

The Cellular Uptake of Anandamide Is Coupled to Its Breakdown by Fatty-acid Amide Hydrolase*

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Anandamide is an endogenous compound that acts as an agonist at cannabinoid receptors. It is inactivated via intracellular degradation after its uptake into cells by a carrier-mediated process that depends upon a concentration gradient. The fate of anandamide in those cells containing an amidase called fatty-acid amide hydrolase (FAAH) is hydrolysis to arachidonic acid and ethanolamine. The active site nucleophilic serine of FAAH is inactivated by a variety of inhibitors including methylarachidonylfluorophosphonate (MAFP) and palmitylsulfonylethyl fluoride. In the current report, the net uptake of anandamide in cultured neuroblastoma (N18) and glioma (C6) cells, which contain FAAH, was decreased by nearly 50% after 6 min of incubation in the presence of MAFP. Uptake in laryngeal carcinoma (Hep2) cells, which lack FAAH, is not inhibited by MAFP. Free anandamide was found in all MAFP-treated cells and in control Hep2 cells, whereas phospholipid was the main product in N18 and C6 control cells when analyzed by TLC. The intracellular concentration of anandamide in N18, C6, and Hep2 cells was up to 18-fold greater than the extracellular concentration of 100 nM, which strongly suggests that it is sequestered within the cell by binding to membranes or proteins. The accumulation of anandamide and/or its breakdown products was found to vary among the different cell types, and this correlated approximately with the amount of FAAH activity, suggesting that the breakdown of anandamide is in part a driving force for uptake. This was shown most clearly in Hep2 cells transfected with FAAH. The uptake in these cells was 2-fold greater than in vector-transfected or untransfected Hep2 cells. Therefore, it appears that FAAH inhibitors reduce anandamide uptake by cells by shifting the anandamide concentration gradient in a direction that favors equilibrium. Because inhibition of FAAH increases the levels of extracellular anandamide, it may be a useful target for the design of therapeutic agents.

Endocannabinoids, such as anandamide (arachidonyl ethanolamide) and 2-arachidonyl glycerol, are endogenous ligands that bind to the cannabinoid receptors (1–3). Emerging evidence suggests that they are involved in many physiological phenomena such as pain, locomotion, memory, learning, blood pressure, immunity, sleep, reproduction, mood, perception, response to stress, and so forth (for review see Ref. 4). Δ^9 -Tetrahydrocannabinol, the active component of marijuana, appears to mimic many of the physiological and pharmacological effects of the endogenous cannabinoids, in some cases to an extreme degree (e.g. a marijuana “high”). Anandamide is transported into the neuroblastoma, glioma, brain neuron, brain astrocyte, cerebellar granule cells, leukocyte, macrophage, leukemia, and lymphoma cells in culture (5–10). The driving force for uptake is substrate concentration (facilitated diffusion) rather than an active cotransport system (11, 12). The transport appears to be carrier-mediated, and specific transport inhibitors have been described (9, 11, 13–16). After being transported into the cell, anandamide is subsequently broken down into arachidonic acid and ethanolamine by an endoplasmic reticular integral membrane-bound enzyme called fatty-acid amide hydrolase (FAAH),¹ anandamide amidase, or anandamide amidohydrolase (for review see Ref. 17). Interestingly, the catalytic site contains at least two important serines and a lysine (not the histidine, serine, and aspartate triad found in many hydrolytic serine active site enzymes) with Ser-241 acting as the nucleophile involved in the bond breaking of substrates (18–20). It has been shown that FAAH is inhibited by a variety of compounds, such as methylarachidonylfluorophosphonate (MAFP) and palmitylsulfonylethyl fluoride (PSF) (21–23). Employing these inhibitors, we show that the net movement of anandamide into the cells is coupled to the activity of intracellular FAAH. We propose that the inhibition of anandamide breakdown results in its intracellular build-up and the attainment of equilibrium between free intracellular and extracellular anandamide, and this disfavors further net uptake.

EXPERIMENTAL PROCEDURES

Cell Culture—N18TG2 neuroblastoma, C6 glioma (kindly provided by Allyn Howlett and Joel Levine, respectively), and human laryngeal carcinoma cells (Hep2), provided by our in-house cell culture facility, were grown in 35 × 10-mm dishes in 2 ml of Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), 1% penicillin/streptomycin, and L-glutamine (Life Technologies, Inc.). All cells were grown at 37 °C with 5% CO₂. For all experiments, the number of cells plated for

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¹ The abbreviations used are: FAAH, fatty-acid amide hydrolase; MAFP, methylarachidonylfluorophosphonate; PSF, palmitylsulfonylethyl fluoride; PMSF, phenylmethylsulfonyl fluoride; N18, N18TG2 neuroblastoma cells; C6, C6 glioma cells; Hep2, human laryngeal carcinoma cells.

Hep2 and C6 was $\sim 1.7 \times 10^6$, and the number of cells plated for N18 was $\sim 7 \times 10^5$. The exact cell numbers were determined for comparison of the uptake rates in N18 neuroblastoma, C6 glioma, and Hep2 carcinoma (see below and Fig. 3).

Time Course of Anandamide Uptake—The effects of MAFP and PSF on anandamide uptake were measured using arachidonyl-[5,6,8,9,11,12,14,15- ^3H]ethanolamide called [^3H]anandamide (172 Ci/mmol, 62.2 nCi/ μl) from PerkinElmer Life Sciences as substrate. Growth medium was removed from the cells and replaced with 750 μl of supplemented Dulbecco's modified Eagle's medium containing 40 nCi [^3H]anandamide (100 nM anandamide). The cells were incubated for 1–6 min at 37 °C with 5% CO_2 . To account for nonspecific binding to cellular membrane, parallel incubations were carried out at 4 °C. The counts from the nonspecific binding were subtracted from the total amount of anandamide taken up after each incubation so only the carrier-mediated transport uptake was represented in the data. To study whether MAFP affected the cellular uptake of anandamide, incubations were carried out after a 10-min preincubation of the cells with 1 μM MAFP (Cayman Chemical, Ann Arbor, MI) or 100 nM pamitylsulfonyl fluoride (provided by Alex Makriyannis, University of Connecticut) in the medium at 37 °C. After the incubation, the medium was immediately removed, and the cells were washed with ice-cold supplemented Dulbecco's modified Eagle's medium. The cells were scraped from the plate after the addition of 0.4 ml of 2 mM EDTA in phosphate-buffered saline. This procedure was repeated two additional times to maximize yield. The labeled compounds were then extracted from both the medium and the cells by the addition of 2 volumes of chloroform:methanol (1:1) (Fisher) mixed thoroughly and spun down in a clinical centrifuge for 5 min. Finally, 100 μl of the organic layer from both the cell and medium extractions were placed in scintillation vials with 3.0 ml of ScintiVerse II scintillation fluid (Fisher). The samples were counted in an LKB Rack beta scintillation counter.

Determination of K_m and $V_{\max}$ —Saturation kinetics were determined in N18, C6, and Hep2 cells plated at a minimal density of 1×10^6 cells. They were incubated for either 3 s (to determine nonspecific binding) or 60 s at 37 °C. For N18 and C6 cells, these experiments were performed with [^3H]anandamide (0.09–3.0 nM) and unlabeled anandamide to yield total concentrations ranging from 0.03 to 1.0 μM anandamide. For Hep2 cells, [^3H]anandamide (0.01–5.5 nM) was added to unlabeled anandamide over a concentration range of 0.25–100 μM anandamide. The same procedure was used for these experiments as for the time course of anandamide uptake with the exception that the experiment was terminated by the addition of 5 ml of ice-cold supplemented Dulbecco's modified Eagle's medium, which was then immediately removed. Experiments were conducted in duplicate or triplicate and repeated two or three separate times. Calculations were performed by subtracting the nonspecific binding at 3 s from the 60-s incubation, and the picomoles taken up were corrected for the number of cells. To determine the K_m and $V_{\max}$, the data were analyzed employing a Lineweaver-Burk plot using the linear regression program of Sigma Plot (SPSS, Chicago, IL).

5-Min Uptake Experiments and TLC Analysis—The same procedure described above for the time course experiments was employed for these 5-min incubations, with the exception that the level of radioactivity was increased to 600 nCi/dish to have enough counts for thin layer chromatography analysis. After the 5-min incubation, the labeled anandamide was extracted from both the medium and the cells by the addition of 2 volumes of chloroform:methanol (1:1) mixed thoroughly and spun down in a clinical centrifuge for 5 min. 100 μl of the organic layer were removed for counting from both the cellular (1.2 ml total) and medium (750 μl) extracts. The remaining portions of the organic extracts from the cells and medium were dried down. The residue was resuspended in 40 μl of chloroform:methanol (1:1), and this was spotted on silica-based thin layer chromatography plates provided by Analtech (Newark, DE). The solvent consisted of a 6:3:1 mixture of ethyl acetate, hexane, and acetic acid (Fisher). Arachidonic acid and anandamide (PerkinElmer Life Sciences) standards were run alongside the experimental samples. The plate was then treated with EN 3 HANCE autoradiography spray provided by PerkinElmer Life Sciences and exposed on Kodak X-Omat AR film. After film development, the areas corresponding to the bands on the x-ray film were scraped off the plates and placed in scintillation vials with scintillation fluid to quantify the products of the TLC analysis (anandamide, phospholipids, and arachidonic acid). Each experiment was performed in triplicate. Following the time course experiments and TLC analysis, the raw results were corrected to account for the percentages of the organic layer, which was taken from each sample. The inhibition of uptake values in Table I was obtained by averaging

the triplicates and assuming that the uptake of controls for each cell line was 100%.

Comparison of Anandamide Uptake in N18 Neuroblastoma, C6 Glioma, and Hep2 Carcinoma—To statistically quantify the uptake rates in these three cell lines, the percentage of the total anandamide taken up by each cell line was compared after 5 min of incubation under identical conditions at the same time. The amount of uptake was then related to the total number of cells, thus allowing the comparison of uptake rates among different cell lines. Each experiment was performed in triplicate on 35-mm plates, which were $\sim 50\%$ confluent. A fourth plate was run in parallel to determine the number of cells/plate.

Enzyme Assay for FAAH—The enzyme assay for FAAH activity in N18, C6, and Hep2 cells was conducted as described previously (24). Cells were washed with ice cold PBS and scraped in ice-cold Tris-EDTA, pH 7.6. The cells were then disrupted by sonication. Incubations were performed in triplicate at 37 °C in a water bath with shaking. Each incubation contained 10 μl of 50 mg/ml defatted bovine serum albumin in H_2O , 50 μl of the cellular extract, 30 μM anandamide (Cayman Chemical Co., Ann Arbor, MI), and 0.01 mCi of 120 mCi/mmol arachidonyl[ethanolamine-1,2- ^{14}C]ethanolamide (PerkinElmer Life Sciences). The control tubes contained everything except the cell extract. The reactions were terminated after 30 min by the addition of 2 volumes of chloroform:methanol (1:1). The radioactivity in the aqueous phase was measured by liquid scintillation counting. The number of cells/plate was determined with a hemacytometer. The specific activity was expressed as nanomoles of anandamide hydrolyzed/ 10^6 cells/h. This was more meaningful than activity based on the amount of protein because protein levels varied widely among the different cell types.

Transfection of Hep2 Cells with Rat FAAH cDNA—Cells were seeded at 3×10^5 cells/35-mm dish on day 1. On day 2, cells were transfected with 2.5 μg of DNA using 2.5 μl of LipofectAMINE according to the manufacturer's instructions (Life Technologies, Inc.). On day 4, cells were either harvested in 150 μl of Tris-EDTA, pH 7.4, for FAAH assays or used for uptake experiments. The control Hep2 cells were either untransfected and serum-starved or were transfected with the pcDNA3 vector (Invitrogen, Carlsbad, CA), and the experimental Hep2 cells were transfected with rat pcDNA3-FAAH cDNA (30).

Comparison of Anandamide Uptake in Hep2, Hep2 Vector-transfected, and Hep2 FAAH-transfected Cells—To quantify the anandamide uptake rates of untransfected, vector-transfected, and FAAH-transfected cells, the total anandamide taken up was compared after 5 min of incubation under identical conditions. The cells were used for uptake 48 h after transfection. The same procedure that was used for the time course experiments was used for these incubations, with the exception that 200 nCi/dish was used, nonspecific binding to the cellular membrane was determined by incubating parallel plates in 37 °C uptake medium for 3 s, and all incubations were stopped by adding 4 °C medium.

RESULTS

Anandamide uptake in N18, C6, and Hep2 cells was saturable and temperature- and time-dependent. The apparent K_m and $V_{\max}$ values were 1.8 μM and 17.4 pmol/min/ 10^6 cells for N18 cells, 0.7 μM and 3.9 pmol/min/ 10^6 cells for C6 cells, and 20.9 μM and 5.9 pmol/min/ 10^6 cells for Hep2 cells, respectively.

Time course experiments with N18, C6, and Hep2 cells were conducted to qualitatively determine the effects of two FAAH inhibitors, MAFP and PSF, on anandamide uptake. Fig. 1A shows that after preincubating C6 cells with MAFP for 10 min, the amount of anandamide that enters the cell is reduced at each time point from 2 to 6 min. At 6 min, cellular net uptake of labeled anandamide in the control without inhibitor is about double the net cellular uptake of labeled anandamide in the presence of MAFP. The results of the N18 cell time course experiment in Fig. 1C demonstrate that the effect of MAFP on cellular uptake is not restricted to C6 cells. After 6 min, the control cells have taken up approximately twice as much labeled anandamide as did the MAFP-treated cells. To study the effects of PSF, the other hydrolase inhibitor used in these studies, on anandamide uptake, identical experiments were conducted in N18 and C6 cell lines. Fig. 1, B and D, shows that preincubating C6 and N18 cells with 100 nM PSF for 10 min also reduces the amount of anandamide entering the cell at each time point from 2 up to 6 min. In experiments similar to

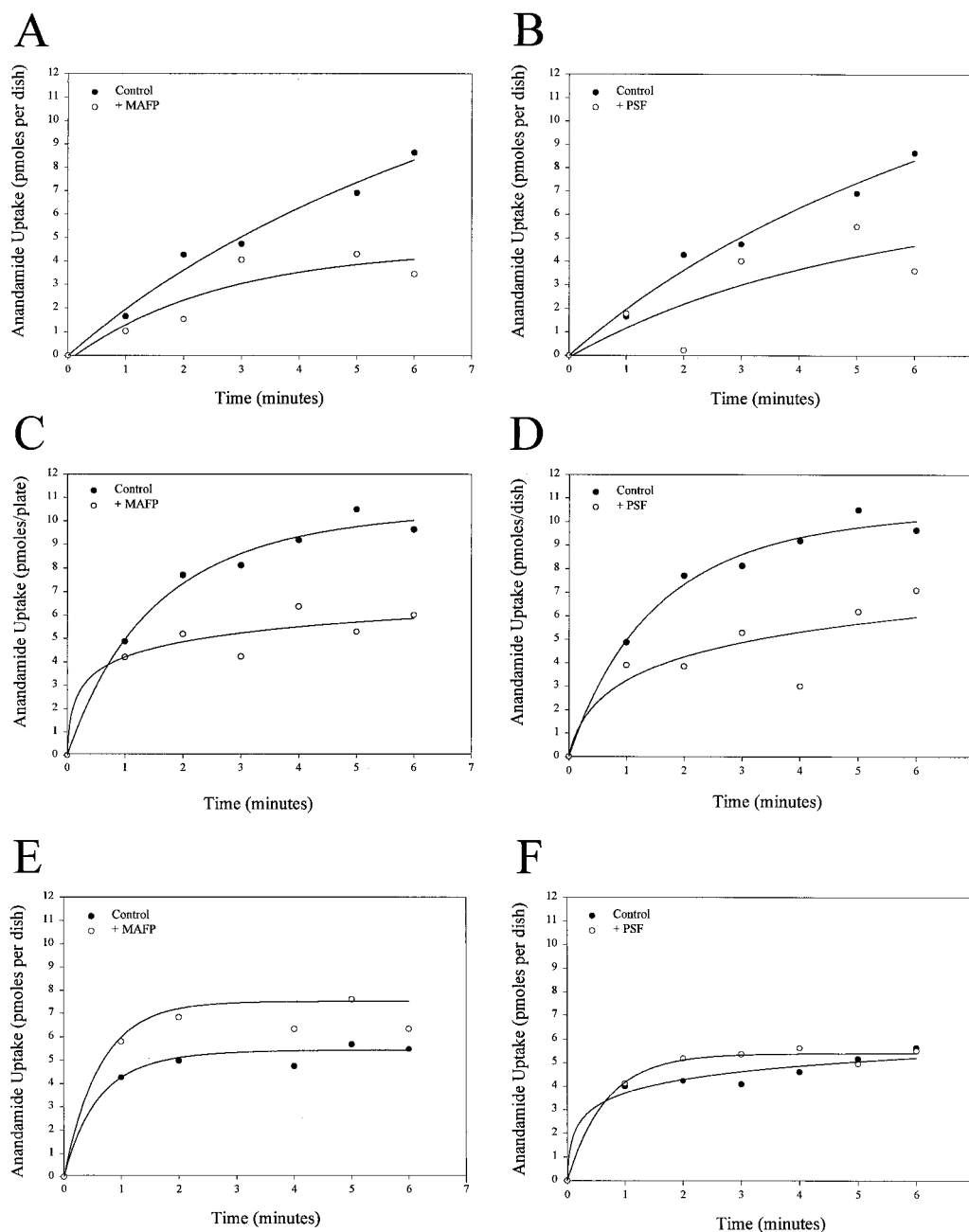


FIG. 1. Time course illustrating the effect of MAFP or PSF on anandamide uptake in C6 glioma cells, N18 neuroblastoma, and Hep2 laryngeal carcinoma cells. ● represents uptake at 37 °C (corrected for nonspecific binding at 4 °C), and ○ represents uptake at 37 °C in the presence of 1 μ M MAFP or 100 nM PSF (corrected for nonspecific binding at 4 °C). Uptake is measured as picomoles of anandamide present inside the cell at a particular time (1–6 min). The points were generated using Sigma Plot, and the curves were fitted using regression analysis (Hyperbola III).

those described above for C6 and N18 cells, uptake in Hep2 cells was characterized in the presence of MAFP and PSF. In contrast to the results found with N18 and C6 cells, net uptake was not decreased in the presence of either inhibitor (Fig. 1, E and F). The shapes of the curves resulting from the time course experiments are interesting. First, anandamide is accumulated at a greater rate during the first min than during the rest of the experiment, and the amount taken up is approximately the same with or without the inhibitor. Second, the cells with uninhibited FAAH show a linear uptake after 1 min, whereas those that are inhibited or lack FAAH appear to level slowly to a plateau. The results in Fig. 1 are qualitatively representative of at least three experiments conducted with N18, C6, and Hep2 cells.

To more accurately characterize the effect of FAAH inhibi-

tion on anandamide uptake, the total cellular radioactivity resulting from anandamide incorporation was quantified in N18, C6, and Hep2 cells after an incubation of 5 min in the absence or presence of MAFP (Table I). MAFP inhibited the incorporation by ~40% in both N18 and C6, and this effect was highly significant for both of these cell lines ($p = 0.0001$). However, MAFP did not inhibit anandamide incorporation in Hep2. In fact, it seemed to cause a slight stimulation, although this effect was not quite significant ($p = 0.0553$). Whereas 1 μ M MAFP was chosen for these experiments, similar results were observed at 100 nM (data not shown). The observed inhibition in N18 and C6 cells in the presence of MAFP was not due to toxicity because cells exposed to MAFP do not exhibit decreased viability as observed after trypan blue staining (data not shown).

TABLE I

The effect of MAFP upon the cellular uptake of anandamide in C6 glioma cells, N18 neuroblastoma, and Hep2 laryngeal carcinoma cells

Quantification of label in cells after 5-min incubation with [³H]anandamide. The total number of counts was determined in the absence (–) and presence (+) of 1 μ M MAFP. These values were corrected for nonspecific binding by the subtraction of counts from a 4 °C incubation. The average of the controls for each cell line was normalized to 100%. A Student's *t* test (two sided) was used to compare the differences between those cells in the presence (+) and absence (–) of MAFP and determine the *p* value. The percent inhibition of uptake was calculated by (dpm in control cells – dpm in MAFP-treated cells) $\times$ 100/dpm in control cells. The remainder of the sample was used for thin layer chromatography analysis as shown in Fig. 2.

Cell line	MAFP	Uptake	Mean $\pm$ S.E.	Student's t-test, 2- sided <i>p</i> value	Inhibition of uptake
		% of control mean	%		%
N18	–	98.3	100.0 $\pm$ 2.0		
	–	97.8			
	–	103.9			
N18	+	56.6	55.7 $\pm$ 0.5	0.0001	44
	+	54.8			
	+	55.7			
C6	–	104.6	100.0 $\pm$ 2.3		
	–	97.7			
	–	97.7			
C6	+	61.9	64.0 $\pm$ 1.2	0.0001	36
	+	66.0			
	+	64.0			
Hep2	–	112.4	100.0 $\pm$ 6.8		
	–	88.8			
	–	98.9			
Hep2	+	128.9	124.0 $\pm$ 5.8	0.0553	–24
	+	130.7			
	+	112.5			

To determine the fate of [³H]anandamide (arachidonyl[5, 6, 8, 9, 11, 12, 14, 15-³H]ethanolamide) after being taken up by the cells, thin layer chromatography was performed on those control and experimental C6, N18, and Hep2 cells, which were quantified in Table I. After incubation of C6 and N18 with anandamide in the absence of inhibitor (–), the main radioactive product formed inside the cell was phospholipids (83% for C6, 93% for N18), which remain at the origin in this TLC development system (Fig. 2). For the C6 and N18 cells, only 14 and 3% of the radioactivity in the cell was free anandamide, respectively, and only 3% of the radioactivity was detected as arachidonic acid in the C6 cells and none in the N18 cells. Because of overexposure, the TLC chromatogram for N18 cells (Fig. 2) gives the impression of quite high anandamide levels in the samples without inhibitor (–), although counting of the samples scraped from the plate actually shows only 3% free anandamide. In the presence of MAFP (+), the entire radioactivity in the C6 and N18 cells is accounted for by anandamide (Fig. 2). Anandamide accumulated in these cells because the breakdown reaction by FAAH was rendered inactive. These experiments raise the possibility that MAFP exerts its effect by inhibiting the transporter. However, it seems unlikely that MAFP exerts its effect in this way as shown by the TLC experiments with Hep2 cells (Fig. 2). The anandamide transport mechanism is operational in these cells in the absence of MAFP (–). However, unlike the situation with control C6 and N18 cells, anandamide is not broken down (>90% of the radioactivity in the cell is anandamide) after being transported into the cells because these cells lack FAAH (5). Significantly, in the presence of MAFP (+) transport is not inhibited because there is no significant difference in the amount of anandamide on the TLC, and as mentioned above (Table I), there seems to be a slight stimulation. The TLC pattern for Hep2 cells with (+) or

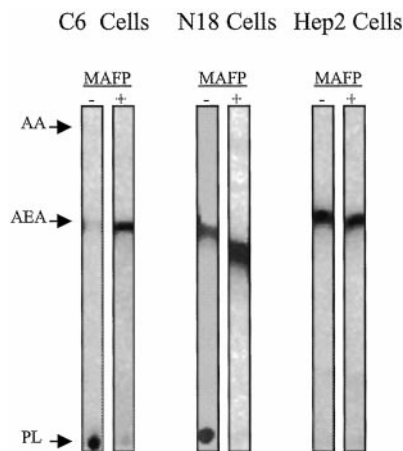


FIG. 2. TLC autoradiogram of C6 glioma cells, N18 neuroblastoma cells, and Hep2 human laryngeal carcinoma cells incubated for 5 min. As indicated, experiments were conducted with or without 1 μ M MAFP. Arrows indicated the position of anandamide (AEA), arachidonic acid (AA), and phospholipids (PL) as determined by standards. The contrast in the N18 autoradiograph was adjusted to make it comparable with the C6 and Hep2 panels. The apparent large amount of anandamide in the control N18 cells is an artifact due to overexposure of the TLC radiogram that caused saturation of the film even at low levels of radioactivity. Exposure times for these autoradiographs are 4 days for C6, 7 days for N18, and 2 days for Hep2. Replicate chromatograms for each experiment were essentially identical.

without (–) MAFP treatment is the same as that observed in N18 and C6 cells that have been treated with MAFP.

An experiment was performed to make a side by side comparison of N18, C6, and Hep2 in terms of their uptake and enzyme activity. Interestingly, the absolute rate of uptake was found to vary among the different cell types when the cell numbers were carefully quantified, and this correlated approx-

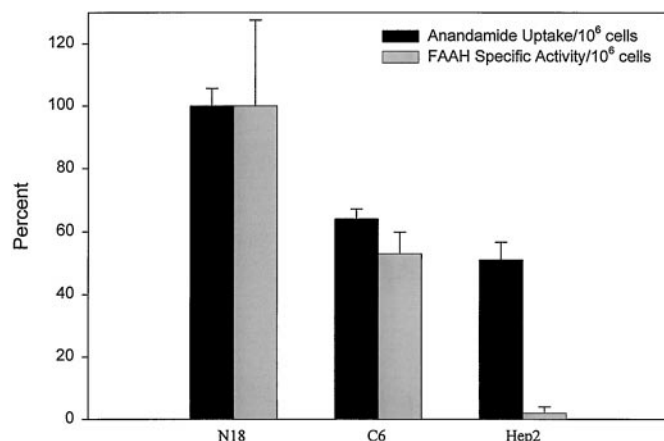


FIG. 3. Comparison of uptake and FAAH activity among N18, C6, and Hep2 cell lines. Each experiment was performed in triplicate (repeated twice for the N18 and C6 and three times for the Hep2 cells), and the graph indicates the mean $\pm$ S.E. of each group. Each experiment was standardized for percentage uptake/10⁶ cells. The graph was generated using Sigma Plot. In N18 cells, the uptake was 29 ± 1.64 pmol/10⁶ cells, and the specific activity of FAAH was 0.87 ± 0.24 nmol/h/10⁶ cells, and these values were normalized to 100% on the bar graph.

imately with the amount of FAAH activity (Fig. 3). The N18 cells had the greatest total uptake with the C6 and Hep2 cells having ~ 64 and 51% of the uptake of the N18, respectively. The FAAH activity in C6 was found to be approximately 60% of that found in N18, although there was no measurable FAAH activity in Hep2 cells as mentioned above.

FAAH transfection experiments were undertaken to demonstrate that uptake is dependent upon FAAH activity in Hep2 cells. The Hep2 cells, which have no measurable FAAH activity, were transfected with a pcDNA3 vector as a control or with pcDNA3-FAAH cDNA (Fig. 4). The vector-transfected Hep2 cells had no measurable FAAH activity, whereas the Hep2 cells transfected with pcDNA3-FAAH cDNA were very active (2.92 ± 0.19 nmol $\pm$ 0.19/h/10⁶ cells), demonstrating the expression of FAAH ($p < 0.0001$). Significantly, anandamide uptake in the transfected cells doubled (8.27 ± 1.0 pmol for transfected *versus* 4.28 ± 0.21 pmol for untransfected cells and 4.29 ± 0.34 pmol for vector-transfected cells) (Fig. 4). This finding demonstrates that uptake is dependent upon FAAH activity.

The intracellular concentration of anandamide was calculated for each of the cell types (Table II). The intracellular concentration of anandamide in N18, C6, and Hep2 cells was determined to be ~ 5 -, 7 -, and 18 -fold greater, respectively, than the extracellular concentration of 100 nM that was used in these experiments. These data strongly suggest some sequestering mechanisms for accumulating anandamide inside cells without FAAH or when FAAH is inhibited with MAFP.

DISCUSSION

It is known that anandamide uptake is an energy-independent, reversible process and is governed by a concentration gradient of unbound anandamide, which exists across the cellular membrane, *i.e.* a facilitated diffusion-mediated transporter (8, 12, 25). The cells employed in the current study also demonstrate the characteristics of facilitated diffusion in that they exhibit saturation kinetics as well as temperature and time dependence. Using 1-min incubation periods to obviate any effects due to metabolism, the uptake of anandamide as a function of concentration exhibited Michaelis-Menten kinetics. The K_m of ~ 1 μ M that we determined for the N18 and C6 cells was similar to that reported for cerebral cortical neurons and astrocytes, astrocytoma, and CHP100 neuroblastoma (13, 14,

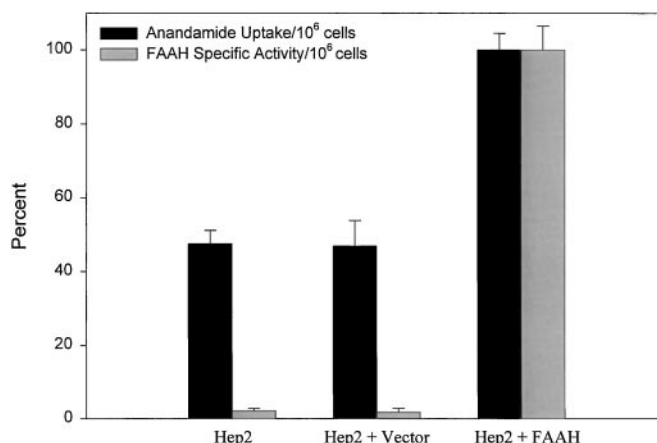


FIG. 4. Comparison of anandamide uptake and FAAH enzymatic activity in pcDNA vector-transfected and pcDNA3-FAAH-transfected Hep2 cells. The values shown are the mean $\pm$ S.E. from two experiments, each experiment was performed in triplicate. The uptake of anandamide in Hep2 cells transfected with pcDNA3-FAAH was 8.2 ± 0.37 pmol/10⁶ cells, the specific activity of FAAH was 2.92 ± 0.19 nmol/h/10⁶ cells, and these values were normalized to 100%.

25). The K_m that we determined for the Hep2 (20 μ M) was similar to reports for cerebellar granule neurons (8). In addition to a facilitated diffusion component of the uptake, a non-saturable proportion of the uptake, particularly with lipophilic molecules such as anandamide, may occur via simple diffusion across the membrane (for review see Ref. 26).

FAAH has been shown by immunohistochemistry to be localized to the endoplasmic reticulum (27, 28). It is the enzyme that is responsible for the inactivation of anandamide in brain, liver, other tissues, and in many cell lines (20, 29, 30). In our experiments we found a correlation between the rate of uptake in cell lines and the amount of FAAH, giving further credence to the hypothesis that anandamide hydrolysis drives uptake (Fig. 3). However, the most unequivocal demonstration that anandamide uptake is coupled to its metabolism by FAAH was shown in Hep2 cells transfected with FAAH. Strikingly, the total amount of anandamide uptake doubled in Hep2 cells transfected with FAAH ($p < 0.0003$). Although this experiment definitively demonstrates that uptake is dependent upon FAAH activity, the nature of the transporter in Hep2 may be structurally different from those of N18 and C6 and other systems reported to transport anandamide. A putative transporter(s) for anandamide has not been characterized. The large variation in K_m values (for review see Ref. 32) for cells that take up anandamide suggests that there may be more than one transporter. Recently, another enzyme was described that hydrolyzes anandamide (31). However, it occurs in a human megakaryoblastic cell line, has a pH optimum of around 5, and is less sensitive to phenylmethylsulfonyl fluoride (PMSF) and MAFP.

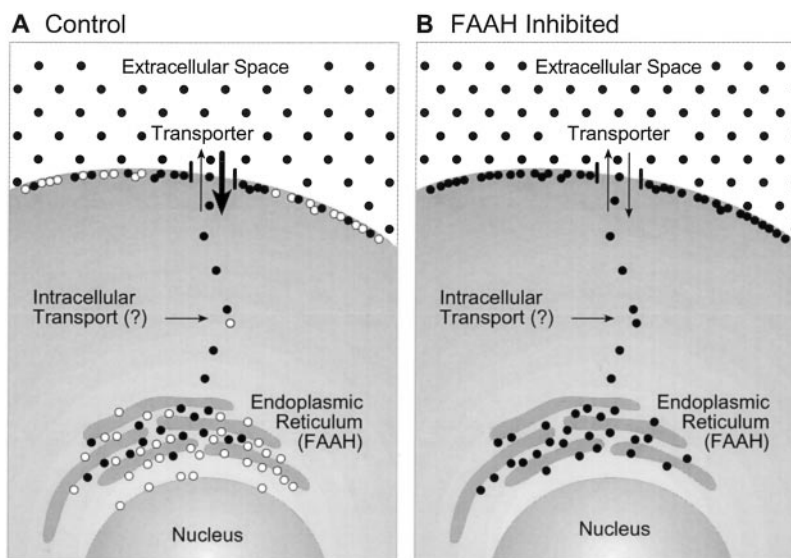
A model that accounts for our observations is shown in Fig. 5. The first step is anandamide uptake into the cell (5–10). In the C6 and N18 control cells, the observed linear increase in disintegrations/min inside the cell (Fig. 5A, *Control*) reflects the continued cellular accumulation of anandamide and its products. The driving force for the continued movement of anandamide inside the cell is its degradation. A breakdown results in low anandamide concentration inside the cell, and this disfavors the establishment of equilibrium between anandamide inside and outside the cell. The linear uptake rates indicate that there are no changes in substrate supply (less than 20% of medium anandamide was taken up in 5 min) or cell viability (enzymatic activity was constant) during the assay. In

TABLE II
Intracellular concentrations of anandamide in the presence of inhibitor

The intracellular concentrations of anandamide were calculated in N18, C6, and Hep2 cells after a preincubation with 1 μ M MAFP for 10 min followed by a 5-min incubation at 37 °C in supplemented Dulbecco's modified Eagle's medium containing 100 nM anandamide and 1 μ M MAFP. Cell volumes of N18 and C6 cells were described previously (37). The intracellular volume of Hep2 cells was kindly provided by Janikke Ludt and Kirsten Sandvig (personal communication) from calculations related to prior experiments (38).

Cell line	Uptake of anandamide/cell	Intracellular volume	Extracellular concentration of anandamide	Intracellular concentration of anandamide	-Fold intracellular accumulation
	<i>pmol</i>	<i>liters</i>	<i>nM</i>	<i>nM</i>	
N18TG2	4.28×10^{-6}	8.5	100	503.8	5
C6	5.31×10^{-6}	7.5	100	707.9	7
Hep2	7.90×10^{-6}	4.5	100	1756.1	18

FIG. 5. **Proposed mechanism for the inhibition of cellular anandamide uptake by MAFP.** A, in the control cells (Control), the degradation of anandamide ($[^3\text{H}]$ anandamide, ●) by FAAH, after being taken up by the carrier from the medium, is illustrated. The presence of FAAH in the endoplasmic reticulum ensures a low concentration of free anandamide in the cell relative to the medium, and this concentration gradient drives the uptake. Large amounts of labeled metabolites ($[^3\text{H}]$ anandamide metabolites, ○) accumulate in the cell. B, the MAFP-treated cells illustrate the effect of MAFP on anandamide uptake (FAAH Inhibited). As the cell takes up anandamide ($[^3\text{H}]$ anandamide, ●), the presence of MAFP prevents its breakdown by FAAH. The intracellular concentration increases, and the attainment of equilibrium between free intracellular and extracellular anandamide prevents further accumulation. Intracellular membranes or a putative anandamide-binding protein act as a "sink" for anandamide.



the control cells, there is only a small amount of anandamide accumulation in the cells after 6 min (Fig. 2), and the same results were observed with longer incubation periods up to 2.5 h (5, 6). The increase of disintegrations/min inside the cell reflects the fact that the bulk of $[^3\text{H}]$ anandamide taken up into the cell is rapidly degraded to other radioactive products containing arachidonic acid (the labeled portion of anandamide) due to the presence of intracellular FAAH. Arachidonic acid does not accumulate because it is rapidly transformed to phospholipid (see Fig. 2). During the incubation period used in these experiments, insignificant amounts of labeled phospholipids and arachidonic acid that are formed inside the cells are exported back into the medium as observed by TLC analysis of lipids in the medium (data not shown). Similarly, only small amounts of anandamide would be expected to be exported into the medium, although the transport is reversible (6, 8). When anandamide is incubated with N18 and C6 cells for longer periods (30 min–2.5 h), anandamide uptake continues to be unabated, and the label from the phospholipid is subsequently incorporated into other compounds, such as triglycerides and cholesterol esters (5, 6).

In C6 and N18 in the presence of inhibitors (Fig. 5, *FAAH Inhibited*), the shape of the uptake curves (Fig. 1) reflects the situation when cellular degradation of anandamide is blocked. The inhibition of anandamide breakdown results in its intracellular build-up and the attainment of equilibrium between free intracellular and extracellular anandamide. The shifting of the anandamide concentration gradient in a direction that favors equilibrium prevents further net movement of anandamide from the outside to the inside of the cell. The shape of the experimental curves in Fig. 1 supports this interpretation of the data because uptake reached a plateau quickly after the

1-min time point, reflecting equilibration of free nonmetabolized anandamide across the membrane. The nearly identical uptake seen after a 1-min incubation in control and FAAH-inhibited cells suggests that the uptake during this time period is entirely due to movement of anandamide across the membrane via the carrier. Furthermore, these data indicate that the inhibitor is specific for FAAH and does not affect the transporter directly. The complete leveling off of anandamide accumulation in FAAH-inhibited cells indicates that the catabolism of anandamide by FAAH is a major driving force for its accumulation in these particular cells. For example, in the case where uptake would have reflected metabolism, rates during the first minute would have to be much higher in control cells because anandamide would be siphoned away and not allowed to equilibrate with extracellular anandamide. This observation suggests that the inhibitor could be very useful in cases where complete suppression of anandamide utilization is desired. However, even in the presence of this inhibitor, because membrane transport is not inhibited, anandamide becomes highly concentrated in the cells (Table II). One possible explanation for this is that after uptake, anandamide was dispersed throughout intracellular membranes and/or to an anandamide-binding protein. (12, 32). It has been observed that long chain fatty acid diffusion within the cytoplasm is slow and lacks selective targeting toward specific organelles. However, fatty acids bound to a cytoplasmic binding protein have increased solubility and targeting (26, 33). It is possible that a binding protein exists that binds anandamide and speeds up the process of anandamide intracellular transport.

Hep2 cells, which lack FAAH, have the same time course and TLC profile as the cells incubated with FAAH inhibitor. MAFP does not inhibit uptake in Hep2, demonstrating that MAFP

does not exert its effect on uptake at the level of the putative transporter in these cells. Beltramo *et al.* (14), using relatively weak FAAH inhibitors with cortical astrocytes in culture, concluded that there was no effect upon uptake of anandamide after a 4-min incubation. Likewise, they did not observe any inhibition of FAAH by *N*-(4-hydroxyphenyl)arachidonylamide, a selective uptake inhibitor. We observed a 15% inhibition of uptake in Hep2 cells in the presence of 10 μ M *N*-(4-hydroxyphenyl)arachidonylamide (data not shown). Bisogno *et al.* (34) reported that PMSF, which strongly inhibited FAAH, gave nearly 50% inhibition of uptake during a 20-min incubation in RBL-2H3 basophils, and this was postulated to occur as a result of alkylating the transporter. Likewise, Maccarrone *et al.* (25) reported that PMSF inhibited uptake 50% in RBL-2H3 cells. The mechanism they postulated was that the transporter may contain a cysteine that was inactivated. In view of our current study, it is possible that the effect of PMSF on uptake may be secondary to its effect on FAAH. This has recently been proposed for PMSF, arachidonyltrifluoromethylketone, and MAFP in RBL-2H3 cells (12). The approach in our study distinguishes between the two possible mechanisms (*i.e.* directly blocking the carrier for transport or the indirect effect of FAAH inhibition). These studies establish a theoretical basis for the use of FAAH inhibitors as therapeutic agents to increase the levels of extracellular anandamide. For example, PSF has been shown to raise exogenous levels of anandamide in brain hippocampal slice (35). Also, analogs of MAFP have recently been shown to be very potent and long acting antinociceptive agents *in vivo* (36).

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