Anandamide Uptake Is Consistent with Rate-limited Diffusion and Is Regulated by the Degree of Its Hydrolysis by Fatty Acid Amide Hydrolase*

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Martin Kaczocha1, Anita Hermann5, Sherrye T. Glaser3, Inge N. Bojesen4, and Dale G. Deutsch1‡

From the 1Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, New York 11794-5215, the 2Center for Translational Neuroimaging, Brookhaven National Laboratory, Upton, New York 11973, and the 3Department of Medical Biochemistry and Genetics, University of Copenhagen, DK-2200 Copenhagen N, Denmark

The uptake of arachidonoyl ethanolamide (anandamide, AEA) in rat basophilic leukemia cells (RBL-2H3) has been proposed to occur via a saturable transporter that is blocked by specific inhibitors. Measuring uptake at 25 s, when fatty acid amide hydrolase (FAAH) does not appreciably affect uptake, AEA accumulated via a nonsaturable mechanism at 37 °C. Interestingly, saturation was observed when uptake was plotted using unbound AEA at 37 °C. Such apparent saturation can be explained by rate-limited delivery of AEA through an unstirred water layer surrounding the cells (1). In support of this, we observed kinetics consistent with rate-limited diffusion at 0 °C. Novel transport inhibitors have been synthesized that are either weak FAAH inhibitors or do not inhibit FAAH in vitro (e.g. UCM707, OMDM2, and AM1172). In the current study, none of these purported AEA transporter inhibitors affected uptake at 25 s. Longer incubation times illuminate downstream events that drive AEA uptake. Unlike the situation at 25 s, the efficacy of these inhibitors was unmasked at 5 min with appreciable inhibition of AEA accumulation correlating with partial inhibition of AEA hydrolysis. The uptake and hydrolysis profiles observed with UCM707, VDM11, OMDM2, and AM1172 mirrored two selective and potent FAAH inhibitors CAY10400 and URB597 (at low concentrations), indicating that weak inhibition of FAAH can have a pronounced effect upon AEA uptake. At 5 min, the putative transport inhibitors did not reduce AEA uptake in FAAH chemical knock-out cells. This strongly suggests that the target of UCM707, VDM11, OMDM2, and AM1172 is not a transporter at the plasma membrane but rather FAAH, or an uncharacterized intracellular component that delivers AEA to FAAH. This system is therefore unique among neuro/immune modulators because AEA, an uncharged hydrophobic molecule, diffuses into cells and partial inhibition of FAAH has a pronounced effect upon its uptake.

Arachidonoyl ethanolamide (AEA)2 is a neuromodulatory lipid that belongs to a family of molecules collectively termed the endocannabinoids. Like Δ⁹-tetrahydrocannabinol, many of the actions of AEA are mediated through the G-protein-coupled cannabinoid (CB, and CB₃) and vanilloid (TRPV1) receptors (2–7). AEA signaling is terminated by a rapid reuptake mechanism followed by its hydrolysis into arachidonic acid and ethanolamine primarily by the intracellular enzyme FAAH (8–10). By metabolizing AEA, FAAH maintains an inward gradient that drives the continued cellular accumulation of AEA (11–13).

There is some controversy regarding the processes mediating AEA uptake. It was widely accepted that AEA transport is a selective, saturable process of facilitated diffusion for which there are selective transport inhibitors (For review, see Refs. 14–16). However, in platelets, neuroblastoma, astrocytoma, primary cortical neurons, and erythrocyte ghost membranes it has been suggested that uptake occurs by passive diffusion (13, 17–20). Experimental variables such as incubation times, inclusion of bovine serum albumin (BSA) in the incubation medium and cell type differences may account for the disparities in results between laboratories (19, 21, 22). For a detailed review of these issues, see Ref. 23. AEA modulates the production and function of a variety of cytokines in mast and basophilic cells through the cannabinoid receptors (24). RBL-2H3 cells are of immune origin and express FAAH (25) and the CB₂ receptor (26). Whereas it is generally believed that AEA uptake in these cells occurs via a putative AEA transporter that can be modulated by both FAAH and transporter inhibitors (11, 25, 27–30), these studies used relatively long incubation times incapable of distinguishing events at the plasma membrane from those occurring downstream.

In the current study, the saturability of AEA transport into RBL-2H3 cells was examined at early time points in the presence of BSA to determine if its accumulation was consistent with an AEA transporter or passive diffusion. In contrast to previous reports, we undertook these studies at 37 and 0 °C employing the equilibrium dissociation constants to calculate free AEA in solution (31). To date many of the putative AEA transport inhibitors have been found to also inhibit FAAH (19, 32, 33). However, the recent characterization of novel inhibitors that display negligible effects upon FAAH activity (34–36), provided an opportunity to more accurately characterize the contributions of FAAH and a putative transporter to the uptake process. These inhibitors were examined in RBL-2H3 cells and in their cognate chemical FAAH knock-out at both early time points for their effects upon transport at the membrane and at later times to determine if they decrease uptake by interfering with the AEA concentration gradient maintained by FAAH. As a positive control for transport, parallel experiments were also conducted on the 5-HT transporter.

**EXPERIMENTAL PROCEDURES**

*Materials—RBL-2H3 cells were obtained from American Type Culture Collection (Manassas, VA). Culture media (MEM), streptomycin, penicillin, non-essential amino acids, and sodium pyruvate were from Invitrogen and fetal bovine serum from Gemini (Woodland, CA). AEA (arachidonoyl

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1 To whom correspondence should be addressed: Dept. of Biochemistry and Cell Biology, SUNY at Stony Brook, Stony Brook, NY 11794-5215. Tel.: 631-632-8595; Fax: 631-632-8575; E-mail: DDeutsch@notes.sunysb.edu.
2 The abbreviations used are: AEA, arachidonoyl ethanolamide; FAAH, fatty acid amide hydrolase; BSA, bovine serum albumin.
ethanolamide), UCM707 (N-(3-furanylmethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), OMDM2 ([(R)-N-(1-(4-hydroxyphenyl)-2-hydroxyethyl)oleamide] and CAY10400 (1-(Oxazolo[4,5-b]pyridin-2-yl)-1-oxo-cis-9-octadecenoic acid 3’-carbamoyl-biphenyl-3-yl ester) was from Biomol International. SR144528 amide) and CAY10400 (1-(Oxazolo[4,5-b]pyridin-2-yl)-1-oxo-cis-9-octadecanoic acid 3’-carbamoyl-biphenyl-3-yl ester) was from Biomol International. SR144528 (N-[(1S)-endo-1,3,3-trimethyl bicyclo-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) was obtained from the National Institute on Drug Abuse (NIDA). Arachidonoyl-5,6,8,9,11,12,14,15-[3H]ethanolamide (204 Ci/mmol, 100 nCi/phenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) was obtained from Tocris Cookson (Ellisville, MO). URB597 (cyclohexylcarboxylic acid 3’-carbamoyl-biphenyl-3-yl ester) was from Biomol International. SR144528 (N-[(1S)-endo-1,3,3-trimethyl bicyclo-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) was obtained from the National Institute on Drug Abuse (NIDA). Arachidonoyl-5,6,8,9,11,12,14,15-[3H]ethanolamide (204 Ci/mmol, 100 nCi/µl) was from Perkin-Elmer Life Sciences and arachidonoyl ethanolamide (ethanolamine-1,2-[14C] (110 mCi/mmol, 100 nCi/µl) from American Radiolabeled Chemicals (St. Louis, MO). Fatty acid-free bovine serum albumin, capsazepine (N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide), 5-hydroxytryptamine creatinine sulfate and fluoxetine ([S]-N-methyl-1-[4-trifluoromethyl]phenoxyl benzenepropamine) were from Sigma and 5-hydroxy-2-tryptamine [3H]trifluoracetate (118 Ci/mmol, 1 µCi/µl) was from Amer sham Biosciences. For the thin layer chromatography (TLC) experiment Baker-flex silica gels IB-F from JT Baker (Phillipsburg, NJ) were used.

**Cell Culture—**RBL-2H3 cells (American Type Culture Collections) were seeded at a density of 5 × 10^4 cells per 35 × 10-mm dishes in 2 ml of MEM (Invitrogen) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% non-essential amino acids, and 1% penicillin/streptomycin. All cells were grown at 37 °C with 5% CO2.

**FAAH Hydrolytic Activity and [3H]AEA Uptake Inhibition**—The use of [14C]AEA allowed for the concurrent measurement of AEA uptake and hydrolysis. This is a modification of a procedure described by Bisogno et al. (25). RBL-2H3 cells were plated at the specified density. Eight hours later, the cells were washed once with 750 µl of supplemented medium (MEM + 0.4% BSA) and pretreated with 750 µl of supplemented medium containing either inhibitor or vehicle (ethanol <0.08%) for 15 min at 37 °C. Preincubation media were removed, and the cells were incubated for 3 or 25 s to 5 min at 37 °C in 750 µl of MEM + 0.4% BSA that was pre-equilibrated for 15 min, 100 nCi of 140 mCi/mmol AEA (ethanolamine-1,2-[14C]) and 100 nCi unlabeled AEA ± inhibitor. At the indicated times, 750 µl of ice-cold MEM + 0.4% BSA were added, and the liquid media separated from the cells, which were scraped three times with 400 µl of ice-cold MEM containing 0.15% BSA and 700 nCi of 204 Ci/mmol of arachidonoyl-5,6,8,9,11,12,14,15-[3H]ethanolamide and unlabeled AEA, ranging from 100 nM to 20 μM. To stop the reaction, 3 ml of ice-cold MEM + 0.4% BSA (or 0.15% BSA) were added, cells were placed on ice, washed with MEM + 0.4% BSA (or 0.15% BSA) and scraped three times with 400 µl of ice-cold EDTA in PBS, and 2.4 ml of 1:1 chloroform/methanol were added. Samples were centrifuged, the organic phase removed, scintillation fluid was added, and the samples were counted. To determine specific AEA uptake at 25 s, a 3 s blank was subtracted to account for nonspecific binding of AEA to cellular membranes and culture plates. The amount of uptake was determined by averaging the triplicates and plotting these values as pmol of AEA uptake per 10^6 cells per s. The experiments were repeated three times, except for the 0 °C experiment, which was repeated twice. The data were analyzed using a linear regression program provided by GraphPad Prism. In order to study uptake in the absence of FAAH activity, cells were preincubated for 15 min with 100 nM CAY10400 prior to the addition of AEA. Unbound AEA concentrations were calculated based on the formula [Aw] = Kd ν(1 − ν) (20, 31, 37). [Aw] = unbound AEA, ν = total [AEA]/total [BSA] using the given Kd at 37 °C (54.92 nm) and at 0 °C (6.87 nm) (20, 31, 37).

**Time Course of [3H]AEA Uptake Inhibition**—Growth medium was removed, and the cells were washed once with supplemented medium (MEM + 0.4% BSA). The cells were subsequently preincubated for 15 min in supplemented medium containing either vehicle (ethanol <0.08%) or inhibitor at the concentration indicated. Preincubation medium was removed, and the cells were incubated for 25 s, 90 s, or 5 min in supplemented medium containing [3H]AEA (and unlabeled AEA for a final concentration of 100 nM) and vehicle or inhibitor as indicated. The reactions were terminated as described above. Nonspecific (3 s) binding values were subtracted from all points. The degree of uptake inhibition was obtained by averaging the triplicates and plotting these values as percent inhibition of control cells. Statistical significance of inhibition was determined using a two-tailed unpaired Student’s t tests between treated samples and untreated controls.

**Effect of Increasing AEA Concentration upon Its Uptake Rate**—The procedure was a modification of that described by Refs. 38 and 39. RBL-2H3 cells were washed and then incubated for 25 s at 37 °C with 750 µl of uptake buffer (10 mM HEPES, 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4 × 7 H2O, 2.2 mM CaCl2 × 2H2O, 10 mM glucose, pH 7.4) containing either 4.5 nM or 6 nM of 5-[3H]HT and non-labeled 5-HT at a concentration range of 31.5–1000 nM. Cells were then washed three times with 750 µl of ice-cold buffer, 2 ml of liquid scintillation mixture was added, and plates were placed on a shaker overnight. Samples were then analyzed in an LKB scintillation counter. Nonspecific uptake was determined by adding 10 µM fluoxetine for 10 min prior to the incubation with 5-HT. The amount of uptake was determined by averaging the triplicates of two separate experiments and plotting these values as pmol of 5-HT uptake per 10^6 cells per s using.
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FIGURE 1. Time course of AEA uptake and hydrolysis in the absence or presence of FAAH inhibitor. RBL-2H3 cells were pretreated for 15 min with medium containing either inhibitor (100 nM CAY10400) or vehicle control (ethanol) and subsequently incubated with [14C]AEA (100 nM) for up to 5 min. AEA uptake and hydrolysis were quantified as described under “Experimental Procedures.” ○, total AEA uptake in the absence of inhibitor; □, total AEA uptake in the presence of 100 nM CAY10400; ●, AEA hydrolysis in the absence of inhibitor; ▼, AEA hydrolysis in the presence of 100 nM CAY10400. Non-enzymatic AEA hydrolysis in the absence of cells was found to account for less than 1% of total AEA metabolism at all time points tested. Data represent means ± S.E. of three independent experiments performed in triplicate.

GraphPad Prism. To determine if there was saturation of uptake, data were analyzed with a Michaelis-Menten plot, and $V_{\text{max}}$ and $K_m$ values were determined in Graphpad Prism.

Enzymatic Assay of Inhibitors in RBL-2H3 Homogenates—FAAH activity assays were performed as previously described (19). Briefly, RBL-2H3 homogenates were incubated with 30 μM AEA + 0.01 μCi [14C]AEA in the presence or absence of inhibitors for 30 min. Reactions were stopped by the addition of two volumes of chloroform/methanol (1:1). The phases were separated, and [14C]ethanolamine measured in the aqueous phase by liquid scintillation counting.

Uptake Experiment and TLC Analysis—A 3, 25, 90 s, and 5 min uptake experiment using [14C]AEA preincubated for 10 min with either vehicle or CAY10400 was performed as described above except the level of radioactivity for this experiment was increased to 180 nCi/dish. After chloroform/methanol extraction the organic extract from the cells was dried down. The residue was suspended in 40 μl of chloroform/methanol (1:1) and spotted on thin layer plates. The standard was [14C]AEA. TLC was performed as previously described (12).

RESULTS

Time Course of AEA Uptake and Metabolism—At 25 and 45 s, the earliest times permitting accurate quantification of AEA uptake, there was already significant AEA hydrolysis (~40% of the AEA taken into the cell). Inhibition of FAAH by CAY10400 had only a slight, non-significant, effect upon AEA uptake, although it completely inhibited AEA hydrolysis. Accordingly, 25 s was chosen to examine AEA uptake at the plasma membrane for measurement of possible AEA transport saturation and effects of putative transport inhibitors.

At longer uptake times, AEA transport and hydrolysis proportionally increased with time. At 90 s and 5 min, ~65 and 89% of the AEA taken up by the cells was hydrolyzed, respectively. However, unlike shorter time points, a significant reduction in AEA uptake was observed in RBL-2H3 cells treated with CAY10400, indicating that AEA metabolism influences its uptake at these times (Fig. 1). For that reason, 5 min was chosen to examine events occurring downstream of the membrane.

Kinetics of AEA and 5-HT Uptake—Based upon the results from the previous experiment (Fig. 1), 25 s was chosen to examine AEA and 5-HT uptake kinetics. RBL-2H3 cells were incubated with increasing concentrations of AEA in medium supplemented with 0.4% BSA at 37 °C (Fig. 24) or 0.15% BSA at 37 °C (Fig. 2C). Expressing the amount of total AEA added versus its uptake, AEA accumulated in a nonsaturable manner ($r^2 = 0.964$ for 0.4% BSA, $r^2 = 0.969$ for 0.15% BSA). AEA uptake utilizing 0.15% BSA was ~2-fold higher than 0.4% BSA. Based upon the $K_r$ of AEA dissociation from BSA (31), the concentration of unbound AEA was calculated and also plotted versus uptake. From these saturation curves (Fig. 2, B and D), the $K_m$ and $V_{\text{max}}$ values were 36.3 nM and 27.7 pmol/106 cells/s, and 51.4 nM and 23.1 pmol/106 cells/s, respectively.

The experiments were also performed at 0 °C. Again the uptake was not saturable (Fig. 2E) when plotting the added amount of AEA versus uptake ($r^2 = 0.922$) whereas saturation was observed with unbound AEA (Fig. 2F), with $K_m = 3.9$ nM and $V_{\text{max}} = 7.9$ pmol/106 cells/sec. In Fig. 2F, the calculated unbound AEA concentrations are lower than those in Fig. 2, B and D since the $K_r$ is temperature-dependent (31). The differences in unbound concentrations of AEA in Fig. 2, B and D result from varying AEA:BSA ratios. Our 3 s blank at 37 °C (Fig. 2C) gives the same result as the 3 s blank at 0 °C (Fig. 2E).

The contribution of FAAH activity toward AEA uptake was also examined. Following treatment with CAY10400, no significant difference in AEA transport kinetics was observed at AEA concentrations ranging from 0.1 μM to 50 μM in 0.4% BSA, confirming that FAAH activity does not affect the AEA uptake rate at 25 s (data not shown).

In contrast to AEA, 5-HT is a water soluble molecule and as a control, 5-HT uptake was examined to validate that carrier-mediated transport could be observed in RBL-2H3 cells at 25 s. As shown in Fig. 3C, 5-HT uptake (31.5–1000 nM) undergoes saturation with $K_m$ and $V_{\text{max}}$ values of 0.2 μM and 2.6 pmol/106 cells/s, respectively.

Effects of AEA and 5-HT Transport Inhibitors upon Uptake at 25 s—To establish the selectivity of newly described AEA transport inhibitors upon a putative AEA transporter, RBL-2H3 cells were incubated with 100 nM [3H]AEA in medium containing 0.4% BSA in the presence or absence of inhibitors. As expected, AEA accumulation was not significantly reduced by 100 nM of the FAAH inhibitor CAY10400 (Fig. 3A). Similarly, the putative transporter inhibitors, 10 μM OMDM2, 10 μM AM1172, 1 and 80 μM UCM707, and 10 μM VDM11, were without effect. Comparable results were obtained in parallel inhibitor studies carried out in the presence of 0.15% BSA (data not shown).

As a positive control, the uptake of 5-HT was also examined with fluoxetine, a documented selective inhibitor of its uptake. Contrary to AEA uptake inhibitors, the transport of 5-HT (250 nM and 500 nM) into RBL-2H3 cells was reduced by ~50 to 60% upon treatment with 10 μM fluoxetine (Fig. 3B). Collectively, these data validate the use of 25 s to examine inhibition of transport and suggest that these putative transport inhibitors are not acting upon an AEA transporter on the plasma membrane.

Effect of Inhibitors and CB2 Antagonist upon AEA Transport at 90 s and 5 Min—Although the putative transport inhibitors do not affect AEA uptake at 25 s (Fig. 3A), others have shown that these compounds act as potent inhibitors of FAAH transport at longer incubation times. Given that FAAH mediates AEA transport at these uptake times and may serve as a target for these inhibitors, cells were first treated with 100 nM CAY10400 (Fig. 4A). With FAAH inhibited, AEA uptake was reduced to ~65 and ~39% of the uninhibited control values at 90 s and 5 min, respectively. Strikingly, similar uptake inhibition patterns were observed in cells treated with 30 μM VDM11 (Fig. 4B) and 80 μM UCM707 (Fig. 4C), both at concentra-
tions high enough to inhibit FAAH (30, 34), but not with 1 μM UCM707 (Fig. 4D). The putative transport inhibitors OMDM2 (Fig. 4E) and AM1172 (Fig. 4F) produced similar but less efficacious uptake inhibition profiles when tested at 10 μM. To examine the possibility that the CB₂ receptor plays a role in the apparent uptake of AEA, SR144528, an antagonist of the CB₂ receptor, was examined and found not to reduce AEA uptake over the entire time course (Fig. 4G).

Putative Transport Inhibitors Reduce AEA Uptake at 5 Min through the Partial Inhibition of AEA Hydrolysis—It was shown that at 25 s, AEA uptake was independent of FAAH activity and unaffected by putative transport inhibitors (Fig. 3A). However, at longer uptake times, transport inhibitors and CAY10400 had a dramatic effect upon AEA accumulation (Fig. 4). To explore the relationship between the effects of these inhibitors upon FAAH activity and uptake, we simultaneously measured AEA transport and breakdown in RBL-2H3 cells incubated...
with 100 nM [14C]AEA and inhibitors. In control cells, ~90% of AEA was hydrolyzed following uptake (Table 1). To calibrate the system, varying concentrations of the selective and potent FAAH inhibitors, CAY10400 and URB597, were tested. With increasing concentrations of both FAAH inhibitors, a dose-dependent reduction in AEA uptake and hydrolysis was observed (Fig. 5A). The uptake of AEA was reduced to ~83%, ~60%, and ~37% of control following preincubation with 0.5, 1.0, and 100 nM CAY10400, respectively (Table 1). Furthermore, the hydrolysis of AEA following uptake was reduced to ~74%, ~71%, and ~17% after CAY10400 treatment. Similar values were observed with URB597, albeit at much lower concentrations. The inhibition of AEA hydrolysis was significant in all cases except for 0.1 nM URB597. As expected, commensurate with an inhibition of hydrolysis, the levels of intact intracellular AEA were elevated following treatment with both FAAH inhibitors at all concentrations tested (Table 1). For CAY10400, intact AEA increased from 2.5 pmol/10^6 cells for the control to 5.3, 4.4 and 7.7 pmol/10^6 cells after treatment with 0.5, 1.0, and 100 nM CAY10400, respectively. Similar values were observed for URB597.

Next, the effects of putative transport inhibitors upon AEA uptake and hydrolysis were examined. Similar to FAAH inhibitors, treatment of cells with most putative transport inhibitors (3 and 10 μM OMDM2, 10 and 30 μM AM1172, 4 and 10 μM VDM11, and 10 and 30 μM UCM707) also significantly reduced AEA hydrolysis. When examined at the lowest concentrations of 3 μM and 10 μM, respectively, OMDM2 and UCM707 were the only exceptions (Fig. 5A). As with the FAAH inhibitors, this inhibition in AEA hydrolysis was also coupled to a reduction in AEA uptake. These results in Fig. 5 using [14C]AEA confirm those obtained using [3H]AEA shown in Fig. 4. Interestingly, the uptake and hydrolysis profiles with these inhibitors mirrored those of FAAH inhibitors at low concentrations. Importantly, the levels of intact intracellular AEA were elevated following treatment with all inhibitors compared with control (Table 1). The efficacy of these inhibitors upon AEA uptake generally correlated with their potencies against FAAH in vitro (Table 2). Specifically, CAY10400, URB597, VDM11, UCM707, and OMDM2 were equipotent inhibitors of AEA hydrolysis in homogenized RBL-2H3 cells and in RBL-2H3 cells in culture. In contrast, AM1172 was ineffec-
Anandamide uptake and hydrolysis in cultured RBL-2H3 cells (Fig. 5A).

A scatter diagram was prepared to correlate the relationship between AEA accumulation and hydrolysis at 5 min using AEA uptake and hydrolysis values from Fig. 5A. A nearly direct correlation was observed between a reduction in AEA hydrolysis and uptake with both FAAH inhibitors, with a significant reduction of AEA uptake (60–80% of control) corresponding to a 15–20% inhibition in AEA hydrolysis (Fig. 5B). Interestingly, the profiles observed with the putative transport inhibitors were similar in magnitude to those obtained with low concentrations of the two FAAH inhibitors, suggesting the partial inhibition of AEA breakdown by both classes of compounds.

**DISCUSSION**

The mechanism(s) governing the cellular transport of AEA is controversial with most reports indicating carrier-mediated uptake whereas a few recent studies propose simple diffusion (13, 18, 19, 22, 40–42). Understanding the mechanism of AEA inactivation is important from a basic science standpoint and for the design of therapeutics (43–48). Experimentally, these two models have been distinguished by testing for...
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TABLE 1
Quantification of inhibitor effects upon AEA uptake and hydrolysis at 5 min

| Inhibitor         | AEA uptake | AEA hydrolyzed/total uptake | Intact AEA |
|-------------------|------------|-----------------------------|------------|
|                   | % average control | % pmol/10⁶ cells |          |
| Average control   | 100 ± 2.7   | 89.9 ± 1.3                  | 2.5 ± 0.3  |
| 0.5 nM CAY10400   | 82.5 ± 6.5  | 74.3 ± 4.3                  | 5.3 ± 0.9  |
| 1 nM CAY10400     | 60.4 ± 1.8  | 70.9 ± 1.5                  | 4.4 ± 0.2  |
| 100 nM CAY10400   | 37.2 ± 1.9  | 17.2 ± 1.7                  | 7.7 ± 0.5  |
| 0.1 nM URB597     | 72.5 ± 4.1  | 75.3 ± 3.8                  | 4.4 ± 0.4  |
| 1 nM URB597       | 41.1 ± 3.1  | 18.8 ± 6.3                  | 8.4 ± 1.2  |
| 3 µM OMDM2        | 80.1 ± 6.6  | 81.9 ± 4.7                  | 3.8 ± 1.2  |
| 10 µM OMDM2       | 65.2 ± 3.2  | 80.9 ± 4.6                  | 3.2 ± 0.8  |
| 10 µM AM1172      | 63.0 ± 9.8  | 70.9 ± 3.8                  | 4.5 ± 0.6  |
| 30 µM AM1172      | 64.0 ± 4.0  | 56.6 ± 9.9                  | 7.1 ± 1.9  |
| 4 µM VDM11        | 54.8 ± 7.5  | 62.5 ± 4.2                  | 5.2 ± 0.9  |
| 10 µM VDM11       | 38.1 ± 3.8  | 38.4 ± 10.0                 | 5.8 ± 0.4  |
| 10 µM UCM707      | 71.0 ± 4.0  | 83.1 ± 3.1                  | 3.0 ± 0.5  |
| 30 µM UCM707      | 51.7 ± 5.6  | 71.7 ± 7.1                  | 3.5 ± 0.6  |

saturability of AEA uptake and its inhibition by selective compounds (14, 16, 19, 23, 49).

AEA uptake into RBL-2H3 is driven by FAAH at time points greater than 1 min (11, 27) whereas at 25 s FAAH activity does not significantly affect AEA accumulation (Fig. 1). Although AEA is already being hydrolyzed at 25 s, its uptake appears to be independent of FAAH activity because of the large initial AEA influx into the cell before approaching equilibrium. This rapid phase of AEA accumulation likely reflects its cellular uptake in vivo where FAAH maintains an AEA concentration gradient and primes cells to quickly take up and inactivate AEA.

In contrast to 25 s, FAAH plays a more prominent role in driving AEA cellular accumulation at longer uptake times. For example, untreated RBL-2H3 cells take up 1.5- and 2.7-fold more AEA than CAY10400-treated cells at 90 s and 5 min, respectively (Fig. 1). At times >5 min, the contribution of FAAH to AEA uptake is likely even greater (with FAAH accounting for the majority of AEA accumulation). Therefore, it is impractical to numerically define the component of uptake driven by FAAH as it is temporally dependent. These findings contrast a recent report suggesting that RBL-2H3 cells do not metabolize AEA appreci-
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TABLE 2

Effects of putative transport inhibitors upon FAAH activity in RBL-2H3 homogenates

Homogenates were incubated with 30 μM [14C]AEA for 30 min. FAAH activity was determined as described previously (19). IC50 values for FAAH were determined from at least two independent experiments performed in triplicate.

| Inhibitor | IC50 (μM) |
|-----------|-----------|
| OMDM2    | >50 μM (16% inhibition at 10 μM) |
| AM1172   | >50 μM |
| CAY10400 | 0.5 ± 0.1 μM |
| URB8979  | 2 ± 0.2 μM |
| VDM11    | 5 ± 0.5 μM |
| UCM707   | 50 ± 5.4 μM |

FIGURE 6. Effects of putative AEA transport inhibitors in FAAH chemical knock-out cells at 5 min. RBL-2H3 cells were pretreated with vehicle, 10 nM URB8979, or 10 nM URB8979 plus the indicated inhibitor for 15 min and subsequently incubated with [3H]AEA (100 nm) for 5 min. AEA uptake was quantified as described under “Experimental Procedures.” Statistical significance was calculated using a two-tailed unpaired Student’s t test against the 10 nM URB8979 treated cells. Data represent means ± S.E. of three independent experiments performed in triplicate.

...bly at 90 s and FAAH activity does not contribute to the uptake process (29). For other cell types, it has been shown that incubation times longer than 1 min cannot isolate AEA uptake occurring at the plasma membrane from downstream processes, including FAAH activity and intracellular sequestration (11, 12, 19, 20, 49).

Short incubation times have been used extensively to determine transport of fatty acids (50) (for review, see Ref. 51), and only recently extended to include AEA (18, 19, 22). In our hands, at 37 °C the rate of AEA accumulation in RBL-2H3 cells displayed linear uptake kinetics at 25 s, when plotted as a function of total AEA in the presence of BSA (Fig. 2, A and C), consistent with previous work implicating simple diffusion. Linear kinetics have also been observed in neuroblastoma, astrocytoma, and P19 embryonic carcinoma cells where uptake of total AEA occurred through rapid and unsaturable processes from 25 s to 45 s (19, 22). A recent report examining the movement of AEA through a membrane (erythrocyte ghosts) lends further credence to this assertion (20). However, when we plotted uptake using unbound AEA, calculated from the Kd for the dissociation of AEA from BSA (31), the resulting curves are saturating at 37 °C (Fig. 2, B and D). This apparent saturation was also observed at 0 °C (Fig. 2F). One of us (I. N. B.) has recently shown that AEA uptake becomes saturated by virtue of AEA, an uncharged hydrophobic molecule, undergoing rate-limited permeation of the hydrophilic unstirred water layer surrounding the cell (1). Our uptake profiles at 0 °C are similar to those observed at 37 °C as predicted from the unstirred water model. Collectively, these data highlight the difficulty of using kinetic data to identify a process of facilitated diffusion since rate-limited permeation of AEA through the unstirred water layer confounds the results. Therefore, the saturable transport kinetics observed here for AEA, a lipophilic molecule, cannot distinguish a carrier-mediated process from permeation through the unstirred water layer.

Our findings contrast previous studies in these cells using longer incubation times (>1 min) concluding that AEA transport is carrier-mediated with apparent Km values ranging from ~9 to 33 μM (11, 25, 27–29). In this study, the Km ranges from 36.3 to 51.4 nM using unbound AEA. Our results may help explain the saturation observed by others who added AEA directly to the cells in ethanol in the absence of BSA (29, 40). However, the shortcoming of such an approach including the inability to determine unbound AEA concentrations owing to AEA adherence to pipette tips, tubes, and plastic culture dishes has been widely discussed (13, 22, 30, 52).

For hydrophilic compounds the situation is different. We took advantage of the well established mechanism of 5-HT transport in RBL-2H3 cells to confirm the validity of our assay conditions (39, 53). Uptake of 5-HT (a hydrophilic molecule) was saturable at 37 °C for 25 s (Fig. 3C), with Km and Vmax values in concordance with previously published data (39). These results demonstrate that carrier-mediated uptake can be observed using analogous temporal conditions to our AEA transport assays.

AEA is unique compared with other hydrophobic molecules studied in transport, since it is not charged. For example, fatty acids, which are charged, have been shown to pass through the membrane by a specific transporter in certain tissues, by simple diffusion in others, or by a combination of both (54, 55). Prostaglandins are charged anions which diffuse poorly through membranes despite their hydrophobicity (56). Therefore, prostaglandins utilize a membrane transporter which has been identified and cloned (57, 58). This transporter displays time and concentration dependent uptake kinetics for prostaglandins which are inhibited by specific transport inhibitors. However, prostaglandin efflux from cells occurs by simple diffusion (59).

Carrier-mediated uptake processes can be modulated by selective compounds and thus many putative AEA transporter inhibitors have been described (32, 34, 35, 41). The inability of UCM707, OMDM2, VDM11, and AM1172 to reduce AEA uptake at 25 s suggests that they are not acting upon an AEA transporter (Fig. 3A). This contrasts previous reports in the literature that used longer incubation times (34–36, 60). In contrast to AEA transport inhibitors, fluoxetine inhibited the uptake of 5-HT at 25 s, confirming that these assay conditions are sufficient to observe inhibition of a transporter.

To probe for inhibitor interactions with sites downstream of the plasma membrane, notably FAAH and trafficking mechanisms, longer incubation times (>1 min) were used. The FAAH inhibitors CAY10400 and URB8979 reduced AEA cellular accumulation by inhibiting FAAH (Figs. 4 and 5), thereby shifting the AEA concentration gradient toward equilibrium as described previously (11, 12). Although FAAH is known to modulate AEA accumulation, it is interesting to note that the two FAAH inhibitors dose-dependently reduced AEA uptake (Fig. 5, A and B and Table 1). To our knowledge, this is the first report to directly correlate an incremental inhibition of AEA hydrolysis and cellular AEA...
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accumulation in the steady state. Consequently, weak FAAH inhibitors may reduce AEA accumulation in the steady state, mimicking the inhibition of a putative transport protein. A potent inhibitor of AEA uptake in RBL-2H3 cells was recently described using long incubation times of 16–20 h (61), and was not tested as a FAAH inhibitor in these cells (62). At high concentrations, the putative transport inhibitors displayed uptake and hydrolysis profiles comparable to the low concentration FAAH inhibitors. Therefore these compounds affect uptake since they are either weak inhibitors of FAAH or interfere with intracellular delivery of AEA to FAAH for hydrolysis.

The result that AM1172 may act as a FAAH inhibitor was surprising, since in contrast to UCM707, VDM11, and OMDM2 (30, 34), it has not been reported to significantly inhibit FAAH in vitro (Table 2) (35, 36). Therefore, the inhibition of FAAH activity observed in intact RBL-2H3 cells may have resulted from intracellular concentration of this inhibitor in FAAH-containing membranes, likely exceeding concentrations examined in vitro. Similarly, biochemical activation of this compound may have ensued following its uptake into cells thereby promoting FAAH inhibition, as has been reported previously for AEA-derived arachidonic acid metabolites (63).

Alternatively, AM1172 may have targeted a putative downstream intracellular trafficking mechanism that mediates AEA delivery to FAAH, thereby limiting AEA availability to FAAH and consequently reducing its hydrolysis and uptake (12, 13, 19, 27, 49). This notion is further supported by the elevated levels of intact intracellular AEA observed following inhibitor treatment. The selective inhibition of a membrane transporter would reduce AEA uptake while retaining FAAH hydrolysis by FAAH, resulting in reduced intact intracellular AEA. However, the opposite is observed in this study (Table 1), suggesting the inhibition of downstream events such as metabolism by FAAH, intracellular AEA binding sites, or AEA trafficking mechanisms.

None of the transport inhibitors significantly reduced AEA uptake in FAAH chemical knock-out cells (Fig. 6), strongly suggesting that transport inhibition is solely dependent upon perturbing AEA metabolism in RBL-2H3 cells at long uptake times. It is noteworthy that URB597 was used to inactivate FAAH as it is structurally distinct from AEA and transport inhibitors based upon the AEA structure (45), and therefore reduces the possibility of it targeting a theoretical AEA transporter or a trafficking protein. URB597 has been shown through a proteomics screen to be selective for FAAH (46). Other groups have shown that inhibition of uptake by UCM707, AM1172, and OMDM2 also involved a FAAH-independent target. For UCM707, this was a postulated binding protein and for AM1172 and OMDM2 an AEA transporter (13, 36, 64). A binding protein has also been postulated as a target for other FAAH transport inhibitors (19). In our hands, the addition of putative transport inhibitors failed to significantly alter AEA uptake in chemical knock-out cells. This suggests that pretreatment with URB597 alone is sufficient to eliminate the concentration gradient driving AEA accumulation. Such conditions may prevent observable inhibition of intracellular AEA trafficking to FAAH.

BSA displays an apparent $K_a$ of ~55 nM at 37 °C for AEA (31) from which, in our study, we may calculate unbound AEA concentrations ranging from ~10 pM to 0.5 μM. These are within the physiological range of AEA concentrations found in blood plasma, averaging ~4 nM (65, 66).

In conclusion, the current study is the first to present evidence that AEA uptake into RBL-2H3 cells may be rate-limited by AEA permeation through an unstirred water layer outside of the cell. This phenomenon, consistent with recent findings in a model system (1), displays saturation similar to what one would expect if there were an AEA transporter. These data highlight the difficulties of using saturating kinetics as the principal determining factor for the presence of transport mediated by an unidentified and uncloned protein. Cellular AEA accumulation is driven by a concentration gradient that is tightly regulated by FAAH, with partial inhibition of FAAH causing a corresponding decrease in AEA uptake in the steady state. To our knowledge, this is the first demonstration of such strong coupling between uptake of neurotransmitters/neuromodulators and their catabolism. This phenomenon likely extends to neuronal cell populations that utilize AEA signaling and express FAAH. While caveolae/lipid raft-mediated endocytosis has been suggested to contribute to AEA uptake (67, 68), it is unlikely to make a major contribution within the time frame of our experiments. On the other hand, whether AEA uptake takes place through defined areas of the cell membrane cannot be excluded by the present work.

Taken together, our findings suggest that FAAH is a viable pharmacological target while an AEA transporter, similar to fatty acid and prostaglandin transporters, will require characterization and cloning prior to gaining acceptance. It was predicted nearly a decade ago that inhibition of AEA breakdown would have significant therapeutic value in the areas of analgesia, mood, nausea, memory, appetite, sedation, locomotion, glaucoma and immune function (69). It is therefore noteworthy that FAAH inhibitors have recently been reported to exert, for example, anxiolytic and angesic effects in vivo (45, 46), confirming earlier predictions. Strikingly, a single nucleotide polymorphism in the FAAH gene (P129T) results in decreased FAAH expression and is linked to drug abuse in humans (70, 71).

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