Review Article

Molecular characterization of a novel lysosomal enzyme degrading the anti-inflammatory lipid mediator *N*-acylethanolamine

Kazuhito Tsuboi

Department of Biochemistry, Kagawa University School of Medicine, Kagawa, Japan

N-Acylethanolamines (NAEs) represent a class of endogenous bioactive lipids generated from glycerophospholipids, and they include *N*-palmitoylethanolamine (an anti-inflammatory substance) and *N*-arachidonoylethanolamine (anandamide, an endogenous ligand of cannabinoid receptor). It is generally accepted that NAEs are hydrolyzed to free fatty acids and ethanolamine by the catalysis of fatty acid amide hydrolase (FAAH) acting principally at alkaline and neutral pH. Several years ago, our laboratory found another enzyme catalyzing the same reaction only at acidic pH. However, the molecular cloning and detailed analysis of this enzyme, termed "*N*-acylethanolamine-hydrolyzing acid amidase (NAAA)", have not yet been performed. In the present study, we purified the enzyme from rat lung, and cloned its cDNA from human, mouse, and rat. The primary structures revealed that NAAA had no homology to FAAH, but revealed 33-35% amino acid identity to lysosomal acid ceramidase, which hydrolyzes ceramide to free fatty acids and sphingosine at acidic pH. Recombinant human NAAA hydrolyzed various NAEs, and exhibited the highest activity toward *N*-palmitoylethanolamine. In addition, a low ceramide-hydrolyzing activity was detected with NAAA. We also observed a lysosome-like distribution of NAAA-green fluorescence protein fusion protein, as expressed in HEK293 cells. The organ distribution of mRNA and enzyme activity in rats revealed its wide distribution with the highest level in lung. Interestingly, macrophages expressed NAAA abundantly, suggesting a role of this enzyme in inflammation. By using an NAAA-selective inhibitor which we developed, we showed that NAAA and FAAH cooperatively degraded NAEs in macrophages. Taken together, our results indicated that NAAA is a novel lysosomal enzyme having structural and functional similarity to acid ceramidase, and functions as a second NAE hydrolase in mammalian tissues.

Rec./Acc.12/1/2006, pp18-27

Correspondence should be addressed to:
Kazuhito Tsuboi, Department of Biochemistry, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793, Japan. Phone: +81-87-891-2104, Fax: +81-87-891-2105, e-mail: ktsuboi@med.kagawa-u.ac.jp

Key words lipid mediator, *N*-acylethanolamine, anandamide, fatty acid amide hydrolase, lysosome

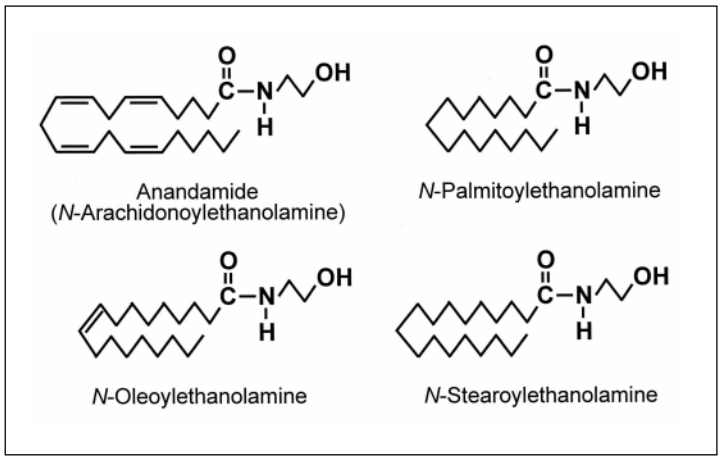

Fig.1 Chemical structures of NAEs

Occurrence and biological activities of *N*-acylethanolamines

N-Acylethanolamines (NAEs) are ethanolamides of long-chain fatty acids, which represent a class of endogenous bioactive lipids[1]. Since the late 1950s, saturated and monounsaturated NAEs such as *N*-palmitoylethanolamine, *N*-stearoylethanolamine, and *N*-oleoylethanolamine (Fig.1) have been recognized to be trace, but ubiquitous components in animal tissues[2]. In 1992, Devane et al. isolated an endogenous ligand of cannabinoid receptor (referred to as "endocannabinoid") from porcine brain, and identified it as *N*-arachidonoylethanolamine (anandamide) (Fig.1)[3]. Thereafter, other polyunsaturated NAEs were also reported to be endocannabinoids[4]. It is now established that anandamide functions as a partial or full agonist of the central cannabinoid receptor CB1, which is abundantly expressed in the nervous system, and shows a variety of cannabimimetic activities[4,5]. Unlike CB1 receptor, the peripheral cannabinoid receptor CB2 is expressed mainly in immune cells. 2-Arachidonoylglycerol is well known to be a full agonist of CB2 as well as CB1, and shows pro-inflammatory actions through CB2[6]. However, the efficacy of anandamide to bind to CB2 is low, and the role of anandamide in inflammation is unclear. More recently, anandamide was also reported to act as an agonist of transient receptor potential vanilloid type 1[7].

In contrast to polyunsaturated NAEs, saturated and monounsaturated NAEs are inactive with cannabinoid receptors. However, these NAEs show a variety of biological activities[1,8,9]. For example, *N*-palmitoylethanolamine exhibits anti-inflammatory[10,11], immunosuppressive[12], neuroprotective[13], and analgesic[14] actions. The molecular targets of *N*-palmitoylethanolamine accounting for these biological actions have been a matter of debate[15]. The neuroprotective action and anti-inflammatory effect were reported to be at least partially mediated by an unknown G protein-coupled receptor in microglial cells[16] and peroxisome proliferator-activated receptor-α (PPAR-α)[17], respectively. Interestingly, in inflammatory pain models, the analgesic actions of *N*-palmitoylethanolamine were partially suppressed by SR144528, a selective CB2 receptor antagonist[18], in spite of the poor affinity of *N*-palmitoylethanolamine for CB2 receptor[19]. This apparently contradictory observation might be explained by the presence of another SR144528-sensitive receptor or by an "entourage" effect of *N*-palmitoylethanolamine, which enhances the action of endogenous endocannabinoids by inhibiting their enzymatic degradation[8]. More recently, *N*-palmitoylethanolamine as well as anandamide was reported to be an agonist of GPR55, a G protein-coupled receptor[20]. In addition, the anorexic action of *N*-oleoylethanolamine was reported and suggested to be mediated by PPAR-α[21]. *N*-Stearoylethanolamine was pro-apoptotic[22] and anorexic[23]. It was also reported that *N*-stearoylethanolamine exerted cannabimimetic actions in the central nervous system through interacting with its specific binding sites in the brain[24].

Biosynthesis and degradation of NAEs

It is generally accepted that NAEs are principally biosynthesized from membrane phospholipids by two steps of enzyme reactions in animal tissues (Fig.2)[1,25]. The first step is the transfer of an acyl group at the *sn*-1 position in glycerophospholipids to the amide group of phosphatidylethanolamine to yield *N*-acylphosphatidylethanolamine (NAPE). In the second step, NAPE is hydrolyzed to NAE and phosphatidic acid by a specific phospholipase D (PLD), termed NAPE-PLD. Our laboratory cloned cDNA of NAPE-PLD from mouse, rat, and human[26]. NAPE-PLD belonged to the metallo-β-lactamase family, and was highly specific for NAPE among various glycerophospholipids[27].

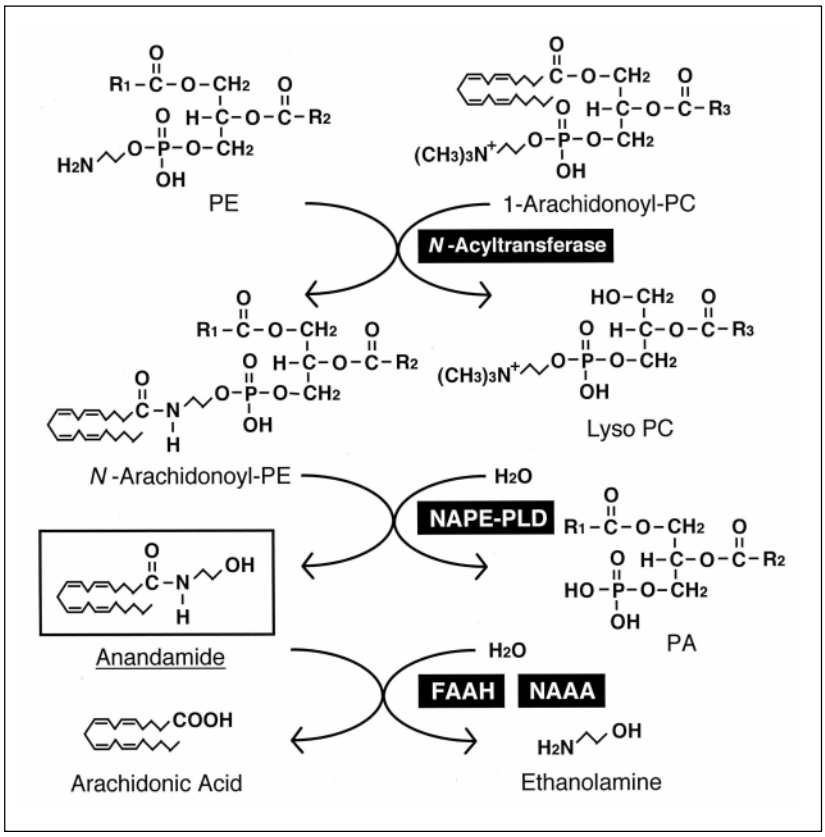

Fig.2 Biosynthesis and degradation of NAE

The pathway for biosynthesis and degradation of anandamide as a representative NAE is shown. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Recent analysis on NAPE-PLD-deficient mice revealed its considerable contribution to the *in vivo* formation of NAEs, but also suggested the involvement of other enzyme(s) or pathway(s) in the NAE formation[28,29].

NAEs including anandamide are hydrolyzed to free fatty acids and ethanolamine after their cellular uptake. This intracellular degradation is mostly attributable to a membrane-bound enzyme, fatty acid amide hydrolase (FAAH)[30,31]. The cDNA was cloned in 1996, and found to be a member of the amidase signature family[32]. FAAH is widely distributed in mammalian tissues including liver and brain, and analysis of FAAH-deficient mice revealed the central role of this enzyme in the degradation of anandamide and other NAEs in the brain[33,34]. The catalysis by FAAH is characterized by an optimal pH value at 8.5-10 and high sensitivity to serine hydrolase inhibitors, such as phenylmethylsulfonyl fluoride and methyl arachidonyl fluorophosphonate. It is also noted that this enzyme prefers anandamide to other NAEs including *N*-palmitoylethanolamine. A number of specific FAAH inhibitors have been developed[35]. They are expected as new therapeutic drugs for anxiety, cancer, and neurodegenerative disorders. Very recently, an isozyme of FAAH was discovered in human, and termed FAAH-2 to distinguish this isozyme from the known FAAH (FAAH-1)[36].

Molecular characterization of NAAA

In 1999, Ueda et al. reported that human megakaryoblastic (CMK) cells have an NAE-hydrolyzing activity, and suggested that the activity is attributable to an enzyme different from FAAH[37]. The most important property of this enzyme was the optimal pH value around 5, which was in sharp contrast to FAAH being active at neutral and alkaline pH. The enzyme was also distinguishable by the less sensitivity to two FAAH inhibitors, phenylmethylsulfonyl fluoride and methyl arachidonyl fluorophosphonate. Later, such an activity was also detected with various rat tissues including lung, spleen, and macrophages[38]. This enzyme, which we later termed "*N*-acylethanolamine-hydrolyzing acid amidase (NAAA)", could be solubilized from the 12000 x g pellet of rat lung homogenate by freezing and thawing, and was purified to apparent homogeneity by the use of four steps of column chromatography[38]. The purified NAAA exhibited a protein band around 31 kDa as analyzed by SDS-PAGE.

We purified NAAA from rat lung by this method, and then determined its N-terminal amino acid sequence by Edman degradation[39]. With the aid of database, we found cDNAs which

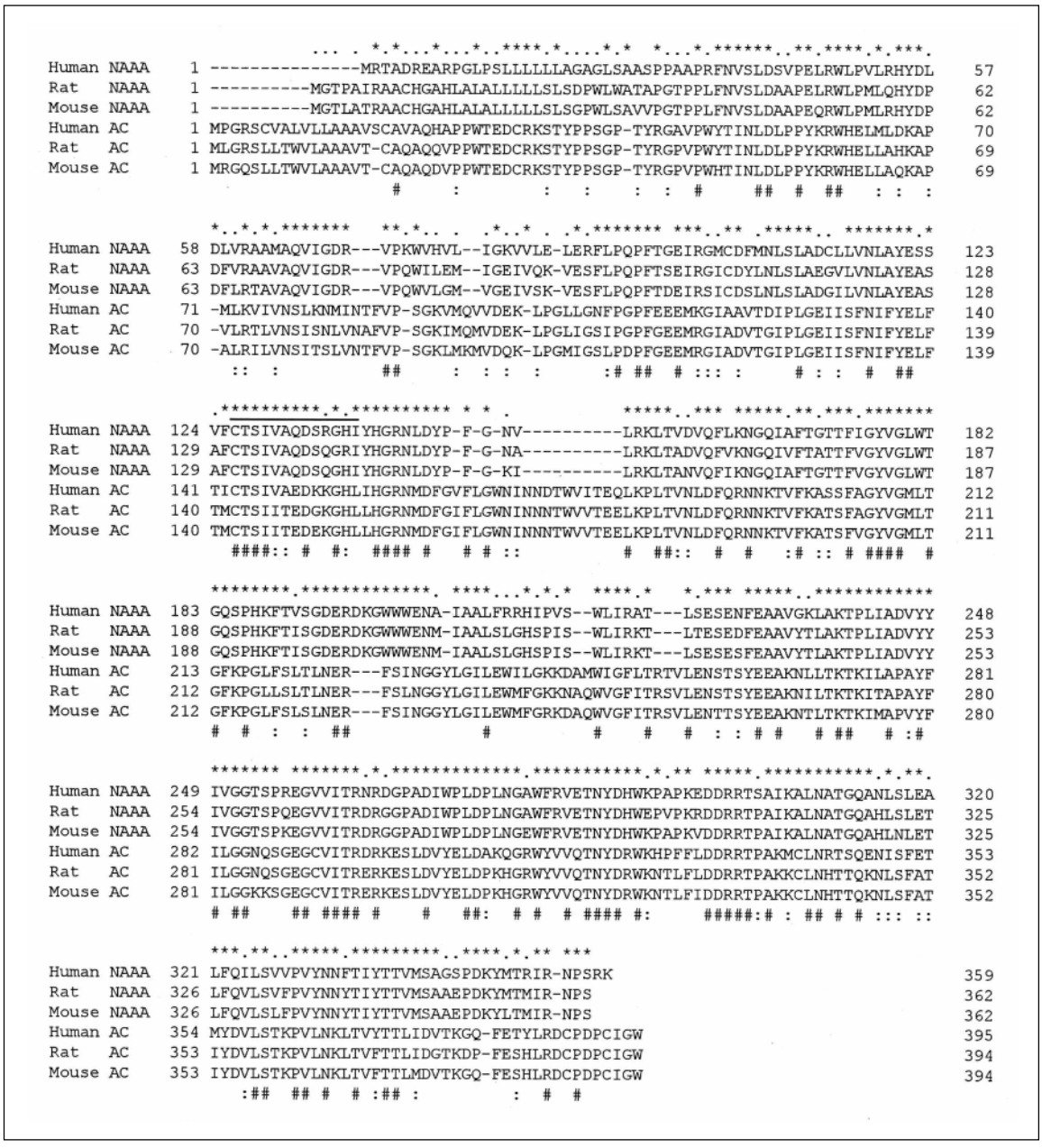

Fig.3 Amino acid sequences deduced from cDNAs for NAAA and acid ceramidase

Amino acid sequences deduced from NAAAs and acid ceramidases (ACs) of human, rat, and mouse are aligned. Asterisks and dots indicate identity shared by three or two NAAA proteins, respectively. Sharps and colons denote identity shared by six proteins or four to five proteins (among NAAAs and acid ceramidases), respectively. A line indicates the sequences corresponding with the N-terminal sequence of the purified rat NAAA protein, which we actually determined.

contained nucleotide sequences corresponding to this peptide sequence from rat, mouse, and human. We cloned the entire coding regions of these cDNAs by PCR, and confirmed NAAA activity of the recombinant protein overexpressed in HEK293 cells. The deduced amino acid sequences of NAAA are shown in Fig.3. The sequences consisted of 362 (rat and mouse) or 359 (human) residues with predicted molecular masses of 40.3 kDa (rat) or 40.1 kDa (mouse and human). The amino acid identities were 90.1% between rat and mouse, 76.5% between rat and human, and 76.7% between mouse and human. Five (rat and mouse) or six (human) potential *N*-glycosylation sites (Asn-Xxx-Ser/Thr) were found in the sequences. Importantly, NAAA had no ho-

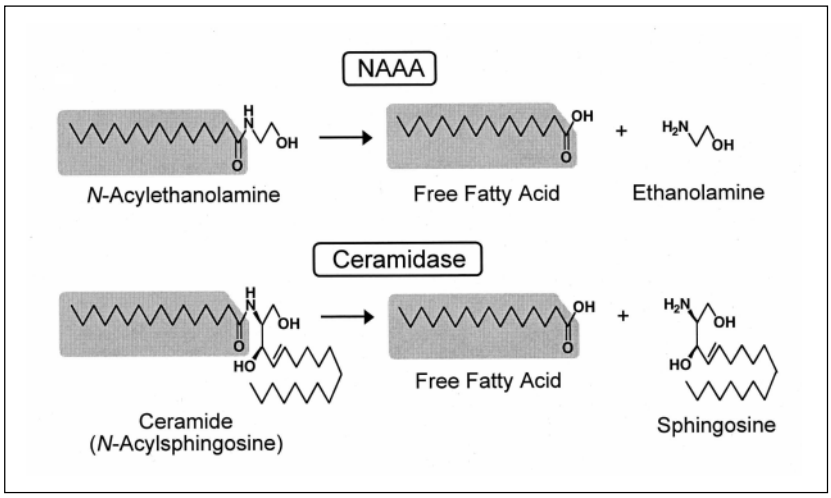

Fig.4 Similarity of the NAE hydrolysis to the ceramide hydrolysis

As a representative NAE and ceramide, *N*-palmitoylethanolamine and *N*-palmitoylsphingosine ($C_{16:0}$-ceramide) are shown, respectively.

mology with FAAH. The human NAAA sequence was essentially identical to that of "acid ceramidase-like protein", which was earlier cloned from human placenta by Hong et al.[40]. They found the gene product as a homologous protein with acid ceramidase, a lysosomal enzyme catalyzing the hydrolysis of ceramide under acidic conditions. However, they could not detect ceramide-hydrolyzing activity with this protein, and its function remained unclear until we identified it as NAAA. The amino acid sequences of acid ceramidase from human, rat, and mouse are also shown in Fig.3. Human NAAA revealed 33% identity and 70% similarity with human acid ceramidase over their entire length at protein level. Both of NAAA and acid ceramidase belong to the choloylglycine hydrolase family, which contains several hydrolases cleaving carbon-nitrogen bonds in linear amides except peptide bonds. As shown in Fig.4, the chemical structure of ceramide (*N*-acylsphingosine) is similar to that of NAE, and acid ceramidase and NAAA hydrolyze the amide bonds of ceramide and NAE, respectively. Such a functional similarity between the two enzymes was in agreement with their high sequence similarity. Human acid ceramidase consists of two different subunits (α and β) that are generated by the cleavage of a single precursor protein[41]. The N-terminal sequence of β-subunit of human acid ceramidase (CTSIVAED) exactly corresponded with the N-terminal sequence of the purified rat NAAA (CTSIVAQD), which we actually determined. Thus, NAAA and acid ceramidase appeared to be subjected to proteolytic cleavage at the same site. However, it remains unclear whether NAAA also exists as a heterodimer.

Catalytic properties of NAAA

As overexpressed in HEK293 cells, human NAAA hydrolyzed various NAEs including anandamide (Fig.5). The activity was stimulated in the presence of non-ionic detergent Nonidet P-40 (0.1%, w/v). The activity was also enhanced by dithiothreitol, and the optimal pH value was around 4.5. Under these assay conditions, *N*-palmitoylethanolamine was by far the most reactive substrate. These catalytic properties were in good agreement with those of native NAAA from rat lung[38], confirming the authenticity of NAAA cDNA. It should be noted that these catalytic properties were in contrast to those of FAAH, which is the most active around pH 8.5-10, is insensitive to dithiothreitol, and prefers anandamide to *N*-palmitoylethanolamine as substrate[37].

Since NAAA showed considerable sequence similarity to acid ceramidase as described above, we examined whether recombinant human acid ceramidase could hydrolyze NAEs. As expected, acid ceramidase hydrolyzed various NAEs at low rates, but *N*-lauroylethanolamine (ethanolamide of lauric acid) was the most reactive (Fig. 5). This substrate specificity regarding *N*-acyl species of NAEs was in good agreement with the previous observation that *N*-lauroylsphingosine ($C_{12:0}$-ceramide) was the best substrate among various ceramides with different *N*-acyl groups[42]. Conversely, we could also detect a low ceramide-hydrolyzing activity with recombinant NAAA. These observations further emphasized analogy of NAAA to acid ceramidase in catalytic functions.

Glycosylation, processing, and intracellular localization of NAAA

As discussed above, the amino acid sequence of human NAAA had six potential *N*-glycosylation sites. Furthermore, the molecular mass (31 kDa) of the purified rat NAAA estimated by SDS-PAGE was larger than the calculated value based on the amino acid

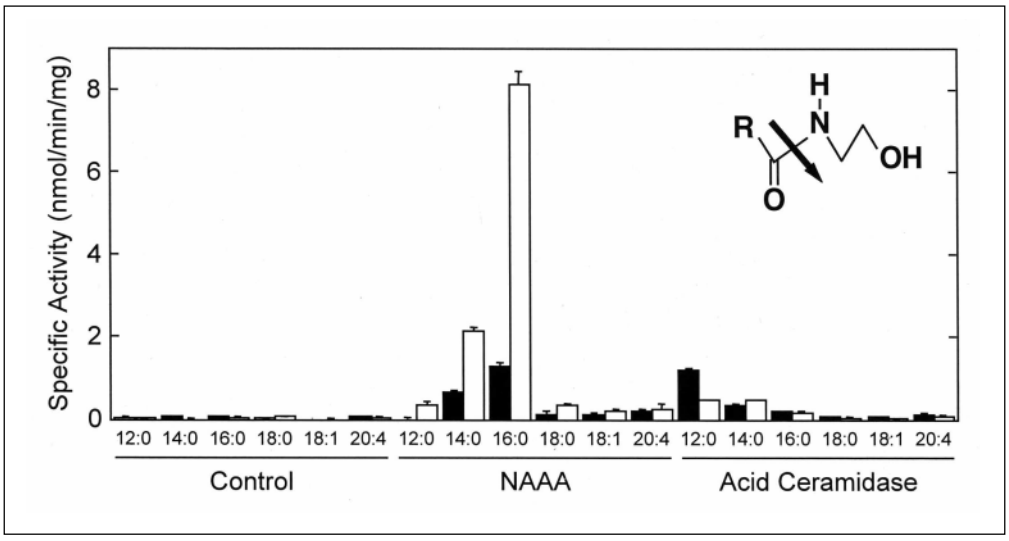

Fig. 5 Reactivity of NAAA and acid ceramidase with various NAEs

The homogenates of HEK293 cells overexpressing recombinant NAAA or acid ceramidase or control cells were allowed to react with various NAEs at pH 4.5 in the presence (open columns) or absence (closed columns) of 0.1% (w/v) Nonidet P-40. Mean ± S.D. are shown (n = 3). 12:0, *N*-lauroylethanolamine; 14:0, *N*-myristoylethanolamine; 16:0, *N*-palmitoylethanolamine; 18:0, *N*-stearoylethanolamine; 18:1, *N*-oleoylethanolamine; 20:4, anandamide.

sequence (26 kDa for the sequence from Cys-131 to Ser-362). These observations suggested the presence of N-glycan chains in NAAA. In fact, the treatment of recombinant hexahistidine-tagged NAAA with an endoglycosidase (peptide: *N*-glycosidase F) caused a decrease in the molecular weight, as examined by Western blotting with anti-hexahistidine antibody[39]. Furthermore, the putative glycosylated NAAA band was not detected when NAAA-transfected cells were cultured in the presence of tunicamycin, an inhibitor of N-type protein glycosylation. The tunicamycin treatment also resulted in the decrease in the specific enzyme activity, suggesting that glycosylation is necessary for maturation or stabilization of NAAA protein.

As mentioned above, native rat NAAA appeared to be cleaved between Phe-130 and Cys-131, and exist as the processed form. We found that recombinant NAAA was present as both the processed form and the unprocessed form when overexpressed in HEK293 cells. Interestingly, the latter form was converted to the former form under acidic conditions in a cell-free system[39]. Similar *in vitro* processing was reported with several lysosomal enzymes including cathepsin L[43] and tripeptidyl-peptidase I[44]. Although the processing of these two enzymes was suggested to be self-catalyzed, it remains unclear whether the cleavage of NAAA is catalyzed by NAAA itself or another enzyme.

We also examined intracellular localization of NAAA by the use of a fusion protein with green fluorescent protein. When cDNA for this fusion protein was constructed and introduced into HEK293 cells, the fluorescent signal showed lysosome-like distribution[39]. Similar intracellular distribution of recombinant "acid ceramidase-like protein" (identical to NAAA) was previously reported with COS-1 cells[40]. These observations suggested that native NAAA also localizes in lysosomes, and this localization was in consistence with its acidic pH optimum and its high sequence homology with lysosomal acid ceramidase.

Expression of NAAA in mammalian tissues

We could detect mRNA and activity of NAAA in a variety of rat organs[38,39]. Among the tested rat organs, the lung showed the highest level, followed by several organs including thymus, spleen, and intestine (Fig.6). Interestingly, Fegley et al. reported that URB597, a FAAH specific inhibitor, did not affect the endogenous levels of anandamide, *N*-palmitoylethanolamine, and *N*-oleoylethanolamine in rat duodenum[45]. Furthermore, an NAAA-like activity was observed with the duodenum of FAAH-deficient mice. Based on these results, the authors suggested a role of NAAA to degrade NAEs in this tissue. We also examined the tissue distribution of NAAA mRNA in mouse[46]. Overall, the distribution in mouse organs was similar to that in rat organs

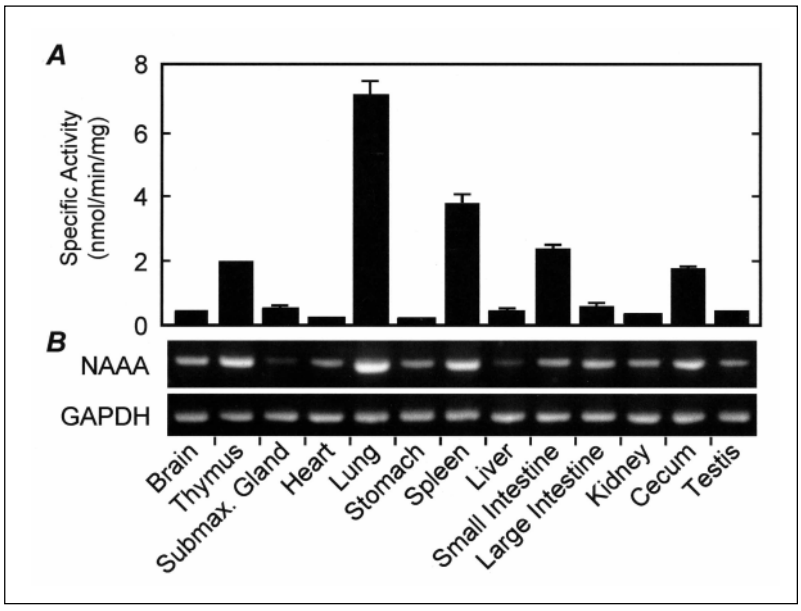

Fig. 6 Organ distribution of NAAA activity and mRNA in rat

(A) proteins were solubilized from the indicated rat organs by freezing and thawing, and were allowed to react with *N*-[^{14}C]palmitoylethanolamine at pH 5 in the presence of 0.1% (w/v) Triton X-100. Mean ± S.D. are shown (n = 3). (B) total RNA was isolated from the indicated rat organs and was subjected to RT-PCR using primers specific for NAAA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

with the highest level in lung. However, the level in mouse lung was not so prominent among various mouse organs. In contrast to the similarity between rat and mouse, the organ distribution of human NAAA appeared to be considerably different according to the previous report on that of mRNA for human "acid ceramidase-like protein"[40]. High expression levels were observed with liver and kidney, which exceeded that in lung. It should be also noted that the tissue distribution of NAAA in rodents is largely different from that of rodent FAAH, which was highly expressed in liver and brain. This difference suggests distinct physiological roles of the two enzymes.

In addition to various organs, NAAA was suggested to be expressed in various blood cells. NAAA activity was reported earlier with human megakaryoblastic cells (CMK)[37], and later with rat alveolar and peritoneal macrophages[38], rat basophilic leukemia cells (RBL-1)[47], and mouse macrophage cells (RAW264.7)[46]. Especially, a very high NAAA activity of rat alveolar and peritoneal macrophages was noted[38]. We also revealed expression of NAAA mRNA in various macrophage or macrophage-like cells of mouse (peritoneal macrophages, RAW264.7, and P388-D1) and human (U937 and THP-1)[46].

Development and application of NAAA inhibitors

Selective inhibitors are useful tools to elucidate the physiological role of an enzyme. We have developed several NAAA inhibitors which had lower inhibitory effects on FAAH[47,48]. The most potent compound was *N*-cyclohexanecarbonylpentadecylamine (CCP), which dose-dependently inhibited rat lung NAAA with an IC_{50} value of 4.5 μM, but did not inhibit rat liver FAAH at concentrations up to 100 μM. The inhibition was reversible and non-competitive. Since macrophage cells express both of NAAA (mentioned above) and FAAH[49,50], we investigated how these two enzymes share in the degradation of NAEs by the use of CCP and URB597[46]. First, we confirmed that intact RAW 264.7 and mouse peritoneal macrophage cells have ability to degrade exogenous radiolabeled NAEs including anandamide, *N*-palmitoylethanolamine, *N*-oleoylethanolamine, and *N*-stearoylethanolamine. Next, we showed that pretreatment of the cells with CCP or URB597 partially inhibited the degradation, respectively, and a combination of the two inhibitors caused more profound inhibition. These results suggested that NAAA and FAAH cooperatively degraded various NAEs in macrophages.

Conclusion

In the present study, we identified cDNA of NAAA for the first time. Its primary structure and catalytic properties demonstrated that NAAA is a second mammalian NAE-hydrolyzing enzyme distinguishable from FAAH. Moreover, NAAA appeared to be a novel lysosomal hydrolase with similarity to acid ceramidase. The high expression of NAAA in macrophages and the anti-inflammatory activity of NAEs suggest a role of this enzyme in inflammation. However, its physiological significance remains to be solved. Further investigation on NAAA, including immunohistochemistry and development of more potent inhibitors, will be required.

Acknowledgements

The present study was supervised by Prof. Natsuo Ueda of Department of Biochemistry, Kagawa University School of Medicine, and was collaborated with Drs. Y. Okamoto, Y.-X. Sun, L.-Y. Zhao (Department of Biochemistry, Kagawa University School of Medicine), N. Araki (Department of Histology and Cell Biology, Kagawa University School of Medicine), T. Tonai (National Hospital Organization Zentsuji National Hospital), and D. M. Lambert (Université catholique de Louvain, Belgium). This work was partially supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Japan Society for the Promotion of Science, and grants from The Uehara Memorial Foundation and Kagawa University.

References

1) Schmid HHO, Schmid PC, Natarajan V: N-Acylated glycerophospholipids and their derivatives. Prog Lipid Res, 29: 1-43, 1990.
2) Bachur NR, Masek K, Melmon KL, Udenfriend S: Fatty acid amides of ethanolamine in mammalian tissues. J Biol Chem, 240: 1019-1024, 1965.
3) Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R: Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science, 258: 1946-1949, 1992.
4) Di Marzo V: 'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance. Biochim Biophys Acta, 1392: 153-175, 1998.
5) Pacher P, Bátkai S, Kunos G: The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol Rev, 58: 389-462, 2006.
6) Sugiura T, Kishimoto S, Oka S, Gokoh M: Biochemistry, pharmacology and physiology of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand. Prog Lipid Res, 45: 405-446, 2006.
7) van der Stelt M, Di Marzo V: Endovanilloids. Putative endogenous ligands of transient receptor potential vanilloid 1 channels. Eur J Biochem, 271: 1827-1834, 2004.
8) Lambert DM, Di Marzo V: The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic? Curr Med Chem, 6: 757-773, 1999.
9) Lambert DM, Vandevoorde S, Jonsson KO, Fowler CJ: The palmitoylethanolamide family: a new class of anti-inflammatory agents? Curr Med Chem, 9: 663-674, 2002.
10) Facci L, Dal Toso R, Romanello S, Buriani A, Skaper SD, Leon A: Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. Proc Natl Acad Sci USA, 92: 3376-3380, 1995.
11) Mazzari S, Canella R, Petrelli L, Marcolongo G, Leon A: N-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation. Eur J Pharmacol, 300: 227-236, 1996.
12) Berdyshev EV, Boichot E, Germain N, Allain N, Anger JP, Lagente V: Influence of fatty acid ethanolamides and Δ^9-tetrahydrocannabinol on cytokine and arachidonate release by mononuclear cells. Eur J Pharmacol, 330: 231-240, 1997.
13) Skaper SD, Buriani A, Dal Toso R, Petrelli L, Romanello S, Facci L, Leon A: The ALIAmide palmitoylethanolamide and cannabinoids, but not anandamide, are protective in a delayed postglutamate paradigm of excitotoxic death in cerebellar granule neurons. Proc Natl Acad Sci USA, 93: 3984-3989, 1996.
14) Calignano A, La Rana G, Piomelli D: Antinociceptive activity of the endogenous fatty acid amide, palmitylethanolamide. Eur J Pharmacol, 419: 191-198, 2001.
15) Darmani NA, Izzo AA, Degenhardt B, Valenti M, Scaglione G, Capasso R, Sorrentini I, Di Marzo V: Involvement of the cannabimimetic compound, N-palmitoyl-ethanolamine, in inflammatory and neuropathic conditions: Review of the available pre-clinical data, and first human studies. Neuropharmacology, 48: 1154-1163, 2005.
16) Franklin A, Parmentier-Batteur S, Walter L, Greenberg DA, Stella N: Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. J Neurosci, 23: 7767-7775, 2003.
17) Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D: The nuclear receptor peroxisome proliferator-activated receptor-α mediates the anti-inflammatory actions of palmitoylethanolamide. Mol Pharmacol, 67: 15-19, 2005.
18) Jaggar SI, Hasnie FS, Sellaturay S, Rice AS: The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain. Pain, 76: 189-199, 1998.
19) Lambert DM, DiPaolo FG, Sonveaux P, Kanyonyo M, Govaerts SJ, Hermans E, Bueb J, Delzenne NM, Tschirhart EJ: Analogues and homologues of N-palmitoylethanolamide, a putative endogenous CB_2 cannabinoid, as potential ligands for the cannabinoid receptors. Biochim Biophys Acta, 1440: 266-274, 1999.
20) Baker D, Pryce G, Davies WL, Hiley CR: In silico patent

searching reveals a new cannabinoid receptor. Trends Pharmacol Sci, 27: 1-4, 2006.

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

22) Maccarrone M, Pauselli R, Di Rienzo M, Finazzi-Agro A: Binding, degradation and apoptotic activity of stearoylethanolamide in rat C6 glioma cells. Biochem J, 366: 137-144, 2002.

23) Terrazzino S, Berto F, Carbonare MD, Fabris M, Guiotto A, Bernardini D, Leon A: Stearoylethanolamide exerts anorexic effects in mice via down-regulation of liver stearoylcoenzyme A desaturase-1 mRNA expression. FASEB J, 18: 1580-1582, 2004.

24) Maccarrone M, Cartoni A, Parolaro D, Margonelli A, Massi P, Bari M, Battista N, Finazzi-Agro A: Cannabimimetic activity, binding, and degradation of stearoylethanolamide within the mouse central nervous system. Mol Cell Neurosci, 21: 126-140, 2002.

25) Schmid HHO: Pathways and mechanisms of N-acylethanolamine biosynthesis: can anandamide be generated selectively? Chem Phys Lipids, 108: 71-87, 2000.

26) Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N: Molecular characterization of a phospholipase D generating anandamide and its congeners. J Biol Chem, 279: 5298-5305, 2004.

27) Wang J, Okamoto Y, Morishita J, Tsuboi K, Miyatake A, Ueda N: Functional analysis of the purified anandamide-generating phospholipase D as a member of the metallo-β-lactamase family. J Biol Chem, 281: 12325-12335, 2006.

28) Leung D, Saghatelian A, Simon GM, Cravatt BF: Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. Biochemistry, 45: 4720-4726, 2006.

29) Simon GM, Cravatt BF: Endocannabinoid biosynthesis proceeding through glycerophospho-N-acyl ethanolamine and a role for α/β-hydrolase 4 in this pathway. J Biol Chem, 281: 26465-26472, 2006.

30) Ueda N: Endocannabinoid hydrolases. Prostaglandins Other Lipid Mediat, 68-69: 521-534, 2002.

31) Cravatt BF, Lichtman AH: Fatty acid amide hydrolase: an emerging therapeutic target in the endocannabinoid system. Curr Opin Chem Biol, 7: 469-475, 2003.

32) Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB: Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature, 384: 83-87, 1996.

33) Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH: Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proc Natl Acad Sci USA, 98: 9371-9376, 2001.

34) Clement AB, Hawkins EG, Lichtman AH, Cravatt BF: Increased seizure susceptibility and proconvulsant activity of anandamide in mice lacking fatty acid amide hydrolase. J Neurosci, 23: 3916-3923, 2003.

35) Bari M, Battista N, Fezza F, Gasperi V, Maccarrone M: New insights into endocannabinoid degradation and its therapeutic potential. Mini Rev Med Chem, 6: 257-268, 2006.

36) Wei BQ, Mikkelsen TS, McKinney MK, Lander ES, Cravatt BF: A second fatty acid amide hydrolase with variable distribution among placental mammals. J Biol Chem, 281: 36569-36578, 2006.

37) Ueda N, Yamanaka K, Terasawa Y, Yamamoto S: An acid amidase hydrolyzing anandamide as an endogenous ligand for cannabinoid receptors. FEBS Lett, 454: 267-270, 1999.

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

39) Tsuboi K, Sun Y-X, Okamoto Y, Araki N, Tonai T, Ueda N: Molecular characterization of N-acylethanolamine-hydrolyzing acid amidase, a novel member of the choloylglycine hydrolase family with structural and functional similarity to acid ceramidase. J Biol Chem, 280: 11082-11092, 2005.

40) Hong SB, Li CM, Rhee HJ, Park JH, He X, Levy B, Yoo OJ, Schuchman EH: Molecular cloning and characterization of a human cDNA and gene encoding a novel acid ceramidase-like protein. Genomics, 62: 232-241, 1999.

41) Koch J, Gartner S, Li CM, Quintern LE, Bernardo K, Levran O, Schnabel D, Desnick RJ, Schuchman EH, Sandhoff K: Molecular cloning and characterization of a full-length complementary DNA encoding human acid ceramidase. Identification of the first molecular lesion causing Farber disease. J Biol Chem, 271: 33110-33115, 1996.

42) Bernardo K, Hurwitz R, Zenk T, Desnick RJ, Ferlinz K, Schuchman EH, Sandhoff K: Purification, characterization, and biosynthesis of human acid ceramidase. J Biol Chem, 270: 11098-11102, 1995.

43) Ishidoh K, Kominami E: Multi-step processing of procathepsin L in vitro. FEBS Lett, 352: 281-284, 1994.
44) Lin L, Sohar I, Lackland H, Lobel P: The human CLN2 protein/tripeptidyl-peptidase I is a serine protease that autoactivates at acidic pH. J Biol Chem, 276: 2249-2255, 2001.
45) Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D: Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleoylethanolamide deactivation. J Pharmacol Exp Ther, 313: 352-358, 2005.
46) Sun Y-X, Tsuboi K, Zhao L-Y, Okamoto Y, Lambert DM, Ueda N: Involvement of N-acylethanolamine-hydrolyzing acid amidase in the degradation of anandamide and other N-acylethanolamines in macrophages. Biochim Biophys Acta, 1736: 211-220, 2005.
47) Tsuboi K, Hilligsmann C, Vandevoorde S, Lambert DM, Ueda N: N-Cyclohexanecarbonylpentadecylamine: a selective inhibitor of the acid amidase hydrolysing N-acylethanolamines, as a tool to distinguish acid amidase from fatty acid amide hydrolase. Biochem J, 379: 99-106, 2004.
48) Vandevoorde S, Tsuboi K, Ueda N, Jonsson KO, Fowler CJ, Lambert DM: Esters, retroesters, and a retroamide of palmitic acid: pool for the first selective inhibitors of N-palmitoylethanolamine-selective acid amidase. J Med Chem, 46: 4373-4376, 2003.
49) Maccarrone M, van der Stelt M, Rossi A, Veldink GA, Vliegenthart JF, Agro AF: Anandamide hydrolysis by human cells in culture and brain. J Biol Chem, 273: 32332-32339, 1998.
50) Di Marzo V, Bisogno T, De Petrocellis L, Melck D, Orlando P, Wagner JA, Kunos G: Biosynthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in circulating and tumoral macrophages. Eur J Biochem, 264: 258-267, 1999.