Mammalian cells stably overexpressing *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D exhibit significantly decreased levels of *N*-acylphosphatidylethanolamines

Yasuo OKAMOTO*, Jun MORISHITA*, Jun WANG*, Patricia C. SCHMID†, Randy J. KREBSBACH†, Harald H. O. SCHMID† and Natsuo UEDA*[1]

*Department of Biochemistry, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793, Japan, and †The Hormel Institute, University of Minnesota, Austin, MN 55912, U.S.A.

In animal tissues, NAEs (*N*-acylethanolamines), including *N*-arachidonoylethanolamine (anandamide), are primarily formed from their corresponding NAPEs (*N*-acylphosphatidylethanolamines) by a phosphodiesterase of the PLD (phospholipase D) type (NAPE-PLD). Recently, we cloned cDNAs of NAPE-PLD from mouse, rat and human [Okamoto, Morishita, Tsuboi, Tonai and Ueda (2004) J. Biol. Chem. **279**, 5298–5305]. However, it remained unclear whether NAPE-PLD acts on endogenous NAPEs contained in the membrane of living cells. To address this question, we stably transfected two mammalian cell lines (HEK-293 and CHO-K1) with mouse NAPE-PLD cDNA, and investigated the endogenous levels and compositions of NAPEs and NAEs in these cells, compared with mock-transfected cells, with the aid of GC-MS. The overexpression of NAPE-PLD caused a decrease in the total amount of NAPEs by 50–90 % with a 1.5-fold increase in the total amount of NAEs, suggesting that the recombinant NAPE-PLD utilizes endogenous NAPE as a substrate in the cell. Since the compositions of NAEs and NAPEs of NAPE-PLD-overexpressing cells and mock-transfected cells were very similar, the enzyme did not appear to discriminate among the N-acyl groups of endogenous NAPEs. These results confirm that overexpressed NAPE-PLD is capable of forming NAEs, including anandamide, in living cells.

Key words: anandamide, fatty acid amide hydrolase, *N*-acylethanolamine, *N*-acylphosphatidylethanolamine, mammalian cell, phospholipase D.

INTRODUCTION

Ethanolamides of long-chain fatty acids, collectively referred to as NAEs (*N*-acylethanolamines), are ubiquitous trace constituents of mammalian cells and tissues (reviewed in [1,2]). In most animal tissues, major NAEs are saturated and mono-unsaturated, whereas polyunsaturated NAEs, including *N*-arachidonoylethanolamine (anandamide), are minor components [3–9]. NAEs often accumulate in large amounts under pathological conditions, such as irreversible myocardial or cerebral ischaemia [10,11], brain trauma [12] and cytotoxicity [7,13]. They can also increase reversibly under nutritional and other stress [9] and they are of additional interest since a variety of biological activities such as anti-inflammatory, analgesic and neuroprotective actions have been ascribed to them (reviewed in [1,14,15] and *N*-oleoylethanolamine appears to have anorexic effects [16]). In addition, anandamide was found to be an endogenous agonist of cannabinoid [17] and vanilloid receptors [18], and was reported to exhibit a variety of cannabimimetic activities (reviewed in [19]).

In animal tissues, NAEs are generated from PE (phosphatidylethanolamine) through the 'transacylation–phosphodiesterase pathway', which is composed of two consecutive enzyme-catalysed reactions (reviewed in [20–23]). The first reaction is the transfer of a fatty acyl chain from the *sn*-1 position of a glycerophospholipid to the amino group of PE by a calcium-dependent, energy-independent *N*-acyltransferase, leading to the formation of NAPE (*N*-acylphosphatidylethanolamine) [24,25]. The second reaction is the hydrolysis of NAPE to NAE and phosphatidic acid by a phosphodiesterase of the PLD (phospholipase D) type (NAPE-PLD) [26–28]. All NAEs, including anandamide, are synthesized by the same pathway [29]. NAEs are hydrolysed to fatty acid and ethanolamine [30] by FAAH (fatty acid amide hydrolase) [30–32] or an acid amidase [33,34].

Recently, we purified NAPE-PLD from the particulate fraction of rat heart and cloned its cDNA from mouse, rat and human [35]. The deduced primary structure showed no homology with other known PLDs, but was suggested to belong to the β-lactamase fold family. The recombinant NAPE-PLD expressed in COS-7 cells produced various long-chain NAEs including anandamide from their corresponding NAPEs with similar Michaelis constant (K_m) and maximal velocity (V_{max}) values [35]. NAPE-PLD is a membrane-bound protein, and the substrate NAPE also exists as a membrane constituent of the cell [25,28,36]. However, it remained unclear whether NAPE-PLD acts towards endogenous NAPEs integrated into the membrane of living cells.

In the present study, we stably transfected two mammalian cell lines, HEK-293 (human embryonic kidney 293) and CHO-K1 (Chinese-hamster ovary-K1), with mouse NAPE-PLD cDNA, and investigated the effect of the overexpressed NAPE-PLD on the levels and compositions of endogenous NAPEs and NAEs with the aid of GC-MS. We found that overexpression of NAPE-PLD caused a decrease in the total amount of NAPEs and an increase

Abbreviations used: CHO cell, Chinese-hamster ovary cell; FAAH, fatty acid amide hydrolase; FCS, fetal calf serum; HEK-293 cell, human embryonic kidney 293 cell; MAFP, methyl arachidonyl fluorophosphonate; NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; PE, phosphatidylethanolamine; PLD, phospholipase D.

[1] To whom correspondence should be addressed (email nueda@med.kagawa-u.ac.jp).

in the total amount of NAEs without showing obvious selectivity for N-acyl species of the endogenous NAPEs and NAEs.

MATERIALS AND METHODS

Materials

[1-^{14}C]Palmitic acid was purchased from PerkinElmer Life Sciences (Boston, MA, U.S.A.), [1-^{14}C]arachidonic acid, horseradish peroxidase-linked anti-rabbit IgG, Hybond P and enhanced chemiluminescence (ECL®) kit from Amersham Biosciences (Piscataway, NJ, U.S.A.), anandamide, MAFP (methyl arachidonyl fluorophosphonate) and URB597 from Cayman Chemical (Ann Arbor, MI, U.S.A.), palmitic acid, 1,2-dioleoyl-PE and PLD (*Streptomyces chromofuscus*) from Sigma (St. Louis, MO, U.S.A.) and ethanolamine and Triton X-100 from Nakalai Tesque (Kyoto, Japan). HEK-293 cells and CHO-K1 cells were obtained from Health Science Research Resources (Osaka, Japan), Dulbecco's modified Eagle's medium, RPMI 1640 medium, Lipofectamine™ and hygromycin B from Invitrogen (Carlsbad, CA, U.S.A.), pIREShyg2 from BD Biosciences (Franklin Lakes, NJ, U.S.A.), FCS (fetal calf serum) from PAA Laboratories (Linz, Austria), protein assay dye reagent concentrate from Bio-Rad (Hercules, CA, U.S.A.) and precoated silica gel 60 F254 aluminium sheets for TLC (20 cm × 20 cm, 0.2 mm thickness) from Merck (Darmstadt, Germany). Both *t*-butyldimethylchlorosilane/imidazole reagent and solid-phase extraction cartridges were from Alltech Associates (Deerfield, IL, U.S.A.). *N*-[^{14}C]palmitoyl-PE was prepared from [1-^{14}C]palmitic acid and 1,2-dioleoyl-PE as described previously [26]. [Arachidonoyl-1-^{14}C]Anandamide was prepared from [1-^{14}C]arachidonic acid and ethanolamine [37]. The products were purified by TLC with a mixture of chloroform/methanol/28 % ammonium hydroxide (40:10:1, by vol.). A full-length mouse NAPE-PLD cDNA (GenBank® accession no. AB112350) was prepared as described previously [35].

Stable expression of NAPE-PLD in HEK-293 and CHO-K1 cells

HEK-293 and CHO-K1 cells were maintained in Dulbecco's modified Eagle's medium or RPMI 1640 medium respectively, with 10 % (v/v) FCS at 37 °C in humidified air containing 5 % CO_2. The full-length mouse NAPE-PLD cDNA was inserted into a pIREShyg2 mammalian expression vector by the use of NaeI and BamHI sites of the multicloning sites. HEK-293 and CHO-K1 cells were transfected with the plasmid by Lipofectamine™. Hygromycin B-resistant cell populations were selected in the medium containing 150 μg/ml hygromycin B. From the selected cell populations, 12 clonal cell lines were isolated by colony lifting and maintained in the hygromycin B-containing medium. The NAPE-PLD activity in each clone was examined with *N*-palmitoyl-PE as substrate. Several clones with a high NAPE-PLD activity were then established, and the clones showing the highest enzyme activity were used in the present study. Control HEK-293 or CHO-K1 cells were prepared by the same method, except that the insert-free pIREShyg2 vector was used for transfection. To prepare cell homogenates, the harvested cells were suspended in 20 mM Tris/HCl (pH 7.4) and sonicated three times each for 3 s. Protein concentrations were determined by the method of Bradford [38] with BSA as the standard.

Enzyme assay

For the NAPE-PLD assay, the cell homogenate was incubated with 100 μM *N*-[^{14}C]palmitoyl-PE (10000 c.p.m. in 5 μl of ethanol) in 100 μl of 50 mM Tris/HCl (pH 7.5) containing 0.1 % Triton X-100 and 1 μM MAFP at 37 °C for 10 min. 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 to a silica gel thin-layer plate (height 10 cm). The sample was then developed in chloroform/methanol/28 % ammonium hydroxide (40:10:1, by vol.) at 4 °C for 20 min. Distribution of radioactivity on the plate was quantified by a BAS1500 bioimaging analyser (Fujix, Tokyo, Japan).

For the FAAH assay, the cell homogenate was incubated with 100 μM [^{14}C]anandamide (10000 c.p.m. in 5 μl of DMSO) in 100 μl of 50 mM Tris/HCl (pH 9.0) at 37 °C for 10 min. A mixture of diethyl ether/methanol/1 M citric acid (30:4:1, by vol., 0.3 ml) was added to the reaction mixture to terminate the reaction. After centrifugation, 100 μl of the upper layer was spotted on to a silica gel thin-layer plate (height 10 cm). TLC and quantification of radioactivity were performed as described for the NAPE-PLD assay.

Western blotting

Western blotting was performed using rabbit anti-NAPE-PLD antiserum [35]. The cell homogenates were subjected to SDS/PAGE (10 % polyacrylamide) and electrotransferred on to a hydrophobic PVDF membrane (Hybond P). The membrane was blocked with PBS containing 0.1 % Tween 20 and 5 % (w/v) dried milk (buffer A) and then incubated with anti-NAPE-PLD antiserum (1:5000 dilution) in buffer A at 4 °C for 1 h, followed by incubation with the horseradish peroxidase-labelled secondary antibody (1:2000 dilution) in buffer A at 4 °C for 1 h. The membrane was finally treated with ECL® reagents and visualized by a LAS1000plus lumino imaging analyser (Fujix).

Lipid extraction, sample preparation and GC-MS analysis

Total lipids were extracted from the cell pellets by the Folch procedure [39] after removing the media. At the beginning of each extraction procedure, the internal standards (20 ng of each [$^2H_4$]-NAE and 19 pmol of C_{17}NAPE) were added to the samples. Aliquots of the final extract were taken to assay for lipid phosphorus [40]. NAEs and NAPEs were isolated with silica solid-phase extraction cartridges using chloroform/methanol mixtures of 49:1 and 4:1 (v/v) respectively. NAPE samples were digested with *S. chromofuscus* PLD [41] and the resulting NAEs were separated by solid-phase extraction chromatography. NAE samples were converted into *t*-butyldimethysilyl derivatives and analysed by GC-MS with a Hewlett–Packard 5890 gas chromatograph equipped with a 5972 mass selective detector and a 7673 autosampler as described in [42]. The HP5MS column, 30 m × 0.25 mm inner diameter (Hewlett–Packard, Palo Alto, CA, U.S.A.) was programmed from 230 to 280 °C at 2.5 °C/min. The M-57 ions were monitored in selected ion monitoring mode.

As a separate experiment, the mock cells and NAPE-PLD-expressing CHO-K1 cells were incubated with RPMI 1640 medium containing 0.1 % FCS at 37 °C for 48 h in the presence of 3 μM URB597 or vehicle (0.1 % DMSO) with one change of medium at 24 h. Total lipids were then extracted from the cell pellets.

RESULTS

To examine whether or not NAPE-PLD utilizes endogenous membrane-bound NAPE as substrate, we stably transfected two mammalian cell lines (HEK-293 and CHO-K1) with mouse NAPE-PLD cDNA. By transfecting the cells with a pIREShyg2 vector harbouring the NAPE-PLD cDNA, followed by selecting for resistance against hygromycin B, we established clonal cell lines

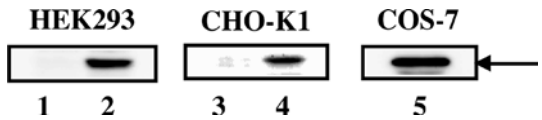

Figure 1 Stable expression of recombinant NAPE-PLD in HEK-293 and CHO-K1 cells

Western blotting was performed with anti-NAPE-PLD antiserum as described in the Materials and methods section. Lane 1, HEK-293 cell homogenates (20 μg of protein) transfected with the insert-free vector; lane 2, HEK-293 cell homogenates (20 μg of protein) transfected with mouse NAPE-PLD cDNA; lane 3, CHO-K1 cell homogenates (20 μg of protein) transfected with the insert-free vector; lane 4, CHO-K1 cell homogenates (20 μg of protein) transfected with mouse NAPE-PLD cDNA; lane 5, COS-7 cell homogenates (5 μg of protein) transiently expressing mouse NAPE-PLD prepared as described previously [35].

Table 1 The NAPE-PLD and FAAH activities of mock cells and NAPE-PLD-expressing cells

		Specific enzyme activity [nmol · min^{-1} · (mg of protein)$^{-1}$]	
Host cell	Vector	NAPE-PLD*	FAAH†
HEK-293	Insert-free vector	0.011 ± 0.010	0.11 ± 0.03
	NAPE-PLD cDNA	2.07 ± 0.08	0.13 ± 0.04
CHO-K1	Insert-free vector	0.023 ± 0.004	1.60 ± 0.28
	NAPE-PLD cDNA	0.95 ± 0.08	1.65 ± 0.23

* The cell homogenate was allowed to react with 100 μM *N*-[^{14}C]palmitoyl-PE in the presence of 0.1 % Triton X-100 and 1 μM MAFP (an FAAH inhibitor). Means ± S.D. are shown ($n = 3$).

† The cell homogenate was allowed to react with 100 μM [^{14}C]anandamide. Means ± S.D. are shown ($n = 3$).

that stably expressed NAPE-PLD. As analysed by Western blotting with anti-NAPE-PLD antibody [35], the homogenates of HEK-293 and CHO-K1 cells stably transfected with the NAPE-PLD cDNA revealed an immunoreactive protein band of approx. 46 kDa (Figure 1, lanes 2 and 4). The recombinant NAPE-PLD transiently expressed in COS-7 cells used as a positive control gave a band at the same position (lane 5). In contrast, the immunoreactive band was hardly detectable with the homogenates of the cells transfected with the insert-free vector (mock cells) (lanes 1 and 3). Reverse transcriptase–PCR, however, revealed mRNA of endogenous NAPE-PLD in control HEK-293 cells (results not shown), suggesting expression of endogenous NAPE-PLD in the cells.

The homogenates of the mock and NAPE-PLD-expressing cells were then allowed to react with *N*-[^{14}C]palmitoyl-PE, and the product, *N*-[^{14}C]palmitoylethanolamine, was separated by TLC. The results indicated that the NAPE-PLD-expressing HEK-293 and CHO-K1 cells had a NAPE-PLD specific activity of 2.07 ± 0.08 or 0.95 ± 0.08 nmol · min^{-1} · (mg of protein)$^{-1}$ at 37 °C respectively (Table 1). Although these values were lower than the specific enzyme activity of COS-7 cells transiently expressing NAPE-PLD [25 nmol · min^{-1} · (mg of protein)$^{-1}$], they were 50–200-fold higher than those of the endogenous NAPE-PLD in mock HEK-293 and CHO-K1 cells [0.011–0.023 nmol · min^{-1} · (mg of protein)$^{-1}$]. Moreover, we examined the endogenous activity of FAAH in these cells, which should contribute to the degradation of NAEs produced by NAPE-PLD. The FAAH activity was assayed by allowing the homogenates of the mock and NAPE-PLD-expressing cells to react with [^{14}C]anandamide, followed by separation of the produced [^{14}C]arachidonic acid by TLC. The results clarified that the endogenous FAAH activity was much higher than the endogenous NAPE-PLD activity in both the cells

Table 2 NAPE content and composition in NAPE-PLD-expressing cells and the mock cells of HEK-293

	NAPE content			
	First experiment*		Second experiment†	
	pmol/μmol phosphorus	%	pmol/μmol phosphorus	%
Mock cells				
16:0	80.92	40.37	47.58	40.76
18:0	38.97	19.44	24.10	20.65
18:1, $n-9$	46.82	23.36	23.26	19.93
18:1, $n-7$	29.91	14.92	18.81	16.12
18:2, $n-6$	2.39	1.19	2.09	1.79
20:4, $n-6$	1.42	0.71	0.88	0.75
Total	200.43	100	116.72	100
NAPE-PLD-expressing cells				
16:0	9.81	51.96	4.13	40.37
18:0	2.22	11.76	1.30	12.71
18:1, $n-9$	4.38	23.20	3.36	32.84
18:1, $n-7$	1.49	7.89	0.72	7.04
18:2, $n-6$	0.64	3.39	0.54	5.28
20:4, $n-6$	0.34	1.80	0.18	1.76
Total	18.88	100	10.23	100

* The values were obtained from a single sample.

† The values are the means for duplicate experiments.

(Table 1). In addition, the specific FAAH activity of CHO-K1 cells was 16-fold higher than that of HEK-293 cells. We also found that the overexpression of NAPE-PLD did not affect the endogenous FAAH activity in these cell lines.

We then examined the contents of NAPE and NAE in the NAPE-PLD-expressing cells and mock cells. Total lipids were extracted from the cells [39], and the NAPE and NAE fractions were separated with a silica cartridge. NAPEs were hydrolysed to their corresponding NAEs with *S. chromofuscus* PLD. NAEs isolated as such or prepared from NAPEs were then subjected to GC-MS analysis as described previously [9,42]. The contents of NAPEs (Tables 2 and 4) and NAEs (Tables 3 and 5) in HEK-293 cells (Tables 2 and 3) or CHO-K1 cells (Tables 4 and 5) are presented. In these analyses, six types of NAPEs with a different N-acyl species and their corresponding NAEs were separately quantified. The amide-linked fatty acids were palmitic acid (shown as 16:0), stearic acid (18:0), oleic acid (18:1, $n-9$), vaccenic acid (18:1, $n-7$), linoleic acid (18:2, $n-6$) and arachidonic acid (20:4, $n-6$). We performed the same experiments twice at different times, and the results are presented separately as 'first experiment' and 'second experiment' in each Table. We chose to list the data separately, because they were obtained with different cell populations and therefore exhibited differences in absolute amounts. However, within the second experiment, we performed two separate analyses, which yielded very similar results that were averaged.

When total amounts of NAPEs were compared between the NAPE-PLD-expressing cells and mock HEK-293 cells (Table 2), the total level in the NAPE-PLD cells was only approx. 9 % of that in the mock cells. Moreover, with CHO-K1 cells (Table 4), the total NAPE level in the NAPE-PLD cells was 50–58 % of that in the mock cells. In contrast, the total level of NAEs in the NAPE-PLD cells was 1.4–1.5-fold (HEK-293 cells; Table 3) or 1.2–1.6-fold (CHO-K1 cells; Table 5) higher than that in the corresponding mock cells. Thus the overexpression of NAPE-PLD in both the cell lines caused a remarkable decrease in the endogenous NAPE levels with a concomitant increase in the NAE levels. These results suggest that recombinant NAPE-PLD

Table 3 NAE content and composition in NAPE-PLD-expressing cells and the mock HEK-293 cells

	NAE content			
	First experiment*		Second experiment†	
	pmol/μmol phosphorus	%	pmol/μmol phosphorus	%
Mock cells				
16:0	5.94	59.82	3.15	46.60
18:0	2.21	22.26	2.08	30.77
18:1, $n-9$	0.70	7.05	0.80	11.83
18:1, $n-7$	0.88	8.86	0.35	5.18
18:2, $n-6$	0.11	1.11	0.21	3.11
20:4, $n-6$	0.09	0.91	0.17	2.51
Total	9.93	100	6.76	100
NAPE-PLD-expressing cells				
16:0	8.54	57.51	4.29	45.93
18:0	2.87	19.33	2.95	31.58
18:1, $n-9$	1.13	7.61	0.65	6.96
18:1, $n-7$	1.73	11.65	0.74	7.92
18:2, $n-6$	0.33	2.22	0.20	2.14
20:4, $n-6$	0.25	1.68	0.51	5.46
Total	14.85	100	9.34	100

* The values were obtained from a single sample.
† The values are the means for duplicate experiments.

Table 4 NAPE content and composition in NAPE-PLD-expressing cells and the mock CHO-K1 cells

	NAPE content			
	First experiment*		Second experiment†	
	pmol/μmol phosphorus	%	pmol/μmol phosphorus	%
Mock cells				
16:0	62.20	33.89	18.38	37.83
18:0	30.11	16.41	5.66	11.65
18:1, $n-9$	40.55	22.10	9.22	18.98
18:1, $n-7$	44.29	24.13	13.57	27.93
18:2, $n-6$	3.96	2.16	1.47	3.03
20:4, $n-6$	2.41	1.31	0.28	0.58
Total	183.52	100	48.58	100
NAPE-PLD-expressing cells				
16:0	31.35	34.17	11.17	39.37
18:0	12.89	14.05	4.54	16.00
18:1, $n-9$	29.01	31.62	6.43	22.66
18:1, $n-7$	14.51	15.82	4.57	16.11
18:2, $n-6$	2.95	3.22	1.40	4.93
20:4, $n-6$	1.03	1.12	0.26	0.92
Total	91.74	100	28.37	100

* The values were obtained from a single sample.
† The values are the means for duplicate experiments.

is active in the intact cells and utilizes endogenous membrane-bound NAPE as substrate.

We next examined whether or not the overexpression of NAPE-PLD affected the composition of the N-acyl species of NAPEs and NAEs. In the mock cells of HEK-293, the most predominant N-fatty acyl moiety of NAPE was palmitic acid, accounting for 40–41 % of the total NAPE amount, followed by oleic acid, stearic acid and vaccenic acid (Table 2). *N*-arachidonoyl-PE (an anandamide precursor) amounted to only 0.7–0.8 %. The mock CHO-K1 cells also revealed a similar composition: the most abundant N-acyl moiety was palmitic acid, accounting for 34–38 %, followed by vaccenic acid, oleic acid and stearic acid (Table 4). Although the total levels of NAPE in the NAPE-PLD-expressing cells were lower than those of the mock cells as indicated above, the overexpression of NAPE-PLD in HEK-293 (Table 2) and CHO-K1 cells (Table 4) did not bring about a remarkable change in the composition of the N-acyl moiety of NAPEs. There was no obvious change in the percentage of *N*-arachidonoyl-PE, whereas the proportion of *N*-vaccenoyl-PE was decreased.

Table 5 NAE content and composition in NAPE-PLD-expressing cells and the mock CHO-K1 cells

	NAE content			
	First experiment*		Second experiment†	
	pmol/μmol phosphorus	%	pmol/μmol phosphorus	%
Mock cells				
16:0	1.56	33.77	2.27	45.58
18:0	0.80	17.32	1.16	23.29
18:1, $n-9$	0.52	11.26	0.43	8.63
18:1, $n-7$	1.42	30.74	0.33	6.63
18:2, $n-6$	0.19	4.11	0.21	4.22
20:4, $n-6$	0.13	2.81	0.58	11.65
Total	4.62	100	4.98	100
NAPE-PLD-expressing cells				
16:0	3.80	51.42	2.56	43.39
18:0	1.14	15.43	1.24	21.02
18:1, $n-9$	0.74	10.01	0.96	16.27
18:1, $n-7$	1.13	15.29	0.38	6.44
18:2, $n-6$	0.46	6.22	0.32	5.42
20:4, $n-6$	0.12	1.62	0.44	7.46
Total	7.39	100	5.90	100

* The values were obtained from a single sample.
† The values are shown as the means for duplicate experiments.

We also compared the NAE composition between the NAPE-PLD cells and mock cells. The most predominant NAE in the mock cells was *N*-palmitoylethanolamine, accounting for 47–60 % (HEK-293 cells; Table 3) or 34–46 % (CHO-K1 cells; Table 5) of the total NAE amounts. Anandamide was a minor component, accounting only for 1–3 % (HEK-293 cells; Table 3) or 3–12 % (CHO-K1 cells; Table 5). When NAPE-PLD was overexpressed in both cells, no obvious change in the composition of NAEs was observed. These results reveal that the N-acyl moieties of NAPE and NAE in HEK-293 and CHO-K1 cells consist primarily of saturated and mono-unsaturated acyl groups, and the expressed NAPE-PLD did not appear to show any substrate selectivity for N-acyl species of the endogenous NAPEs in cells.

DISCUSSION

NAPE-PLD was described as a phosphodiesterase hydrolysing NAPE to NAE and phosphatidic acid more than 20 years ago [43], and has been characterized with crude enzyme preparations [26,27,44]. A series of enzymological studies strongly suggested that NAPE-PLD is distinct from PLDs of the HKD/phosphatidyltransferase family that are involved in signal transduction [3,27,44]. Our recent cDNA cloning clarified that NAPE-PLD is a novel type of PLD belonging to the β-lactamase fold family and is not structurally or functionally related to the known PLDs [35]. We also demonstrated that the recombinant enzyme does not have selectivity for N-acyl groups of NAPEs, as was also shown with rat brain microsomes [3]. Furthermore, we showed that the mRNA and protein of NAPE-PLD are widely expressed in various mouse tissues, strongly suggesting an important role for NAPE-PLD in the formation of NAEs, including anandamide, in animal tissues.

However, since NAPE represents only a very minor component of cellular membrane phospholipids, it was necessary to examine whether NAPE-PLD utilizes the endogenous membrane-bound NAPE of living cells. In the present study, we successfully established clones stably overexpressing NAPE-PLD from HEK-293 cells and CHO-K1 cells, which showed a high *N*-palmitoyl-PE-hydrolysing activity [1–2 nmol · min^{-1} · (mg of protein)$^{-1}$]. When the NAPE-PLD-expressing cells were compared with the mock cells, we found that overexpression of NAPE-PLD caused a decrease in the total amount of NAPEs by 50–90 % and a 1.5-fold increase in the total amount of NAEs, without major changes in the N-acyl compositions of these lipids. These results confirm that overexpressed NAPE-PLD is capable of forming NAEs, including anandamide, in living cells.

As shown in Tables 2–5, total levels of NAPE and NAE on a phospholipid basis in the mock HEK-293 and CHO-K1 cells were 48.6–200.4 pmol/μmol phosphorus and 4.62–9.93 pmol/μmol phosphorus respectively. This means that the NAPE levels were 9.8–39.7 times higher than the NAE levels. In a variety of normal human tissues, NAPE ranged from 21.26 to 221.1 pmol/μmol phosphorus, whereas NAE ranged from 6.60 to 27.68 pmol/μmol phosphorus [6,45]. These NAPE levels were 3–17 times higher than the NAE levels. In addition, mouse peritoneal macrophages and mouse epidermal JB6 P+ cells showed 127.0–187.4 pmol/μmol phosphorus of NAPE and 16.84–57.92 pmol/μmol phosphorus of NAE [8,9,42]. The ratios of NAPE to NAE ranged from 3 to 11. Thus the endogenous levels of NAPE and NAE in HEK-293 and CHO-K1 cells in the present study were within the range of values reported previously, and the considerably higher NAPE levels compared with NAE levels in the two cell lines were also consistent with data from other animal tissues or cells.

As expected, the NAPE-PLD-overexpressing cells showed lower levels of NAPE and higher levels of NAE when compared with the mock cells. However, the decrease in NAPE was much greater than the increase in NAE. This difference might be explained by the rather fast metabolic turnover of these lipids [8]. NAE is hydrolysed to fatty acid and ethanolamine by either FAAH [31,32] or an acid amidase [33,34] and we actually detected an endogenous FAAH activity in HEK-293 and CHO-K1 cells (Table 1). Furthermore, we examined the contribution of FAAH to the regulation of NAE levels by incubating CHO-K1 cells for 48 h in the presence of the FAAH inhibitor URB597 [46]. This treatment resulted in an approx. 5-fold increase in total NAE in both the mock cells and NAPE-PLD cells (results not shown). However, the inhibitor did not cause a remarkable difference in NAE levels between the mock cells and NAPE-PLD cells. This result suggests that FAAH prevents NAE accumulation in cells, but its role in regulating NAE levels appeared to be limited in our system. Also, while NAPEs remain in the membrane, NAEs can be released outside the cell [9,13,29] and may bind to proteins in the aqueous environment [47,48]. The mechanisms for the moderate increase in NAE levels, in spite of a significant decrease of its immediate precursor, remained unclarified, but this finding supported the concept that NAEs may be messenger molecules of potential physiological significance [21,23,49], whose levels must be controlled.

Due to their precursor–product relationship, the N-acyl compositions of NAPE and NAE of most mammalian cells and tissues investigated so far are very similar, with saturated and mono-unsaturated moieties predominating over polyunsaturated ones [3–9], and the same was observed with the HEK-293 and CHO-K1 cells in the present study. Since saturated and mono-unsaturated NAEs do not act as cannabinoid receptor agonists, it has been a matter of debate whether the transacylation–phosphodiesterase pathway can contribute to the selective formation of anandamide [20,23]. Sugiura et al. [3] previously reported that NAPE-PLD of rat brain microsomes is actually somewhat less active with *N*-arachidonoyl-PE than other NAPEs. Recently, we confirmed that the recombinant NAPE-PLD does not discriminate between various NAPEs including *N*-arachidonoyl-PE as substrates [35]. However, we could not rule out the possibility that intact cells possess mechanisms to influence substrate specificity of NAPE-PLD, resulting in the selective generation of anandamide. We were therefore interested in finding whether the overexpression of NAPE-PLD causes a selective decrease in *N*-arachidonoyl-PE or a selective generation of anandamide in intact cells. Because, in the present study, we observed no remarkable change in the relative amounts of *N*-arachidonoyl-PE or anandamide with the NAPE-PLD-overexpressing cells, it appears that the NAPE-PLD in these cells acts almost equally on all NAPEs, as previously observed with cell-free preparations [35].

It is believed that the transacylation–phosphodiesterase pathway is primarily regulated by the activation of a Ca^{2+}-dependent *N*-acyltransferase that generates NAPE [2,20]. Hansen et al. previously reported that, in cortical neurons in primary culture, the formation of NAE increased in parallel with NAPE formation on exposure to glutamate, calcium ionophore and sodium azide [13,50]. Taken together, it was suggested that NAPE-PLD is constitutively active and that the availability of the substrate NAPE determines the rate of NAE formation. However, our present results suggest that the expression level of NAPE-PLD could be another regulatory mechanism. Thus the cellular stimuli inducing NAPE-PLD at the transcriptional level may lead to an increase in NAE production. In fact it was reported recently that lipopolysaccharide enhances the NAPE-PLD activity in RAW264.7 macrophages, and the involvement of a transcriptional mechanism was suggested [51].

Alternatively, Di Marzo et al. [52] reported that the treatment of J774 macrophages with ionomycin resulted in the production of a peak corresponding to anandamide as well as a decrease in the levels of NAPE-like metabolites, suggesting that NAPE-PLD is activated by Ca^{2+} in cells; however, other macrophages did not respond to ionophore treatment with any change in NAE/NAPE turnover [8]. Also, we previously showed that NAPE-PLD, partially purified from rat heart, could be stimulated with Ca^{2+} [53,54]. However, since millimolar concentrations of Ca^{2+} were required for a significant activation of the enzyme and several other bivalent cations could fully substitute for Ca^{2+}, the physiological importance of Ca^{2+} as an NAPE-PLD activator remains uncertain and further studies are required to identify the possible mechanism that activates NAPE-PLD. A purified recombinant enzyme will help us to answer this question at the molecular level. Moreover, for the comprehensive understanding of regulatory mechanism in the transacylase–phosphodiesterase pathway, cDNA cloning and functional expression of *N*-acyltransferase will be needed.

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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 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

4 Sugiura, T., Kondo, S., Sukagawa, A., Tonegawa, T., Nakane, S., Yamashita, A. and Waku, K. (1996) Enzymatic synthesis of anandamide, an endogenous cannabinoid receptor ligand, through *N*-acylphosphatidylethanolamine pathway in testis: involvement of Ca^{2+}-dependent transacylase and phosphodiesterase activities. Biochem. Biophys. Res. Commun. **218**, 113–117

5 Cadas, H., di Tomaso, E. and Piomelli, D. (1997) Occurrence and biosynthesis of endogenous cannabinoid precursor, *N*-arachidonoyl phosphatidylethanolamine, in rat brain. J. Neurosci. **17**, 1226–1242

6 Deutsch, D. G., Goligorsky, M. S., Schmid, P. C., Krebsbach, R. J., Schmid, H. H. O., Das, S. K., Dey, S. K., Arreaza, G., Thorup, C., Stefano, G. et al. (1997) Production and physiological actions of anandamide in the vasculature of the rat kidney. J. Clin. Invest. **100**, 1538–1546

7 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

8 Kuwae, T., Shiota, Y., Schmid, P. C., Krebsbach, R. and Schmid, H. H. O. (1999) Biosynthesis and turnover of anandamide and other *N*-acylethanolamines in peritoneal macrophages. FEBS Lett. **459**, 123–127

9 Berdyshev, E. V., Schmid, P. C., Dong, Z. and Schmid, H. H. O. (2000) Stress-induced generation of *N*-acylethanolamines in mouse epidermal JB6 P^+ cells. Biochem. J. **346**, 369–374

10 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

11 Berger, C., Schmid, P. C., Schabitz, W.-R., Wolf, M., Schwab, S. and Schmid, H. H. O. (2004) Massive accumulation of *N*-acylethanolamines after stroke. Cell signaling in acute cerebral ischemia? J. Neurochem. **88**, 1159–1167

12 Hansen, H. H., Schmid, P. C., Bittigau, P., Lastres-Becker, I., Berrendero, F., Manzanares, J., Ikonomidou, C., Schmid, H. H. O., Fernándes-Ruiz, J. J. and Hansen, H. S. (2001) Anandamide, but not 2-arachidonoylglycerol, accumulates during *in vivo* neurodegeneration. J. Neurochem. **78**, 1415–1427

13 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

14 Hansen, H. S., Moesgaard, B., Peterson, G. and Hansen, H. H. (2002) Putative neuroprotective actions of *N*-acyl-ethanolamines. Pharmacol. Ther. **95**, 119–126

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 Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodriguez de Fonseca, F., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G. et al. (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. Nature (London) **425**, 90–93

17 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

18 Van Der Stelt, M. and Di Marzo, V. (2004) Endovanilloids. Putative endogenous ligands of transient receptor potential vanilloid 1 channels. Eur. J. Biochem. **271**, 1827–1834

19 Di Marzo, V. (1998) 'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance. Biochim. Biophys. Acta **1392**, 153–175

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

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

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

23 Schmid, H. H. O., Schmid, P. C. and Berdyshev, E. V. (2002) Cell signaling by endocannabinoids and their congeners: questions of selectivity and other challenges. Chem. Phys. Lipids **121**, 111–134

24 Natarajan, V., Reddy, P. V., Schmid, P. C. and Schmid, H. H. O. (1982) *N*-acylation of ethanolamine phospholipids in canine myocardium. Biochim. Biophys. Acta **712**, 342–355

25 Natarajan, V., Schmid, P. C., Zuzarte-Augustin, M. L. and Schmid, H. H. O. (1983) Biosynthesis of *N*-acylethanolamine phospholipids in dog brain preparations. J. Neurochem. **41**, 1303–1312

26 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

27 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

28 Natarajan, V., Schmid, P. C. and Schmid, H. H. O. (1986) *N*-acylethanolamine phospholipid metabolism in normal and ischemic rat brain. Biochim. Biophys. Acta **878**, 32–41

29 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

30 Schmid, P. C., Zuzarte-Augustin, M. L. and Schmid, H. H. O. (1985) Properties of rat liver *N*-acylethanolamine amidohydrolase. J. Biol. Chem. **260**, 14145–14149

31 Patricelli, M. P. and Cravatt, B. F. (2001) Proteins regulating the biosynthesis and inactivation of neuromodulatory fatty acid amides. Vitam. Horm. **62**, 95–131

32 Ueda, N. (2002) Endocannabinoid hydrolases. Prostaglandins Other Lipid Mediators **68–69**, 521–534

33 Ueda, N., Yamanaka, K., Terasawa, N. and Yamamoto, S. (1999) An acid amidase hydrolyzing anandamide as an endogenous ligand for cannabinoid receptors. FEBS Lett. **454**, 267–270

34 Ueda, N., Yamanaka, K. and Yamamoto, S. (2001) Purification and characterization of an acid amidase selective for *N*-palmitoylethanolamine, a putative endogenous anti-inflammatory substance. J. Biol. Chem. **276**, 35552–35557

35 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

36 Cadas, H., Schinelli, S. and Piomelli, D. (1996) Membrane localization of *N*-acylphosphatidylethanolamine in central neurons: studies with exogenous phospholipases. J. Lipid Mediators Cell Signalling **14**, 63–70

37 Ueda, N., Yamamoto, K., Yamamoto, S., Tokunaga, T., Shirakawa, E., Shinkai, H., Ogawa, M., Sato, T., Kudo, I., Inoue, K. et al. (1995) Lipoxygenase-catalyzed oxygenation of arachidonylethanolamide, a cannabinoid receptor agonist. Biochim. Biophys. Acta **1254**, 127–134

38 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

39 Folch, J., Lees, M. and Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**, 497–509

40 Bartlett, G. R. (1959) Phosphorus assay in column chromatography. J. Biol. Chem. **234**, 466–468

41 Schmid, P. C., Natarajan, V., Weis, B. K. and Schmid, H. H. O. (1986) Hydrolysis of *N*-acylated glycerophospholipids by phospholipases A_2 and D: a method of identification and analysis. Chem. Phys. Lipids **41**, 195–207

42 Schmid, P. C., Kuwae, T., Krebsbach, R. J. and Schmid, H. H. O. (1997) Anandamide and *N*-acylethanolamines in mouse peritoneal macrophages. Chem. Phys. Lipids **87**, 103–110

43 Natarajan, V., Reddy, P. V., Schmid, P. C. and Schmid, H. H. O. (1981) On the biosynthesis and metabolism of *N*-acylethanolamine phospholipids in infarcted dog heart. Biochim. Biophys. Acta **664**, 445–448

44 Petersen, G. and Hansen, H. S. (1999) *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D lacks the ability to transphosphatidylate. FEBS Lett. **455**, 41–44

45 Schmid, P. C., Wold, L. E., Krebsbach, R. J., Berdyshev, E. V. and Schmid, H. H. O. (2002) Anandamide and other *N*-acylethanolamines in human tumors. Lipids **37**, 907–912

46 Kathuria, S., Gaetani, S., Fegley, D., Valino, F., Duranti, A., Tontini, A., Mor, M., Tarzia, G., La Rana, G., Calignano, A. et al. (2003) Modulation of anxiety through blockade of anandamide hydrolysis. Nat. Med. **9**, 76–81

47 Giuffrida, A. and Piomelli, D. (1998) Isotope dilution GC/MS determination of anandamide and other fatty acylethanolamides in rat blood plasma. FEBS Lett. **422**, 373–376

48 Leweke, F. M., Giuffrida, A., Wurster, U., Emrich, H. M. and Piomelli, D. (1999) Elevated endogenous cannabinoids in schizophrenia. Neuroreport **10**, 1665–1669

49 Berdyshev, E. V., Schmid, P. C., Krebsbach, R. J., Hillard, C. J., Huang, C., Chen, N., Dong, Z. and Schmid, H. H. O. (2001) Cannabinoid receptor-independent cell signaling by *N*-acylethanolamines. Biochem. J. **360**, 67–75

50 Hansen, H. S., Lauritzen, L., Strand, A. M., Vinggaard, A. M., Frandsen, A. and Schousboe, A. (1997) Characterization of glutamate-induced formation of *N*-acylphosphatidylethanolamine and *N*-acylethanolamine in cultured cortical neurons. J. Neurochem. **69**, 753–761
51 Liu, J., Bátkai, S., Pacher, P., Harvey-White, J., Wagner, J. A., Cravatt, B. F., Gao, B. and Kunos, G. (2003) Lipopolysaccharide induced anandamide synthesis in macrophages via CD14/MAPK/phosphoinositide 3-kinase/NF-κB independently of platelet-activating factor. J. Biol. Chem. **278**, 45034–45039
52 Di Marzo, V., De Petrocellis, L., Sepe, N. and Buono, A. (1996) Biosynthesis of anandamide and related acylethanolamides in mouse J774 macrophages and N_{18} neuroblastoma cells. Biochem. J. **316**, 977–984
53 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
54 Liu, Q., Tonai, T. and Ueda, N. (2002) Activation of *N*-acylphosphatidylethanolamine-releasing phospholipase D by polyamines. Chem. Phys. Lipids **115**, 77–84

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