Endocannabinoid Biosynthesis Proceeding through Glycerophospho-N-acyl Ethanolamine and a Role for α/β-Hydrolase 4 in This Pathway*1

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N-Acyl ethanolamines (NAEs) are a large class of signaling lipids implicated in diverse physiological processes, including nociception, cognition, anxiety, appetite, and inflammation. It has been proposed that NAEs are biosynthesized from their corresponding N-acyl phosphatidylethanolamines (NAPEs) in a single enzymatic step catalyzed by a phospholipase D (NAPE-PLD). The recent generation of NAPE-PLD(−/−) mice has revealed that these animals possess lower brain levels of saturated NAEs but essentially unchanged concentrations of polyunsaturated NAEs, including the endogenous cannabionoid anandamide. These findings suggest the existence of additional enzymatic routes for the production of NAEs in vivo. Here, we report evidence for an alternative pathway for NAE biosynthesis that proceeds through the serine hydrolase-catalyzed double-decaylation of NAPE to generate glycerophospho-NAE, followed by the phosphodiesterase-mediated cleavage of this intermediate to liberate NAE. Furthermore, we describe the functional proteomic isolation and identification of a heretofore uncharacterized enzyme α/β-hydrolase 4 (Abh4) as a lysophospholipase/phospholipase B that selectively hydrolyzes NAPEs and lysNAPEs. Abh4 accepts lysNAPEs bearing both saturated and polyunsaturated N-acyl chains as substrates and displays a distribution that closely mirrors lysNAPE-lipase activity in mouse tissues. These results support the existence of an NAPE-PLD-independent route for NAE biosynthesis and suggest that Abh4 plays a role in this metabolic pathway by acting as a (lyso)NAPE-selective lipase.

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2 The abbreviations used are: CB, cannabinoid receptor; Abh4, α/β-hydrolase 4; ABPP-MudPIT, activity-based protein profiling-multidimensional protein identification technology; CNS, central nervous system; GP, glycerophospho, MAFP, methoxy arachidonyl fluorophosphonate; NAE, N-acyl ethanolamine; NAPE, N-acyl phosphatidylethanolamine; PLD, phospholipase D; LC, liquid chromatography; MS, mass spectrometry; FP, fluorophosphonate.
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respectively. These results suggest the existence of multiple biosynthetic pathways for NAEs in vivo, with NAPE-PLD being principally responsible for generating very long chain saturated NAEs and other, as of yet, unidentified enzymes contributing to the production of long chain saturated and polyunsaturated NAEs. Consistent with this premise, tissues from NAPE-PLD (−/−) mice possessed a “residual” calcium-independent enzymatic activity that catalyzed the conversion of NAPEs to NAEs (20).

When considering the potential enzymatic basis for the conversion of NAPEs to NAEs in NAPE-PLD (−/−) tissues, it is instructive to revisit previously proposed routes for this process. Studies by Schmid and co-workers (21) in the 1980s provided in vitro biochemical evidence for enzymatic activities capable of producing NAEs from NAPEs via two- or three-step pathways involving the hydrolysis of one or both O-acyl chains from NAPEs (see Fig. 1, reactions II and III, respectively), followed by cleavage of the phosphodiester bond of the resulting N-acyl lysophosphatidylethanolamine (lysoNAPE) or glycerophospho (GP)-NAE, respectively (see Fig. 1, reactions IV and V).

Previously, a secreted phospholipase A2 was shown to catalyze the hydrolysis of an O-acyl chain of NAPE to yield lysoNAPE in vitro; however, the restricted expression profile of this enzyme (predominantly expressed in stomach, pancreas, and small intestine) suggests the existence of additional enzymes responsible for the calcium-independent NAPE hydrolase activity detected in brains and testes of NAPE-PLD (−/−) mice (22). Here we report evidence that the NAPE-PLD-independent formation of NAPEs in brain tissue involves the serine hydrolase-mediated cleavage of both O-acyl chains of NAPEs to give GP-NAEs, which are then converted to NAEs and glycerol-3-phosphate by a metal-dependent phosphodiesterase. Additionally, we describe the functional proteomic isolation and identification of a previously uncharacterized enzyme α/β-hydrolase 4 (Abh4) as a major lysoNAPE-lipase activity present in both the CNS and the peripheral tissues of mice.

EXPERIMENTAL PROCEDURES

Materials—[1'-14C]Palmitic acid was purchased from Moravek Biochemicals (Brea, CA). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine, 1-oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine, 1-oleoyl-2-hydroxy-sn-glycerol-3-phosphocholine, 1-oleoyl-2-hydroxy-sn-glycerol-3-phosphoserine, and 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (di-ether phosphatidylethanolamine) were purchased from Avanti Polar Lipids (Alabaster, AL). Methyl arachidonoyl fluorophosphate (MAFP) was purchased from Cayman Chemicals (Ann Arbor, MI). HiTrap Q FF and HiTrap SP FF ion exchange columns and PD-10 desalting columns were purchased from GE Healthcare (Piscataway, NJ).

Enzyme Assays—Enzyme assays were performed in 4-ml glass vials in 50 mM Tris/HCl, pH 7.5, in a total volume of 100 μl using 1 mg/ml protein (0.5 mg/ml for LC-MS assays). The reactions were incubated at 37 °C for 30 min (1 h for LC-MS assays) with 100 μM synthetic substrate (5 μl of 2 mM stock in EtOH), with or without 2 mM EDTA. For MAFP experiments, the samples were pretreated with 5 μl of 100 μM MAFP in Me2SO (final concentration, 5 μM) or Me2SO alone for 30 min at room temperature before the addition of substrate. The reactions were stopped by the addition of 1.5 ml of 2:1 CHCl3:MeOH and 0.4 ml of 50 mM Tris containing 1% formic acid, mixed and then centrifuged for 3 min at 1,400 × g to separate phases. The organic phase was extracted and concentrated to dryness under a stream of N2. For radiochromatographic assays, the dried reaction mixtures were dissolved in 20 μl of 2:1 CHCl3:MeOH, spotted on thin layer silica plates, and developed in CHCl3:MeOH:28% NH4OH at 40:10:1. Distribution of radioactivity on the plate was quantified by a phosphorimaging device (Packard), and products were identified by comparison with 14C-radiolabeled synthetic standards. Resulting product (NAE or GP-NAE) concentration was calculated from percentage values of the total radioactivity on the TLC plates. All of the radiochromatographic assays included heat-denatured controls, and these values were subtracted as background. For the analysis of the substrate selectivity of Abh4, nonradiolabeled substrates were used. The dried reactions were dissolved in 200 μl of 2:1 CHCl3:MeOH, and 50 μl were injected onto an Agilent 1100 series LC-MS. Oleic acid release was measured by comparison with a pentadecanoic acid standard. All of the assays were conducted with n ≥ 3, and the error bars represent the standard error of the mean.

Cell Culture and Transfection—COS-7 cells were grown at 37 °C and 5% CO2 to ~70% confluence in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in 15-cm dishes and transfected with 15 μg of plasmid DNA (or empty vector control) using the FuGENE 6 (Roche Applied Science) transfec-
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![Diagram of potential metabolic pathways for the biosynthesis of NAEs.](Image)

**FIGURE 1.** Potential metabolic pathways for the biosynthesis of NAEs. Reaction I is mediated by an NAPE-selective PLD (19). Pathways II-III-IV and II-V are NAPE-PLD-independent. G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid.

**RESULTS**

A Biosynthetic Pathway for NAEs That Proceeds through a GP-NAE Intermediate—In addition to the direct NAPE-PLD-mediated biosynthetic pathway for the production of NAEs, it has also been suggested that phosphodiester cleavage is preceded by de-acylation of one or both of the O-acyl chains of NAPEs (Fig. 1, reactions II and III, respectively) (21). The enzymes involved in this putative O-deacylation-phosphodiesterase route for endocannabinoid biosynthesis remain unknown. The cellular hydrolysis of acyl chains on phospholipids is commonly catalyzed by phospholipase enzymes from the serine hydrolase superfamily (25). The catalytic nucleophile of serine hydrolases can be covalently modified by fluorophosphate (FP) inhibitors (26), providing a useful screen to test the contribution that these enzymes make to specific metabolic pathways. In contrast, most PLD enzymes, including NAPE-PLD (19, 22), do not use serine nucleophiles (27) and are therefore not sensitive to FP reagents (22). To investigate whether serine hydrolyses are involved in NAE biosynthesis, we measured the conversion of NAPE to NAE in NAPE-PLD(+/+) and (-/-) brains in the presence of methoxy arachidonyl FP (MAFP), a general inhibitor of this enzyme class (Fig. 2A) (28, 29). The residual NAE biosynthetic activity in NAPE-PLD(-/-) brains was strongly inhibited (>80%) by MAFP, suggesting the requirement for a serine hydrolyase-mediated step(s) in this pathway. Interestingly, MAFP treatment also reduced the calcium-independent conversion of NAPEs to NAEs by ~60% in NAPE-PLD(+/+) brains (Fig. 2A), indicating that in the absence of millimolar calcium, which is required for maximal activation of NAPE-PLD (30), a significant fraction of NAE biosynthesis proceeds through a serine hydrolyase-mediated pathway.

Phospholipase-mediated conversion of NAPE to NAE could occur by removing either one or both O-acyl chains to produce lysoNAPE (Fig. 1, reaction II) or GP-NAE (Fig. 1, reaction III), respectively. To distinguish between these two routes, we synthesized radiolabeled C16:0-lysoNAPE and measured its conversion to C16:0-NAE in the presence or absence of MAFP (Fig. 2A). Treatment with MAFP (5 μM in Me₂SO) blocked greater than 80% of the conversion of lysoNAPE to NAE in both NAPE-PLD(+/+) and (-/-) brains, suggesting that both O-acyl chains of NAPEs must be hydrolyzed for NAPE-PLD-independent NAE biosynthesis to occur. Considering further that the extent of hydrolysis of lysoNAPE was equivalent between NAPE-PLD(+/+) and (-/-) brains, these results support previous studies indicating that NAPE-PLD does not possess significant lysoNAPE-PLD activity (19).

To provide additional evidence that the MAFP-sensitive pathway for NAE biosynthesis requires hydrolysis of the O-acyl chains of NAPE, we synthesized a radiolabeled NAPE analogue that contains sn-1 and sn-2 ether linkages in place of the typical ester bonds. This hydrolysis-resistant NAPE analogue was not converted to NAE in NAPE-PLD(-/-) brains (Fig. 2B). In NAPE-PLD(+/+) brains, the amount of NAE product derived from diether-NAPE was comparable with that generated with diester-NAPE in MAFP-treated samples (Fig. 2, compare A and B), indicating that NAPE-PLD can accept NAPE substrates bearing ester or ether bonds in the sn-1 and sn-2 positions. These results confirm that the NAPE-PLD-independent bio-
synthesis of NAEs requires hydrolysis of the O-acyl chains of NAPE.

Previous studies have shown that EDTA blocks conversion of lysoNAPE to NAE (21, 22). In the course of our studies, we observed serendipitously that upon the addition of EDTA (2 mM), not only is NAE production blocked, but there is a concomitant accumulation of a slower migrating species on silica gel thin layer chromatography (Fig. 2C). We speculated that this more polar intermediate might represent GP-NAE, which is a predicted metabolic intermediate for certain candidate pathways for NAE biosynthesis (Fig. 1, reaction III) (21). We confirmed the identity of this intermediate as GP-NAE by comparing its migration on a TLC plate to a synthetic standard and by LC-MS analysis, which showed its EDTA-dependent accumulation following incubation of lysoNAPE with brain extracts (Fig. 2D). We also observed the presence of an EDTA-sensitive enzymatic activity in brain tissue that rapidly cleaves the phosphodiester bond of GP-NAE to generate NAE (Fig. 2E). Taken together, these data provide strong evidence that the major NAE-PLD-independent pathway for NAE biosynthesis in brain involves the serine hydrolase-catalyzed double deacylation of NAPE to yield GP-NAE, followed by phosphodiesterase-mediated conversion to NAE (Fig. 1, pathways II–IV). A similar pathway was also characterized in the testes of NAPE-PLD(-/-) mice (supplemental Fig. S1), suggesting that this route may contribute to NAE biosynthesis in both the CNS and peripheral tissues.

Identification of α/β-Hydrolase 4 as a LysoNAPE-Lipase That Participates in NAE Biosynthesis—We next attempted to purify the lysoNAPE-lipase activity involved in NAE biosynthesis from mouse testis, which was previously observed to contain the highest activity for conversion of NAPEs to NAEs in a panel of tissues from NAPE-PLD(-/-) mice (20). Testis tissues from NAPE-PLD(+/+) and (-/-) mice were found to possess equivalent levels of lysoNAPE-lipase activity, with significant activity distributed between both membrane and soluble frac-
tions (supplemental Fig. S2). We therefore pursued the enrichment of the lysoNAPE-lipase activity from the soluble fraction of wild type testis. Passage of soluble testis extract over sequential ion exchange columns (see “Experimental Procedures”) led to a nearly 300-fold enrichment of lysoNAPE-lipase activity (Table 1). Enriched fractions were pooled and incubated with FP-biotin, an activity-based probe that reacts selectively with enzymes from the serine hydrolase superfamily (24). FP-biotin-labeled hydrolases were isolated by affinity purification using avidin-conjugated beads, subjected to on-bead digestion, and analyzed by multidimensional LC-MS/MS. This functional proteomic method, referred to as activity-based protein profiling-multidimensional protein identification technology (ABPP-MudPIT) (23), identified three serine hydrolases in the lysoNAPE-lipase enriched fractions: hormone-sensitive lipase, group VI calcium-independent phospholipase A2 (Pla2g6), and the uncharacterized enzyme Abh4 (Table 2). Hormone-sensitive lipase is an interfacially activated neutral lipase selective for triacylglycerols and cholesterol esters (31). Strong signals for this enzyme were also observed in ABPP-MudPIT profiles of heart (Table 2), a tissue that lacked significant lysoNAPE-lipase activity (Fig. 3A). This result indicated that hormone-sensitive lipase was unlikely to account for the observed lysoNAPE-lipase activity. Additional ABPP-MudPIT studies of brain tissue, which displayed strong lysoNAPE-lipase activity (Fig. 3A), identified Abh4, but not Pla2g6 in these samples (Table 2). Based on these findings, we pursued the characterization of Abh4 as a candidate lysoNAPE-lipase.

Biochemical Characterization of Abh4—The Abh4 cDNA was subcloned from a commercially available expressed sequence tag into the mammalian expression vector pCDNA3 and transiently transfected into COS-7 cells. Labeling of extracts from Abh4-transfected and mock transfected cells with a rhodamine-tagged FP probe (32) confirmed high levels of Abh4 expression in the former samples (Fig. 4A, left panel). Extracts of Abh4-transfected cells displayed robust lysoNAPE-lipase activity as compared with mock transfected controls (Fig. 4A, right panel). Interestingly, Abh4-transfected cells also exhibited significant NAPE-lipase activity compared with

| Purification step   | Total protein mg | Total activity units* | Specific activity unit/mg | Fold purification |
|---------------------|------------------|-----------------------|---------------------------|------------------|
| Soluble fraction    | 17.1             | 0.07                  | 0.004                     | 1                |
| HiTrap Q FF         | 3.28             | 0.17                  | 0.05                      | 11.95            |
| HiTrap SP FF        | 0.11             | 0.14                  | 1.24                      | 291.96           |

*1 unit is defined as the amount of enzyme required to hydrolyze 10 nmol of lysoNAPE in 1 min at 37 °C.

TABLE 2

ABPP-MudPIT analysis of proteomes with high (brain, activity-enriched testis fraction) and low (heart) lysoNAPE-lipase activity

The numerical values correspond to average spectral counts for each enzyme plus or minus standard error (for brain and heart, n ≥ 3; for activity-enriched testis fraction, n = 1). Pla2g6, cytosolic phospholipase A2 group VI.

| Enzyme                  | Brain | Heart | Activity-enriched testis fraction |
|-------------------------|-------|-------|----------------------------------|
| Hormone-sensitive lipase| 8 ± 4 | 9 ± 2 | 9                                |
| Pla2g6                  | 0 ± 0 | 0 ± 0 | 9                                |
| Abh4                    | 17 ± 5| 2 ± 1 | 9                                |

FIGURE 3. Tissue distribution of lysoNAPE-lipase activity. A, lysoNAPE-lipase activity of homogenates of mouse tissues measured in the presence of EDTA (2 mM) to prevent further conversion of GP-NAE to NAE. B, tissue distribution of Abh4 as measured by reverse transcription-PCR. Reverse transcription-PCR analysis of the ubiquitously expressed enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control to confirm integrity of tissue cDNA.

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FIGURE 4. Recombinant Abh4 exhibits NAPE- and lysoNAPE-lipase activity. A, left panel, COS-7 cells transiently transfected with a cDNA for mouse Abh4 possess high levels of this enzyme (arrow) as judged by labeling with the serine hydrolase-directed activity-based probe FP-rhodamine. Right panel, Abh4-transfected cells display robust lysoNAPE-lipase activity compared with mock-transfected cells. Shown for N-palmitoyl-lysoNAPE. B, thin layer radiochromatogram showing that Abh4 also possesses NAPE-lipase activity. Abh4- or mock-transfected cell lysates were incubated with 14C-N-palmitoyl-NAPE in the presence or absence of EDTA (2 mM). EDTA blocked the conversion of lysoNAPE to NAE, with concomitant accumulation of GP-NAE. Heat-denaturation (Δ) and MAFP (5 μM) (data not shown) blocked both the NAPE- and lysoNAPE-lipase activity of Abh4-transfected cells. The Rf of indicated products were verified with synthetic standards. The structures of products were also confirmed by LC-MS analysis of reactions and comparison with synthetic standards.
mock transfected cells (Fig. 4B), indicating that this enzyme possesses phospholipase B-type activity (33) and is capable of catalyzing both O-deacylation steps in the conversion of NAPE to GP-NAE (Fig. 1, reactions II and III).

Mouse Abh4 is a 342-amino acid protein that shares very high sequence identity with its rat and human orthologues (99 and 96%, respectively; Fig. 5A). Abh4 is a member of the α/β-hydrolase family, so named for a highly conserved tertiary fold consisting of alternating α helices and β sheets (34). The rodent and human genomes contain at least 15 orthologous sets of predicted α/β-hydrolase proteins (Fig. 5, B and C), none of which, to date, have been functionally annotated in terms of their catalytic activities or substrate selectivities. Alignment with other α/β-hydrolases identified Ser146 as the putative catalytic nucleophile of Abh4 (Fig. 5, A and C), a prediction that was supported by the characterization of this residue as the site of FP labeling (supplemental Table 1). This alignment also revealed that Abh5, the nearest sequence homologue of Abh4 (55% identity), lacks a serine nucleophile, suggesting that it may not possess hydrolytic activity. Consistent with this premise, recombinantly expressed Abh5 did not exhibit lysoNAPE-lipase activity (data not shown).

We next investigated the substrate selectivity of Abh4 by screening the enzyme for activity against a panel of lysophospholipids. Abh4 was remarkably selective for lysoNAPE, showing no significant activity with other lysophospholipids, including lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidylserine (Fig. 6A). Within the lysoNAPE family, Abh4 hydrolyzed substrates bearing saturated (C16:0, C18:0, and C20:0), monounsaturated (C18:1), and polyunsaturated (C20:4) N-acyl chains (Fig. 6B), indicating that this enzyme could contribute to the production of a wide range of NAEs, including the endocannabinoid anandamide. Finally, the tissue distribution of mouse Abh4 was evaluated by reverse transcription-PCR and found to correlate well with the levels of lysoNAPE-lipase activity, with the highest expression being observed in the CNS and testis, followed by liver and kidney, with negligible signals in heart (Fig. 3B). These data suggest that Abh4 is a primary source for the lysoNAPE-lipase activity observed in both the CNS and peripheral tissues of mice.

**DISCUSSION**

A central tenant of chemical signaling is that the levels of small molecule transmitters should be tightly controlled by a balance of biosynthetic and degradative enzymes in vivo (35). For many neurotransmitters, such as acetylcholine and the monoamines, cognate enzymes responsible for their production and catabolism have been delineated (35). In contrast, our knowledge of the metabolic pathways that regulate lipid mes-
sengers and, in particular, the endocannabinoids remains incomplete. Termination of anandamide/NAE signaling is principally performed by the integral membrane enzyme fatty acid amide hydrolase (15), the inactivation of which leads to elevated levels of NAEs and analgesic, anxiolytic, and anti-inflammatory phenotypes (6, 7, 16, 36, 37). The enzymatic basis for NAE biosynthesis has proven more challenging to decipher.

One postulated route for NAE biosynthesis invokes the sequential action of an NAPE-generating transacylase and NAPE-hydrolyzing phospholipase D (17, 21). The molecular characterization of a PLD enzyme from the metallo-lactamase family that selectively accepts NAPEs as substrates has lent support to this model (19). However, targeted disruption of the gene was found to lower the levels of only a subset of NAEs (16), which all contained ω-linked oleic acid chains in the sn-1 position, and hydrolytic activity was quantified by measuring oleic acid release in the presence of EDTA (2 mM) by LC-MS. To avoid contaminating signals from endogenous lipids, soluble extracts of transfected cells were assayed. LysoNAPE contained a C16:0 lysoNAPE being set to 100%.

Taken together, our findings suggest that Abh4 is a dedicated (lyso)NAPE-lipase that participates in an NAPE-PLD-independent pathway for NAE biosynthesis. Determining the unique contribution that this Abh4-mediated route makes to the production of NAEs in vivo, including the endocannabinoid anandamide, will require the generation of genetic or pharmacological tools that selectively perturb this pathway. Nonetheless, its high activity in tissues with enriched expression of the CB1 receptor (e.g. CNS, testis) is provocative and suggests a potentially major contribution to endocannabinoid signaling. The continued pursuit of additional enzymes involved in NAE biosynthesis, including the NAPE-generating transacylase, GP-NAE phosphodiesterase, and possibly other NAPE-lipases, should further enrich our understanding of the complex metabolic network that supports the endocannabinoid/NAE system in vivo. From a therapeutic perspective, any of these enzymes could represent an attractive drug target for a range of human disorders in which disruption of endocannabinoid signaling by CB1 antagonists has proven beneficial (38).

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**FIGURE 6. Substrate selectivity of Abh4.** A. Abh4- and mock-transfected COS-7 cell extracts (0.5 mg/ml) were incubated with the indicated lysophospholipids (100 μM), which all contained ω-linked oleic acid chains in the sn-1 position, and hydrolytic activity was quantified by measuring oleic acid release in the presence of EDTA (2 mM) by LC-MS. To avoid contaminating signals from endogenous lipids, soluble extracts of transfected cells were assayed. LysoNAPE contained a C16:0 N-acyl chain. B. Abh4 showed similar activity with lysoNAPEs bearing saturated (C16:0, C18:0, and C20:0), monounsaturated (C18:1), and polyunsaturated (C20:4) N-acyl chains. LysoNAPE lipase activities have been normalized, with the activity observed with N-C16:0 lysoNAPE being set to 100%.

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A multi-step pathway, which can be detected in both the CNS and peripheral tissues (e.g. testis), appears to be driven by the sequential actions of an FP-sensitive serine hydrolase(s) and metal-dependent phosphodiesterase.
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