

**REVIEW****Biosynthetic Pathways of the Endocannabinoid Anandamide**by **Yasuo Okamoto, Jun Wang, Jun Morishita, and Natsuo Ueda\***Department of Biochemistry, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki,  
Kagawa 761-0793, Japan(phone: +81-87-891-2102; fax: +81-87-891-2105; e-mail: [nueda@med.kagawa-u.ac.jp](mailto:nueda@med.kagawa-u.ac.jp))

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Anandamide (= *N*-arachidonylethanolamine) is the first discovered endocannabinoid, and belongs to the class of bioactive, long-chain *N*-acylethanolamines (NAEs). In animal tissues, anandamide is principally formed together with other NAEs from glycerophospholipid by two successive enzymatic reactions: 1) *N*-acylation of phosphatidylethanolamine to generate *N*-acylphosphatidylethanolamine (NAPE) by Ca<sup>2+</sup>-dependent *N*-acyltransferase; 2) release of NAE from NAPE by a phosphodiesterase of the phospholipase D type (NAPE-PLD). Although these anandamide-synthesizing enzymes were poorly understood until recently, our cDNA cloning of NAPE-PLD in 2004 enabled molecular-biological approaches to the enzymes. NAPE-PLD is a member of the metallo- $\beta$ -lactamase family, which specifically hydrolyzes NAPE among glycerophospholipids, and appears to be constitutively active. Mutagenesis studies suggested that the enzyme functions through a mechanism similar to those of other members of the family. NAPE-PLD is widely expressed in animal tissues, including various regions in rat brain. Its expression level in the brain is very low at birth, and remarkably increases with development. Analysis of NAPE-PLD-deficient mice and other recent studies revealed the presence of NAPE-PLD-independent pathways for the anandamide formation. Furthermore, calcium-independent *N*-acyltransferase was discovered and characterized. In this article, we will review recent progress in the studies on these enzymes responsible for the biosynthesis of anandamide and other NAEs.

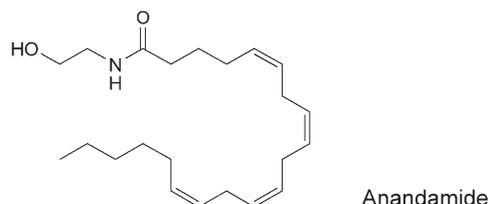
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**1. Introduction<sup>1)</sup>**. – Endocannabinoids are endogenous ligands of cannabinoid receptors in animal tissues [1]. Shortly after cDNA cloning of the central cannabinoid receptor CB1 [2], anandamide was isolated from porcine brain as the first endocannabinoid, and its chemical structure was determined as *N*-arachidonylethanolamine (= *N*-(2-hydroxyethyl)arachidonamide)<sup>2)</sup> [3]. Later, anandamide was reported to show a variety of cannabimimetic activities such as the induction of

<sup>1)</sup> *Abbreviations* (in alphabetic order): Abh4,  $\alpha/\beta$ -hydrolase 4; CB1, cannabinoid receptor type 1; FAAH, fatty acid amide hydrolase; GP-NAE, glycerophospho-*N*-acylethanolamine; GST, glutathione *S*-transferase; LPS, lipopolysaccharide; LRAT, lecithin retinol acyltransferase; NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; NAPE-PLD, NAPE-hydrolyzing phospholipase D; NAPE-PLD<sup>-/-</sup>, NAPE-PLD-deficient mice; NAT, *N*-acyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipase; PPAR, peroxisome proliferator-activated receptor; PS, phosphatidylserine; RLP, rat LRAT-like protein; SNP, single-nucleotide polymorphism; sPLA<sub>2</sub>-IB, group-IB secretory phospholipase A<sub>2</sub>; TRPV1, transient receptor potential vanilloid type 1.

<sup>2)</sup> Systematic name: (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(2-hydroxyethyl)icosa-5,8,11,14-tetraenamide.

hypothermia, analgesia, and motor defects [1]. In addition, anandamide was found to be a ligand of the transient receptor potential vanilloid type 1 (TRPV1) [4]. However, anandamide is a relatively minor component among various *N*-acylethanolamines (NAEs) in tissues [5]. Saturated and monounsaturated long-chain NAEs are major components [5], and have been reported to exhibit a variety of biological activities such as antinociceptive and anti-inflammatory effects of *N*-palmitoylethanolamine [6][7], anorexic effects of *N*-oleoylethanolamine [8], and pro-apoptotic and anorexic effects of *N*-stearoylethanolamine [9][10]. Although these NAEs do not bind to or activate cannabinoid receptors [11], recent studies suggested that they activate peroxisome proliferator-activated receptor (PPAR)- $\alpha$  [12][13] and TRPV1 [14]. Moreover, *N*-palmitoylethanolamine and *N*-oleoylethanolamine were reported to bind to the G-protein-coupled receptors GPR55 [15] and GPR119 [16], respectively.



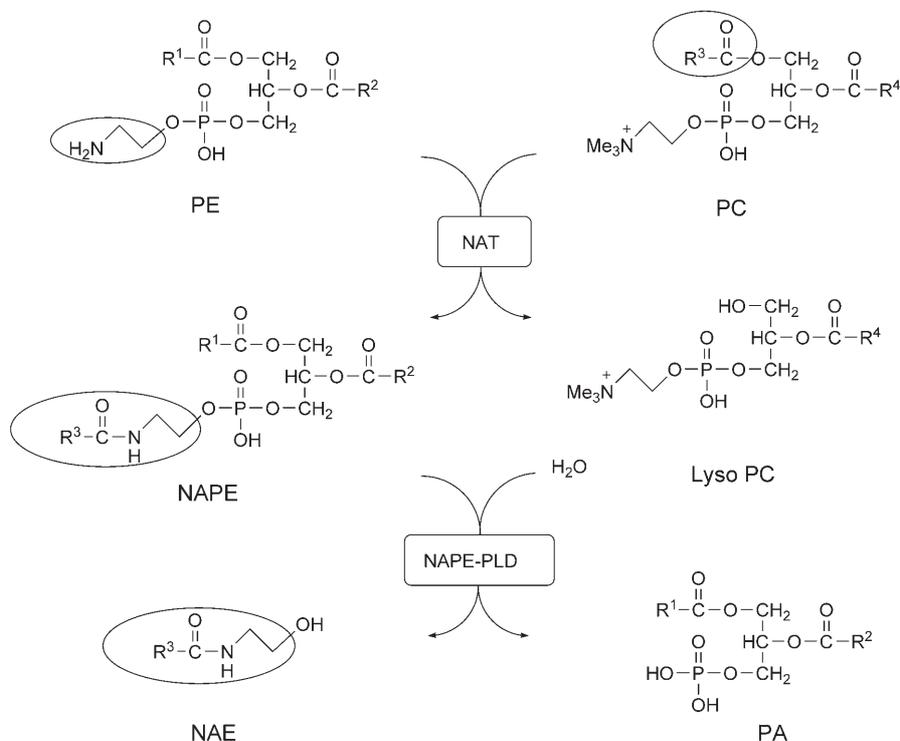
As a member of the NAE class, anandamide is generally thought to be formed by a pathway common to all NAEs in animal tissues. Earlier studies confirmed the principal pathway of the NAE biosynthesis, often called ‘the transacylation-phosphodiesterase pathway’, which is composed of two steps of enzyme reactions, starting from membrane glycerophospholipids (see *Scheme 1* below) [5][17][18]. The first step is the transfer of an acyl chain from the *sn*-1 position of a glycerophospholipid to the amino group of the hydroxyethyl moiety of phosphatidylethanolamine (PE), catalyzed by calcium-dependent *N*-acyltransferase (NAT), and the second step is the hydrolysis of the generated *N*-acylphosphatidylethanolamine (NAPE) to NAE and phosphatidic acid by a phosphodiesterase of the phospholipase (PL) D-type (NAPE-hydrolyzing PLD, usually abbreviated as NAPE-PLD). These anandamide-synthesizing enzymes have been poorly understood until recently. In 2004, our group succeeded in cDNA cloning and functional expression of NAPE-PLD, and for the first time introduced molecular-biological approaches in the study of the biosynthesis of anandamide [19]. More recently, further studies, including the analysis of NAPE-PLD-deficient mice, suggested the presence of alternative pathways for the formation of anandamide [20].

In this article, we will review recent advances in the studies on the enzymes involved in the biosynthesis of anandamide and other NAEs, with special reference to molecular and catalytic characterization of NAPE-PLD.

**2. *N*-Acyltransferase (NAT).** – NAT Catalyzes the first reaction of the transacylation-phosphodiesterase pathway, and transfers a fatty acyl chain at *sn*-1 position of various glycerophospholipids to the amino group of ethanolamine phospholipids, leading to the formation of NAPE (*Scheme 1*). NAT is generally considered to be the rate-limiting step in the NAE biosynthesis, which is probably regulated by intracellular

$\text{Ca}^{2+}$  [5][17][18][21]. Although its cDNA has not yet been cloned, NAT has been characterized with crude or partially purified enzyme preparations from the brain [22–24] and testis [25] of rats, and the brain [26] and heart [27][28] of dogs. Phosphatidylcholine (PC), 1-acyl-lyso-PC, PE, and cardiolipin were used as acyl donors, while free fatty acid or acyl-CoA were not used [22–30].

Scheme 1. *The Biosynthesis of Anandamide and Other N-Acylethanolamines via the Transacylation-Phosphodiesterase Pathway*



It should be noted that the acyl chain was exclusively abstracted from the *sn*-1 position of these phospholipids. It seemed that the enzyme has no substrate specificity in terms of the fatty acid species at the *sn*-1 position of the acyl donors [25]. Various ethanolamine phospholipids (diacyl-PE, alkylacyl-PE, ethanolamine plasmalogen and lyso-PE) acted as acyl-acceptor substrates [22–30]. NAT is a membrane-bound protein [24][27], and can be solubilized with *Nonidet P-40* [24]. As for the subcellular distribution of NAT, the activity of canine brain was the highest in microsomal fractions, followed by synaptosomes and mitochondria [27]. The optimal pH for the enzyme reaction was in the range 7–10, depending on different preparations and different assay conditions [22][23][27][28]. High-energy compounds such as ATP were not necessary for the transacylation [27].  $\text{Ca}^{2+}$  potently stimulated the activity, and it could be replaced with  $\text{Sr}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Ba}^{2+}$  [24][28]. Although treatment of cultured cells with calcium ionophores leads to accumulation of NAPEs and NAEs [31][32], it is



expression level of RLP-1 mRNA was by far the highest in testis among various rat organs, and the cytosol of testis actually exhibited a  $\text{Ca}^{2+}$ -independent, cytosolic activity of *N*-acylation. To distinguish RLP-1 from the known  $\text{Ca}^{2+}$ -dependent NAT ( $\text{Ca}^{2+}$ -NAT), we propose to refer to this novel enzyme as  $\text{Ca}^{2+}$ -independent NAT (iNAT). Although the physiological significance of iNAT currently remains unclear, the enzyme may be responsible for the effective formation of anandamide in testis.

### 3. *N*-Acylphosphatidylethanolamine-Hydrolyzing Phospholipase D (NAPE-PLD).

– 3.1. *Molecular Properties*. Although NAPE-PLD has been recognized for more than 20 years, its molecular cloning was only recently achieved by our group [19]. We highly purified this enzyme from the particulate fraction of rat heart using four steps of column chromatography [19]. When the final preparation was analyzed by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE), a 46-kDa protein was a promising candidate for NAPE-PLD. This protein was digested with trypsin, and the resulting peptides were micro-sequenced. The determined sequences of two peptides were then found in a single cDNA clone of mouse with the aid of a database. By further database search, rat and human homologous sequences of this mouse cDNA were also found.

We next cloned the putative NAPE-PLD cDNAs from mouse, rat, and human, and confirmed their nucleotide sequences. The deduced amino acid sequences (*Fig. 2*) from the three animal species were composed of 396 (mouse and rat) or 393 (human) residues, and their amino acid identity was 95.5% between mouse and rat, 89.1% between mouse and human, and 90.4% between rat and human, respectively. The molecular masses were calculated to be in the range 45–46 kDa, in good agreement with the value estimated on the basis of SDS-PAGE.

The sequences of NAPE-PLD did not show any homology with those of PLDs so far reported from various organisms. Instead, NAPE-PLD was suggested to belong to the metallo- $\beta$ -lactamase family, a superfamily including a wide variety of hydrolases [35][36]. Members of this superfamily are characterized by a highly conserved motif: H-X-[E/H]-X-D-[C/R/S/H]-X<sub>(50–70)</sub>-H-X<sub>(15–30)</sub>-[C/S/D]-X<sub>(30–70)</sub>-H [35][36]. The aspartic acid and histidine residues, highly conserved in this motif, have been suggested to be involved in binding and processing of substrates [35][36]. Based on the multiple sequence alignment, three aspartic acid residues (Asp<sup>147</sup>, Asp<sup>189</sup>, and Asp<sup>284</sup>) and five histidine residues (His<sup>185</sup>, His<sup>187</sup>, His<sup>190</sup>, His<sup>253</sup>, and His<sup>331</sup>) of NAPE-PLD were presumed to be such conserved residues, being completely conserved among rat, mouse, and human NAPE-PLDs [19].

When various aspartic acid and histidine residues of rat NAPE-PLD were separately replaced with asparagine by site-directed mutagenesis [37], the NAPE-PLD activity was abrogated with D147N, H253N, and D284N, and was remarkably reduced (<0.1% of the activity of the wild type) with H185N, H187N, D189N, and H190N. However, H331N still showed a significant activity, but H321N was completely inactive. These results of our mutagenesis studies on NAPE-PLD were in good agreement with those obtained for other members of the metallo- $\beta$ -lactamase family such as *Escherichia coli* and *Arabidopsis thaliana* ribonuclease Z [38][39], human Artemis [40][41], *Bacillus thuringiensis* *N*-acyl-L-homoserine lactone hydrolase [42], and IMP-1 metallo- $\beta$ -lactamase [43–45]. In *Fig. 2*, the catalytically important aspartic

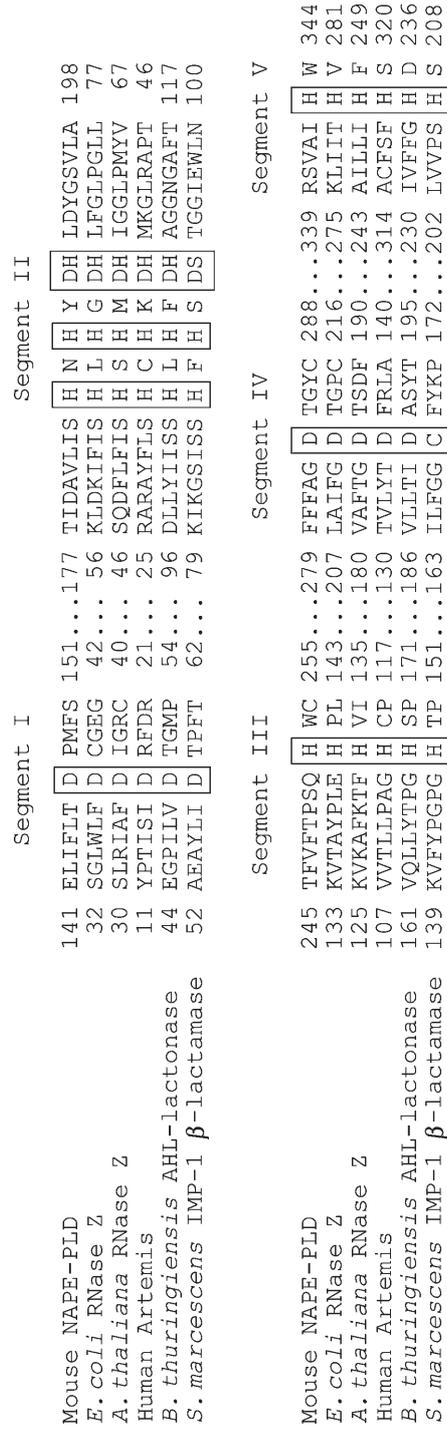


Fig. 2. Amino acid sequences of NAPE-PLD with representative members of the metallo- $\beta$ -lactamase family. Highly conserved Asp (D) and His (H) residues are boxed. Alignment over five segments of the highly conserved motif is shown. GenBank accession numbers of mouse NAPE-PLD, *Escherichia coli*, and *Arabidopsis thaliana* ribonuclease (RNase) Z, human Artemis, *Bacillus thuringiensis* N-acyl-L-homoserine lactone hydrolase (AHL-lactonase), and *Serratia marcescens* IMP-1  $\beta$ -lactamase are BAD02397, P0A8V0, Q8L GU7, Q96SD1, ABD93924, and BAD34719, resp.

acid and histidine residues conserved among NAPE-PLD and these family members are shown. Thus, the catalytic mechanism of NAPE-PLD was suggested to be similar to those of the well-characterized members of this family.

With the aid of ModBase [46] and 3D-JIGSAW [47], we performed homology-based protein modeling of the conserved domain spanning L125-D389 of NAPE-PLD using the known crystal structure of *Bacillus subtilis* ribonuclease Z (PDB code 1y44) [48] as a template (Fig. 3) [37]. This model predicted that His<sup>185</sup>, His<sup>187</sup>, and His<sup>253</sup> of NAPE-PLD bind to the first metal ion, Asp<sup>189</sup>, His<sup>190</sup>, and His<sup>343</sup> bind to the second metal ion, and Asp<sup>284</sup> forms a bridge between these two metal ions. Because we found that H343N is as active as the wild type, further examination is required to clarify the role of His<sup>343</sup> in metal binding. His<sup>321</sup> may be involved in positioning of the phosphate moiety of the substrate NAPE. Asp<sup>147</sup> was assumed to form salt bridges to the conserved motif backbone, probably stabilizing the conformation of the motif to coordinate optimally the metal ions [35].

Zn<sup>2+</sup> is generally contained in the members of the metallo- $\beta$ -lactamase family [35][36]. However, we were not able to determine the precise number of Zn<sup>2+</sup> ions per NAPE-PLD molecule by means of atomic-absorption spectroscopy (AAS), but revealed that the Zn<sup>2+</sup> content of each preparation of the purified enzyme is well-correlated with its specific activity, suggesting the presence of catalytically crucial Zn<sup>2+</sup> in NAPE-PLD [37].

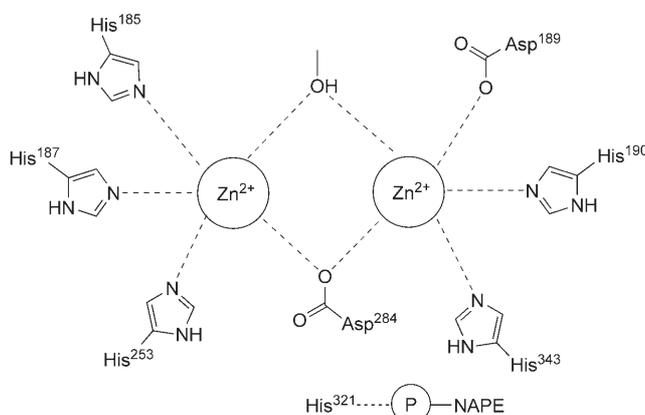


Fig. 3. Predicted model of Zn coordination in NAPE-PLD. The conserved domain spanning L<sup>125</sup>–D<sup>389</sup> was modeled based on the crystal structure of *Bacillus subtilis* ribonuclease Z (PDB ID 1y44) [48] using ModBase [46] and 3D-JIGSAW [47].

Recombinant NAPE-PLD was inhibited by the SH blocker *para*-chloromercuribenzoic acid, with an  $IC_{50}$  value of *ca.* 3  $\mu$ M [49], which suggested the presence of catalytically important cysteine residue(s) in NAPE-PLD. Six cysteine residues (Cys<sup>170</sup>, Cys<sup>222</sup>, Cys<sup>224</sup>, Cys<sup>237</sup>, Cys<sup>255</sup>, and Cys<sup>288</sup>) – conserved among rat, mouse, and human NAPE-PLDs – were replaced individually with serine by site-directed mutagenesis, and these mutants were examined for their NAPE-PLD activities. The results showed that only C224S caused a considerable reduction in activity, suggesting that Cys<sup>224</sup> is a target for development of inhibitors [37]. The NCBI human database for single nucleotide

polymorphism (SNP) revealed four SNPs in the open reading frame of the human NAPE-PLD gene. Although these SNPs did not appear to link to any diseases according to the database, they caused the substitution of an amino acid residue, and generated S152A, L207F, H380R, and D389N. Importantly, these four amino acid residues were completely conserved among human, rat, and mouse NAPE-PLDs, and L207F and H380R revealed a markedly reduced NAPE-hydrolyzing activity [37].

By further database search, we found that *Caenorhabditis elegans* Y37E11AR.3c and *Saccharomyces cerevisiae* YPL103c show considerable homology with NAPE-PLD [50]. These genes had 42.5% and 30.5% identity with mouse NAPE-PLD at amino acid level, respectively, when the amino acid residues 52–361 of mouse NAPE-PLD were compared with the corresponding regions of these genes. Although the functions of the gene products remain unclear, *Merkel et al.* [51] reported the endogenous NAPE and NAE levels with a yeast strain lacking the YPL103c gene. The deletion of the YPL103c gene caused a slight increase in the total amount of NAPE, and a concomitant decrease in that of NAEs by 60% compared with the wild-type, suggesting that the YPL103c product partially contributes to the conversion of NAPE to NAE in yeast. A recent report based on BLAST search also suggested that the genes of *S. cerevisiae* and *C. elegans* are functional NAPE-PLD orthologues [52].

Information on the locus and intron–exon structure of the NAPE-PLD gene is also available from database [53]. The NAPE-PLD genes from human (GeneID 222236) and mouse (242864) are assigned to chromosomes 7 and 5, respectively. The coding regions of both human and mouse NAPE-PLD cDNAs are composed of four exons, and the highly conserved metallo- $\beta$ -lactamase motif is localized in exons 3 and 4.

**3.2. Catalytic Properties.** Earlier characterization of NAPE-PLD has been performed with crude preparations or partially purified preparations [22][25][54–58]. We intended the characterization of a purified recombinant NAPE-PLD. However, our initial attempt to purify the recombinant NAPE-PLD from *E. coli* by a conventional method was unsuccessful, because the expressed protein was mostly insoluble and inactive [19]. Later, we expressed a fusion protein of rat NAPE-PLD and glutathione *S*-transferase (GST) in *E. coli*, together with the chaperone protein GroEL and cochaperonin GroES [37]. The expressed fusion protein was solubilized with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and was purified by chromatography using glutathione-*Sepharose 4B*. By the digestion with *PreScission* protease and the second cycle of glutathione-affinity chromatography, the GST tag was removed, and the resultant GST-free NAPE-PLD was further purified by chromatography on hydroxyapatite. With *N*-palmitoyl-PE as substrate, the purified enzyme showed a specific activity of *ca.* 2.0  $\mu\text{mol}/\text{min}/\text{mg}$  protein [37].

The activity of the purified enzyme was enhanced in a dose-dependent manner up to 15- to 30-fold by increasing concentrations of  $\text{MgCl}_2$  and  $\text{CaCl}_2$ , with  $EC_{50}$  values of 2.3 and 1.4 mM, respectively. The effect of  $\text{Mg}^{2+}$  could be replaced not only with  $\text{Ca}^{2+}$ , but also with other divalent cations such as  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ba}^{2+}$ , and  $\text{Sr}^{2+}$ , although their stimulatory effects varied. In contrast,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Hg}^{2+}$ , and  $\text{Zn}^{2+}$  were found inhibitory. These results with the purified recombinant enzyme were in agreement with our previous findings for the partially purified NAPE-PLD from rat heart [57][58]. Considering the intracellular  $\text{Mg}^{2+}$ , present at a concentration of 20 mM, the enzyme appeared to be constitutively active in the cell. *Triton X-100* [22][54][55][57] and

spermine [58] were also reported to stimulate NAPE-PLD. However, a synergistic effect of  $\text{Ca}^{2+}$ , *Triton X-100*, and spermine was not observed [58]. Octyl glucoside (0.1%, w/v) acted as a weak activator for the purified recombinant enzyme, and showed a synergistic effect with  $\text{Mg}^{2+}$  [37].

Earlier studies with crude enzyme preparations suggested high specificity of NAPE-PLD toward NAPEs [54]. We investigated substrate specificity of the pure recombinant enzyme [37]. The enzyme was almost inactive with major glycerophospholipids in biomembranes (PC, PE, phosphatidylserine (PS), and phosphatidylinositol). As compared at 25  $\mu\text{M}$ , the enzyme reacted with these lipids at rates of less than 0.1% of that with NAPE. Moreover, the enzyme hardly hydrolyzed phospholipids structurally related to NAPE, including *N*-acyl-PS, phosphatidylethanol, and phosphatidylbutanol. Such high substrate specificity may be critical for constitutively active NAPE-PLD to minimize damage of membrane phospholipids. Crude preparations of NAPE-PLD were previously reported not to show selectivity with respect to long-chain *N*-acyl groups of NAPEs [19][22]. Our extensive studies with the pure recombinant enzyme demonstrate that NAPE-PLD does not discriminate the length ( $\text{C}_4$ – $\text{C}_{20}$ ) of medium- and long-chain *N*-acyl species of NAPEs, suggesting that NAPE-PLD is responsible for the degradation of a variety of NAPEs with different *N*-acyl groups *in vivo* [37].

To examine the role in the substrate recognition of the *sn*-1 and *sn*-2 acyl chains and glycerol structure of NAPE, partially digested compounds were prepared from *N*-palmitoyl-PE [37]. The specific activities with *N*-palmitoyl-lyso-PE and glycerophospho(*N*-palmitoyl)ethanolamine were only 4% and 1% of that with *N*-palmitoyl-PE, respectively. Furthermore, *N*-palmitoylethanolamine phosphate was totally inactive. Thus, enzymes other than NAPE-PLD may be responsible for rather high reactivities with these partially digested compounds in rat brain [37].

**3.3. Distribution in Organs.** NAPE-PLD Activity has been shown to be widely distributed in mammalian tissues. With homogenates from various rat organs, the heart exhibited the highest activity, followed by brain, testis, kidney, spleen, liver, and lung [54]. Our group also showed a similar organ distribution in rat with proteins solubilized from microsomes [58]. Among bovine organs, the brain exhibited the highest specific activity, followed by kidney, spleen, lung, heart, and liver [59]. Moreover, we detected NAPE-PLD activity in almost all of the mouse organs, with higher specific activities in brain, kidney, and testis [19]. As examined by RT-PCR and Western blotting, the expression levels of the mRNA and protein of NAPE-PLD showed a similar distribution to that of the NAPE-PLD activity [19]. It should be noted that the brain consistently exhibits a high NAPE-PLD activity in all animal species.

We further investigated regional distribution of NAPE-PLD in rat brain by enzyme assay, Western blotting, and real-time PCR [49]. The results revealed wide distribution of this enzyme in various brain regions, with the highest expression level in thalamus. In rat brain, the expression level of fatty acid amide hydrolase (FAAH), the principal NAE-hydrolyzing enzyme, was previously reported to be significantly high in hippocampus, cortex, and cerebellum [60][61], and we also observed a similar distribution pattern of the FAAH activity [49]. This distribution is known to be similar to that of the CB1 receptor [60][62]. Therefore, the regional distribution of NAPE-

PLD was not necessarily consistent with those of FAAH and CB1 receptor, suggesting a role of NAPE-PLD not related to the endocannabinoid system.

Age-dependent increase of the NAPE-PLD activity was previously reported with brains of rat [23][33] and mouse [63]. We showed that the NAPE-PLD activity of rat brain increased dramatically with development, being very low at birth, and the increase of the NAPE-PLD activity was in parallel with the increase of expression levels of NAPE-PLD protein and mRNA, suggesting induction of NAPE-PLD at transcriptional level [49]. This was in contrast with the NAT activity in rat brain, which decreased age-dependently, as described above. The opposite age dependency of the NAPE-PLD and NAT levels may rationalize the remarkable postmortem accumulation of NAPEs in the brains of infant rats, but not in those of adult rats [33]. Age-dependent increase in FAAH activity was also reported with rat brain [23]. However, when compared between 1-day- and 56-day-old rats, the increase in the NAPE-PLD activity was much larger than that in the FAAH activity (40-fold vs. 3-fold) [49]. Earlier, it was noted that NAPEs as well as NAPEs remarkably accumulated in the lesion of canine models of cardiac infarction [64][65]. However, unlike the brain, obvious species difference was observed in the NAPE-PLD activity of heart, being very high in rat and guinea pig, but relatively low in mouse, rabbit, frog, cat, dog, cow, mini pig, and human [66]. Interestingly, the species difference in the NAT activity of heart was considerably different from that of NAPE-PLD, being high only in dog and cat [66]. In contrast to the brain, the NAPE-PLD activity in rat heart did not change for 70 d after birth [66]. Although we reported an age-dependent increase in the NAPE-PLD level with rat heart [49], this result was not clearly reproduced in our later assays (unpublished data).

Recently, the role of the endocannabinoid system in the regulation of female and male fertility in mammals has attracted much attention [67–73]. NAPE-PLD was expressed in mouse oviduct on days one to four of pregnancy and pre-implantation embryos [70]. NAPE-PLD was detected in the oviduct epithelium at the isthmus region, with much lower levels in the ampullary region by *in situ* hybridization, and in embryos at all stages from fertilized one-cell embryos to blastocysts by RT-PCR and Western blotting [70]. In contrast, FAAH was expressed at higher levels in the epithelium at the ampullary region, with lower levels in the isthmus, and its expression was dramatically upregulated at the morula and blastocyst stages after first detected in two-cell embryos [70]. These different expression patterns of NAPE-PLD and FAAH suggested that a coordination of the anandamide synthesis and degradation contributes to pre-implantation embryo development and oviductal transport of developing embryos [70]. It was also reported that a low level of NAPE-PLD was detected primarily in the luminal and glandular epithelia in mouse uterus prior to implantation (on days one to four of pregnancy) by *in situ* hybridization, and that, in inter-implantation sites, its expression level was increased significantly after the initiation of implantation (on days five to seven) [68]. The endogenous anandamide level in uterus was also changed in parallel with the NAPE-PLD activity during implantation. Furthermore, it was shown that ovarian steroid hormones including progesterone and 17 $\beta$ -estradiol down-regulated the uterine NAPE-PLD expression *via* their nuclear receptors. These results suggested that NAPE-PLD is a major player in regulating the anandamide level in the uterus during early pregnancy [68].

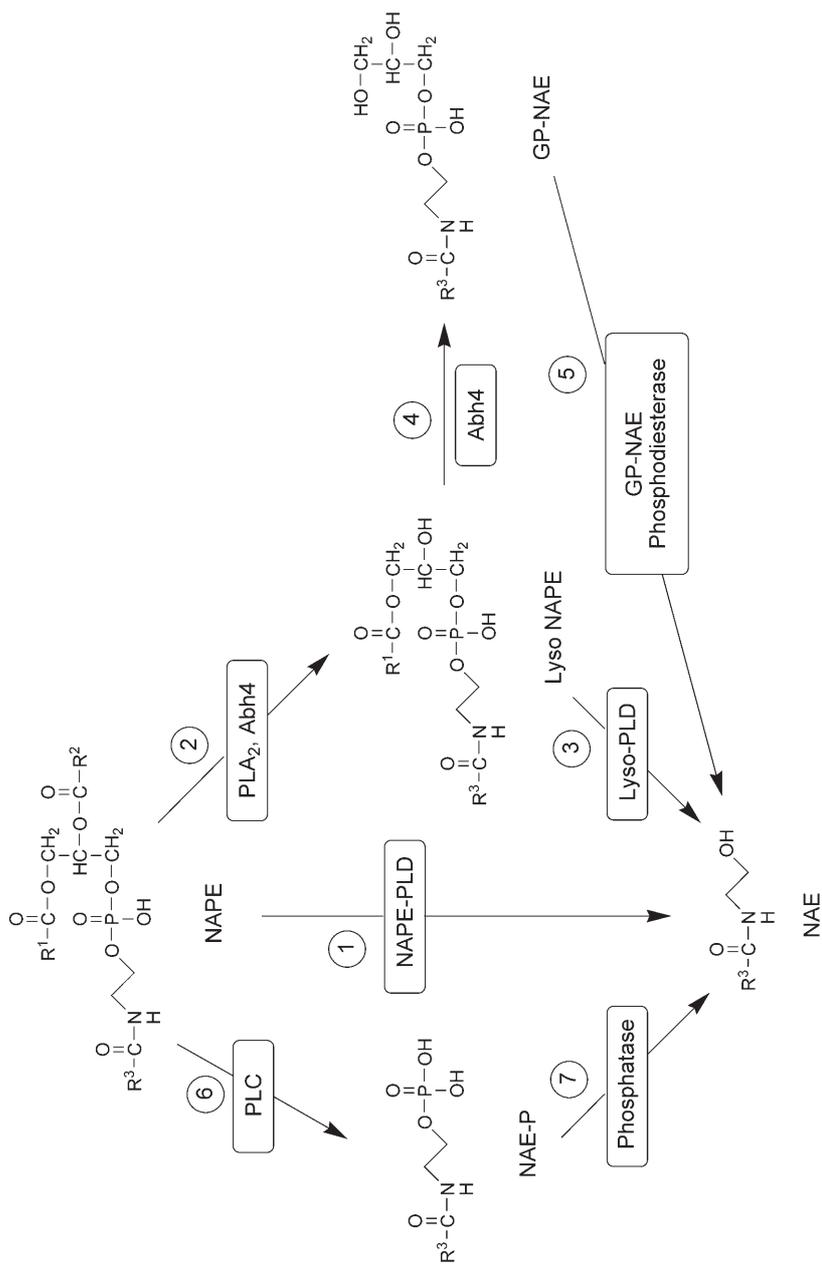
In addition, boar sperm cells were shown to have NAPE-PLD as well as other endocannabinoid-related proteins, including CB1, TRPV1, anandamide membrane transporter, and FAAH [69]. Endogenous anandamide was in fact detected in sperm cells as well as seminal plasma [69][73]. A high concentration of anandamide appeared to inhibit capacitation *via* a cAMP-dependent pathway, but to be associated with the stabilization of acrosome membranes by activating TRPV1 once the capacitation is completed [69]. These findings suggested regulation of mammalian sperm functions by anandamide signaling.

**3.4. NAPE-PLD-Deficient Mice.** Recently, *Leung et al.* [20] successfully generated mice that possess a targeted disruption of the NAPE-PLD gene (NAPE-PLD<sup>-/-</sup>). Western blotting with anti-NAPE-PLD antibody confirmed the defect of NAPE-PLD in the brain and spinal cord of the mice. These NAPE-PLD<sup>-/-</sup> mice were viable and healthy, and showed no obvious abnormality in their cage behavior. The activity forming *N*-palmitoylethanolamine from *N*-palmitoyl-PE was found to decrease to varying degrees with homogenates of the brain, heart, kidney, testis and spinal cord. The activity forming anandamide from *N*-arachidonoyl-PE was also significantly reduced in brain, but was unchanged in testis. Interestingly, differences in endogenous levels of NAPes and NAEs between NAPE-PLD<sup>-/-</sup> mice and wild-type mice were dependent on *N*-acyl species of NAPes and NAEs. Namely, disruption of NAPE-PLD caused a remarkable increase in the endogenous brain levels of saturated and monounsaturated NAPes, with a concomitant decrease in the levels of their corresponding NAEs. In contrast, little or no changes were observed for the levels of polyunsaturated NAPes and NAEs. These results suggested that NAPE-PLD is actually responsible for the conversion of NAPes to NAEs *in vivo*, but other enzyme(s) or pathway(s) are also involved in the syntheses of NAEs, especially polyunsaturated NAEs, including anandamide.

**4. Other Biosynthetic Pathways of Anandamide and Other *N*-Acylethanolamines from *N*-Acylphosphatidylethanolamines.** – As discussed above, our molecular cloning and subsequent analysis of NAPE-PLD confirmed the presence of an enzyme specifically hydrolyzing NAPes. This finding supported the physiological importance of the transacylation-phosphodiesterase pathway in the biosynthesis of anandamide and other NAEs. However, considering the existence of multiple pathways for the phospholipid metabolism, it was likely that NAEs are formed from NAPes by different enzymes or pathways. Earlier, *Natarajan et al.* [55] referred to such pathways, which were composed of two or three steps of hydrolysis reactions (*Scheme 2*). In these pathways, NAPes were hydrolyzed to release one or two *O*-acyl chains from the glycerol backbone, followed by hydrolytic cleavage of the phosphodiester bond of the resulting *N*-acyl-lyso-PE or glycerophospho-NAE (GP-NAE) [55]. In rat brain, the PLD-type hydrolyzing activity for *N*-acyl-lyso-PE and GP-NAE was actually observed [37][55]. Since NAPE-PLD has poor or no activities for these compounds, as described above, it was suggested that phosphodiesterases other than NAPE-PLD are mostly responsible for the PLD-type hydrolysis of these compounds [37].

Our group reported a PLA<sub>1</sub>/A<sub>2</sub> activity to hydrolyze *N*-palmitoyl-PE to *N*-palmitoyl-lyso-PE in various rat tissues, with by far the highest activity in stomach. The stomach enzyme was purified and identified as a group-IB secretory PLA<sub>2</sub> (sPLA<sub>2</sub>-IB)

Scheme 2. Multiple Biosynthetic Pathways Involved in the Formation of Anandamide and Other NAEs from NAPEs. NAE-P refers to N-acylethanolamine phosphate.



[74]. Recombinant preparations of group IB, IIA, and V of sPLA<sub>2</sub> were also active with *N*-palmitoyl-PE, while group X PLA<sub>2</sub> and cytosolic PLA<sub>2</sub>- $\alpha$  were much less active. We also detected a lyso PLD-like activity releasing *N*-palmitoylethanolamine from *N*-palmitoyl-lyso-PE in various rat tissues, with higher activities in brain and testis. The lyso PLD-like enzyme was membrane-associated, and its catalytic properties were different from those of NAPE-PLD. However, we did not perform its further purification or characterization. *N*-Arachidonoyl-PE and *N*-arachidonoyl-lyso-PE, the anandamide precursors, were also active substrates for sPLA<sub>2</sub>-IB and the lyso-PLD-like enzyme. These results could indicate that the pathway composed of steps 2 and 3 in *Scheme 2* contributes to the biosynthesis of NAEs, including that of anandamide.

As mentioned above, based on the metabolic analysis of NAPE-PLD-deficient mice, *Cravatt's* group [20] suggested the presence of another pathway(s) in the conversion of NAEs to NAEs. Especially, the anandamide formation appeared to be independent of NAPE-PLD. Furthermore, the same group proposed a new pathway in which a phospholipase catalyzes double-deacylation of NAPE, followed by the hydrolysis of the resultant GP-NAE to NAE and glycerol-3-phosphate (steps 2, 4, and 5 in *Scheme 2*) [75]. Inhibition by methyl arachidonyl fluorophosphonate suggested that the phospholipase is a serine hydrolase. Using functional-proteomic isolation, they identified  $\alpha/\beta$ -hydrolase-4 (Abh4) as a lipase to deacylate both *N*-acyl-lyso-PE and NAPE. Distribution of the *N*-acyl-lyso-PE lipase activity in mouse organs showed the highest activity in brain, spinal cord, and testis, followed by liver, kidney, and heart. The mRNA level of Abh4 was similarly distributed in mice, suggesting that Abh4 is responsible for this activity in various mouse organs. Abh4 showed a high reactivity with *N*-acyl-lyso-PE as compared with other lysophospholipids, but did not discriminate different *N*-acyl species of *N*-acyl-lyso-PE, including *N*-arachidonoyl-lyso-PE. Furthermore, it was shown that mouse brain and testis have an enzyme to rapidly cleave the phosphodiester bond of GP-NAE to generate NAE. Thus, the combination of Abh4 and a GP-NAE phosphodiesterase might participate in the formation of NAEs from NAEs as an NAPE-PLD-independent pathway. However, it remained unclear whether the anandamide formation in NAPE-PLD<sup>-/-</sup> mice can be rationalized by this pathway. The above-mentioned lyso PLD-like activity (step 3 in *Scheme 2*) may be explained, at least in part, by this pathway (steps 4 and 5).

*Liu et al.* [76] recently reported another anandamide biosynthetic pathway from *N*-arachidonoyl-PE with lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages and mouse brain [76]. They suggested that the pathway comprises a PLC-like enzyme generating anandamide phosphate from *N*-arachidonoyl-PE and a phosphatase dephosphorylating anandamide phosphate (steps 6 and 7 in *Scheme 2*). The expression of NAPE-PLD mRNA in RAW264.7 cells was downregulated by LPS, while the PLC inhibitor neomycin reduced the anandamide-generating activity. Thus, this pathway appeared to contribute to the increase of anandamide level by LPS. Moreover, the extracts from RAW264.7 cells and mouse brain converted anandamide phosphate to anandamide in a sodium orthovanadate (a non-selective tyrosine phosphatase inhibitor)-sensitive manner. Using the cDNA subtraction method between LPS-treated and untreated RAW264.7 cells, they identified PTPN22, a non-receptor protein tyrosine phosphatase, as a candidate for the enzyme dephosphorylating anandamide

phosphate. However, since this reaction was not completely eliminated even in the absence of PTPN22, other enzymes might also be involved.

**5. Perspectives.** – cDNA Cloning and characterization of NAPE-PLD strongly suggested the physiological significance of the transacylation-phosphodiesterase pathway in the formation of bioactive NAEs. Further studies, including the analysis of NAPE-PLD<sup>-/-</sup> mice, revealed the presence of other NAE-generating pathways independent of NAPE-PLD in animal tissues. It will be of interest to clarify whether or not an anandamide-specific pathway exists *in vivo*. Identification of key enzymes involved in these novel pathways such as lyso-PLD, GP-NAE phosphodiesterase, and NAPE-hydrolyzing PLC will be essential to elucidate the roles of these pathways. Although we discovered Ca<sup>2+</sup>-independent NAT, the cDNA of the known Ca<sup>2+</sup>-dependent NAT has not been cloned yet. Its cDNA cloning and detailed analysis on the structure and function are expected.

#### REFERENCES

- [1] V. Di Marzo, *Biochim. Biophys. Acta* **1998**, 1392, 153.
- [2] L. A. Matsuda, S. J. Lolait, M. J. Brownstein, A. C. Young, T. I. Bonner, *Nature* **1990**, 346, 561.
- [3] W. A. Devane, L. Hanus, A. Breuer, R. G. Pertwee, L. A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, R. Mechoulam, *Science* **1992**, 258, 1946.
- [4] M. van der Stelt, M. Trevisani, V. Vellani, L. De Petrocellis, A. Schiano Moriello, B. Campi, P. McNaughton, P. Geppetti, V. Di Marzo, *EMBO J.* **2005**, 24, 3026.
- [5] H. H. O. Schmid, P. C. Schmid, E. V. Berdyshev, *Chem. Phys. Lipids* **2002**, 121, 111.
- [6] A. Calignano, G. La Rana, A. Giuffrida, D. Piomelli, *Nature* **1998**, 394, 277.
- [7] D. M. Lambert, S. Vandevoorde, K.-O. Jonsson, C. J. Fowler, *Curr. Med. Chem.* **2002**, 9, 663.
- [8] F. Rodríguez de Fonseca, M. Navarro, R. Gomez, L. Escuredo, F. Nava, J. Fu, E. Murillo-Rodríguez, A. Giuffrida, J. LoVerme, S. Gaetani, S. Kathuria, C. Gall, D. Piomelli, *Nature* **2001**, 414, 209.
- [9] M. Maccarrone, R. Pauselli, M. Di Rienzo, A. Finazzi-Agrò, *Biochem. J.* **2002**, 366, 137.
- [10] S. Terrazzino, F. Berto, M. Dalle Carbonare, M. Fabris, A. Guiotto, D. Bernardini, A. Leon, *FASEB J.* **2004**, 18, 1580.
- [11] C. C. Felder, E. M. Briley, J. Axelrod, J. T. Simpson, K. Mackie, W. A. Devane, *Proc. Natl. Acad. Sci. U.S.A.* **1993**, 90, 7656.
- [12] J. Fu, S. Gaetani, F. Oveisi, J. LoVerme, A. Serrano, F. Rodríguez de Fonseca, A. Rosengarth, H. Luecke, B. Di Giacomo, G. Tarzia, D. Piomelli, *Nature* **2003**, 425, 90.
- [13] J. LoVerme, J. Fu, G. Astarita, G. La Rana, R. Russo, A. Calignano, D. Piomelli, *Mol. Pharmacol.* **2005**, 67, 15.
- [14] P. Movahed, B. A. G. Jönsson, B. Birnir, J. A. Wingstrand, T. D. Jorgensen, A. Ermund, O. Sterner, P. M. Zygumt, E. D. Högestatt, *J. Biol. Chem.* **2005**, 280, 38496.
- [15] D. Baker, G. Pryce, W. L. Davies, C. R. Hiley, *Trends Pharmacol. Sci.* **2006**, 27, 1.
- [16] H. A. Overton, A. J. Babbs, S. M. Doel, M. C. Fyfe, L. S. Gardner, G. Griffin, H. C. Jackson, M. J. Procter, C. M. Rasamison, M. Tang-Christensen, P. S. Widdowson, G. M. Williams, C. Reynet, *Cell Metab.* **2006**, 3, 167.
- [17] H. H. O. Schmid, P. C. Schmid, V. Natarajan, *Prog. Lipid Res.* **1990**, 29, 1.
- [18] H. S. Hansen, B. Moesgaard, H. H. Hansen, G. Petersen, *Chem. Phys. Lipids* **2000**, 108, 135.
- [19] Y. Okamoto, J. Morishita, K. Tsuboi, T. Tonai, N. Ueda, *J. Biol. Chem.* **2004**, 279, 5298.
- [20] D. Leung, A. Saghatelian, G. M. Simon, B. F. Cravatt, *Biochemistry* **2006**, 45, 4720.
- [21] M. van der Stelt, H. H. Hansen, W. B. Veldhuis, P. R. Bar, K. Nicolay, G. A. Veldink, J. F. G. Vliegthart, H. S. Hansen, *Neurotox. Res.* **2003**, 5, 183.

- [22] T. Sugiura, S. Kondo, A. Sukagawa, T. Tonegawa, S. Nakane, A. Yamashita, Y. Ishima, K. Waku, *Eur. J. Biochem.* **1996**, *240*, 53.
- [23] V. Natarajan, P. C. Schmid, H. H. O. Schmid, *Biochim. Biophys. Acta* **1986**, *878*, 32.
- [24] H. Cadas, E. di Tomaso, D. Piomelli, *J. Neurosci.* **1997**, *17*, 1226.
- [25] T. Sugiura, S. Kondo, A. Sukagawa, T. Tonegawa, S. Nakane, A. Yamashita, K. Waku, *Biochem. Biophys. Res. Commun.* **1996**, *218*, 113.
- [26] V. Natarajan, P. C. Schmid, P. V. Reddy, M. L. Zuzarte-Augustin, H. H. O. Schmid, *J. Neurochem.* **1983**, *41*, 1303.
- [27] V. Natarajan, P. V. Reddy, P. C. Schmid, H. H. O. Schmid, *Biochim. Biophys. Acta* **1982**, *712*, 342.
- [28] P. V. Reddy, P. C. Schmid, V. Natarajan, T. Muramatsu, H. H. O. Schmid, *Biochim. Biophys. Acta* **1984**, *795*, 130.
- [29] P. V. Reddy, P. C. Schmid, V. Natarajan, H. H. O. Schmid, *Biochim. Biophys. Acta* **1983**, *751*, 241.
- [30] P. V. Reddy, V. Natarajan, P. C. Schmid, H. H. O. Schmid, *Biochim. Biophys. Acta* **1983**, *750*, 472.
- [31] H. Cadas, S. Gaillet, M. Beltramo, L. Venance, D. Piomelli, *J. Neurosci.* **1996**, *16*, 3934.
- [32] H. S. Hansen, L. Lauritzen, A. M. Strand, B. Moesgaard, A. Frandsen, *Biochim. Biophys. Acta* **1995**, *1258*, 303.
- [33] B. Moesgaard, G. Petersen, J. W. Jaroszewski, H. S. Hansen, *J. Lipid Res.* **2000**, *41*, 985.
- [34] X.-H. Jin, Y. Okamoto, J. Morishita, K. Tsuboi, T. Tonai, N. Ueda, *J. Biol. Chem.* **2007**, *282*, 3614.
- [35] L. Aravind, *In Silico Biol.* **1999**, *1*, 69.
- [36] H. Daiyasu, K. Osaka, Y. Ishino, H. Toh, *FEBS Lett.* **2001**, *503*, 1.
- [37] J. Wang, Y. Okamoto, J. Morishita, K. Tsuboi, A. Miyatake, N. Ueda, *J. Biol. Chem.* **2006**, *281*, 12325.
- [38] A. Vogel, O. Schilling, W. Meyer-Klaucke, *Biochemistry* **2004**, *43*, 10379.
- [39] B. Späth, S. Kirchner, A. Vogel, S. Schubert, P. Meinlschmidt, S. Aymanns, J. Nezzar, A. Marchfelder, *J. Biol. Chem.* **2005**, *280*, 35440.
- [40] U. Pannicke, Y. Ma, K.-P. Hopfner, D. Niewolik, M. R. Lieber, K. Schwarz, *EMBO J.* **2004**, *23*, 1987.
- [41] C. Poinsignon, D. Moshous, I. Callebaut, R. de Chasseval, I. Villey, J.-P. de Villartay, *J. Exp. Med.* **2004**, *199*, 315.
- [42] M. H. Kim, W. C. Choi, H. O. Kang, J. S. Lee, B. S. Kang, K.-J. Kim, Z. S. Derewenda, T.-K. Oh, C. H. Lee, J.-K. Lee, *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 17606.
- [43] S. Haruta, H. Yamaguchi, E. T. Yamamoto, Y. Eriguchi, M. Nukaga, K. O'Hara, T. Sawai, *Antimicrob. Agents Chemother.* **2000**, *44*, 2304.
- [44] I. C. Materon, T. Palzkill, *Protein Sci.* **2001**, *10*, 2556.
- [45] I. C. Materon, Z. Beharry, W. Huang, C. Perez, T. Palzkill, *J. Mol. Biol.* **2004**, *344*, 653.
- [46] U. Pieper, N. Eswar, H. Braberg, M. S. Madhusudhan, F. P. Davis, A. C. Stuart, N. Mirkovic, A. Rossi, M. A. Marti-Renom, A. Fiser, B. Webb, D. Greenblatt, C. C. Huang, T. E. Ferrin, A. Sali, *Nucleic Acids Res.* **2004**, *32*, 217.
- [47] P. A. Bates, L. A. Kelley, R. M. MacCallum, M. J. E. Sternberg, *Proteins* **2001**, *45* (Suppl. 5), 39.
- [48] C. Frazão, G. Silva, C. M. Gomes, P. Matias, R. Coelho, L. Sieker, S. Macedo, M. Y. Liu, S. Oliveira, M. Teixeira, A. V. Xavier, C. Rodrigues-Pousada, M. A. Carrondo, J. Le Gall, *Nat. Struct. Biol.* **2000**, *7*, 1041.
- [49] J. Morishita, Y. Okamoto, K. Tsuboi, M. Ueno, H. Sakamoto, N. Maekawa, N. Ueda, *J. Neurochem.* **2005**, *94*, 753.
- [50] N. Ueda, Y. Okamoto, K. Tsuboi, *Curr. Med. Chem.* **2005**, *12*, 1413.
- [51] O. Merkel, P. C. Schmid, F. Paltauf, H. H. O. Schmid, *Biochim. Biophys. Acta* **2005**, *1734*, 215.
- [52] J. M. McPartland, I. Matias, V. Di Marzo, M. Glass, *Gene* **2006**, *370*, 64.
- [53] N. Ueda, Y. Okamoto, J. Morishita, *Life Sci.* **2005**, *77*, 1750.
- [54] P. C. Schmid, P. V. Reddy, V. Natarajan, H. H. O. Schmid, *J. Biol. Chem.* **1983**, *258*, 9302.
- [55] V. Natarajan, P. C. Schmid, P. V. Reddy, H. H. O. Schmid, *J. Neurochem.* **1984**, *42*, 1613.
- [56] G. Petersen, H. S. Hansen, *FEBS Lett.* **1999**, *455*, 41.
- [57] N. Ueda, Q. Liu, K. Yamanaka, *Biochim. Biophys. Acta* **2001**, *1532*, 121.
- [58] Q. Liu, T. Tonai, N. Ueda, *Chem. Phys. Lipids* **2002**, *115*, 77.
- [59] G. Petersen, K. D. Chapman, H. S. Hansen, *J. Lipid Res.* **2000**, *41*, 1532.
- [60] M. Egertova, D. K. Giang, B. F. Cravatt, M. R. Elphick, *Proc. R. Soc. Lond., Sect. B* **1998**, *265*, 2081.

- [61] E. A. Thomas, B. F. Cravatt, P. E. Danielson, N. B. Gilula, J. G. Sutcliffe, *J. Neurosci. Res.* **1997**, *50*, 1047.
- [62] L. Iversen, *Brain* **2003**, *126*, 1252.
- [63] B. Moesgaard, H. H. Hansen, S. L. Hansen, S. H. Hansen, G. Petersen, H. S. Hansen, *Lipids* **2003**, *38*, 387.
- [64] D. E. Epps, P. C. Schmid, V. Natarajan, H. H. O. Schmid, *Biochem. Biophys. Res. Commun.* **1979**, *90*, 628.
- [65] D. E. Epps, V. Natarajan, P. C. Schmid, H. H. O. Schmid, *Biochim. Biophys. Acta* **1980**, *618*, 420.
- [66] B. Moesgaard, G. Petersen, S. A. Mortensen, H. S. Hansen, *Comp. Biochem. Physiol., Sect. B* **2002**, *131*, 475.
- [67] B. Park, J. M. McPartland, M. Glass, *Prostaglandins Leukot. Essent. Fatty Acids* **2004**, *70*, 189.
- [68] Y. Guo, H. Wang, Y. Okamoto, N. Ueda, P. J. Kingsley, L. J. Marnett, H. H. O. Schmid, S. K. Das, S. K. Dey, *J. Biol. Chem.* **2005**, *280*, 23429.
- [69] M. Maccarrone, B. Barboni, A. Paradisi, N. Bernabò, V. Gasperi, M. G. Pistilli, F. Fezza, P. Lucidi, M. Mattioli, *J. Cell Sci.* **2005**, *118*, 4393.
- [70] H. Wang, H. Xie, Y. Guo, H. Zhang, T. Takahashi, P. J. Kingsley, L. J. Marnett, S. K. Das, B. F. Cravatt, S. K. Dey, *J. Clin. Invest.* **2006**, *116*, 2122.
- [71] H. Wang, S. K. Dey, M. Maccarrone, *Endocr. Rev.* **2006**, *27*, 427.
- [72] H. Schuel, L. J. Burkman, *Biol. Reprod.* **2005**, *73*, 1078.
- [73] H. Schuel, L. J. Burkman, J. Lippes, K. Crickard, E. Forester, D. Piomelli, A. Giuffrida, *Chem. Phys. Lipids* **2002**, *121*, 211.
- [74] Y.-X. Sun, K. Tsuboi, Y. Okamoto, T. Tonai, M. Murakami, I. Kudo, N. Ueda, *Biochem. J.* **2004**, *380*, 749.
- [75] G. M. Simon, B. F. Cravatt, *J. Biol. Chem.* **2006**, *281*, 26465.
- [76] J. Liu, L. Wang, J. Harvey-White, D. Osei-Hyiaman, R. Razdan, Q. Gong, A. C. Chan, Z. Zhou, B. X. Huang, H. Y. Kim, G. Kunos, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 13345.

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