Evidence against the presence of an anandamide transporter

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On the basis of temperature dependency, saturability, selective inhibition, and substrate specificity, it has been proposed that an anandamide transporter exists. However, all of these studies have examined anandamide accumulation at long time points when downstream effects such as metabolism and intracellular sequestration are operative. In the current study, we have investigated the initial rates (<1 min) of anandamide accumulation in neuroblastoma and astrocytoma cells in culture and have determined that uptake is not saturable with increasing concentrations of anandamide. However, anandamide hydrolysis, after uptake in neuroblastoma cells, was saturable at steady-state time points (5 min), suggesting that fatty acid amide hydrolase (FAAH) may be responsible for observed saturation of uptake at long time points. In general, arvanil, olvanil, and *N*-(4-hydroxyphenyl)arachidonylamide (AM404) have been characterized as transport inhibitors in studies using long incubations. However, we found these ''transport inhibitors'' did not inhibit anandamide uptake in neuroblastoma and astrocytoma cells at short time points (40 sec or less). Furthermore, we confirmed that these inhibitors *in vitro* were actually inhibitors of FAAH. Therefore, the likely mechanism by which the transport inhibitors raise anandamide levels to exert pharmacological effects is by inhibiting FAAH, and they should be reevaluated in this context. Immunofluorescence has indicated that FAAH staining resides mainly on intracellular membranes of neuroblastoma cells, and this finding is consistent with our observed kinetics of anandamide hydrolysis. In summary, these data suggest that anandamide uptake is a process of simple diffusion. This process is driven by metabolism and other downstream events, rather than by a specific membrane-associated anandamide carrier.

The endocannabinoids, including anandamide, are a class of neurotransmitters, similar to Δ^9-tetrahydracannabinol, involved in multiple physiological events including nociception, memory, blood pressure, locomotion, and immunity (for review, see ref. 1). These compounds bind the CB1 and CB2 cannabinoid receptors, which are G_i-coupled receptors that modulate ion channels and signal transduction pathways (2–4).

Anandamide is readily taken up into cells. The first step of this process has been characterized by several laboratories as a process of facilitated diffusion (for reviews, see refs. 5 and 6). Although an anandamide transporter has never been isolated, its existence is based on an anandamide uptake process that is temperature-dependent, selective, and saturable. In addition, several studies identified compounds that inhibit anandamide accumulation, including *N*-(4-hydroxyphenyl)arachidonylamide (AM404) and the vanilloids arvanil and olvanil (7–16).

After uptake, if fatty acid amide hydrolase (FAAH) is present, anandamide is hydrolyzed to arachidonic acid and ethanolamine (for review, see ref. 17). A recent report investigating the role of FAAH in anandamide metabolism showed that $FAAH^{-/-}$ mice had 15-fold higher anandamide concentrations within the brain relative to wild-type mice and, after administration of exogenous anandamide, had reduced pain sensation and rectal temperature, and spontaneous activity and catalepsy. Also, brain homogenate from $FAAH^{-/-}$ mice had 100-fold less anandamide hydrolysis activity than wild type (18). This finding suggests that FAAH is the main enzyme responsible for metabolizing anandamide within the mouse.

FAAH activity has been identified as a factor affecting anandamide uptake in cell culture. Time courses of anandamide uptake showed that in the first minute, initial rates of anandamide uptake are greater than later steady-state time points. At time points long enough to allow for metabolism, cells with FAAH take up significantly more anandamide than cells exposed to an FAAH inhibitor (10, 13, 14, 19). In addition, cells transfected with FAAH accumulated significantly more anandamide than vector-transfected cells at time points allowing for metabolism (10). The activity of FAAH drives continued anandamide accumulation by maintaining the anandamide concentration gradient across the plasma membrane (9, 10).

Some questions concerning anandamide uptake remain unanswered, however. Why does every cell type studied have the ability to take up anandamide (K_m range = 0.1–190 μM) (7–15)? Are ''transport inhibitors'' actually functioning as FAAH inhibitors? Finally, because all prior studies examined only anandamide accumulation at time points when downstream effects such as metabolism and intracellular sequestration could be observed, do the initial anandamide uptake rates exhibit the characteristics of a carrier-mediated process?

In the current study, we have investigated whether initial anandamide uptake is a saturable process in a manner similar to that used for fatty acid transporters (for review, see ref. 20). We also examined the saturability of anandamide metabolism by FAAH at long time points similar to those commonly used in the literature. Several compounds characterized as transport inhibitors were examined at both early uptake (to investigate transport inhibition) and late time points (to investigate inhibition of downstream events such as hydrolysis and intracellular sequestration). These compounds were then analyzed as possible FAAH inhibitors. In addition, all transport assays were performed in the presence of BSA to increase the solubility of anandamide in the uptake medium, similar to FAAH assays and cannabinoid receptor binding assays, but unlike most transport studies.

In contrast to prior studies, we propose that anandamide uptake is a process of simple diffusion. In addition, we believe the transport inhibitors used in this study do not directly act on an anandamide transporter but rather inhibit downstream effects such as metabolism and intracellular sequestration. This inhibition results in an attainment of equilibrium across the membrane and prevents any further net accumulation of anandamide.

Methods

Cell Culture. N18TG2 neuroblastoma cells (kindly provided by Allyn Howlett, North Carolina Central University, Durham)

Abbreviations: AM404, *N*-(4-hydroxyphenyl)arachidonylamide; FAAH, fatty acid amide hydrolase; MAFP, methylarachidonylfluorophosphonate.

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were seeded at a density of 8×10^5 cells per 35×10-mm dishes in 2 ml of DMEM (GIBCO/BRL) + 10% FBS and 1% penicillin/streptomycin. CCF-STTG1 astrocytoma cells (American Type Culture Collection) were seeded at a density of 7×10^5 cells in RPMI medium 1640 (GIBCO/BRL) supplemented with +10% FBS, 1% sodium pyruvate, 1% Hepes, and 1% penicillin/streptomycin. All cells were grown at 37°C and with 5% CO_2.

Determination of Transport Kinetics. Neuroblastoma and astrocytoma cells were incubated for either 3 or 25–40 sec at 37°C in 750 μl of supplemented medium [DMEM or RPMI medium 1640 + 0.4% fatty acid-free BSA (Sigma)] containing 40 nCi (1 Ci = 37 GBq) of arachidonoyl-5,6,8,9,11,12,14,15-[^{3}H]ethanolamide [172 Ci/mmol, 62.2 nCi/μl (NEN)] and unlabeled anandamide (Cayman Chemical, Ann Arbor, MI) at final concentrations of 0.01–200 μM anandamide. Ice-cold supplemented DMEM (3 ml) was added. Cells were washed and scraped three times with 400 μl of 2 mM EDTA in PBS. Next, 2.5 ml of 1:1 chloroform/methanol was added, samples were spun down, and the organic phase was counted in an LKB Beta scintillation counter. Specific anandamide uptake was determined by subtracting the pmol anandamide in 3-sec samples from 25- to 40-sec samples. To determine whether the data fit a saturating curve and whether a K_m and V_{max} could be determined, data were analyzed with a Michaelis Menten plot in ENZPACK for WINDOWS (Biosoft, Cambridge, U.K.) and by fitting the data to a rectangular hyperbola and a linear curve using the linear regression program SIGMAPLOT (SPSS, Chicago). Experiments were repeated five times. Representative graphs are shown.

Enzyme Assay of FAAH Inhibitors. The enzyme assay was conducted as described (10). AM404, methylarachidonylfluorophosphonate (MAFP), arvanil, and olvanil were purchased from Cayman Chemical. Incubations were performed in triplicate at 37°C while shaking. Each sample contained 10 μl of 50 mg/ml fatty acid-free BSA (Sigma–Aldrich), 180 μg of rat brain homogenate, test compounds in ethanol, and 30 μM anandamide (Cayman Chemical) + 0.01 mCi of 120 mCi/mmol arachidonoyl ethanolamide [ethanolamine-1,2-^{14}C] (NEN). Negative control tubes lacked cell extract. The reactions were terminated after 30 min by the addition of 2 volumes chloroform:methanol (1:1). Samples were spun, and the aqueous phase was measured by liquid scintillation counting. Data were graphed by using SIGMAPLOT as percent inhibition of untreated homogenate activity. IC_{50} values were calculated from the graphs. Experiments were repeated three times. Representative curves are shown.

Saturation of FAAH Activity After Uptake by Neuroblastoma Cells. The transport kinetics protocol described previously was used, using 0.01 mCi of 120 mCi/mmol arachidonoyl ethanolamide [ethanolamine-1,2-^{14}C], with a final concentration range of 10–200 μM. Cells were incubated for 5 min or 25 sec. After the addition of 1:1 chloroform/methanol, the aqueous phase was counted to measure ethanolamine production. The presence of unhydrolyzed ethanolamine in samples was estimated by incubating cells for 3 sec, and this value was subtracted from all time points to determine specific metabolism. Data were analyzed as described previously. Experiments were repeated three times. Representative curves are shown.

Inhibition of Anandamide Uptake. Growth media were removed, and cells were washed once. Cells were preincubated for 10 min in supplemented medium containing inhibitor at the concentration specified. Medium was removed, and cells were incubated for 25 sec, 40 sec, or 5 min in supplemented medium containing inhibitor, anandamide, and [^{3}H]anandamide so that 40 nCi (100 nM anandamide) bathed the cells. The reaction was terminated, and cells were processed as described previously. Nonspecific binding values were subtracted from all points. The degree of uptake inhibition was obtained by averaging the triplicates and plotting these values as a percent inhibition (of control cells) by using SIGMAPLOT. Statistical significance of inhibition was determined with Student's t test calculations by using Microsoft EXCEL. All experiments were repeated three times.

FAAH Immunoreactivity in Neuroblastoma Cells. Neuroblastoma cells were plated on poly(L-lysine)-coated coverslips (VWR Scientific) at a density of 4×10^5 cells per 35-mm plate. The next day, cells were treated with 1 μg/ml wheat germ agglutinin Alexa Fluor 594 conjugate (Molecular Probes) for 15 min on ice. Cells were washed for 10 min three times in cold PBS + Ca^{2+} + Mg^{2+} (pH 7.2, 133 mM NaCl, 6.6 mM K_2HPO_4 + 1.5 mM KH_2PO_4 +1 mM $CaCl_2$ + 1 mM $MgCl_2$) and then fixed in 3% paraformaldehyde in PBS + Ca^{2+} + Mg^{2+} at room temperature for 15 min. Cells were washed three times with PBS + Ca^{2+} + Mg^{2+} and then permeabilized on ice for 5 min with 0.5% Triton X-100 + 0.5% normal goat serum (NGS) in PBS + Ca^{2+} + Mg^{2+}. Cells were washed three times with 2% NGS in PBS + Ca^{2+} + Mg^{2+} and then incubated for 1 h at room temperature with 1:1,000 rabbit anti-rat FAAH IgG (gift from Benjamin Cravatt, The Scripps Research Institute, La Jolla, CA) PBS + Ca^{2+} + Mg^{2+} + 5% NGS. Cells were washed three times and incubated for 1 h at room temperature in 1:625 goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes) in PBS + Ca^{2+} + Mg^{2+} + 5% NGS. Cells were washed three times and mounted with VECTASHIELD (Vector Laboratories) and stored at −20°C. Images were taken with a Leica TCS SP2 laser scanning confocal microscope. The final magnification was ×345 (×100 with the objective and ×3.45 electronically). TIFF images were optimized for brightness and contrast by using PHOTOSHOP (Adobe Systems, Mountain View, CA).

Results

Initial Anandamide Uptake Rates in Astrocytoma and Neuroblastoma Cells Are Unsaturable. Anandamide transport was examined in two cell types by using short incubation times to isolate membrane transport activity from downstream metabolic effects. Astrocytoma cells, which lack hydrolase activity (7), were compared with neuroblastoma cells, which have high levels of FAAH activity (10). Astrocytoma cells were examined after incubation in uptake medium for 40 sec, which is before the attainment of a steady state (data not shown). After incubation with increasing concentrations (up to 100 μM) of anandamide, astrocytoma cells had initial uptake rates that yielded a straight line ($r^2 = 0.999$; Fig. 1). These data indicate that anandamide uptake is not saturable and therefore is not carrier-mediated. Neuroblastoma cells were examined after incubation for 25 sec, which is before hydrolysis of anandamide by FAAH (data not shown). Steady-state rates of uptake in neuroblastoma cells occur after 1 min (10). Similarly, neuroblastoma cells also had a linear anandamide accumulation rate ($r^2 = 0.993$) after incubation at short time points with increasing concentrations (up to 200 μM) of anandamide (Fig. 2*A*).

Anandamide Metabolism After Uptake Is Saturable. The effect of metabolism on anandamide uptake was studied in neuroblastoma cells. Cells were allowed to incubate for 5 min in uptake medium containing increasing concentrations of anandamide (10–200 μM). This extended incubation time permitted FAAH to drive uptake through metabolism of intracellular anandamide (10). The observed rate of anandamide metabolism was saturating at high anandamide concentrations, the calculated K_m was 28.1 μM, and the V_{max} was 19.0 pmol/sec per 10^6 cells (Fig. 2*B*). When the same experiment was conducted for 25 sec, no

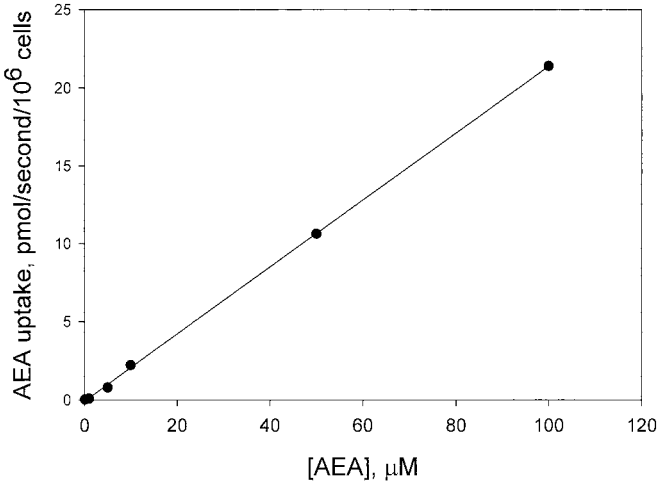

Fig. 1. Astrocytoma anandamide uptake kinetics studies. Accumulation of [^{3}H]anandamide was examined after a series of 40-sec incubations containing increasing concentrations (1–100 μM) of [^{3}H]anandamide. Uptake was stopped, and the cells were washed, scraped, and collected. [^{3}H]Anandamide was extracted and counted. Nonspecific binding was subtracted from each point. The uptake rates were linear ($r^2 = 0.999$), indicating a lack of saturable uptake.

significant anandamide metabolism was observed ($P > 0.07$; data not shown).

Transport Inhibitors Arvanil, Olvanil, and AM404 Inhibit Anandamide Hydrolysis. Other laboratories have reported that transport inhibitors also inhibit FAAH (for reviews, see refs. 5 and 6). We tested this theory by using an FAAH activity assay to examine the effects of AM404, arvanil, and olvanil on anandamide hydrolysis. Rat brain homogenate was incubated with 30 μM anandamide and increasing concentrations of each transport inhibitor. All compounds tested inhibited FAAH activity in this system. The IC_{50} values of arvanil, olvanil, and AM404 are 3, 20, and 6 μM, respectively (Fig. 3).

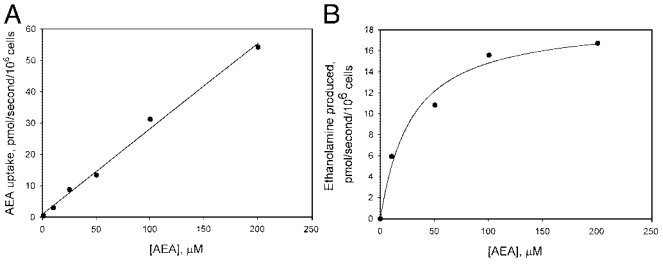

Fig. 2. Anandamide transport (*A*) and metabolism (*B*) in neuroblastoma cells. (*A*) Accumulation of [^{3}H]anandamide and its breakdown products within neuroblastoma cells was examined after a series of 25-sec incubations in increasing concentrations (1–200 μM) of [^{3}H]anandamide. Uptake was stopped, and the cells were washed, scraped, and collected. [^{3}H]Anandamide was extracted and counted. Nonspecific binding was subtracted from each point. The rate of uptake remained linear ($r^2 = 0.993$), indicating a lack of saturable uptake. (*B*) Accumulation of [^{14}C]ethanolamine in neuroblastoma cells was examined after a series of 5-min incubations in increasing concentrations (10–200 μM) of [^{14}C]anandamide. Uptake was stopped, and cells were washed, scraped, and collected. [^{14}C]Ethanolamine was extracted and counted. Nonmetabolized ethanolamine levels were subtracted from each point. The rates of [^{14}C]ethanolamine production fit a saturating hyperbola plot, indicating that metabolism can be saturated in uptake experiments with 5-min incubation times. The calculated K_m was 28.1 μM and the V_{max} was 19.0 pmol/sec per 10^6 cells.

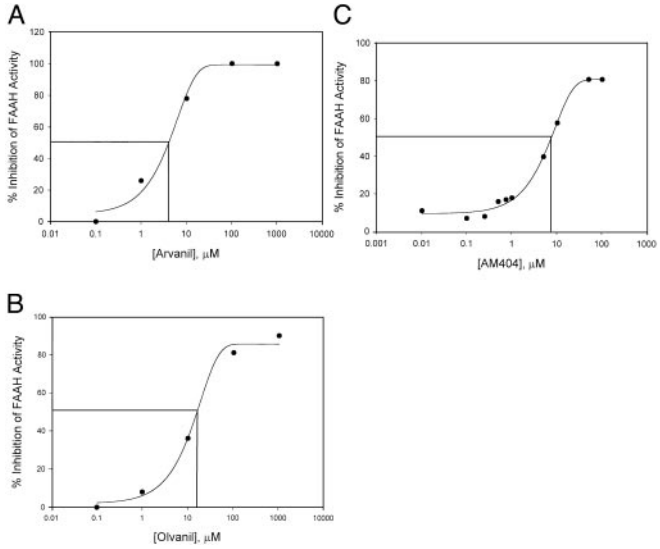

Fig. 3. Inhibition of FAAH by arvanil, olvanil, and AM404. Inhibition of anandamide hydrolysis by rat brain homogenate was examined after the addition of increasing concentrations of arvanil (*A*), olvanil (*B*), and AM404 (*C*). Each sample contained BSA, rat brain homogenate, test compounds in ethanol, and 30 μM arachidonoyl ethanolamide [ethanolamine-1,2-^{14}C]. The samples were incubated at 37°C for 30 min. Data were graphed as percent inhibition of untreated homogenate activity. IC_{50} values were calculated from the graphs and were 3 μM for arvanil, 20 μM for olvanil, and 6 μM for AM404.

MAFP, AM404, Arvanil, and Olvanil Affect Downstream Events, Not Initial Anandamide Uptake Rates. Because we established that these compounds inhibit anandamide metabolism, we examined the effects of a preincubation with these and another FAAH inhibitor, MAFP (22, 23), on anandamide accumulation. A time point was chosen that was long enough to allow for downstream mechanisms to influence uptake (10). After a 5-min incubation in medium containing anandamide, astrocytoma (which lack hydrolysis activity) and neuroblastoma (which contain active FAAH) cells both showed a statistically significant reduction in anandamide accumulation ($P < 0.05$) relative to cells treated only with vehicle (Fig. 4). In sharp contrast, when similar experiments were performed by using short time points that represented initial uptake rates, very different results were observed. A preincubation with every compound tested had no significant effect on [^{3}H]anandamide accumulation within astrocytoma cells at 40-sec uptake time points ($P > 0.5$; Fig. 5*A*).

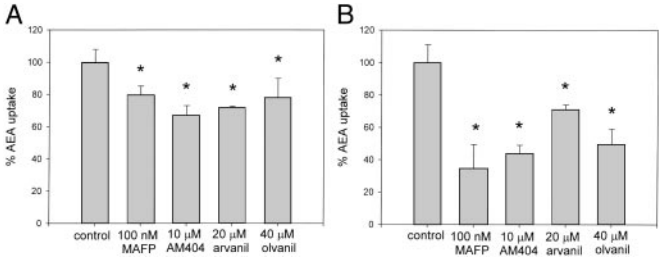

Fig. 4. [^{3}H]Anandamide accumulation at 5 min in astrocytoma (*A*) and neuroblastoma (*B*) cells after a preincubation in MAFP, AM404, arvanil, and olvanil. Accumulation of [^{3}H]anandamide was examined after a 10-min preincubation in medium + 0.4% BSA ± inhibitor followed by 5-min incubations in 100 nM [^{3}H]anandamide ± inhibitor. Uptake was stopped, and cells were washed, scraped, and collected. [^{3}H]Anandamide was extracted and counted. Nonspecific binding was subtracted from each point. A statistically significant decrease ($P < 0.05$) in anandamide accumulation after 5 min of uptake was observed after a pretreatment with all compounds.

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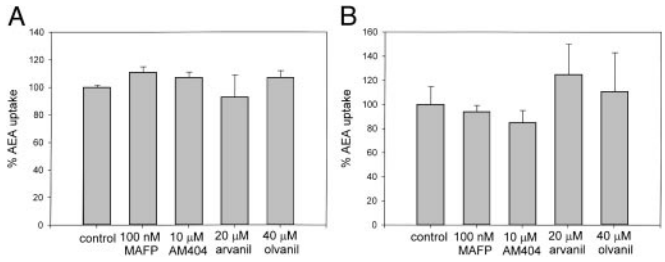

Fig. 5. [^{3}H]Anandamide accumulation in astrocytoma (*A*) and neuroblastoma (*B*) cells after a preincubation in MAFP, AM404, arvanil, and olvanil and a 40- or 25-sec incubation in uptake medium. Accumulation of [^{3}H]anandamide was examined after a 10-min preincubation in medium + 0.4% BSA ± inhibitor followed by 40-sec (astrocytoma) or 25-sec (neuroblastoma) incubations in 100 nM [^{3}H]anandamide ± inhibitor. Uptake was stopped, and cells were washed, scraped, and collected. [^{3}H]Anandamide was extracted and counted. Nonspecific binding values were subtracted from each point. No statistically significant decrease ($P > 0.5$) in anandamide accumulation after uptake was observed after a pretreatment with all compounds, at short time points.

Similarly, no significant difference on accumulation was observed when neuroblastoma cells were incubated in uptake medium for 25 sec ($P > 0.5$; Fig. 5*B*).

FAAH Is Immunolocalized Perinuclearly in Neuroblastoma Cells. Because FAAH activity plays a key role in anandamide uptake, its localization relative to the plasma membrane is of interest. Neuroblastoma cells were treated with wheat germ agglutinin to mark the plasma membrane (21) and immunostained for FAAH with an Ab used previously in immunolocalization studies (24). Confocal fluorescence microscopy identified punctate FAAH immunoreactivity perinuclearly. In addition, a lack of colocalization with wheat germ agglutinin indicated that FAAH was not located in the plasma membrane (Fig. 6). Identical experiments using astrocytoma cells were immunonegative for FAAH (data not shown).

Discussion

The concept of an anandamide transporter has become widely accepted with the exception of one theoretical article (25). It has been characterized as a process of facilitated diffusion, meaning that uptake is mediated by a carrier, and is driven by a concentration gradient across the plasma membrane (for review, see refs. 5 and 6). Although the transport protein has never been isolated, this process has been defined as being protein-mediated, because it is temperature-dependent, saturable, selective for anandamide, and able to be inhibited.

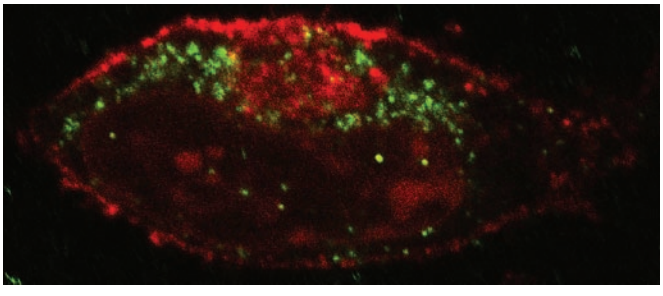

Fig. 6. FAAH immunolocalization in neuroblastoma cells. The FAAH immunoreactivity pattern of neuroblastoma cells was compared with the pattern generated by the membrane marker wheat germ agglutinin. Neuroblastoma cells were treated with wheat germ agglutinin Alexa Fluor 594 conjugate, washed, permeabilized with 0.5% Triton X-100, and then processed for immunofluorescence with rabbit anti-rat FAAH IgG according to *Methods*. FAAH immunoreactivity was perinuclear in neuroblastoma cells. In addition, no colocalization between FAAH immunoreactivity and wheat germ agglutinin occurred, indicating that FAAH is not located on the plasma membrane.

Temperature dependency has been used as an indicator of carrier-mediated anandamide transport (8, 26–28). Problems arise, however, when using this criterion to determine protein involvement in uptake. First, hydrolysis by FAAH is also temperature-dependent. All prior studies of anandamide uptake used incubation time points that were long enough to allow for its metabolism (10, 25). If FAAH is present within the cell, its activity will maintain the anandamide concentration gradient across the membrane (10) in a temperature-dependent manner. Another complication that arises when using temperature dependence to determine protein involvement in anandamide uptake is that it also affects simple diffusion (29). Because temperature affects the fluidity of membranes, we chose to use 3-sec incubations at 37°C to more accurately reflect the nonspecific binding of anandamide to the plasma membrane, as opposed to 4°C as commonly used by others.

Saturability is a second hallmark for protein-mediated transport. Multiple laboratories, including ours, have published articles characterizing saturable transport of anandamide in a variety of cell types (for reviews, see refs. 5 and 6), including the neuroblastoma and astrocytoma cell lines used in this study (10, 12). The kinetics were always determined by using time points >1 min. As a result, the saturation of uptake in these studies could not only be due to the presence of a transport protein, but also could be due to downstream processes such as hydrolysis by FAAH or possibly intracellular sequestration.

In this study, we used time points short enough to avoid such complications so that the initial transport of anandamide could be examined for saturability and specific inhibition by transport inhibitors. In data presented previously (Figs. 1 and 2*A*), this reduction in the incubation time resulted in unsaturable anandamide accumulation in both neuroblastoma and astrocytoma cells. These results suggest that no transport protein is involved, and that anandamide uptake is due to simple diffusion, a process that is not saturable. These kinetic values measure only the total concentration of anandamide in the uptake medium, rather than the amount remaining uncomplexed to albumin, which is the common practice in the fatty acid field. Unlike in fatty acids, however, there are no direct means of examining free anandamide levels in solution, such as the use of a fluorescent probe. In addition, the limited solubility of anandamide in aqueous solutions prohibits the use of uptake solutions lacking albumin. Our examination of initial kinetics of anandamide transport suggests a mechanism of simple diffusion.

Lending further credence to the hypothesis that uncharged, hydrophobic anandamide can easily enter a membrane unaided, the crystal structure of FAAH indicates that the hydrophobic entryway to the active site of the enzyme is adjacent to the membrane from which anandamide is recruited (30). In addition, preliminary studies examining the process of CB1 cannabinoid receptor activation suggest that anandamide is able to approach the receptor by entering the lipid (P. Reggio, personal communication) and may first interact with the lipid face of receptor helix TMH6 (31).

Saturation was observed, however, when neuroblastoma uptake was permitted to continue for long time points, and metabolite production was examined after uptake (Fig. 2*B*). These data suggest that many prior studies using long time points may have been measuring rates of continued accumulation driven by downstream protein-mediated effects, rather than transport. This observation that FAAH activity could be falsely identified as protein-mediated transport is supported further by the literature where the reported K_m range of FAAH (0.8–180 μM; refs. 8, 28, and 32–37) is in the same broad range as the reported K_m of transport (0.1–190 μM; refs. 7–15).

Substrate specificity is the third indicator of protein-mediated transport and has been examined in anandamide analog competition studies (12, 38). By varying structural features of analogs and coincubating cells with these analogs and anandamide, they observed changes in the ability of a cell to accumulate anandamide. From these experiments, it was concluded that anandamide uptake is a protein-mediated process, because there seems to be a degree of specificity for the anandamide structure. Although one study did investigate the FAAH inhibitory effects of these compounds (38), both failed to address how these structural variations could influence uptake by affecting simple diffusion, metabolism, and intracellular sequestration. To account for the slower rate of cellular uptake of palmitoyl ethanolamide relative to anandamide, it has been suggested that separate transporters may be involved (8). However, an alternate explanation is that palmitoyl ethanolamide is also transported by simple diffusion and its uptake is less efficient because it is a poorer substrate than anandamide for FAAH (17).

The final feature of facilitated diffusion is selective inhibition of transport. Numerous compounds have been tested as anandamide transport inhibitors. Steady-state measurements of anandamide accumulation after exposure to known protein-modifying transport inhibitors, such as 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) (11), phloretin (7, 11), and *N*-ethylmaleimide (NEM) (6, 8, 28), have resulted in either no effect on anandamide transport or controversial results. However, many compounds have been shown to inhibit anandamide accumulation, including AM404, arvanil, and olvanil. We found that these compounds also inhibit hydrolysis (Fig. 3). Our results confirmed earlier studies where AM404 was shown to inhibit FAAH with an IC_{50} range of 500 nM to 3.6 μM (38, 39), and arvanil with an IC_{50} of 630 nM (39). These studies are in sharp contrast to those indicating that AM404 (7), arvanil (40), and olvanil (41) had minimal FAAH inhibitory effects and therefore must be inhibiting an anandamide transporter. The enzyme IC_{50} values of AM404, arvanil, and olvanil are extremely similar to reported transport IC_{50} values (all ranging from 2.2 to 9 μM; refs. 7, 38, and 42–44). Because many transport inhibition studies used cells that express FAAH and used long uptake times, their observations may be due to these compounds reducing anandamide accumulation through the inactivation of FAAH, rather than direct inhibition of a transporter. As originally proposed by our laboratory (45), FAAH inhibitors raise anandamide levels to produce pharmacological effects (46), and this is probably the mechanism by which "anandamide transport inhibitors" produce their response *in vivo* (47–49). These inhibitors have therefore been suggested as being therapeutically useful in areas affected by the endocannabinoid system such as analgesia, nausea, appetite, sedation, memory, locomotion, glaucoma, and immune function (45).

Although this current study confirmed that these compounds are able to inhibit anandamide uptake by cells expressing FAAH when time points are long enough to allow for hydrolysis (Fig. 4*B*), it also demonstrated that at time points before significant hydrolysis, no inhibition was observed (Fig. 5*B*). These observations suggest that these compounds do not inhibit a transporter but rather an event downstream. This inhibition leads to a loss of the concentration gradient across the plasma membrane that is necessary to drive anandamide uptake.

Our inhibitor studies using astrocytoma cells, which lack anandamide hydrolase activity (data not shown and ref. 7), suggest that other downstream mechanism(s) besides hydrolysis by FAAH may also affect anandamide uptake. The addition of transport inhibitors induced a slight, but significant, inhibition observed at longer time points (Fig. 4*A*). Although these compounds could be inhibiting a carrier at long time points, it is improbable because no inhibition was observed at short time points (Fig. 5*A*). It is likely that these compounds, which are all structurally similar to anandamide, are affecting other downstream machinery, including intracellular sequestration by membranes or proteins. This mechanism may explain the saturating kinetics observed by others.

Prior immunohistochemical studies of the rat central nervous system described FAAH localization as punctate within the cell bodies and dendrites of pyramidal and Purkinje cells (24, 50). The perinuclear, punctate pattern observed by us in neuroblastoma cells (Fig. 6) is very similar to that reported for COS-7 cells transfected with rat FAAH, and this pattern has been interpreted to be consistent with endoplasmic and/or Golgi localization (51, 52). The observed exclusion of FAAH from the plasma membrane may function to physically separate the activity of anandamide at cannabinoid receptor 1 from its site of hydrolysis.

For plasma membrane-bound anandamide to be hydrolyzed, it is required to traverse the aqueous cytoplasm. This process could be accomplished either by diffusing directly through the cytoplasm in a rather inefficient manner to reach FAAH or by binding an unidentified intracellular anandamide binding protein that would serve to increase the solubility of anandamide in the cytoplasmic space. Long-chain fatty acids have been determined to cross a membrane by means of simple diffusion at rates ranging from 0.5 to 10 sec (29). Diffusion through the cytoplasm, even with the help of a binding protein, would result in a time lag before the start of anandamide metabolism, explaining why hydrolysis is not observed in neuroblastoma cells at 25 sec.

Anandamide metabolism driving uptake is supported further by anandamide uptake autoradiography studies in intact goldfish retinal tissue where accumulation of anandamide overlapped with FAAH immunoreactivity (S.T.G., D.G.D., K.M.S., F.F., and S. Yazulla, unpublished data). Therefore, FAAH activity is the regulative force of anandamide clearance in tissue.

In summary, these data portray a simple and efficient system in neuroblastoma and astrocytoma cells for anandamide clearance from the extracellular space independent of a transporter. The lack of saturation and inhibition of uptake indicate that anandamide could be entering cell membranes by means of simple diffusion. Once intracellular, anandamide is subsequently either metabolized by FAAH or sequestered by mechanisms such as binding intracellular membranes (10). Continuous hydrolysis by FAAH would serve to maintain the concentration gradient necessary to drive the process of simple diffusion. Although this study does not conclusively exclude the existence of a transporter, it does examine rates of initial anandamide uptake and suggest a lack of protein mediation. These data suggest that metabolism by FAAH is the regulating force of anandamide inactivation.

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1. Sugiura, T., Kobayashi, Y., Oka, S. & Waku, K. (2002) *Prostaglandins Leukotrienes Essent. Fatty Acids* **66,** 173–192.
2. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. & Mechoulam, R. (1992) *Science* **258,** 1946–1949.
3. Di Marzo, V. & Fontana, A. (1995) *Prostaglandins Leukotrienes Essent. Fatty Acids* **53,** 1–11.
4. Mechoulam, R., Hanus, L. & Martin, B. R. (1994) *Biochem. Pharmacol.* **48,** 1537–1544.
5. Fowler, C. J. & Jacobsson, S. O. (2002) *Prostaglandins Leukotrienes Essent. Fatty Acids* **66,** 193–200.
6. Hillard, C. J. & Jarrahian, A. (2000) *Chem. Phys. Lipids* **108,** 123–134.
7. Beltramo, M., Stella, N., Calignano, A., Lin, S. Y., Makriyannis, A. & Piomelli, D. (1997) *Science* **277,** 1094–1097.

8. Bisogno, T., Maurelli, S., Melck, D., De Petrocellis, L. & Di Marzo, V. (1997) *J. Biol. Chem.* **272,** 3315–3323.
9. Day, T. A., Rakhshan, F., Deutsch, D. G. & Barker, E. L. (2001) *Mol. Pharmacol.* **59,** 1369–1375.
10. Deutsch, D. G., Glaser, S. T., Howell, J. M., Kunz, J. S., Puffenbarger, R. A., Hillard, C. J. & Abumrad, N. (2001) *J. Biol. Chem.* **276,** 6967–6973.
11. Hillard, C. J., Edgemond, W. S., Jarrahian, A. & Campbell, W. B. (1997) *J. Neurochem.* **69,** 631–638.
12. Piomelli, D., Beltramo, M., Glasnapp, S., Lin, S. Y., Goutopoulos, A., Xie, X. Q. & Makriyannis, A. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 5802–5807.
13. Rakhshan, F., Day, T. A., Blakely, R. D. & Barker, E. L. (2000) *J. Pharmacol. Exp. Ther.* **292,** 960–967.
14. Jacobsson, S. O. & Fowler, C. J. (2001) *Br. J. Pharmacol.* **132,** 1743–1754.
15. Lopez, R., Viso, A., Ortega, G., Lastres, B., Gonzalez, S., Fernandez, R. & Ramos, J. A. (2001) *J. Med. Chem.* **44,** 4505–4508.
16. Maccarrone, M., Bari, M., Lorenzon, T., Bisogno, T., Di Marzo, V. & Finazzi-Agro, A. (2000) *J. Biol. Chem.* **275,** 13484–13492.
17. Deutsch, D. G., Ueda, N. & Yamamoto, S. (2002) *Prostaglandins Leukotrienes Essent. Fatty Acids* **66,** 201–210.
18. Cravatt, B. F., Demarest, K., Patricelli, M. P., Bracey, M. H., Giang, D. K., Martin, B. R. & Lichtman, A. H. (2001) *Proc. Natl. Acad. Sci. USA.* **98,** 9371–9376.
19. Deutsch, D. G. & Chin, S. A. (1993) *Biochem. Pharmacol.* **46,** 791–796.
20. Abumrad, N., Harmon, C. & Ibrahimi, A. (1998) *J. Lipid Res.* **39,** 2309–2318.
21. Gonzalez, J. E. & Tsien, R. Y. (1995) *Biophys. J.* **69,** 1272–1280.
22. Deutsch, D. G., Omeir, R., Arreaza, G., Salehani, D., Prestwich, G. D., Huang, Z. & Howlett, A. (1997) *Biochem. Pharmacol.* **53,** 255–260.
23. De, P., Melck, D., Ueda, N., Maurelli, S., Kurahashi, Y., Yamamoto, S., Marino, G. & Di, M. (1997) *Biochem. Biophys. Res. Commun.* **231,** 82–88.
24. Egertova, M., Giang, D. K., Cravatt, B. F. & Elphick, M. R. (1998) *Proc. R. Soc. London Ser. B* **265,** 2081–2085.
25. Patricelli, M. P. & Cravatt, B. F. (2001) *Vitam. Horm. (San Francisco)* **62,** 95–131.
26. Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C. & Piomelli, D. (1994) *Nature* **372,** 686–691.
27. Hillard, C. J. & Campbell, W. B. (1997) *J. Lipid Res.* **38,** 2383–2398.
28. Maccarrone, M., van der Stelt, M., Rossi, A., Veldink, G. A., Vliegenthart, J. F. & Finazzi-Agro, A. (1998) *J. Biol. Chem.* **273,** 32332–32339.
29. Kleinfeld, A. M., Storms, S. & Watts, M. (1998) *Biochemistry* **37,** 8011–8019.
30. Bracey, M. H., Hanson, M. A., Masuda, K. R., Stevens, R. C. & Cravatt, B. F. (2002) *Science* **298,** 1793–1796.
31. Barnett, N., Hurst, D. P., Lynch, D. L., Guarnieri, F., Makriyannis, A. & Reggio, P. H. (2002) *J. Med. Chem.* **45,** 3649–3659.
32. Fowler, C. J., Stenstrom, A. & Tiger, G. (1997) *Pharmacol. Toxicol. (Copenhagen)* **80,** 103–107.
33. Hillard, C. J., Wilkison, D. M., Edgemond, W. S. & Campbell, W. B. (1995) *Biochim. Biophys. Acta* **1257,** 249–256.
34. Lang, W., Qin, C., Lin, S., Khanolkar, A. D., Goutopoulos, A., Fan, P., Abouzid, K., Meng, Z., Biegel, D. & Makriyannis, A. (1999) *J. Med. Chem.* **42,** 896–902.
35. Omeir, R. L., Chin, S., Hong, Y., Ahern, D. G. & Deutsch, D. G. (1995) *Life Sci.* **56,** 1999–2005.
36. Watanabe, K., Ogi, H., Nakamura, S., Kayano, Y., Matsunaga, T., Yoshimura, H. & Yamamoto, I. (1998) *Life Sci.* **62,** 1223–1229.
37. Maurelli, S., Bisogno, T., De Petrocellis, L., Di Luccia, A., Marino, G. & Di Marzo, V. (1995) *FEBS Lett.* **377,** 82–86.
38. Jarrahian, A., Manna, S., Edgemond, W. S., Campbell, W. B. & Hillard, C. J. (2000) *J. Neurochem.* **74,** 2597–2606.
39. Jonsson, K. O., Vandevoorde, S., Lambert, D. M., Tiger, G. & Fowler, C. J. (2001) *Br. J. Pharmacol.* **133,** 1263–1275.
40. Di Marzo, V., Griffin, G., De Petrocellis, L., Brandi, I., Bisogno, T., Williams, W., Grier, M. C., Kulasegram, S., Mahadevan, A., Razdan, R. K. & Martin, B. R. (2002) *J. Pharmacol. Exp. Ther.* **300,** 984–991.
41. Di Marzo, V., Bisogno, T., Melck, D., Ross, R., Brockie, H., Stevenson, L., Pertwee, R. & De Petrocellis, L. (1998) *FEBS Lett.* **436,** 449–454.
42. De Petrocellis, L., Bisogno, T., Davis, J. B., Pertwee, R. G. & Di Marzo, V. (2000) *FEBS Lett.* **483,** 52–56.
43. De Petrocellis, L., Bisogno, T., Maccarrone, M., Davis, J. B., Finazzi-Agro, A. & DiMarzo, V. (2001) *J. Biol. Chem.* **276,** 12856–12863.
44. Melck, D., Bisogno, T., De Petrocellis, L., Chuang, H., Julius, D., Bifulco, M. & Di Marzo, V. (1999) *Biochem. Biophys. Res. Commun.* **262,** 275–284.
45. Koutek, B., Prestwich, G. D., Howlett, A. C., Chin, S. A., Salehani, D., Akhavan, N. & Deutsch, D. G. (1994) *J. Biol. Chem.* **269,** 22937–22940.
46. Martin, B. R., Beletskaya, I., Patrick, G., Jefferson, R., Winckler, R., Deutsch, D. G., Di Marzo, V., Dasse, O., Mahadevan, A. & Razdan, R. K. (2000) *J. Pharmacol. Exp. Ther.* **294,** 1209–1218.
47. Baker, D., Pryce, G., Croxford, J. L., Brown, P., Pertwee, R. G., Huffman, J. W. & Layward, L. (2000) *Nature* **404,** 84–87.
48. Giuffrida, A., Beltramo, M. & Piomelli, D. (2001) *J. Pharmacol. Exp. Ther.* **298,** 7–14.
49. Goutopoulos, A. & Makriyannis, A. (2002) *Pharmacol. Ther.* **95,** 103–117.
50. Tsou, K., Nogueron, M. I., Muthian, S., Sanudo-Pena, M. C., Hillard, C. J., Deutsch, D. G. & Walker, J. M. (1998) *Neurosci. Lett.* **254,** 137–140.
51. Arreaza, G. & Deutsch, D. G. (1999) *FEBS Lett.* **454,** 57–60.
52. Giang, D. K. & Cravatt, B. F. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2238–2242.