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Endogenous Molecules Stimulating *N*-Acylethanolamine-Hydrolyzing Acid Amidase (NAAA)

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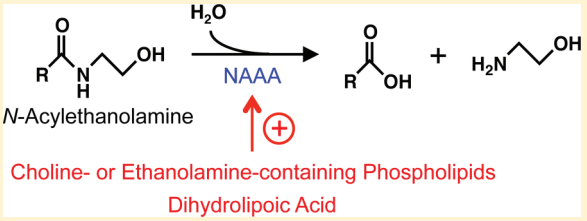
ABSTRACT: Fatty acid amide hydrolase (FAAH) plays the central role in the degradation of bioactive *N*-acylethanolamines such as the endocannabinoid arachidonoylethanolamide (anandamide) in brain and peripheral tissues. A lysosomal enzyme referred to as *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) catalyzes the same reaction with preference to palmitoylethanolamide, an endogenous analgesic and neuroprotective substance, and is therefore expected as a potential target of therapeutic drugs. In the *in vitro* assays thus far performed, the maximal activity of NAAA was achieved in the presence of both nonionic detergent (Triton X-100 or Nonidet P-40) and the SH reagent dithiothreitol. However, endogenous molecules that might substitute for these synthetic compounds remain poorly understood. Here, we examined stimulatory effects of endogenous phospholipids and thiol compounds on recombinant NAAA. Among different phospholipids tested, choline- or ethanolamine-containing phospholipids showed potent effects, and 1 mM phosphatidylcholine increased NAAA activity by 6.6-fold. Concerning endogenous thiol compounds, dihydrolipoic acid at 0.1−1 mM was the most active, causing 8.5−9.0-fold stimulation. These results suggest that endogenous phospholipids and dihydrolipoic acid may contribute in keeping NAAA active in lysosomes. Even in the presence of phosphatidylcholine and dihydrolipoic acid, however, the preferential hydrolysis of palmitoylethanolamide was unaltered. We also investigated a possible compensatory induction of NAAA mRNA in brain and other tissues of FAAH-deficient mice. However, NAAA expression levels in all the tissues examined were not significantly altered from those in wild-type mice.



KEYWORDS: *N-Acylethanolamine-hydrolyzing acid amidase, NAAA, palmitoylethanolamide, dihydrolipoic acid, phospholipid, fatty acid amide hydrolase*

Ethanolamides of long-chain fatty acids are referred to as *N*-acylethanolamines (NAEs) and ubiquitously occur in a variety of organisms including mammals.[1] NAEs exert various biological activities in the central nervous system as well as peripheral tissues by binding to and activating several receptors. For example, arachidonoylethanolamide (anandamide, AEA) behaves as an endogenous agonist for the cannabinoid receptors CB1 and CB2 as well as the vanilloid receptor TRPV1 (transient receptor potential vanilloid 1).[2−4] Palmitoylethanolamide (PEA) shows analgesic and anti-inflammatory effects through peroxisome proliferator-activated receptor α (PPARα).[5−7] Moreover, oleoylethanolamide (OEA) causes a decrease in food intake and gain of body weight,[8,9] which have been suggested to be mediated by PPARα, TRPV1, or the G-protein coupled receptor GPR119.[10−12] NAEs are also neuroprotective.[9,13]

These NAEs are enzymatically biosynthesized from membrane glycerophospholipids via their corresponding *N*-acylphosphatidylethanolamines (NAPEs).[14] NAEs are released from NAPEs by NAPE-hydrolyzing phospholipase D (NAPE-PLD)[15] or through multistep reactions catalyzed by two or more hydrolases.[16,17] Concerning the degradation of NAEs, the membrane-associated enzyme fatty acid amide hydrolase (FAAH) is considered to play the central role.[18,19] In fact, endogenous brain levels of AEA, PEA, and OEA were much higher in FAAH-deficient ($FAAH^{-/-}$) mice than in wild-type mice.[20,21] Similarly, the treatment of mice with specific FAAH inhibitors such as OL-135, URB597, and PF-04457845 caused significant increases in endogenous brain levels of these NAEs.[22−24]

However, we isolated and cloned a lysosomal enzyme that hydrolyzes NAEs at acidic pH in human and rodent tissues, and referred to this enzyme as NAE-hydrolyzing acid amidase (NAAA).[25−28] NAAA is a cysteine hydrolase belonging to the N-terminal nucleophile (Ntn) hydrolase superfamily,[28] and human NAAA has four *N*-glycosylation sites.[29,30] Similar to other members of this family, NAAA is produced as an inactive proenzyme and is activated by autocatalytic cleavage at a

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specific site of the peptide chain.[29] The mature form exists as a heterodimer composed of the resultant two peptides.[30] NAAA is detected in various tissues with its predominant expression in macrophages.[31,32] Among human tissues, prostate showed a relatively high expression level of NAAA.[33] Several selective NAAA inhibitors have been reported.[34−37] Since NAAA hydrolyzes PEA at a much higher rate than other NAEs at least *in vitro*,[26,27] selective NAAA inhibitors are expected to elevate local levels of PEA, which may focally exert anti-inflammatory and analgesic effects.[35,36]

To elucidate the physiological role of NAAA, its catalytic properties must be clarified in detail. In NAAA assays, both dithiothreitol (DTT) and nonionic detergents such as Triton X-100 and Nonidet P-40 are required for full catalytic activity.[25−27] Since these compounds are not biomolecules, we were interested in naturally occurring substances that can stimulate NAAA activity. In the present study, we report choline- or ethanolamine-containing phospholipids and dihydrolipoic acid (the reduced form of α-lipoic acid) as substitutes for nonionic detergent and DTT, respectively. Furthermore, since both FAAH and NAAA are expressed in mice and may share the degradation of NAEs *in vivo*, we investigated whether compensatory induction of NAAA occurs in FAAH$^{-/-}$ mice.

RESULTS AND DISCUSSION

Stimulation of NAAA by Phospholipids. Under our standard assay conditions for NAAA, the reaction mixture contained nonionic detergent of the polyoxyethylene *p-t*-octylphenyl ether type (Triton X-100 or Nonidet P-40) at 0.1% (w/v) as a stimulator.[26,27] We first examined the effect of various widely used detergents at 0.1% (w/v) on the PEA-hydrolyzing activity of recombinant rat NAAA (Figure 1). Triton X-100 and Nonidet P-40 increased the enzyme activity 4.4 ± 0.2- and 4.8 ± 0.4-fold, respectively. 3-[(3-Cholamidopropyl)dimethylammonio]-propanesulfonate (CHAPS) and octyl glucoside hardly affected the activity, while Tween 20 was inhibitory. These results were in agreement with our previous results using NAAA isolated from rat lung,[26] confirming that the polyoxyethylene *p-t*-octylphenyl ether-type detergents are the most effective.

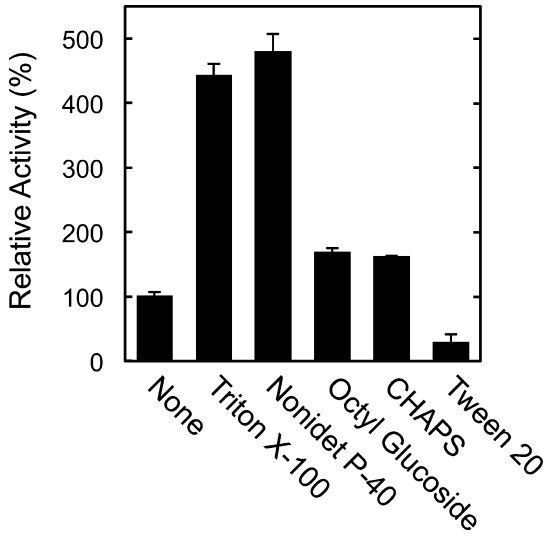

Figure 1. Effects of various detergents on NAAA activity. Recombinant NAAA (300 ng of protein) was allowed to react with 100 μM [^{14}C]PEA in the presence of 3 mM DTT and the indicated detergent at 0.1%. The enzyme activity in the absence of detergent (39.0 nmol/min/mg of protein) is expressed as 100%, and relative activities are shown (means ± SD, n = 3).

We next replaced the detergent with representative phospholipids at 100 μM. As shown in Figure 2A, choline- or ethanolamine-containing phospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM)) were the most effective and increased the activity 4.8 ± 0.2-, 4.5 ± 0.1-, and 4.2 ± 0.1-fold, respectively. However, acidic phospholipids were less stimulatory (phosphatidylinositol (PI) and phosphatidylglycerol (PG)) or inhibitory (phosphatidylserine (PS)). In the assays shown in Figure 2, the reaction mixture contained 5% ethanol, which was used as the vehicle of PC, PE, SM, and PG. Under these conditions, 0.1% (1.66 mM) Nonidet P-40 stimulated the activity 6.1 ± 0.3-fold. When different concentrations (3 μM−1 mM) of PC were present, NAAA activity was dose-dependently increased up to 6.6-fold (Figure 2B). Although the physiological significance of this stimulatory effect remains unclear, choline- or ethanolamine-containing phospholipids as major components of biomembrane

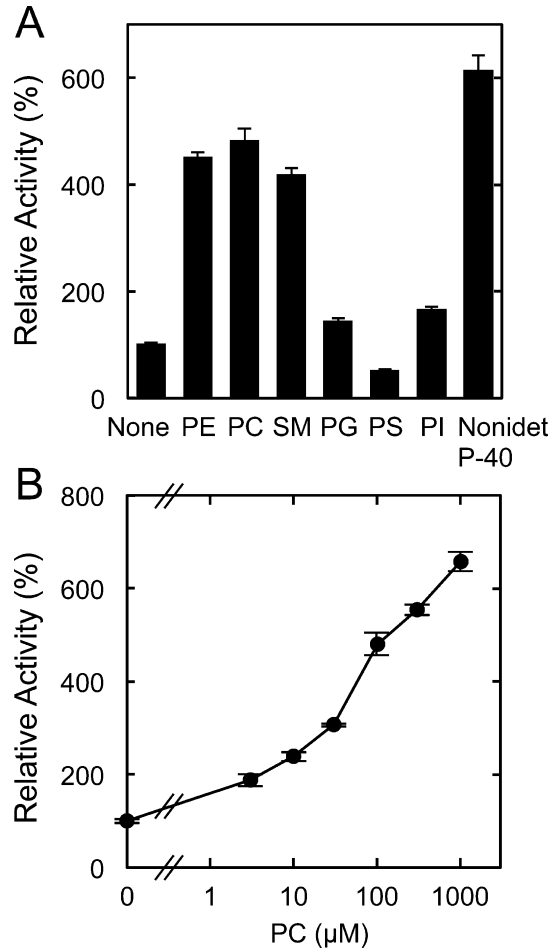

Figure 2. Effects of various phospholipid classes on NAAA activity. Recombinant NAAA (300 ng of protein) was allowed to react with 100 μM [^{14}C]PEA. (A) The reactions were carried out in the presence of 3 mM DTT and either Nonidet P-40 at 0.1% (w/v) or the indicated phospholipid at 100 μM. (B) Varying concentrations of PC in the presence of 3 mM DTT. The enzyme activity in the absence of phospholipid or detergent (44.5 nmol/min/mg of protein) was expressed as 100%, and relative activities are shown (means ± SD, n = 3).

may help keep NAAA active in lysosomes. Previously, we[38] and others[39] reported the stimulatory effect of PE on NAPE-PLD.

Enhancement of NAAA Activity by Dihydrolipoic Acid. Earlier, we reported the thiol requirement of NAAA activity.[25] When various thiol compounds were tested with rat lung NAAA, DTT increased NAAA activity dose-dependently up to 6-fold, while another sulfhydryl-reducing agent (2-mercaptoethanol) and two endogenous thiol compounds (glutathione and cysteine) were weak stimulators.[26] In accordance with these previous results, DTT stimulated recombinant NAAA much more potently than 2-mercaptoethanol, glutathione, and cysteine (Figure 3A). Homocysteine, another endogenous thiol compound, also increased the activity weakly.

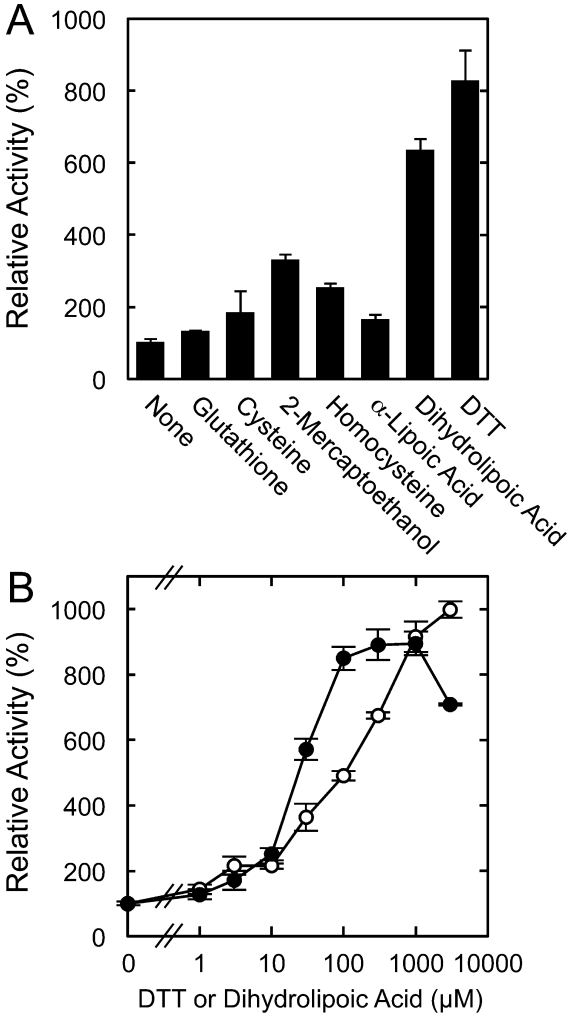

Figure 3. Effects of various thiol compounds on NAAA activity. Recombinant NAAA (300 ng of protein) was allowed to react with 100 μM [^{14}C]PEA. (A) The reactions were carried out in the presence of 0.1% Nonidet P-40 and the indicated compound at 3 mM. (B) Varying concentrations of DTT (○) and dihydrolipoic acid (●) both with 0.1% Nonidet P-40. The enzyme activities in the absence of thiol compound (29.0 (A) and 28.5 (B) nmol/min/mg of protein) are expressed as 100%, and relative activities are shown (means ± SD, n = 3).

α-Lipoic acid is an endogenous disulfide derivative of octanoic acid, and dihydrolipoic acid is the reduced form of α-lipoic acid. α-Lipoic acid and dihydrolipoic acid are enzymatically interchangeable in the cell. α-Lipoic acid is well-known to serve as a cofactor of α-keto acid dehydrogenase complexes and the glycine cleavage system.[40] The molecule also attracts attention due to its antioxidant effect,[40] and dihydrolipoic acid activates prostaglandin E synthase-2.[41] Dihydrolipoic acid at 3 mM stimulated NAAA activity 6.3-fold, being the most potent among the endogenous thiol compounds examined, while α-lipoic acid was much less effective (Figure 3A). When the assay was performed in the presence of increasing concentrations (1 μM−3 mM) of dihydrolipoic acid, the highest activity was seen at 0.1−1 mM (Figure 3B). Interestingly, dihydrolipoic acid at 0.03−0.3 mM was more potent than the corresponding concentrations of DTT. When α-lipoic acid acts as a coenzyme of α-keto acid dehydrogenase complexes and the glycine cleavage system, it is covalently bound to the proteins through an amide linkage.[40] Therefore, it is interesting that the free form of dihydrolipoic acid stimulated NAAA.

Since the sulfhydryl blockers p-chloromercuribenzoic acid and $HgCl_2$ inhibit NAAA,[26] thiol compounds may be necessary to protect the cysteine residue functioning as the catalytic nucleophile (cysteine-131 in rat NAAA). α-Lipoic acid has a disulfide bond, while in dihydrolipoic acid, this disulfide bond is reduced to two sulfhydryl groups. The potent stimulation by dihydrolipoic acid may be explained by the presence of two sulfhydryl groups per molecule and its high hydrophobicity, which may enhance its affinity for the NAAA protein. NAAA is thought to be present inside lysosomes, based on morphological observations, its acidic pH optimum, and inclusion of mannose 6-phosphate necessary for targeting to lysosomes.[32,42] Since the high hydrophobicity allows α-lipoic acid/dihydrolipoic acid to permeate biomembranes at a high rate,[40] dihydrolipoic acid may be a candidate for an endogenous NAAA stimulator.

Substrate Specificity of NAAA in the Presence of Different Stimulators. NAAA hydrolyzes PEA at by far the highest rate among various NAEs. This substrate specificity becomes evident when the assay is performed in the presence of 0.1% Triton X-100[26] or 0.1% Nonidet P-40.[27] When the detergent was removed from the reaction mixture, the reactivities with saturated NAEs were remarkably decreased, while those with unsaturated NAEs were unchanged or slightly decreased.[26,27] Thus, we were interested whether different combinations of stimulators affect the substrate specificity of NAAA, and tested PEA, stearoylethanolamide (SEA), OEA, and AEA as substrates. As shown in Figure 4, however, in the presence of any combination of Nonidet P-40 or PC with DTT or dihydrolipoic acid, substrate specificity was essentially the same, consistently showing by far the highest reactivity with PEA. Although the reactivities with OEA were much lower than those with PEA, it should be noted that the hydrolysis of OEA in the presence of DTT proceeded 3.7−4.0-fold faster than that in the presence of dihydrolipoic acid. In agreement with the preference of PEA *in vitro*, the selective NAAA inhibitor *N*-[(3*S*)-2-oxo-3-oxetanyl]-3-phenylpropanamide increased the endogenous level of PEA in ionomycin-stimulated NAAA-overexpressing human embryonic kidney 293 (HEK293) cells but not that of AEA in lipopolysaccharide-stimulated NAAA-overexpressing HEK293 cells.[35] However, since macrophage cells endogenously expressing NAAA degraded ^{14}C-labeled PEA, OEA, and AEA at similar rates in the presence of the FAAH inhibitor URB597,[31] we cannot rule out a possibility that unknown endogenous factors affect the substrate specificity of NAAA.

Effect of Buffers on NAAA Activity. NAAA shows the highest activity at pH 4.5−5 and is almost inactive at neutral and alkaline pH.[26,27,43] The assay has been performed with

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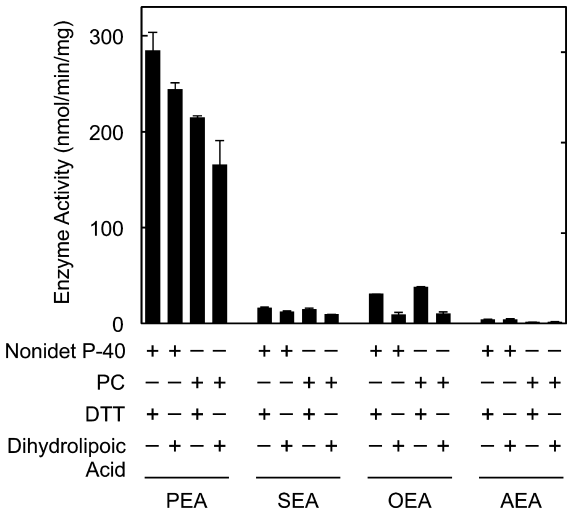

Figure 4. Substrate specificity of NAAA in the presence of different stimulators. Recombinant NAAA (0.3−3 μg of protein) was allowed to react with the indicated ^{14}C-labeled NAEs at 100 μM in the presence or absence of 0.1% Nonidet P-40, 100 μM PC, 3 mM DTT, and 0.1 mM dihydrolipoic acid. Means ± SD of the enzyme activities are shown (n = 3).

citrate-Na_2HPO_4 buffer (pH 4.5, 5.0, or 5.2)[26,27,36] or sodium hydrogen phosphate buffer (pH 5.0).[35] However, it has not been examined whether different acids in buffers affect NAAA activity. We therefore compared NAAA activities in eight buffers containing different acids, all of which were adjusted to pH 4.5 (Figure 5). When the NAAA activity in the presence of

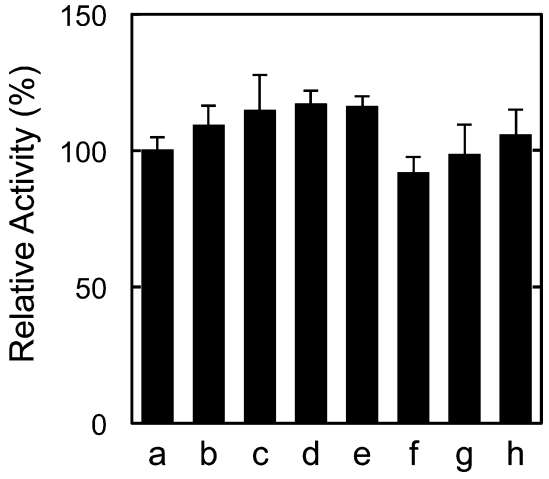

Figure 5. Effects of various buffers on NAAA activity. Recombinant NAAA (300 ng of protein) was allowed to react with 100 μM [^{14}C]PEA in the presence of 3 mM DTT and 0.1% Nonidet P-40 at pH 4.5. The pH was adjusted with the following buffers (100 mM): citrate-Na_2HPO_4 (a), sodium citrate (b), 3,3-dimethylglutaric acid-NaOH (c), sodium acetate (d), sodium succinate (e), sodium phthalate (f), sodium tartrate (g), and sodium formate (h). The enzyme activity in citrate-Na_2HPO_4 buffer (219 nmol/min/mg of protein) is expressed as 100%, and relative activities are shown (means ± SD, n = 3).

citrate-Na_2HPO_4 buffer was normalized to 100%, the other seven buffers gave 92−117% of the activity. Thus, NAAA activity was hardly affected by different acids.

Expression of NAAA in FAAH$^{-/-}$ Mice. We examined the expression levels of NAAA mRNA in the brain, lung, spleen, liver, small intestine, kidney, and testis in wild-type mice by real-time reverse transcription-PCR (Figure 6). The lung and

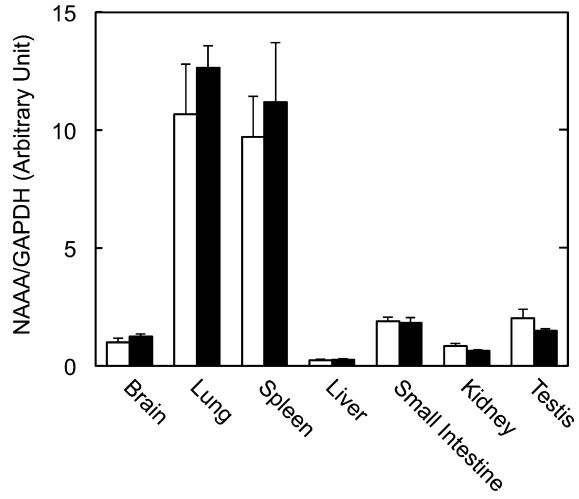

Figure 6. Expression levels of NAAA mRNA in various tissues of wild-type and FAAH$^{-/-}$ mice. Total RNAs were isolated from the indicated tissues of wild-type (open columns) and FAAH$^{-/-}$ (closed columns) mice and were reverse-transcribed. The resultant cDNAs were analyzed by real-time quantitative PCR. The obtained NAAA mRNA levels are normalized to GAPDH mRNA levels, and the NAAA/GAPDH ratios are shown (means ± SE, n = 3).

spleen showed higher mRNA expressions than other tissues. The liver exhibited the lowest expression. These results were similar to our previous results analyzed by conventional reverse transcription-PCR.[31] When the NAAA levels were compared between wild-type and FAAH$^{-/-}$ mice, there were no significant differences in all the tissues. These results suggest that FAAH$^{-/-}$ mice do not have a molecular mechanism by which the accumulating NAEs enhance the levels of NAAA mRNA and are in agreement with the previous reports that endogenous levels of NAEs, including PEA, dramatically increase in the brain and other tissues of FAAH$^{-/-}$ mice.[20,21]

In conclusion, we found for the first time that the endogenous molecules, choline- or ethanolamine-containing phospholipids and dihydrolipoic acid, potently stimulate NAAA as substitutes for polyoxyethylene p-t-octylphenyl ether-type detergent and DTT, respectively. These molecules may contribute to keeping NAAA active in lysosomes. Preferential hydrolysis of PEA over other NAEs was seen even with these new simulators. In addition, a compensatory increase in the expression level of NAAA was not observed in FAAH$^{-/-}$ mice.

METHODS

Materials. [1-^{14}C]Palmitic acid was purchased from PerkinElmer Life Science (Boston, MA); [1-^{14}C]stearic and [1-^{14}C]arachidonic acids from GE Healthcare (Piscataway, NJ); [1-^{14}C]oleic acid from Moravek Biochemicals (Brea, CA); nonradiolabeled NAEs from Cayman Chemical (Ann Arbor, MI); bovine serum albumin, 1,2-dioleoyl-PE, 1,2-dioleoyl-PC, 1,2-dipalmitoyl-PG, bovine liver PI, 1,2-dioleoyl-PS, bovine brain SM, 2-mercaptoethanol, dihydrolipoic and α-lipoic acids, and fetal calf serum from Sigma-Aldrich (St. Louis, MO); Triton X-100, Tween 20, DTT, L-cysteine hydrochloride, glutathione, formic and succinic acids, sodium (+)-tartrate, 3(2)-t-butyl-4-hydroxyanisole, ethanolamine, and Dulbecco's modified Eagle's medium from Wako Pure Chemical (Osaka, Japan); Nonidet P-40, DL-homocysteine, potassium hydrogen phthalate, and L-(+)-tartaric and 3,3-dimethylglutaric acids from Nacalai Tesque (Kyoto, Japan);

n-octyl-β-D-glucoside and CHAPS from Dojindo (Kumamoto, Japan); protein assay dye reagent concentrate from Bio-Rad (Hercules, CA); precoated silica gel 60 F_{254} aluminum sheets for thin-layer chromatography (20 × 20 cm, 0.2-mm thickness) from Merck (Darmstadt, Germany); Lipofectamine 2000, TRIzol, and pcDNA3.1(+) from Life Technologies (Carlsbad, CA); PrimeScript RT reagent kit and SYBR Premix Ex *Taq*II from Takara Bio (Ohtsu, Japan); and HEK293 cells from Health Science Research Resources Bank (Osaka, Japan). [1-^{14}C]NAEs were chemically prepared from ethanolamine and their corresponding ^{14}C-labeled fatty acids.[44]

Preparation of Recombinant NAAA. The expression vector pcDNA3.1(+) harboring cDNA for rat NAAA was prepared as described previously.[27] HEK293 cells were cultured at 37 °C to 70% confluency in a poly-L-lysine-coated 100-mm dish containing Dulbecco's modified Eagle's medium with 10% fetal calf serum and 0.1 mM nonessential amino acids in a humidified 5% CO_2/95% air incubator. The cells were then transfected with 8 μg of pcDNA3.1(+) harboring rat *NAAA* cDNA using 36 μL of Lipofectamine 2000 according to the manufacturer's instructions (Life Technologies). After 48 h, the cells were harvested with the aid of trypsin, washed twice, and sonicated three times each for 3 s in phosphate-buffered saline. The cell homogenate was then centrifuged at 12 000*g* for 30 min. The obtained pellet was suspended in phosphate-buffered saline and subjected to two cycles of freezing and thawing. The sample was centrifuged at 105 000*g* for 50 min, and the resulting supernatant was used for enzyme assays as recombinant NAAA. Protein concentration was determined by the method of Bradford[45] with bovine serum albumin as standard.

Enzyme Assay. Recombinant NAAA was incubated with 100 μM [^{14}C]NAE (1000 cpm/nmol, dissolved in 5 μL of dimethylsulfoxide) at 37 °C for 30 min. Unless otherwise stated, the reactions were carried out in 100 μL of 100 mM citrate-Na_2HPO_4 buffer (pH 4.5) containing 3 mM DTT and 0.1% Nonidet P-40. The reaction mixture also contained 0.05% bovine serum albumin and 150 mM NaCl, and the final concentration of dimethylsulfoxide was adjusted to 10%. In the assays shown in Figures 2 and 4, the reaction mixture contained 5% ethanol, which was used to dissolve phospholipids. The reaction was terminated by the addition of 0.32 mL of a mixture of diethyl ether/methanol/1 M citric acid (30:4:1, by vol.). In the assays shown in Figure 4, 5 mM 3(2)-*t*-butyl-4-hydroxyanisole was also in the stop solution to suppress autoxidation of unsaturated NAEs. After centrifugation, 100 μL of the organic phase was spotted on a silica gel thin-layer plate (10-cm height) and developed at 4 °C for 25 min with a mixture of chloroform/methanol/28% ammonium hydroxide (80:20:2, by vol.). Distribution of radioactivity on the plate was quantified by a BAS1500 bioimaging analyzer (Fujix, Tokyo, Japan). All the enzyme assays were performed in triplicate.

Real-Time Quantitative PCR Analyses. Total RNA was isolated using TRIzol from various tissues of FAAH$^{-/-}$[20] and control C57BL/6 wild-type mice. cDNAs were reverse-transcribed from total RNA (0.5 μg) by using PrimeScript RT reagent kit according to the manufacturer's instructions (Takara Bio). Real-time quantitative PCR analyses were then performed with ABI Prism 7000 Sequence Detection System (Life Technologies) using SYBR Premix Ex *Taq*II. Primers for NAAA were 5′-AAGGCTGGTGGTGGGAGAA-3′ (sense) and 5′-TCAGCAATGAGGGGAGTCTTG-3′ (antisense), and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-AACTCCCACTCTTCCACCTTCGATG-3′ (sense) and 5′-CCTGTTGCTGTAGCCGTATTCATTG-3′ (antisense). The PCR condition was as follows: denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 31 s (40 cycles). Statistical significance was evaluated by one-way ANOVA followed by the Bonferroni post-hoc test.

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Author Contributions

T.T. prepared recombinant NAAA, performed enzyme assays, and also contributed to writing the article. K.T. designed the experiment protocols, performed real-time quantitative PCR analyses, and contributed to writing the article. T.U. and H.H. provided guidance and advice. K.M. prepared the tissues of FAAH$^{-/-}$ mice. B.F.C. oversaw FAAH$^{-/-}$ mouse work and provided important help with writing the article. N.U. cordinated the research project and wrote the article.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AEA, anandamide; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate; DTT, dithiothreitol; FAAH, fatty acid amide hydrolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK293, human embryonic kidney 293; NAAA, *N*-acylethanolamine-hydrolyzing acid amidase; NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; NAPE-PLD, *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D; OEA, oleoylethanolamide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, palmitoylethanolamide; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SEA, stearoylethanolamide; SM, sphingomyelin.

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) Devane, W. A., Hanuš, 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.

(3) Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H., Sørgård, M., Di Marzo, V., Julius, D., and Högestätt, E. D. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature 400*, 452−457.

(4) Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P. H, Di Marzo, V., Elphick, M. R., Greasley, P. J., Hansen, H. S., Kunos, G., Mackie, K., Mechoulam, R., and Ross, R. A. (2010) International union of basic and clinical pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB_1 and CB_2. *Pharmacol. Rev. 62*, 588−631.

(5) Calignano, A., La Rana, G., Giuffrida, A., and Piomelli, D. (1998) Control of pain initiation by endogenous cannabinoids. *Nature 394*, 277−281.

(6) 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.

(7) LoVerme, J., Russo, R., La Rana, G., Fu, J., Farthing, J., Mattace-Raso, G., Meli, R., Hohmann, A., Calignano, A., and Piomelli, D. (2006) Rapid broad-spectrum analgesia through activation of peroxisome proliferator-activated receptor-α. *J. Pharmacol. Exp. Ther. 319*, 1051−1061.
(8) Rodríguez de Fonseca, F., Navarro, M., Gómez, R., Escuredo, L., Nava, F., Fu, J., Murillo-Rodríguez, E., Giuffrida, A., LoVerme, J., Gaetani, S., Kathuria, S., Gall, C., and Piomelli, D. (2001) An anorexic lipid mediator regulated by feeding. *Nature 414*, 209−212.
(9) Pavón, F. J., Serrano, A., Romero-Cuevas, M., Alonso, M., and Rodríguez de Fonseca, F. (2010) Oleoylethanolamide: A new player in peripheral control of energy metabolism. Therapeutic implications. *Drug Discovery Today: Dis. Mech. 7*, e175−e183.
(10) Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Fonseca, R. D., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G., and Piomelli, D. (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. *Nature 425*, 90−93.
(11) Ahern, G. P. (2003) Activation of TRPV1 by the satiety factor oleoylethanolamide. *J. Biol. Chem. 278*, 30429−30434.
(12) Overton, H. A., Babbs, A. J., Doel, S. M., Fyfe, M. C. T., Gardner, L. S., Griffin, G., Jackson, H. C., Procter, M. J., Rasamison, C. M., Tang-Christensen, M., Widdowson, P. S., Williams, G. M., and Reynet, C. (2006) Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. *Cell Metab.*, 167−175.
(13) Garg, P., Duncan, R. S., Kaja, S., Zabaneh, A., Chapman, K. D., and Koulen, P. (2011) Lauroylethanolamide and linoleoylethanolamide improve functional outcome in a rodent model for stroke. *Neurosci. Lett. 492*, 134−138.
(14) Ueda, N., Tsuboi, K., and Uyama, T. (2010) Enzymological studies on the biosynthesis of *N*-acylethanolamines. *Biochim. Biophys. Acta 1801*, 1274−1285.
(15) 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.
(16) Sun, Y.-X., Tsuboi, K., Okamoto, Y., Tonai, T., Murakami, M., Kudo, I., and Ueda, N. (2004) Biosynthesis of anandamide and *N*-palmitoylethanolamine by sequential actions of phospholipase A_2 and lysophospholipase D. *Biochem. J. 380*, 749−756.
(17) Simon, G. M., and Cravatt, B. F. (2006) 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.
(18) Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., and Gilula, N. B. (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature 384*, 83−87.
(19) McKinney, M. K., and Cravatt, B. F. (2005) Structure and function of fatty acid amide hydrolase. *Annu. Rev. Biochem. 74*, 411−432.
(20) Cravatt, B. F., Demarest, K., Patricelli, M. P., Bracey, M. H., Giang, D. K., Martin, B. R., and Lichtman, A. H. (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc. Natl. Acad. Sci. U.S.A. 98*, 9371−9376.
(21) Clement, A. B., Hawkins, E. G., Lichtman, A. H., and Cravatt, B. F. (2003) Increased seizure susceptibility and proconvulsant activity of anandamide in mice lacking fatty acid amide hydrolase. *J. Neurosci. 23*, 3916−3923.
(22) Lichtman, A. H., Leung, D., Shelton, C. C., Saghatelian, A., Hardouin, C., Boger, D. L., and Cravatt, B. F. (2004) Reversible inhibitors of fatty acid amide hydrolase that promote analgesia: evidence for an unprecedented combination of potency and selectivity. *J. Pharmacol. Exp. Ther. 311*, 441−448.
(23) Fegley, D., Gaetani, S., Duranti, A., Tontini, A., Mor, M., Tarzia, G., and Piomelli, D. (2005) 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.
(24) Ahn, K., Smith, S. E., Liimatta, M. B., Beidler, D., Sadagopan, N., Dudley, D. T., Young, T., Wren, P., Zhang, Y., Swaney, S., Van Becelaere, K., Blankman, J. L., Nomura, D. K., Bhattachar, S. N., Stiff, C., Nomanbhoy, T. K., Weerapana, E., Johnson, D. S., and Cravatt, B. F. (2011) Mechanistic and pharmacological characterization of PF-04457845: a highly potent and selective fatty acid amide hydrolase inhibitor that reduces inflammatory and noninflammatory pain. *J. Pharmacol. Exp. Ther. 338*, 114−124.
(25) Ueda, N., Yamanaka, K., Terasawa, Y., and Yamamoto, S. (1999) An acid amidase hydrolyzing anandamide as an endogenous ligand for cannabinoid receptors. *FEBS Lett. 454*, 267−270.
(26) 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.
(27) Tsuboi, K., Sun, Y.-X., Okamoto, Y., Araki, N., Tonai, T., and Ueda, N. (2005) 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.
(28) Ueda, N., Tsuboi, K., and Uyama, T. (2010) *N*-acylethanolamine metabolism with special reference to *N*-acylethanolamine-hydrolyzing acid amidase (NAAA). *Prog. Lipid Res. 49*, 299−315.
(29) Zhao, L.-Y., Tsuboi, K., Okamoto, Y., Nagahata, S., and Ueda, N. (2007) Proteolytic activation and glycosylation of *N*-acylethanolamine-hydrolyzing acid amidase, a lysosomal enzyme involved in the endocannabinoid metabolism. *Biochim. Biophys. Acta 1771*, 1397−1405.
(30) West, J. M., Zvonok, N., Whitten, K. M., Wood, J. T., and Makriyannis, A. (2012) Mass spectrometric characterization of human *N*-acylethanolamine-hydrolyzing acid amidase. *J. Proteome Res. 11*, 972−981.
(31) Sun, Y.-X., Tsuboi, K., Zhao, L.-Y., Okamoto, Y., Lambert, D. M., and Ueda, N. (2005) Involvement of *N*-acylethanolamine-hydrolyzing acid amidase in the degradation of anandamide and other *N*-acylethanolamines in macrophages. *Biochim. Biophys. Acta 1736*, 211−220.
(32) Tsuboi, K., Zhao, L.-Y., Okamoto, Y., Araki, N., Ueno, M., Sakamoto, H., and Ueda, N. (2007) Predominant expression of lysosomal *N*-acylethanolamine-hydrolyzing acid amidase in macrophages revealed by immunochemical studies. *Biochim. Biophys. Acta 1771*, 623−632.
(33) Wang, J., Zhao, L.-Y., Uyama, T., Tsuboi, K., Wu, X.-X., Kakehi, Y., and Ueda, N. (2008) Expression and secretion of *N*-acylethanolamine-hydrolysing acid amidase in human prostate cancer cells. *J. Biochem. 144*, 685−690.
(34) Tsuboi, K., Hilligsmann, C., Vandevoorde, S., Lambert, D. M., and Ueda, N. (2004) *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.
(35) Solorzano, C., Zhu, C., Battista, N., Astarita, G., Lodola, A., Rivara, S., Mor, M., Russo, R., Maccarrone, M., Antonietti, F., Duranti, A., Tontini, A., Cuzzocrea, S., Tarzia, G., and Piomelli, D. (2009) Selective *N*-acylethanolamine-hydrolyzing acid amidase inhibition reveals a key role for endogenous palmitoylethanolamide in inflammation. *Proc. Natl. Acad. Sci. U.S.A. 106*, 20966−20971.
(36) Saturnino, C., Petrosino, S., Ligresti, A., Palladino, C., De Martino, G., Bisogno, T., and Di Marzo, V. (2010) Synthesis and biological evaluation of new potential inhibitors of *N*-acylethanolamine hydrolyzing acid amidase. *Bioorg. Med. Chem. Lett. 20*, 1210−1213.
(37) Solorzano, C., Antonietti, F., Duranti, A., Tontini, A., Rivara, S., Lodola, A., Vacondio, F., Tarzia, G., Piomelli, D., and Mor, M. (2010) Synthesis and structure-activity relationships of *N*-(2-oxo-3-oxetanyl)-amides as *N*-acylethanolamine-hydrolyzing acid amidase inhibitors. *J. Med. Chem. 53*, 5770−5781.
(38) Wang, J., Okamoto, Y., Tsuboi, K., and Ueda, N. (2008) The stimulatory effect of phosphatidylethanolamine on *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD). *Neuropharmacology 54*, 8−15.

(39) Petersen, G., Pedersen, A. H., Pickering, D. S., Begtrup, M., and Hansen, H. S. (2009) Effect of synthetic and natural phospholipids on *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D activity. *Chem. Phys. Lipids 162*, 53−61.
(40) Bustamante, J., Lodge, J. K., Marcocci, L., Tritschler, H. J., Packer, L., and Rihn, B. H. (1998) α-Lipoic acid in liver metabolism and disease. *Free Radical Biol. Med. 24*, 1023−1039.
(41) Watanabe, K., Ohkubo, H., Niwa, H., Tanikawa, N., Koda, N., Ito, S., and Ohmiya, Y. (2003) Essential ^{110}Cys in active site of membrane-associated prostaglandin E synthase-2. *Biochem. Biophys. Res. Commun. 306*, 577−581.
(42) Lübke, T., Lobel, P., and Sleat, D. E. (2009) Proteomics of the lysosome. *Biochim. Biophys. Acta 1793*, 625−635.
(43) Wang, J., Zhao, L.-Y., Uyama, T., Tsuboi, K., Tonai, T., and Ueda, N. (2008) Amino acid residues crucial in pH regulation and proteolytic activation of *N*-acylethanolamine-hydrolyzing acid amidase. *Biochim. Biophys. Acta 1781*, 710−717.
(44) Ueda, N., Yamamoto, K., Yamamoto, S., Tokunaga, T., Shirakawa, E., Shinkai, H., Ogawa, M., Sato, T., Kudo, I., Inoue, K., Takizawa, H., Nagano, T., Hirobe, M., Matsuki, N., and Saito, H. (1995) Lipoxygenase-catalyzed oxygenation of arachidonylethanolamide, a cannabinoid receptor agonist. *Biochim. Biophys. Acta 1254*, 127−134.
(45) 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.