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ACCELERATED COMMUNICATION

Role of Fatty Acid Amide Hydrolase in the Transport of the Endogenous Cannabinoid Anandamide

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ABSTRACT

A facilitated transport process that removes the endogenous cannabinoid anandamide from extracellular spaces has been identified. Once transported into the cytoplasm, fatty acid amide hydrolase (FAAH) is responsible for metabolizing the accumulated anandamide. We propose that FAAH contributes to anandamide uptake by creating and maintaining an inward concentration gradient for anandamide. To explore the role of FAAH in anandamide transport, we examined anandamide metabolism and uptake in RBL-2H3 cells, which natively express FAAH, as well as wild-type HeLa cells that lack FAAH. RBL-2H3 and FAAH-transfected HeLa cells demonstrated a robust ability to metabolize anandamide compared with vector-transfected HeLa cells. This activity was reduced to that observed in wild-type HeLa cells upon the addition of the FAAH inhibitor methyl arachidonyl fluorophosphonate. Anandamide uptake was reduced in a dose-dependent manner by various FAAH inhibitors in both RBL-2H3 cells and wild-type HeLa cells. Anandamide uptake studies in wild-type HeLa cells showed that only FAAH inhibitors structurally similar to anandamide decreased anandamide uptake. Because there is no detectable FAAH activity in wild-type HeLa cells, these FAAH inhibitors are probably blocking uptake via actions on a plasma membrane transport protein. Phenylmethylsulfonyl fluoride, a FAAH inhibitor that is structurally unrelated to anandamide, inhibited anandamide uptake in RBL-2H3 cells and FAAH-transfected HeLa cells, but not in wild-type HeLa cells. Furthermore, expression of FAAH in HeLa cells increased maximal anandamide transport 2-fold compared with wild-type HeLa cells. These results suggest that FAAH facilitates anandamide uptake but is not solely required for transport to occur.

The discovery of the G protein-coupled CB1 and CB2 cannabinoid receptors (Matsuda et al., 1990; Munro et al., 1993) that are activated by Δ^9-tetrahydrocannabinol prompted the search for an endogenous agonist for these receptors. Several endocannabinoids were discovered in the early 1990s, the first being *N*-arachidonylethanolamide, or anandamide (Devane et al., 1992). Anandamide is a long-chain fatty acid amide that is believed to have therapeutic potential similar to marijuana (Devane et al., 1992). Some of these potential medicinal properties include: suppression of nausea and vomiting; appetite stimulation; alleviation of side effects associated with Parkinson's disease and multiple sclerosis; reduction of intraocular pressure from glaucoma; treatment of pain, especially migraines; and regulation of memory, cognition, fever, blood pressure, and the immune system (Mechoulam et al., 1998; Mechoulam and Ben Shabat, 1999).

Anandamide is considered a putative neurotransmitter, with its metabolism occurring intracellularly by a fatty acid amide hydrolase (FAAH) that cleaves anandamide into arachidonic acid and ethanolamine (Schmid et al., 1985; Deutsch and Chin, 1993; Desarnaud et al., 1995; Ueda et al., 1995; Cravatt et al., 1996). FAAH metabolizes several other fatty acid amides and esters, such as oleamide and the endocannabinoid 2-arachidonyl glycerol (Cravatt et al., 1996; Patterson et al., 1996). Although anandamide is capable of being synthesized by FAAH from its components arachidonic acid and ethanolamine in vitro (Devane and Axelrod, 1994; Ueda

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ABBREVIATIONS: FAAH, fatty acid amide hydrolase; PMSF, phenylmethylsulfonyl fluoride; MAFP, methyl arachidonyl fluorophosphonate; ECL, enhanced chemiluminescence; KRH, Krebs-Ringer-HEPES; AM404, *N*-(4-hydroxyphenyl)-arachidonamide; ATFK, arachidonyl trifluoromethyl ketone; A.5-HT, arachidonoyl serotonin.

et al., 1995; Arreaza et al., 1997), the physiological concentrations of ethanolamine and arachidonic acid are not large enough to make this a plausible route in vivo (Schmid et al., 1990; Piomelli, 1994). Increases in intracellular calcium concentrations have been shown to stimulate the formation, cleavage, and release of anandamide from a membrane phospholipid precursor, *N*-arachidonoyl phosphatidylethanolamine (Di Marzo et al., 1994, 1996; Cadas et al., 1996, 1997). Once released from the membrane into the extracellular space, anandamide can activate CB1 receptors in the central nervous system, or CB2 receptors in the periphery (Devane et al., 1992; Felder et al., 1993; Pertwee et al., 1993; Mackie et al., 1993).

Termination of anandamide signaling at the cannabinoid receptors occurs through an uptake mechanism that transports anandamide into the cell where it subsequently undergoes rapid degradation by FAAH (Ueda et al., 1995; Cravatt et al., 1996; Hillard et al., 1997; Beltramo et al., 1997; Piomelli et al., 1999). Current evidence suggests that anandamide uptake is a carrier-mediated process that is time- and temperature-dependent, saturable, and inhibited with a unique pharmacologic profile (Di Marzo et al., 1994; Beltramo et al., 1997; Rakhshan et al., 2000). Colocalization of FAAH and CB1 receptors in rat brain may indicate FAAH's participation in anandamide signaling and uptake (Thomas et al., 1997; Egertova et al., 1998; Yazulla et al., 1999). The putative transmembrane domain and SH3-domain-binding sequence of FAAH suggests that FAAH may localize with the plasma membrane and associated proteins (Cravatt et al., 1996). We propose that FAAH may establish facilitated anandamide uptake by creating and maintaining an inward concentration gradient of anandamide.

To determine the role of FAAH in anandamide uptake, we have studied anandamide uptake and FAAH enzymatic activity in RBL-2H3 cells that natively express FAAH and wild-type HeLa cells that lack FAAH. Although HeLa cells lack FAAH, we detected anandamide transport activity that had kinetics similar to RBL-2H3 cells. Additionally, our results revealed that FAAH inhibitors structurally similar to anandamide not only inhibited FAAH, but also decreased anandamide uptake, possibly by recognizing a distinct extracellularly accessible plasma membrane transporter. Phenylmethylsulfonyl fluoride (PMSF), a FAAH inhibitor that is structurally unrelated to anandamide, did not inhibit anandamide uptake in wild-type HeLa cells. However, FAAH-transfected HeLa cells and RBL-2H3 cells showed reduced anandamide uptake in the presence of PMSF, suggesting that FAAH inhibition may also reduce transport. Furthermore, expression of FAAH in HeLa cells increased maximal anandamide transport compared with wild-type HeLa cells, thus confirming a role for FAAH in facilitated anandamide uptake. Our data using wild-type and FAAH-transfected HeLa cells indicated that although FAAH is not required for anandamide uptake, FAAH may work in conjunction with other membrane proteins to facilitate anandamide transport.

Materials and Methods

FAAH Enzymatic Activity Assay. Experiments were performed on HeLa cells as described previously (Rakhshan et al., 2000). Wild-type HeLa cells, or HeLa cells transiently transfected with rat FAAH cDNA (generous gift from Dr. Ben Cravatt, Scripps Research Institute)/pBluescript II SK$^-$ (Cravatt et al., 1996) using the vaccinia virus T7 expression system (Fuerst et al., 1986; Blakely et al., 1991), were washed with KRH buffer, scraped into 1.5-ml tubes with Tris-EDTA buffer (20 mM Tris-HCl, 1 mM EDTA, pH 9.0, 0.7 μg/ml pepstatin A, and 0.5 μg/ml leupeptin), and homogenized. Lysed cell enzymatic assays were performed by a modification of a method published previously (Omeir et al., 1995). Membrane preparations were incubated with 5 nM anandamide (ethanolamine 1-^{3}H) (American Radiolabeled Chemicals, St. Louis, MO) for 5 min in the presence or absence of 500 nM methyl arachidonyl fluorophosphonate (MAFP). Reactions were terminated with the addition of 2× volume of chloroform/methanol (1:1, v/v). Production of [^{3}H]ethanolamine in the aqueous phase was compared with intact anandamide (ethanolamine 1-^{3}H) in the organic phase by liquid scintillation counting on a TopCount scintillation plate analyzer (Packard, Meriden, CT).

Saturation kinetics were determined by using 125 μg of homogenized cell lysate from RBL-2H3 cells or FAAH-transfected HeLa cells and increasing concentrations of anandamide (ethanolamine 1-^{3}H) (American Radiolabeled Chemicals), with the specific activity diluted to ~0.9 Ci/mmol with nonisotopic anandamide. After a 5-min incubation at 37°C in the presence or absence of 10 μM MAFP, the reaction was terminated with the addition of 2× volume of chloroform/methanol (1:1, v/v). Production of [^{3}H]ethanolamine in the aqueous phase was compared with intact anandamide (ethanolamine 1-^{3}H) in the organic phase by liquid scintillation counting on a Packard TopCount scintillation plate analyzer. FAAH V_{max} and K_m values were derived by nonlinear least-square fits with Prism software (v. 3.0; GraphPad, San Diego, CA).

Western Blot Analysis. Total postnuclear and plasma membrane enriched cell lysates were prepared using a method published previously (Stuhlsatz-Krouper et al., 1998). Briefly, HeLa or RBL-2H3 cells were grown to confluence, washed with KRH buffer, and homogenized in 255 mM sucrose, 20 mM Tris, pH 7.4, 1 mM EDTA, and 1 μg/ml each of pepstatin A and leupeptin. For total postnuclear protein preparations, nuclei were removed by centrifugation at 1,000*g* for 10 min, followed by centrifugation of the supernatant at 356,000*g* for 30 min at 4°C. The pellet was resuspended in 1% Triton X-100, 50 mM Tris, pH 7.4, 2 mM EDTA, 150 mM NaCl with 1 μg/ml each of pepstatin and leupeptin. For plasma membrane enriched protein preparations, homogenized cell lysates were pelleted at 16,000*g* for 20 min, resuspended in the sucrose buffer mentioned above, placed on a 1.12 M sucrose layer, and centrifuged at 99,000*g* for 20 min, which resulted in an interfacial plasma membrane protein fraction. Protein samples were quantified by the Pierce bicinchoninic acid assay (Rockford, IL) and prepared for gel electrophoresis with the addition of 1× volume Laemmli buffer (62.5 mM Tris-HCl, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 5% bromphenol blue). Protein samples were loaded onto a 10% SDS-polyacrylamide gel electrophoresis Tris-HCl gel and electrophoresed at 150 V for ~1 h using the Mini-Protean 3 system (Bio-Rad, Hercules, CA). Resolved proteins were transferred to a polyvinylidene difluoride membrane using the Bio-Rad Mini Trans-Blot system. The membrane was blocked overnight in phosphate-buffered saline containing 0.1% Tween-20 and 5% (w/v) nonfat dry milk at 4°C. To determine the presence of FAAH, α-FAAH polyclonal 1° antibody (gift from Dr. Ben Cravatt, Scripps Research Institute), horseradish peroxidase-labeled goat-anti-rabbit 2° antibody (Bio-Rad), and ECL detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) were used, followed by exposure to X-ray film (Amersham Pharmacia Biotech).

To determine the presence of Mcl-1 and BiP/GRP78, membranes previously blotted with FAAH antibody were stripped for 1 h at 50°C in stripping buffer (62.5 mM Tris-HCl, 2% SDS, and 100 mM β-mercaptoethanol), rinsed in phosphate-buffered saline containing 0.1% Tween-20 buffer for 5 min, blocked overnight (as mentioned above), and probed with α-Mcl-1 monoclonal 1° antibody (BD Transduction Laboratories, Lexington, KY) for 1 h at room temperature. After horseradish peroxidase-labeled goat-anti-mouse 2° antibody labeling

for 1 h at room temperature, ECL detection, and exposure to X-ray film, membranes were stripped once again (as mentioned above) and probed with α-BiP/GRP78 monoclonal 1° antibody (BD Transduction Laboratories) for 1 h at room temperature. After horseradish peroxidase-labeled goat-anti-mouse 2° antibody labeling for 1 h at room temperature and ECL detection, membranes were exposed to X-ray film.

[^{3}H]Anandamide Transport Assay. Experiments were performed on RBL-2H3 and HeLa cells as described previously (Rakhshan et al., 2000). Briefly, cells (2×10^5 RBL-2H3 and 1×10^5 HeLa) were plated in 24-well culture dishes 16–24 h before the assay. HeLa cells were transiently transfected with rat FAAH cDNA (generous gift from Dr. Ben Cravatt, Scripps Research Institute, CA)/pBluescript II SK- (Cravatt et al., 1996) or the catalytically inactive FAAH mutant S217A-FAAH cDNA (Omeir et al., 1999)/pBluescript II SK$^-$ using the vaccinia virus T7 expression system (Fuerst et al., 1986; Blakely et al., 1991). Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was replaced with serum-free medium 1 h before the assay. Uptake and FAAH inhibitors (100 μM) were diluted in Krebs-Ringer-HEPES (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM HEPES, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, pH 7.4) and added 10 min before the incubation with [^{3}H]anandamide (1 nM) at 37°C for 5 min. [^{3}H]Anandamide uptake was terminated by three washes with KRH containing 1% bovine albumin. Cells were solubilized in liquid scintillant overnight before counting using a Packard TopCount scintillation plate analyzer. Nonspecific uptake was determined by the addition of 100 μM AM404 10 min before [^{3}H]anandamide uptake. Saturation kinetics were determined using increasing concentrations of [^{3}H]anandamide with the specific activity diluted to $\sim 5 \times 10^{-3}$ Ci/mmol with nonisotopic anandamide. Substrate K_m and antagonist IC_{50} values were derived by nonlinear least-square fits with GraphPad Prism v. 3.0 using the Hill equation or the four-parameter logistic equation. K_i values were obtained according to the equation of Cheng and Prusoff (1973).

Supplies. The following cell culture supplies were used: Dulbecco's modified Eagle's medium (Fisher, Pittsburgh, PA); fetal bovine serum (Hyclone, Logan, UT); RBL-2H3 and HeLa cells (American Type Culture Collection, Manassas, VA); trypsin, glutamine, penicillin, and streptomycin (Life Technologies, Grand Island, NY); and cell culture plates (Falcon/Becton-Dickinson Labware, Mountain View, CA, and Packard). [^{3}H]Anandamide (217.0 Ci/mmol) was purchased from PerkinElmer Life Science Products (Boston, MA) and anandamide[ethanolamine 1-^{3}H] (60 Ci/mmol) from American Radiolabeled Chemicals Inc. Uptake and FAAH inhibitors arachidonyl trifluoromethyl ketone (ATFK), MAFP, and arachidonoyl serotonin (A.5-HT) were obtained from Cayman Chemical Co. (Ann Arbor, MI), and PMSF was purchased from Sigma (St. Louis, MO). Nonisotopic anandamide and AM404 were purchased from RBI-Sigma Aldrich (Natick, MA). Microscint 20 scintillation cocktail was obtained from Packard. All other chemicals were obtained from either Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA).

Results and Discussion

Determination of FAAH Activity in RBL-2H3 and HeLa Cells. In the present study, we determined the presence, activity, and cellular location of FAAH in RBL-2H3 and HeLa cells. Previous work has demonstrated FAAH activity in RBL-2H3 cells (Bisogno et al., 1997; Rakhshan et al., 2000). An FAAH enzymatic assay using lysed cells showed that wild-type HeLa cells lacked detectable FAAH activity; however, FAAH-transfected HeLa cells had an approximately 10-fold increased ability to metabolize anandamide compared with vector-transfected HeLa cells (Fig. 1A). Furthermore, the addition of MAFP, a potent irreversible FAAH inhibitor (Deutsch et al., 1997b), inhibited the ability of FAAH to cleave anandamide in both FAAH-transfected HeLa cells (Fig. 1A) and wild-type RBL-2H3 cells (Rakhshan et al., 2000). Because vector-transfected HeLa cells lacked detectable FAAH activity, the addition of MAFP in these cells had no effect on apparent anandamide metabolism (Fig. 1A). Evaluation of FAAH kinetics for anandamide metabolism revealed a 2-fold increase in V_{max} value for FAAH-transfected HeLa cells with no change in K_m value compared with RBL-2H3 cells (Table 1).

Western blot analysis of RBL-2H3 total postnuclear and plasma membrane cell lysates showed that RBL-2H3 cells contained endogenous FAAH that seems to be concentrated in the plasma membrane (Fig. 1B). These results support the localization of endogenous FAAH at the plasma membrane; however, this does not exclude the possibility that FAAH may be found in other cellular compartments. FAAH has been found in intracellular compartments, although these

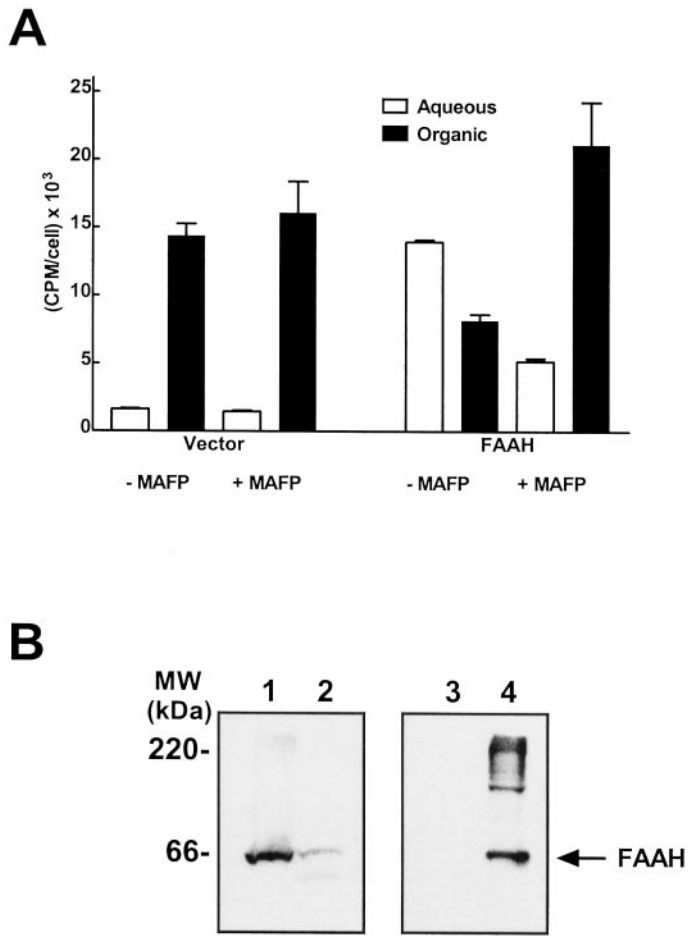

Fig. 1. RBL-2H3 and HeLa cells differ in the expression of FAAH. A, metabolism of anandamide in HeLa cells. FAAH enzymatic assays were performed on pBluescript II SK$^-$-transfected (Vector) and FAAH-transfected (FAAH) HeLa cells as described under *Materials and Methods*. Aqueous indicates cpm of [^{3}H]ethanolamine, or metabolized anandamide, in the aqueous phase. Organic indicates cpm of anandamide[ethanolamine 1-^{3}H], or intact anandamide, in the organic phase. MAFP at a concentration of 500 nM was used to inhibit FAAH. Data plotted are the means ± S.E. of triplicate determinations and are representative of three separate experiments. B, Western blot analysis to detect FAAH immunoreactivity in RBL-2H3, wild-type HeLa, and FAAH-transfected HeLa cells. Lane 1, RBL-2H3 plasma membrane enriched fraction (122 μg); lane 2, RBL-2H3 total postnuclear fraction (100 μg); lane 3, wild-type HeLa total postnuclear fraction (100 μg); and lane 4, FAAH-transfected HeLa total postnuclear fraction (20 μg). Results are representative of three separate experiments.

studies relied exclusively on cells that were transiently transfected with FAAH cDNA (Patricelli and Cravatt, 2000). To further evaluate the contents of our FAAH-containing plasma membrane enriched preparations, RBL-2H3 cell lysates (plasma membrane and total postnuclear) were analyzed for the presence of a mitochondrial protein, Mcl-1, and the BiP/GRP78 endoplasmic reticulum chaperone via Western blot analysis. Results indicated that both cell lysate fractions contained the endoplasmic reticulum protein, but lacked the mitochondrial marker (data not shown). These data suggest that our plasma membrane cell lysate protocol may have enriched for all cellular membranes, leaving open the possibility that native FAAH may be found in intracellular compartments such as the endoplasmic reticulum. Although HeLa cells transiently transfected with vector (pBluescript II SK$^-$) lacked FAAH expression, HeLa cells transiently transfected with FAAH cDNA are capable of expressing large amounts of FAAH (Fig. 1B). Plasma membrane enriched wild-type HeLa cell lysate also lacked FAAH expression (data not shown). To further confirm the lack of FAAH expression in wild-type HeLa cells, reverse transcription-polymerase chain reaction of HeLa RNA was performed using oligonucleotides specific for both rat and human FAAH, which revealed that RBL-2H3 cells, but not HeLa cells, contain endogenous FAAH (data not shown). Taken together, these data not only confirm the plasma membrane localization of FAAH, but also the absence of FAAH expression in wild-type HeLa cells.

FAAH Inhibitors Have Differing Effects on Anandamide Uptake in RBL-2H3 and HeLa Cells. Although FAAH has been shown to play an important role in the metabolism of various fatty acid amide and ester substrates, such as anandamide, there are few commercially available selective inhibitors of this enzyme. In our study, we examined the roles of both reversible [ATFK, AM404, and A.5-HT (Bisogno et al., 1998b)] and irreversible FAAH inhibitors [MAFP and PMSF (Deutsch et al., 1997b)] on anandamide uptake in RBL-2H3 and wild-type HeLa cells. The most potent FAAH inhibitor used in this study is MAFP, an arachidonyl binding site directed phosphonylation reagent (Deutsch et al., 1997b). Other more recent FAAH inhibitors have been shown to also be very potent trifluoromethyl inhibitors (Deutsch et al., 1997a; Boger et al., 2000).

If FAAH maintains an inward concentration gradient necessary for anandamide uptake, then inhibitors of FAAH should reduce transport. To evaluate the role of FAAH in anandamide uptake, we examined the various reversible and irreversible inhibitors of FAAH for their ability to reduce anandamide transport. MAFP and structurally related FAAH inhibitors (ATFK, A.5-HT, and AM404) reduced anandamide uptake in a dose-dependent manner in RBL-2H3 cells (Fig. 2A and Table 2). Because wild-type HeLa cells lack FAAH expression, we did not expect a reduction of anandamide transport upon the addition of the various FAAH inhibitors. However, we observed a reduction in anandamide transport in wild-type HeLa cells in the presence of all the FAAH inhibitors examined except for PMSF (Fig. 2B and Table 2). Interestingly, PMSF inhibited approximately 60% of anandamide uptake in RBL-2H3 cells, but had no effect on anandamide transport in HeLa cells (Fig. 2B). These results suggest that MAFP and related compounds inhibit not only FAAH enzymatic activity but also an earlier step in the

TABLE 1
FAAH enzyme kinetics for anandamide metabolism in FAAH-expressing cells
All values represent means ± S.E. of three separate experiments performed in duplicate. Statistical comparisons of V_{max} and K_m values were performed using a two-tailed t test.

	V_{max}	K_m
	(mol/min/mg protein) × 10^{11}	*μM*
RBL-2H3	2.3 ± 0.3	4.0 ± 1.3
FAAH/HeLa	4.3 ± 0.5[a]	2.7 ± 0.1

[a] $p < 0.05$ compared with RBL-2H3.

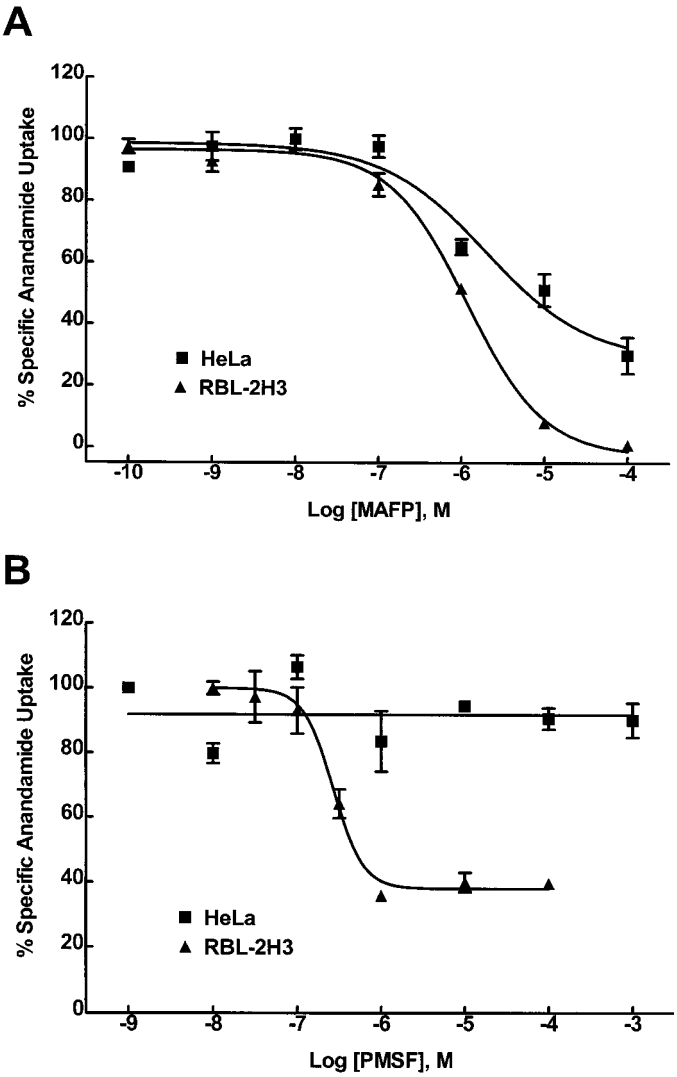

Fig. 2. FAAH inhibitors have different effects on anandamide uptake in RBL-2H3 and HeLa cells. A, anandamide uptake inhibition by MAFP. B, anandamide uptake inhibition by PMSF. [^{3}H]Anandamide uptake assays were performed on HeLa (■) and RBL-2H3 (▲) cells as described under *Materials and Methods*. Data were plotted as percentage of specific anandamide uptake. All data plotted represent means ± S.E. of triplicate determinations and are representative of three to four separate experiments. Nonspecific uptake was determined in the presence of 100 μM AM404 and subtracted from total uptake. Mean values ± S.E. for total and nonspecific anandamide uptake before correction to percentage of specific anandamide are as follows: A, HeLa, 7,004 ± 45 CPM (total) and 1,413 ± 72 CPM (nonspecific); RBL-2H3, 11,816 ± 76 CPM (total) and 2,545 ± 78 CPM (nonspecific). B, HeLa, 7,385 ± 3 CPM (total) and 1,950 ± 85 CPM (nonspecific); RBL-2H3, 13,813 ± 349 CPM (total) and 4,133 ± 298 CPM (nonspecific).

TABLE 2
Inhibition of anandamide uptake by FAAH inhibitors
All values represent means ± S.E. of three or four separate experiments.

K_i values	MAFP	PMSF	A. 5-HT	ATFK	AM404
μM					
HeLa	8.9 ± 2.1	NA	7.4 ± 0.8	30.0 ± 11.0	2.0 ± 0.4
RBL-2H3	2.8 ± 1.7	3.5 ± 1.7	5.6 ± 0.5	2.8 ± 0.1	14.0 ± 2.5[a]

NA = Not active at concentrations up to 1 mM.
[a] Rakhshan et al. (2000)

anandamide transport mechanism, because uptake in both FAAH-containing (RBL-2H3) and FAAH-null (HeLa) cell lines was reduced by these FAAH inhibitors. In contrast, PMSF seemed to be more specific for FAAH inhibition because it did not disrupt anandamide uptake in wild-type HeLa cells that lack endogenous FAAH (Fig. 2B). Taken together, the specificity of the PMSF effect, the partial effect of PMSF on uptake in RBL-2H3 cells, and the existence of high-affinity anandamide transport in the wild-type HeLa cells suggests that FAAH activity is not an absolute requirement for transport. Such findings are consistent with the existence of a distinct transporter protein for anandamide.

Anandamide Uptake Inhibition by FAAH Inhibitors Is Reversible in HeLa Cells, but not in RBL-2H3 Cells. Potential actions of FAAH inhibitors that would result in inhibition of anandamide uptake include: 1) an irreversible mechanism whereby FAAH inhibitors covalently modify proteins involved in transport or 2) a reversible mechanism in which FAAH inhibitors competitively block anandamide recognition at an extracellularly accessible target. To examine whether or not the various FAAH inhibitors reduce anandamide uptake in a reversible or irreversible mechanism, HeLa and RBL-2H3 cells were washed after a 30-min FAAH inhibitor preincubation, followed by the addition of [^{3}H]anandamide (Fig. 3). After the washes, anandamide uptake in HeLa cells was not significantly different from untreated control cells indicating that the effect of the FAAH inhibitors was rapidly reversible (Fig. 3A). In contrast, the effects of MAFP, A.5-HT, or ATFK on anandamide uptake in RBL-2H3 cells were only partially reversed by the washout step (Fig. 3B). These studies suggest that irreversible FAAH inhibitors, which operate via an intracellular phosphonylation mechanism, do not have a similar action at the extracellularly accessible transporter site in HeLa cells. Furthermore, the effects of FAAH inhibitors on anandamide transport in RBL-2H3 cells may be caused in part by inhibition of intracellular FAAH.

Heterologous Expression of FAAH in HeLa Cells Increases Maximal Anandamide Transport and Restores Uptake Inhibition by PMSF. If FAAH is involved in anandamide transport, then expression of FAAH in HeLa cells should increase anandamide transport capacity. Transient expression of FAAH in HeLa cells induced a 2-fold increase in the maximal anandamide transport rate compared with wild-type HeLa cells (Table 3). To verify that FAAH catalytic activity is required for increased anandamide transport, the catalytically inactive FAAH mutant S217A (Omeir et al., 1999) was transiently expressed in HeLa cells. Western blot analysis of S217A-FAAH-transfected HeLa cells revealed robust expression of mutant FAAH comparable with wild-type FAAH (Fig. 4A). However, expression of this mutant did not alter V_{max} and K_m anandamide uptake kinetics compared with wild-type HeLa cells (Table 3). Surprisingly, RBL-2H3 cells that endogenously express FAAH had a lower maximal anandamide transport capacity than wild-type HeLa cells (Table 3). This discrepancy in anandamide uptake in FAAH-expressing cell lines may be attributed to the possibility that there are additional transport mechanisms operating in different cell types, or that anandamide transport is differen-

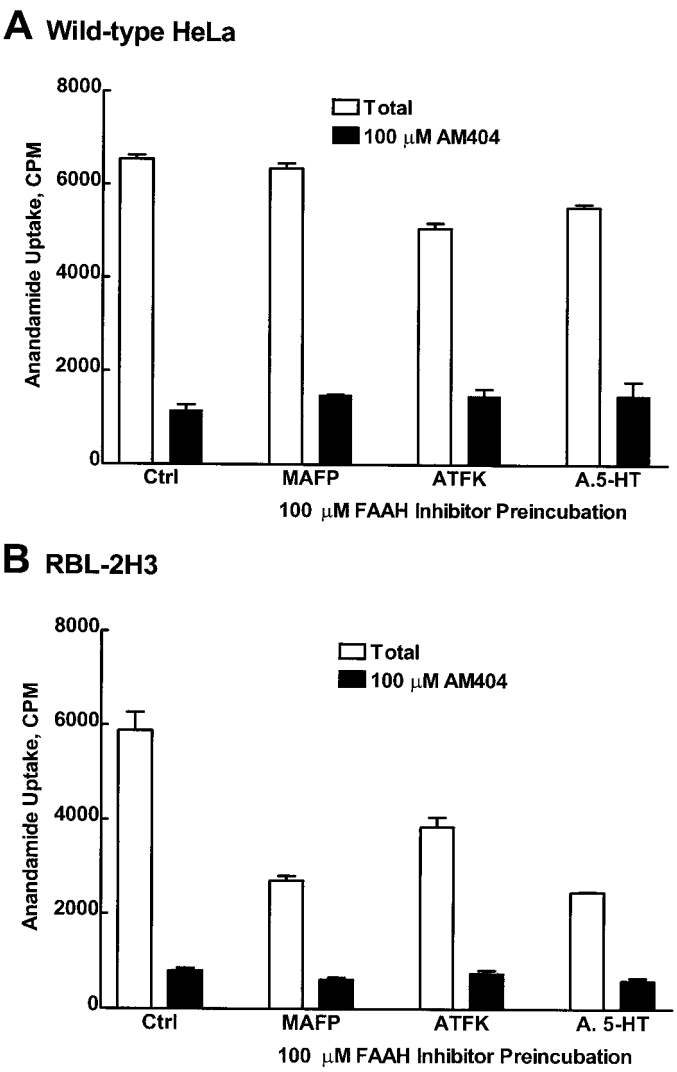

Fig. 3. Anandamide uptake inhibition by FAAH inhibitors is reversible in HeLa cells, but not in RBL-2H3 cells. Anandamide uptake after preincubation with various FAAH inhibitors in wild-type HeLa cells (A) and RBL-2H3 cells (B). [^{3}H]Anandamide uptake assays were performed as described under *Materials and Methods*. Briefly, cells were pretreated for 30 min with the specified FAAH inhibitors, washed 3× with 37°C KRH buffer, then incubated with [^{3}H]anandamide for 5 min. All data plotted represent means ± S.D. of duplicate determinations and are representative of two to three separate experiments. Ctrl, pretreatment with buffer.

tially regulated in various cell types. Further support for additional mechanisms involved in anandamide transport comes from results showing that the FAAH inhibitors, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one and linoleyl trifluoromethyl ketone, do not inhibit anandamide transport in primary cultures of rat cortical astrocytes (Beltramo et al., 1997). Thus, expression levels of other proteins, such as the putative plasma membrane anandamide transporter, may influence uptake in these cells. Finally, anandamide transport in FAAH-transfected HeLa cells was inhibited by PMSF similar to RBL-2H3 cells, with IC_{50} values of 7.6 ± 1.0 μM and 3.5 ± 1.7 μM, respectively (Figs. 4B and 2; Table 2). This 50% reduction in anandamide transport by PMSF is expected because the kinetic data in Table 3 shows a 2-fold increase in anandamide transport for FAAH-transfected HeLa cells compared with wild-type HeLa cells. These data support the idea that the inward concentration gradient created by FAAH's metabolism of anandamide can work to facilitate anandamide transport in certain cell systems.

In summary, we confirmed that endogenous FAAH expression in RBL-2H3 cells is concentrated in the plasma membrane, which supports earlier findings that RBL-2H3 cells metabolize anandamide similar to brain FAAH (Bisogno et al., 1997), and that the RBL-2H3 cognate cell line, RBL-1, contains FAAH mRNA as determined by Northern blot analysis (Bisogno et al., 1998a). Wild-type HeLa cells, that lack endogenous FAAH expression and activity, mimicked RBL-2H3 FAAH expression and enzymatic activity when transiently transfected with FAAH cDNA. In HeLa cells, FAAH inhibitors that are structurally similar to anandamide (MAFP, ATFK, A.5-HT, and AM404) inhibited anandamide transport, perhaps by recognizing an extracellularly accessible plasma membrane transporter. In contrast, PMSF, which is structurally unrelated to anandamide, disrupted anandamide uptake in FAAH-transfected HeLa cells, but not wild-type HeLa cells, confirming the contributions of FAAH in the uptake process. Whereas our studies reveal a role for FAAH in anandamide transport, the partial inhibition of uptake by PMSF in RBL-2H3 and FAAH-transfected HeLa cells, as well as the inhibition of uptake by FAAH inhibitors in wild-type HeLa cells, supports the existence of a yet unidentified plasma membrane transport protein that may act in concert with FAAH to promote anandamide uptake. Further exploration of the role of FAAH in anandamide uptake will allow for the discovery of drugs to efficiently and selectively block anandamide clearance and permit anandamide to remain in the synapse for potential therapeutic activation of cannabinoid receptors. Additionally, control of anandamide accumulation in peripheral tissues via FAAH may also provide a mechanism for the regulation of immune responses, such as T-cell proliferation, antibody formation, and inflammation.

During the review process for this article, Deutsch et al. (2001) reported anandamide transport sensitivity to FAAH inhibitors and expression of FAAH in Hep2 cells increases anandamide uptake.

TABLE 3

Anandamide transport kinetics in HeLa and FAAH-expressing cells

All values represent means ± S.E. of three to eight separate experiments. Statistical comparisons of V_{max} and K_m values were performed using one-way analysis of variance followed by Bonferroni's post test.

	V_{max}	K_m
	$(mol/min/cell) \times 10^{17}$	μM
RBL-2H3	23.7 ± 2.6	16.4 ± 4.4
HeLa	43.7 ± 4.6[a]	12.1 ± 2.6
S217A-FAAH/HeLa	39.7 ± 4.1[a]	12.3 ± 0.5
FAAH/HeLa	76.7 ± 1.3	18.5 ± 1.6

[a] $p < 0.01$ compared to RBL-2H3 and FAAH/HeLa

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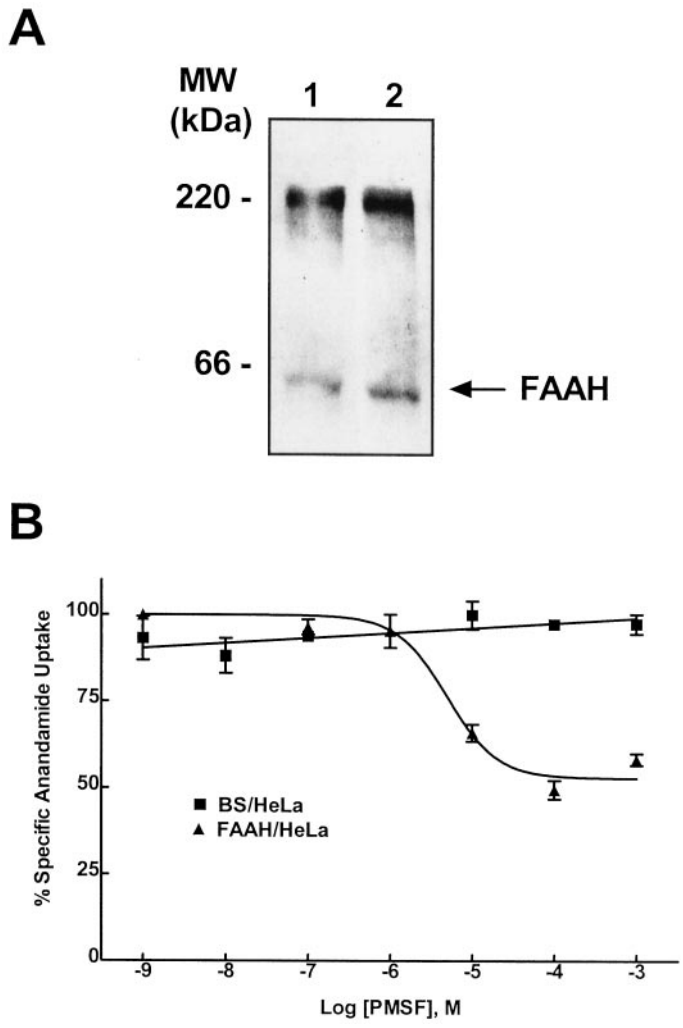

Fig. 4. Expression of catalytically active FAAH in HeLa cells is required for increased anandamide transport and PMSF sensitivity. A, Western blot analysis of FAAH expression in HeLa cells transiently transfected with FAAH and catalytically inactive FAAH (S217A-FAAH). Lane 1, FAAH-transfected HeLa cells (20 μg); lane 2, S217A-FAAH-transfected HeLa cells (20 μg). B, anandamide uptake inhibition by PMSF in FAAH-transfected HeLa cells. [^{3}H]Anandamide uptake assays were performed on pBluescript II SK$^-$ transfected HeLa cells (BS/HeLa, ■) and FAAH-transfected HeLa cells (FAAH/HeLa, ▲) as described under *Materials and Methods*. Data were plotted as percentage of specific anandamide uptake. All data plotted represent means ± S.E. of triplicate determinations and are representative of three separate experiments. Nonspecific uptake was determined in the presence of 100 μM AM404 and subtracted from total uptake. Mean values ± S.E. for total and nonspecific anandamide uptake before correction to percent specific anandamide are as follows: BS/HeLa, 4363 ± 43 CPM (total) and 805 ± 139 CPM (nonspecific); FAAH/HeLa, 4699 ± 60 CPM (total) and 920 ± 129 CPM (nonspecific).

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