Its Congeners

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