Identification of N-acylethanolamines in Dictyostelium discoideum and confirmation of their hydrolysis by fatty acid amide hydrolase

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Abstract N-acylethanolamines (NAEs) are endogenous lipid-based signaling molecules best known for their role in the endocannabinoid system in mammals, but they are also known to play roles in signaling pathways in plants. The regulation of NAEs in vivo is partly accomplished by the enzyme fatty acid amide hydrolase (FAAH), which hydrolyses NAEs to ethanolamine and their corresponding fatty acid. Inhibition of FAAH has been shown to increase the levels of NAEs in vivo and to produce desirable phenotypes. This has led to the development of pharmaceutical-based therapies for a variety of conditions targeting FAAH. Recently, our group identified a functional FAAH homolog in Dictyostelium discoideum, leading to our hypothesis that D. discoideum also possesses NAEs. In this study, we provide a further characterization of FAAH and identify NAEs in D. discoideum for the first time. We also demonstrate the ability to modulate their levels in vivo through the use of a semispecific FAAH inhibitor and confirm that these NAEs are FAAH substrates through in vitro studies. We believe the demonstration of the in vivo modulation of NAE levels suggests that D. discoideum could be a good simple model organism in which to study NAE-mediated signaling.—Hayes, A. C., J. Stupak, J. Li, and A. D. Cox. Identification of N-acylethanolamines in Dictyostelium discoideum and confirmation of their hydrolysis by fatty acid amide hydrolase. J. Lipid Res. 2013. 54: 457–466.

Supplementary key words N-acylethanolamine • endocannabinoid • anandamide

N-acylethanolamines (NAEs) are lipid-based molecules that play a role in many cell signaling pathways. The most renowned NAE, anandamide (NAE20:4), plays a central role in the endocannabinoid system by being an endogenous agonist for the cannabinoid receptors (1). The enzyme fatty acid amide hydrolase (FAAH) plays an important role in regulating the concentration of anandamide, as well as other NAEs, in vivo by hydrolyzing NAEs to ethanolamine and their corresponding fatty acid (2). FAAH knockout studies in mice have demonstrated that inhibition of FAAH can produce analgesic (3), anxiolytic (4), neuroprotective (5), and anti-inflammatory (6, 7) effects. This has resulted in considerable research into developing highly specific FAAH inhibitors as potential pharmaceutical-based therapies (8–11).

Although much emphasis has been placed on studying NAE-mediated cannabinoid signaling in mammals, NAE signaling is also involved in noncannabinoid signaling pathways in humans and other eukaryotic organisms. For example, N-palmitoylethanolamine (NAE16:0) and N-stearoyl-ethanolamine (NAE18:0) have been shown to be involved in noncannabinoid receptor anti-inflammatory signaling pathways (12, 13). Evidence from plant studies has indicated that NAE signaling may be involved in pathogen defense, as evidenced by increased levels of N-myristoylethanolamine (NAE14:0) in the presence of the fungal elicitor xylanase (14) and the down-regulation of defense-related transcripts and increased pathogen susceptibility in FAAH over-expressing plants (15). NAE signaling, specifically N-oleylethanolamine (NAE12:0), has also been shown to inhibit phospholipase Dα activity and absciscic acid-induced stomata closure (16) and to induce microtubule, actin, and endomembrane reorganization (17, 18).

NAEs and their precursors have also been shown to be present in several lower eukaryotic organisms. It has long been known that the precursors of NAEs, Nacylphosphatidylethanolamines, are present in Dictyostelium discoideum (19), and NAEs and N-acylphosphatidylethanolamines have been shown to be present in the yeast Saccharomyces cerevisiae (20). Recently, it has been shown that the ciliate Tetrahymena thermophila possesses numerous NAEs...
Along with evidence of an enzyme similar to FAAH present in _Tetrahymena pyriformis_ (22), this suggests that NAE signaling is carried out in lower eukaryotes. Furthermore, cannabinoid and cannabinoid-like molecules have been shown to elicit responses in lower eukaryotes, with δ₂-tetrahydrocannabinol affecting cellular growth, movement, and division of _T. pyriformis_ (23, 24) and 2-arachidonoyl glycerol inhibiting cellular growth in free-living amoebae (25). Finally, arachidonic acid, the product of anandamide and arachidonoyl glycerol hydrolysis by FAAH and monoacylglycerol lipase, respectively, is a known chemoattractant for _D. discoideum_ (26).

It is not known whether NAEs are present in _D. discoideum_. The identification of FAAH in _D. discoideum_ by our group (27) led to our hypothesis that these lipids are present in this organism. To test this hypothesis, we further characterized the _D. discoideum_ FAAH enzyme and then, using a targeted lipidomics approach, identified NAEs native to _D. discoideum_ in the presence and absence of a semispecific FAAH inhibitor. These substrates were then confirmed as FAAH substrates through in vitro studies. We believe that this establishes _D. discoideum_ as a suitable model organism in which to study NAE-mediated signaling, which may help elucidate these signaling pathways with broader implications on human health.

### MATERIALS AND METHODS

#### Chemicals

- N-arachidonoylthanolamine (NAE20:4), N-arachidonoylthanolamine-Δ(4), N-palmitoylthanolamine (NAE16:0), N-palmitoylthanolamine-Δ(4) (PEA-Δ(4)), N-stearoylthanolamine (NAE18:0), Noleoyethanolamine (NAE18:1), N-ninoleoythanolamine (NAE18:2), arachidonyl β-nitroaniline (AγNA), CAY10435 (1-oxazolo [4, 5-b]pyridin-2-yl-1-dodecanone), and URBS97 (5'-aminocarbonyl)[1,1'-biphenyl]-3)-4-cyclohexycarbamate) were purchased from Cayman Chemicals (Ann Arbor, MI).
- Arachidonoyltrifluoromethyl ketone (ATFMK), methoxyarachidonoyl fluorophosphonate (MAFP), LY2183240 (5-[(1,1'-CAY10435 (1-oxazolo [4, 5-b]pyridin-2-yl-1-dodecanone), and URBS97 (5'-aminocarbonyl)[1,1'-biphenyl]-3)-4-cyclohexycarbamate) were purchased from Cayman Chemicals (Ann Arbor, MI).
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#### Preparation of N-acylthanolamines

NAE standards were prepared for NAE12:0 and NAE14:0 as described by Williams et al. (28). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.6 mmol) was reacted with a solution containing the corresponding fatty acid (0.5 mmol), ethanolamine (0.75 mmol), and 4-dimethylaminopyridine (0.5 mmol) in 20 ml ice-cold acetonitrile. The reaction was warmed to 21°C and stirred for 6 h. The acetonitrile solvent was then exchanged for dichloromethane to fully dissolve solids before adding SO₂ to adsorb the NAE. Dichloromethane was then evaporated, and the SO₂ was packed into a column, dry-loaded onto a SO₃ flash chromatography column, and purified using a flash chromatography system (Teledyne Isoe, Lincoln, NE) using an acetone/hexane gradient from 5-80%. Fractions containing NAEs as determined by UV absorbance were pooled and the solvent evaporated.

#### Enzyme purification

The Dd2 gene from _D. discoideum_ AX3 was cloned into _Escherichia coli_ BL21 (DE3) as previously described (27). Enzyme was produced by inducing a fresh overnight culture of _E. coli_ BL21 (DE3) containing pCMWαET-FAAH vector in LB medium containing 0.2% glucose and 100 μg/ml ampicillin. The culture was grown at 25°C and 200 rpm to an OD₆₀₀ of 0.6, at which time it was induced with 0.1 mM isopropyl-1-thio-

#### Bioinformatics

FAAH protein sequences were selected based on previous work performed by McPartland et al. (29). FAAH 1 sequences (or proposed FAAH 1 homologs) were obtained for _Homo sapiens_ (NP_014342), _Mus musculus_ (NP_034303), _Takifugu rubripes_ (FRUPO0000014610000), _Ciona intestinalis_ (c1000153926), _Caenorhabditis elegans_ (NP_501508), _Saccharomyces cerevisiae_ S288c (NP_010528), and _Tetrahymena thermophila_ SB210 (159.m.00087). FAAH 2 sequences (or proposed FAAH 2 homologs) were obtained for _Homo sapiens_ (NP_777572), _Takifugu rubripes_ (SINFRUP0000168827), and _Drosophila melanogaster_ (NP_725139).

Another FAAH sequence was obtained for _Arabidopsis thaliana_ (NP_201249). Other amide signature sequence proteins, most annotated as glutamyl-rNA<sub>5</sub> amidotransferases, were obtained for _Homo sapiens_ (NP_066762), _Mycobacterium tuberculosis_ (NP_335746), _Arachoglobus fulgidus_ DSM 4304 (NP_0970778), and _Plasmodium falciparum_ 3D7 (NP_702811). Amidase signature sequences-containing protein sequences from _D. discoideum_ AX3, Dd1 (XM_632408), and Dd2 (XM_638290) were obtained from previous sequencing studies by our group (27). _Takifugu_ and _Ciona_ sequences were obtained from the Joint Genome Institute (http://www.jgi-psf.org), and the _Tetrahymena_ sequence originated from the The Institute for Genomic Research (http://www.tigr.org). All other sequences were obtained from GenBank (www.ncbi.nlm.nih.gov). Protein sequences were aligned with ClustalX (30) using default parameters, and phylogenetic trees were generated using a minimum evolution algorithm using MEGA4 (31). Confidence values for trees were generated by bootstrapping based on 500 resampling replicates. 
CA). In the case of further purification of MBP-FAAH, MBP was removed via an overnight digestion at 4°C using a recombinant human thrombin (EMD Chemicals, Gibbstown, NJ). The resulting protein consisted of Dictyostelium FAAH with a hexahistidine tag located at the N-terminus (His$_{6}$-FAAH), which was constructed in the initial cloning of the gene. Protein was then batch bound to a Ni-NTA resin (Qiagen, Mississauga, Ontario, Canada) for 1 h at 4°C before being packed onto a column. Protein-bound Ni-NTA resin was washed with 10 column volumes of wash buffer (20 mM Tris-Cl [pH 9.0], 400 mM NaCl, 10 mM imidazole, and 10 mM β-mercaptoethanol). To elute recombinant FAAH, a linear gradient was applied from 10 to 100 mM imidazole in wash buffer over 30 volumes followed by a final pulse of 10 volumes of wash buffer containing 200 mM imidazole. Fractions containing recombinant FAAH, as determined by absorbance at 280 nm, were pooled and dialyzed against a dialysis buffer (20 mM Tris-Cl [pH 9.0] and 50 mM NaCl). Protein size was verified using SDS-PAGE, and protein yield was determined by Bradford assay (Bio-Rad Laboratories).

**Inhibitor studies**

Inhibitor studies were carried out using ApNA as the substrate. The rate of hydrolysis by FAAH was determined by monitoring the production of p-nitroaniline at 380 nm with a microplate reader (PowerWave X; Biotech Instruments Inc., Winooski, VT). Substrate conversion was extrapolated from $A_{405}$ versus mass of p-nitroaniline using the microplate reader. Reactions were carried out in 96-well plates where ApNA (in DMSO) was added to 100 µL reaction buffer (20 mM Tris-Cl [pH 9.0], 50 mM NaCl and 0.5% Triton X-100) to a final concentration of 0–300 µM. Wells were preincubated with inhibitor and preheated to 37°C. Reactions were initiated through the addition of 30 µg of MBP-FAAH (in a 20 mM Tris-Cl [pH 9.0], 50 mM NaCl storage buffer). Final reaction volumes were 200 µL, and reactions were performed in duplicate. Kinetic constants ($V_{max}$, $K_m$, and $K_i$) were determined using three different inhibitor concentrations and calculated using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). Results of inhibitor studies were verified on thrombin-digested MBP-FAAH to demonstrate that the maltose binding protein played no significant role in enzyme inhibition.

**Dictyostelium strain growth and lipid extraction**

*D. discoideum* AX3 cells were grown axenically in liquid culture (18 g/L maltose, 14.3 g/L peptone, 7.15 g/L yeast extract, 0.486 g/L KH$_2$PO$_4$, and 0.616 g/L Na$_2$HPO$_4$·2H$_2$O) at 150 rpm and 21°C to a density of 5 x $10^6$ cells/ml. To study the effect of FAAH on NAEs production, neutral lipids were extracted from Dictyostelium cells pellets using a mixture of ethyl acetate and hexane (9:1, v/v) (32). PEA-d$_4$ and AEA-d$_4$ (1 ng of each) were added to Dictyostelium cell pellets containing 5 x $10^5$ cells and resuspended in 250 µL of PBS. A 9:1 (v/v) mixture of ethyl acetate and hexane (750 µL) was then added, and the solution was vortexed at maximum speed for 30 s before separating the phases via centrifugation at 4,500 g for 5 min. The organic layer (top) was then transferred to a clean glass vial and dried under nitrogen and stored at -20°C until analysis by LC-MS/MS, at which time dried samples were reconstituted in a 50% ethanol/water mixture.

**LC-MS/MS analysis**

Analyses were performed on an LC-MS/MS system consisting of an Ultimate3000 HPLC system (Thermo Scientific, Sunnyvale, CA) linked to a 4000 Qtrap triple quadrupole linear ion trap mass spectrometer equipped with a Micro Ion Spray interface (AB/Sciex, Concord, Ontario, Canada). Samples were loaded from an autosampler that was kept at ambient temperature. Chromatographic separation was performed on a Kappa BioBasic C18 column (Thermo Scientific) with dimensions of $150 \times 0.32$ mm (5 µm particle size with 300 Å pore size). Mobile phase A consisted of 10 mM ammonium acetate in water. Mobile phase B consisted of 10 mM ammonium acetate in methanol. Gradient elution was performed at a flow rate of 5 µL/min. The initial composition of the gradient was 50% B for 3 min before a ballistic gradient raised the composition to 96% B over 1 min. The gradient was held at 96% B for 26 min. At 30 min, the gradient was returned to 50% B to reequilibrate the column for 10 min. The total run time was 40 min. The mass spectrometer was operated in positive ionization mode. Nitrogen was used as the curtain and nebulizer gas with the following source conditions: capillary voltage: 5.1 kV, temperature: 100°C, GS1: 12. The declustering potential was set at 70 eV for all masses. The collision energy and dwell times were set at 30 eV and 100 ms, respectively, for all multiple reaction monitoring transitions. Q1 and Q3 were operated at unit resolution. The following MS parameters were used to measure NAEs (precursor ion, product ion): NAE12:0 (244.5, 62.1), NAE14:0 (272.5, 62.1), NAE16:0 (300.5, 62.1), NAE18:0 (328.5, 62.1), NAE18:1 (326.5, 62.1), NAE18:2 (324.5, 62.1), NAE20:4 (348.5, 62.1), PEA-d$_4$ (304.5, 66.1), and AEA-d$_4$ (352.5, 66.1). Detection limits for all NAEs were determined during standard curve construction and were 5 fmol on column for NAE12:0 and 1 fmol on column for NAE14:0, NAE16:0, NAE18:0, NAE18:1, NAE18:2, and NAE20:4.

**Substrate consumption assays**

Measurement of FAAH activity was determined through a modified LC-MS/MS based assay (33). The rate of hydrolysis of putative FAAH substrates was determined by measuring the depletion of reactant via LC-MS/MS. NAEs tested include NAE12:0, NAE14:0, NAE16:0, NAE18:0, NAE18:1, and NAE20:4. Reactions were carried out in 1.5 ml microcentrifuge tubes where the addition of the corresponding NAE (in DMSO) to a final concentration of 300 µM was added to reaction buffer (20 mM Tris-Cl [pH 9.0], 50 mM NaCl, 10% DMSO, and 0.5% Triton X-100) to a final volume of 1.0 ml and preincubated at 21°C. Enzymatic reactions were initiated through the addition of 10 µg of MBP-FAAH (in a 20 mM Tris-Cl [pH 9.0], 50 mM NaCl storage buffer) and incubated at 21°C. Aliquots (100 µL) were taken at 0, 1, 2, 5, 10, and 20 min. Another 100 µL aliquot was taken for an overnight reaction (~16 h) to determine reaction completion. Enzymatic reactions were stopped by placing aliquots in 300 µL of a 9:1 (v/v) mixture of ethyl acetate and hexane and purified and stored as described previously. For analysis by LC-MS/MS, dried samples were reconstituted in a 50% ethanol/water mixture. Kinetic assays were performed in triplicate.

**RESULTS**

**Phylogenetic characterization of Dictyostelium FAAH**

Two putative genes (Dd1, Dd2) found in the *D. discoideum* genome containing the amidase signature sequence were compared with confirmed and potential FAAH protein sequences as identified in *silico* by McPartland et al. (29). The analysis resulted in the formation of five distinct phylogenetic groupings (Fig. 1). These groupings can be described as FAAH 1 and its putative homologs, FAAH 2
the proteins were analyzed using SDS-PAGE to verify protein size. The calculated molecular weight of Dictyostelium FAAH was approximately 70 kDa. Given that the maltose binding protein has an approximate molecular weight of 42.5 kDa, the approximate molecular weight of the MBP-FAAH fusion protein is 112.5 kDa. All proteins migrated near their theoretical weights on the SDS-PAGE gel (Fig. 2).

**Inhibition of Dictyostelium FAAH**

The effect of known mammalian FAAH inhibitors on Dictyostelium FAAH was determined. The rate of hydrolysis of A\textsubscript{p}NA was measured by monitoring the release of \(\textit{p}\)-nitroaniline at 380 nm with each inhibitor being tested at three different concentrations. The mode of inhibition was determined through Lineweaver-Burk analysis (Fig. 3), whereas \(K_i\) was determined via a nonlinear regression of reaction velocity versus substrate concentration plots. Dictyostelium FAAH had a \(V_{\text{max}}\) and \(K_M\) on A\textsubscript{p}NA of 127 nmoles/min/mg and 74 µM, respectively. Results of the inhibitor studies are summarized in Table 1.

First-generation mammalian FAAH inhibitors ATFMK and MAFP inhibited Dictyostelium FAAH with a \(K_i\) of 15.4 µM and 658 nM, respectively. ATFMK and MAFP are less effective on Dictyostelium FAAH than mammalian homologs of the enzyme, where IC\textsubscript{50} values of 1.9 µM and 2.5 nM have been reported, respectively (33). CAY10435, an \(\alpha\)-keto oxazolopyridine, was a strong inhibitor of Dictyostelium FAAH with a \(K_i\) of 835 nM. This compares with a \(K_i\) of 0.57 nM for the mammalian enzyme. Furthermore, CAY10435 was shown to be a noncompetitive inhibitor of Dictyostelium FAAH, whereas for the mammalian enzyme the inhibition was competitive (8).
NAE candidates were identified via a precursor scan (9:1, v/v) followed by LC-MS/MS analysis. Initially, potential neutral lipids with a mixture of ethyl acetate and hexane were further purified via an overnight digestion at 4°C with a recombinant thrombin, which separated the maltose binding protein from His₆-FAAH. His₆-FAAH could then be further purified using a Ni-NTA column. MBP-FAAH could be further purified using a Ni-NTA column. Lane 1, protein standard; Lane 2, MBP-FAAH post amyllose column; Lane 3, thrombin-digested MBP-FAAH; Lane 4, His₆-FAAH post Ni-NTA column.

Other mammalian FAAH inhibitors tested in this study were far less effective inhibitors of Dictyostelium FAAH. The irreversible serine protease inhibitor PMSF inhibited Dictyostelium FAAH with a Ki of 4.2 mM (compared with 900 nM for the mammalian version [34]), whereas the highly potent mammalian FAAH inhibitors JNJ1661010 (a thiadiazolopiperazinyl urea-based inhibitor), URB597 (a carbamate-based inhibitor), and LY2183240 (a heterocyclic urea-based inhibitor) were all poor inhibitors of Dictyostelium FAAH, with negligible inhibition at inhibitor concentrations of 500 µM, 150 µM, and 250 µM, respectively. These values compare with a Ki value of 34 nM for JNJ1661010 (11) and with the IC₅₀ values of 4.6 nM and 12.4 nM for URB597 and LY2183240, respectively (9, 35), on mammalian FAAH homologs.

**Dictyostelium AX3 NAEs**

NAEs were identified in *Dictyostelium* AX3 through a targeted lipidomics approach consisting of the extraction of neutral lipids with a mixture of ethyl acetate and hexane (9:1, v/v) followed by LC-MS/MS analysis. Initially, potential NAE candidates were identified via a precursor scan (m/z = 62.1), with electrospray ionization carried out in positive mode of uninhibited and MAFP-inhibited (10 µM) AX3 neutral lipid extracts. These scans revealed peaks consistent with the expected peaks of NAE12:0, NAE14:0, NAE16:0, NAE18:0, NAE18:1, NAE18:2, and NAE20:4. To confirm the identity of these peaks as NAEs, LC-MS/MS was performed on lipid extracts from an MAFP-infected culture. One sample was spiked with a 1 nmol mixture of the seven NAE standards, and the other was not. In terms of lipophilicity, all seven peaks in the unspiked sample had the same retention time as their corresponding NAE standard (Fig. 4). Also, the two deuterated internal standards, PEA-d₄ and AEA-d₄, coeluted with their corresponding nondeuterated NAE (data not shown). Furthermore, MS/MS was carried out on each putative NAE to produce a collision-induced dissociation spectrum (Fig. 5; supplementary Fig. 1). The peaks in the unspiked sample produce a similar spectrum to that of the synthetic NAE standards for six out of the seven NAEs. The lone exception was NAE20:4, where the MS/MS spectral quality of unspiked sample did not allow for a confident spectral match to the standard. The presence of NAEs in *D. discoideum* has been demonstrated by lipophilicity (i.e., retention time in HPLC) and gas-phase ion chemistry (i.e., MS/MS fragmentation patterns).

After the confirmation of the identity of NAEs in *D. discoideum*, they were quantified in uninhibited and MAFP-inhibited lipid extracts using multiple reaction monitoring (Table 2). The uninhibited AX3 strain produced quantifiable levels of NAE12:0, NAE14:0, NAE16:0, and NAE18:0, with quantities ranging from 0.4 pmol per 10⁸ cells for NAE16:0 to 9.0 pmol per 10⁸ cells for NAE12:0. There were no detectable levels of NAE18:1, NAE18:2, or NAE20:4 in the uninhibited AX3 extracts. As for the MAFP-inhibited extracts, addition of 10 µM MAFP for 24 h resulted in detectable levels of NAE18:1 and NAE18:2 (3.6 and 9.3 pmol per 10⁸ cells, respectively). The addition of MAFP also resulted in a significant increase in the level of NAE16:0 from 0.41 to 1.2 pmol per 10⁸ cells (P < 0.0001).

**Substrate consumption rate of Dictyostelium FAAH**

An attempt to determine the in vitro kinetics of Dictyostelium FAAH on a variety of NAE substrates was made by measuring the rate of substrate depletion in 300 µM reactions via LC-MS/MS. The determination of true initial reaction rates was difficult due to the variance of LC-MS/MS measurements at low substrate consumption levels. To reduce this variance, we report our results as a substrate consumption rate from time 0 to 20 min. At this time, approximately 30% of the substrate was consumed. All seven NAE substrates tested were completely degraded by FAAH in overnight reactions, demonstrating that Dictyostelium FAAH is capable of hydrolyzing NAE substrates with a variety of acyl chain lengths and levels of unsaturation. Substrate consumption rates were determined for six of the seven substrates (Table 3). A consumption rate was not reported for NAE12:0 because there was no significant difference in the amount of the NAE during the 20 min time course.

Dictyostelium FAAH displayed a preference for unsaturated NAE substrates, with consumption rates of 111.4, 83.4, and 112.2 nmol/min/mg for NAE20:4, NAE18:2, and NAE18:1, respectively. By comparison, saturated NAE substrates were hydrolyzed at less than half those rates at 39.9, 10.1, and 20.7 nmol/min/mg for NAE18:0, NAE16:0, and NAE14:0, respectively. The preference of Dictyostelium FAAH for unsaturated NAEs is consistent with results reported for the Arabidopsis and rat FAAH homologs. However, although anandamide is still a preferred NAE substrate for all three homologs, neither Dictyostelium

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**Fig. 2.** SDS-PAGE of purification of FAAH from *D. discoideum*. A recombinant N-terminal hexahistidine tagged FAAH from *D. discoideum* linked to a maltose-binding protein (MBP-FAAH) was expressed in *E. coli* BL21 (DE3). After lysis, MBP-FAAH was purified using an amyllose column. MBP-FAAH could be further purified via an overnight digestion at 4°C with a recombinant thrombin, which separated the maltose binding protein from His₆-FAAH. His₆-FAAH could then be further purified using a Ni-NTA column. Lane 1, protein standard; Lane 2, MBP-FAAH post amyllose column; Lane 3, thrombin-digested MBP-FAAH; Lane 4, His₆-FAAH post Ni-NTA column.
higher reaction velocity on NAE16:0 than NAE14:0 (37), suggesting the opposite.

**DISCUSSION**

Previous work by our group had identified two amidase signature sequence genes in the *D. discoideum* genome, which were named *Dd1* (GenBank: XM_632408) and *Dd2* (GenBank: XM_638290). Initial cloning and expression of these genes as recombinant proteins in *E. coli* had revealed that only *Dd2* was indeed a FAAH homolog being most closely related to *Arabidopsis* FAAH. Although the presence of an unsaturated site in the acyl chain had a definitive impact on reaction velocity, there was no clear effect of acyl chain length on reaction velocity. The hydrolysis of NAE18:0 was higher than NAE16:0 and NAE14:0. However, NAE14:0 had a higher reaction velocity than NAE16:0. Previous reports have shown that rat FAAH slightly prefers shorter acyl chain substrates (36), whereas *Arabidopsis* FAAH had a higher reaction velocity on NAE16:0 than NAE14:0 (37), suggesting the opposite.

**TABLE 1.** Summary of inhibitor studies detailing mode of inhibition on *Dictyostelium* FAAH, $K_i$ on *Dictyostelium* FAAH, and either $K_i$ or IC$_{50}$ on mammalian versions of FAAH

| Inhibitor | Mode of Inhibition for *Dictyostelium* FAAH | $K_i$ *Dictyostelium* | $K_i$ or IC$_{50}$ Mammalian |
|-----------|---------------------------------------------|------------------------|-------------------------------|
| ATFMK     | Competitive                                 | 15,400                 | IC$_{50}$ = 1900 (34)         |
| MAFP      | Irreversible                                | 648                    | IC$_{50}$ = 2.5 (34)          |
| CAY10435  | Noncompetitive                              | 835                    | $K_i$ = 0.57 (8)              |
| PMSF      | Irreversible                                | 4,160,000              | IC$_{50}$ = 900 (34)          |

**Fig. 3.** Inhibitor studies of *Dictyostelium* FAAH. Saturation curves of the reaction of *Dictyostelium* FAAH hydrolyzing arachidonoyl-$p$-nitroaniline to arachidonic acid and $p$-nitroaniline with four different inhibitors at three different inhibitor concentrations. Lines of best fit determined by nonlinear regression of either a competitive or noncompetitive inhibition model and errors bars represent ± 1 SD. Inset: Lineweaver-Burk plot of inhibition of *Dictyostelium* FAAH. A: MAFP (squares, 0 µM; triangles, 100 nM inverted triangles, 1 µM; diamonds 2.5 µM). B: CAY10435 (squares, 0 µM; triangles, 100 nM; inverted triangles, 1 µM; diamonds, 2.5 µM). C: ATFMK (squares, 0 µM; triangles, 14 µM; inverted triangles, 70 µM; diamonds, 140 µM). D: PMSF (squares, 0 mM; triangles, 1 mM; inverted triangles, 5 mM; diamonds, 10 mM).
eukaryotic amidotransferases. The presence of a functional FAAH in D. discoideum led to the hypothesis that the natural substrates of FAAH, NAEs, are also present in D. discoideum. To verify this, we further characterized FAAH to identify inhibitors of FAAH, which should elevate NAE levels in D. discoideum and aid in identifying NAEs before attempting targeted lipidomic analyses of D. discoideum lipid extracts.

Inhibitor studies were carried out in vitro to verify if known FAAH inhibitors were effective on Dictyostelium FAAH and to identify inhibitors that could potentially allow for the modulation of NAE levels in vivo. Although the first-generation substrate-based inhibitors ATFMK and MAFP inhibited Dictyostelium FAAH, they were slightly less effective than on mammalian FAAH homologs. The serine protease inhibitor PMSF was also capable of inhibiting Dictyostelium FAAH but with a $K_i$ of several orders of magnitude higher than mammalian FAAH homologs. These results are similar to results observed in inhibitor studies of Arabidopsis FAAH (35). Of the four highly specific mammalian FAAH inhibitors tested, only CAY10435 inhibited Dictyostelium FAAH, although through a noncompetitive mechanism as opposed to the competitive mechanism in which it inhibits mammalian FAAH homologs.

Targeted lipidomic analyses of D. discoideum lipid extracts identified several NAEs as native to D. discoideum that

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**Fig. 4.** Multiple reaction monitoring (MRM) chromatograms for each of the seven putative NAEs identified in D. discoideum. The sample was obtained from D. discoideum culture that had been grown to a density of $5 \times 10^6$ cells and then exposed to MAFP at a concentration of 10 µM for 24 h. A: Chromatograms from a sample that was not spiked with a 1 nmol mixture of each NAE. B: Chromatograms from a sample from the same culture spiked with a 1 nmol mixture. Elution times are denoted in minutes, and MRM transitions are indicated for each run.
In conjunction with a similar NAE profile, substrate kinetic studies revealed that *Dictyostelium* FAAH, like other FAAH homologs, hydrolyzes a broad spectrum of NAE substrates. The principal difference in substrate kinetics is that *Dictyostelium* FAAH hydrolyzes NAE20:4 at a similar rate to other NAEs, whereas mammalian homologs hydrolyze NAE20:4 at a 3-fold elevated rate compared with other NAE substrates. This could be due to the need for mammals

have been reported to be present in a variety of plant species (40), human tissues (41), and the ciliate *Tetrahymena thermophila* (21). Although the short-chain saturated NAEs NAE12:0 and NAE14:0 are typically associated with plants where, functionally, NAE12:0 has been shown to competitively inhibit lipoxygenase activity and manipulate oxy-lipin metabolism (42), NAE16:0, NAE18:0, NAE18:1, and NAE18:2 are widespread in plants, animals, and *T. thermophila*.

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Table 2. Quantitation of NAEs in *D. discoideum* in the presence and absence of 10 µM MAFP

| NAE Concentration | NAE12:0 | NAE14:0 | NAE16:0 | NAE18:0 | NAE18:1 | NAE18:2 |
|-------------------|---------|---------|---------|---------|---------|---------|
| AX3 (n = 8)       | 9.0 (4.5)* | 2.6 (2.2) | 0.41 (0.21) | 1.1 (0.5) | —       | —       |
| MAFP (n = 6)      | 4.5 (3.1) | 1.3 (1.2) | 1.2* (0.3) | 0.88 (0.14) | 3.6* (1.9) | 9.3* (6.7) |

* P < 0.0001.
* Numbers in parentheses represent SD.
to tightly regulate NAE20:4 due to its binding to the cannabinoid receptors, which have yet to be identified in plants and lower eukaryotes. With a similar NAE profile and FAAH activity, this raises the possibility that many of the NAE-signaling pathways present in D. discoideum may be conserved in higher eukaryotic organisms.

The characterization of *Dictyostelium* FAAH, the identification of native NAEs, and the ability to modulate their levels in vivo provides an opportunity to use *D. discoideum* as a model system in which to study NAE-mediated signaling. The use of *D. discoideum* as a model system has many advantages. *D. discoideum* is already an extensively studied protozoan in developmental biology due to its unusual life cycle that sees it switch from a unicellular grazer to a multicellular slug upon starvation (43) and has been used as a model in other areas, including the study of mitochondrial and neurological diseases (44, 45), pathogen-host interactions (46), and chemotaxis (47). Due to its well-characterized nature, genetic and biochemical techniques in *D. discoideum* are already well developed, including gene knockout by homologous recombination, RNAi-mediated knockdowns, and genetic overexpression (48). Finally, many of the roles linked to NAEs in higher eukaryotes, such as the elevated levels of NAE14:0 in the presence of fungal xylanase in plants (14), the anti-inflammatory effects linked to NAE16:0 (49), the role of NAE18:0 as an immunomodulator and apoptosis-inducer (50), and NAE18:1 as a regulator of food intake (51), are subsets of larger research areas already being modeled in *D. discoideum*.

We have demonstrated that the slime mold *D. discoideum* possesses a similar NAE profile and a FAAH that acts on a similarly broad set of NAE substrates as those found in higher eukaryotic organisms. Furthermore, we have demonstrated the ability to modulate the levels of NAEs in vivo. Given its genetic tractability and broad use as a model organism to study many cellular processes, we propose that *D. discoideum* would be a suitable model in which to study NAE-mediated signaling. Given the large number of human physiological conditions associated with NAE-mediated signaling, the elucidation of these pathways in *D. discoideum* could have much broader implications on human health.

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**Table 3. Substrate consumption rate of *Dictyostelium* FAAH on NAE substrates at 300 µM substrate concentration and comparison of acyl chain length on FAAH reaction rates for three different species (n = 3)**

| Substrate Substrate Consumption Rate | Dictyostelium | Arabidopsis (35) | Rat* (36) | Relative Rate to FAAH20:4 |
|-------------------------------------|---------------|-----------------|----------|-------------------------|
| N-arachidonoyl ethanolamine (NAE20:4) | 111.4 (58.9) | 1.00            | 1.00     | 1.00                     |
| N-linoleoyl ethanolamine (NAE18:2)  | 83.4 (58.5)  | 0.75            | 0.79     | 0.33                     |
| N-oleoyl ethanolamine (NAE18:1)     | 112.2 (56.3) | 1.01            | n.d.     | 0.32                     |
| N-stearoyl ethanolamine (NAE18:0)   | 39.9 (18.7)  | 0.36            | n.d.     | 0.22                     |
| N-palmitoyl ethanolamine (NAE16:0)  | 10.1 (0.5)   | 0.09            | 0.68     | 0.23                     |
| N-myristoyl ethanolamine (NAE14:0)  | 20.7 (6.6)   | 0.19            | 0.51     | 0.27                     |

n.d., not determined.

*In this study, corresponding amide substrates were used (i.e., arachidonamide).

*Numbers in parentheses represent standard deviations.
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