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ARTICLE *in* BIOCHEMISTRY · JULY 2004

Impact Factor: 3.02 · DOI: 10.1021/bi049395f · Source: PubMed

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Comparison of Anandamide Transport in FAAH Wild-Type and Knockout Neurons: Evidence for Contributions by both FAAH and the CB1 Receptor to Anandamide Uptake†

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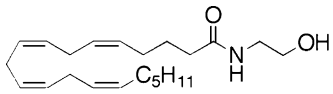
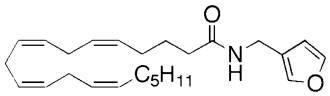
Received March 29, 2004; Revised Manuscript Received April 28, 2004

ABSTRACT: The cellular inactivation of the endogenous cannabinoid (endocannabinoid) anandamide (AEA) represents a controversial and intensely investigated subject. This process has been proposed to involve two proteins, a transporter that promotes the cellular uptake of AEA and fatty acid amide hydrolase (FAAH), which hydrolyzes AEA to arachidonic acid. However, whereas the role of FAAH in AEA metabolism is well-characterized, the identity of the putative AEA transporter remains enigmatic. Indeed, the indirect pharmacological evidence used to support the existence of an AEA transporter has been suggested also to be compatible with a model in which AEA uptake is driven by simple diffusion coupled to FAAH metabolism. Here, we have directly addressed the contribution of FAAH to AEA uptake by examining this process in neuronal preparations from FAAH(−/−) mice and in the presence of the uptake inhibitor UCM707. The results of these studies reveal that (i) care should be taken to avoid the presence of artifacts when studying the cellular uptake of lipophilic molecules like AEA, (ii) FAAH significantly contributes to AEA uptake, especially with longer incubation times, and (iii) a UCM707-sensitive protein(s) distinct from FAAH also participates in AEA uptake. Interestingly, the FAAH-independent component of AEA transport was significantly reduced by pretreatment of neurons with the cannabinoid receptor 1 (CB1) antagonist SR141716A. Collectively, these results indicate that the protein-dependent uptake of AEA is largely mediated by known constituents of the endocannabinoid system (FAAH and the CB1 receptor), although a partial contribution of an additional UCM707-sensitive protein is also suggested.

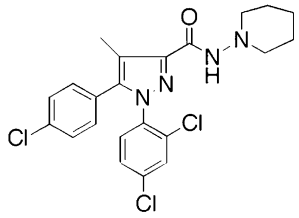
Anandamide (AEA)[1] (Figure 1) is a member of a large class of endogenous signaling lipids called the fatty acid amides that modulate a number of physiological processes in both the nervous system and the periphery (*1*, *2*). Many of the actions of AEA are mediated by binding and activation of the central cannabinoid receptor CB1 (*3*). One hallmark of signaling molecules such as AEA is that enzymatic mechanisms exist for their biosynthesis and inactivation, and the elucidation of both metabolic pathways for AEA constitutes a major focus of current research. AEA appears to be produced from phospholipids in a stimulus-dependent manner by the consecutive action of two enzymes, a calcium-dependent transacylase and a phospholipase D (*4*), and



Anandamide (AEA)



UCM707



SR141716A

FIGURE 1: Structures of the endogenous cannabinoid anandamide (AEA) (*3*), the AEA uptake inhibitor UCM707 (*18*), and the CB1 receptor antagonist SR141716A (*30*).

† This work was supported by the National Institute of Drug Abuse of the National Institutes of Health (DA15197), Allergan Inc., the Helen L. Dorris Institute for the Study of Neurological and Psychiatric Disorders, the Skaggs Institute for Chemical Biology, and Spanish Ministerio de Ciencia y Tecnología Grant BQU 2001-1459. S.O.-G. is a predoctoral fellow from the Spanish Ministerio de Educación, Cultura y Deporte.

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[1] Abbreviations: AEA, anandamide; BSA, bovine serum albumin; CB1, brain cannabinoid receptor; FAAH, fatty acid amide hydrolase; SEM, standard error of the mean.

10.1021/bi049395f CCC: $27.50
Published on Web 06/03/2004

inactivated by a two-step process involving the transport of this lipid into cells (*5*) followed by intracellular hydrolysis by the integral membrane enzyme fatty acid amide hydrolase (FAAH) (*6*, *7*). However, with respect to the inactivation of AEA, only FAAH has been molecularly characterized and structurally studied (*8*, *9*). Indeed, the actual nature and mechanism of AEA uptake or, more generally, the movement of this fatty acid amide not only through biological membranes but also through aqueous media (extracellular and cytoplasmic) remains an enigmatic and controversial subject (*10*).

The existence of a protein transporter involved in the uptake of AEA from the extracellular medium to the cytoplasm has been postulated on the basis of the temperature dependence, saturability, substrate specificity, and pharmacological inhibition of this uptake process (*5*, *11*, *12*). However, these biochemical characteristics are also compatible with other mechanisms for AEA uptake and cannot be viewed as unequivocal evidence of a protein transporter. For example, the simple diffusion of hydrophobic molecules across cell membranes or lipid vesicles is temperature-dependent (*10*). Likewise, saturability could reflect the insolubility of AEA in aqueous media or the solubility limit of AEA in cell membranes. Finally, all four features could also be compatible with a diffusion-controlled uptake mechanism followed by FAAH metabolism. Consistent with this latter possibility, several studies have already described not only the existence of cross regulation between the AEA transporter and FAAH (*13*, *14*) but also the absence of any protein-mediated, FAAH-independent component of AEA transport (*15*, *16*). These findings, together with the fact that most of the AEA uptake inhibitors described to date exhibit similar affinities for FAAH (*17*), have hindered experimental efforts to distinguish between the contribution of FAAH and any potential "FAAH-independent" component of AEA transport.

Given the aforementioned issues, efforts to study the cellular uptake of AEA would clearly benefit from (i) selective inhibitors of this process and (ii) cellular preparations devoid of FAAH. With the recent development of the first uptake inhibitors that show minimal activity against FAAH (*18*, *19*) and the generation of the first FAAH knockout [(−/−)] mice (*20*), these two criteria appear to have been met. Here, we have examined AEA uptake in primary neuronal cultures obtained from FAAH(+/+) and -(−/−) mice and tested the sensitivity of this process to UCM707 (Figure 1), an inhibitor of AEA uptake with only weak activity against FAAH [IC_{50} (AEA uptake) = 0.8 μM; IC_{50} (FAAH)/IC_{50} (AEA uptake) $\approx$ 40] (*18*). The results of these studies indicate that AEA uptake is mediated by a combination of FAAH-dependent and FAAH-independent mechanisms, with at least a portion of the latter process being sensitive to inhibition by UCM707 and the CB1 receptor antagonist SR141716A (Figure 1).

EXPERIMENTAL PROCEDURES

Reagents. N-Arachidonoyl[5,6,8,9,11,12,14,15-^{3}H]ethanolamine ([^{3}H]AEA, 200 Ci/mmol) was obtained from Moravec Biochemicals, Inc. Unlabeled anandamide and UCM707 were synthesized as previously described (*8*, *18*). The CB1 antagonist SR141716A was kindly provided by the National Institute on Drug Abuse. Culture reagents were purchased from Gibco, and all other reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Cell Culture. Neuronal cortical cell cultures were prepared from 15-day-old mouse embryos. FAAH(+/+) and FAAH-(−/−) mice were developed as described previously (*20*) and represented inbred animals backcrossed for at least five generations onto the C57BL/6 background. Dissociated cortical cells were plated on six-well culture plates precoated with poly(D-lysine) and laminin. Plating media consisted of Eagles' MEM supplemented with 5% horse serum, 5% fetal bovine serum (FBS), 2 mM glutamine, 2 g/L $NaHCO_3$, and 20 mM glucose. Cortical neurons were tested after being cultured *in vitro* for 5−6 days.

Determination of the Rate of Cellular Uptake of [^{3}H]-Anandamide. Incorporation of AEA into neurons was assessed by incubating the cells in DMEM (1 mL) with 100 nM radiolabeled AEA (0.45 nM [^{3}H]AEA, brought to 100 nM with nonradioactive AEA), in the presence or absence of different concentrations of fatty acid-free bovine serum albumin (BSA) and UCM707 as indicated in the experiments. Incubations were stopped by placing the culture plates on ice and rapidly aspirating the media. Cells were rinsed three times with 1.5 mL of ice-cold Krebs-Hepes buffer [118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20.0 mM $NaHCO_3$, 2.4 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 4.0 μM $EDTANa_2$, 110 μM ascorbic acid, 11.1 mM glucose, and 10.0 mM Hepes (pH 7.4)] containing 1% (w/v) BSA. Then, the cells were scraped and subjected to lipid extraction with methanol. Radioactivity in the extracts was measured by liquid scintillation counting. For a typical experiment (5% BSA, incubation for 10 min), the specific quantity of AEA uptake into neurons was approximately 0.5 pmol/well, or 0.5% of the total AEA in the assay.

For experiments carried out in the presence of UCM707 or SR141716A, cells were preincubated at 37 °C for 10 min in the presence of these agents and then the rate of uptake of AEA was measured as indicated above.

IC_{50} values were calculated after subtraction of the noncellular adsorption of AEA for each point, and the curves were obtained by nonlinear least-squares fitting of the data (one-site competition) with GraphPad Prism (GraphPad Software, San Diego, CA).

The noncellular uptake of AEA was assessed by running the identical experiments in parallel using plates without cells. Each graph point represents duplicate determinations, and results are based on two or three independent experiments.

Western Blot Analysis. Neurons were washed with ice-cold PBS and lysed in TBS (pH 7.6) containing 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, and Complete protease inhibitor cocktail (Roche). Brain homogenates were obtained as previously reported (*20*). Equal amounts of protein (100 μg) were resolved on SDS−PAGE and transferred to nitrocellulose, and FAAH Western blots were conducted using polyclonal anti-FAAH antibodies as described previously (*20*).

RESULTS

Characterization of a Noncellular Component for AEA Uptake. To compare the uptake of AEA in FAAH(+/+) and

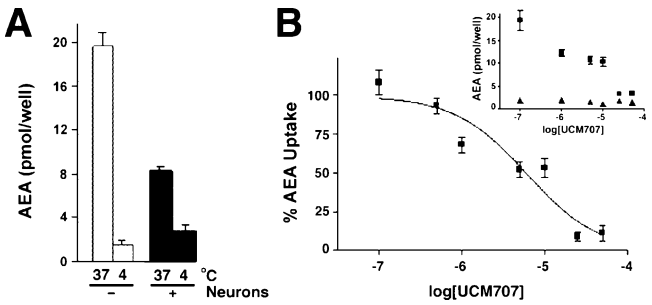

FIGURE 2: Binding of AEA to plastic cell culture plates in the presence or absence of cells. (A) AEA uptake measured at 37 and 4 °C in plates without (□) or with FAAH(+/+) cortical neuronal cultures (■). All uptake measurements were conducted for 4 min with 100 nM [^{3}H]AEA in cell culture media. Data represent averages and standard errors (SEM) of at least three independent experiments performed in duplicate. (B) UCM707 blocks uptake of AEA onto plates without cells in a dose-dependent manner (100% uptake corresponds to 14.1 pmol of AEA/well). Culture plates without cells were incubated at 37 °C for 4 min with 100 nM [^{3}H]-AEA in culture media in the presence of different concentrations of UCM707. Points represent the subtraction of the extent of [^{3}H]-AEA accumulation at 4 °C from the extent of [^{3}H]AEA accumulation at 37 °C. The curve was generated using the sigmoidal dose−response fit (GraphPad Prism). The inset shows the quantity of AEA bound to wells at 37 (■) or 4 °C (▲). Results are expressed as means ± SEM of at least two independent experiments performed in duplicate.

-(−/−) neurons, we first established conditions that allowed reliable measurement of the accumulation of this lipid into neuronal cultures. Considering that AEA is highly hydrophobic, we were concerned about potential nonspecific interactions, such as the adsorption of this lipid onto plastic culture plates. Indeed, when AEA was incubated in serum-free media with plates lacking cells, a strong level of nonspecific binding was observed that was, surprisingly, even greater than the level of binding of this lipid to plates containing wild-type mouse cortical neurons (Figure 2A). Notably, this absorption of AEA onto culture plates lacking cells was temperature-dependent (Figure 2A) and blocked by the uptake inhibitor UCM707 in a dose-dependent manner that exhibited a sigmoid-like curve characteristic of specific interactions between a protein and its ligand (Figure 2B). These data indicate that many of the controls typically used to indicate the presence of an AEA transporter (*5*, *11*) are not capable of distinguishing specific (i.e., binding to protein) from nonspecific (i.e., binding to culture plates) components of AEA uptake. Accordingly, an alternative set of conditions was required to accurately assess the uptake of AEA into cultured cells.

The high degree of nonspecific binding of AEA (and apparently UCM707 as well) to plastic culture plates mandated the inclusion of a protein carrier for maintenance of these lipophilic molecules in solution. In this regard, bovine serum albumin (BSA) has been successfully used in other lipid transport studies [e.g., fatty acid transport (*21*, *22*)] to reduce the level of nonspecific interactions of lipids with surfaces, thereby facilitating the assessment of their interactions with specific binding sites. Notably, however, BSA has not been included in most of the previous investigations of AEA transport (*5*, *11*), with an exception being a recent study that measured AEA uptake in the presence of low levels of BSA (0.4%) (*15*). Therefore, we incubated AEA with increasing amounts of BSA in media

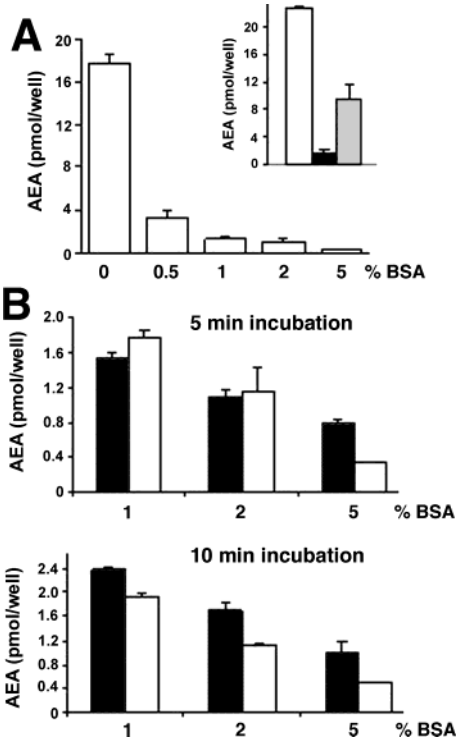

FIGURE 3: AEA uptake in the presence of bovine serum albumin (BSA). (A) Culture plates without cells were incubated with [^{3}H]-AEA (100 nM) at 37 °C for 4 min in the presence of the indicated concentrations of BSA. The inset shows the quantity of AEA bound to wells in the absence of BSA (white bar), in the presence of 1% BSA (black bar), or after blocking with 1% BSA followed by washing in BSA-free culture media (gray bar). (B) Culture plates with FAAH(+/+) neurons (black bars) or without cells (white bars) were incubated with [^{3}H]AEA (100 nM) at 37 °C for 5 or 10 min in the presence of the indicated concentrations of BSA. Results are expressed as means ± SEM of at least two independent experiments performed in duplicate.

on empty culture plates and identified a concentration of BSA (5%) at which the nonspecific binding of AEA to plates was largely eliminated (Figure 3A). Under these conditions (5% BSA, incubation for 5−10 min), uptake of AEA into FAAH-(+/+) cortical neurons was found to be significantly greater than the nonspecific uptake of this lipid onto plates with no cells (Figure 3B). In contrast, similar incubations carried out in the presence of 1% BSA failed to differentiate the cellular and noncellular components of AEA uptake (Figure 3B). Therefore, for all subsequent experiments, the uptake of AEA was measured in the presence of 5% BSA, where specific (cellular) uptake was determined by subtracting measurements of uptake in neuronal cultures from measurements of uptake in plates with no cells.

AEA Uptake in FAAH(+/+) and -(−/−) Neurons. We next examined the uptake of AEA into cultured preparations of cortical neurons from FAAH(+/+) and -(−/−) mice. Western blotting confirmed that FAAH was strongly expressed in FAAH(+/+) neurons, but absent from FAAH(−/−) neurons (Figure 4A). The time course of AEA uptake into FAAH(+/+) neurons showed that total AEA uptake remained greater than noncellular AEA uptake throughout the experiment (Figure 4B, left panel). Subtraction of noncellular AEA uptake from total uptake revealed that the specific (cellular) uptake of AEA exhibited saturation kinetics (Figure 4B, right panel). In an analogous set of experiments conducted with FAAH(−/−) neurons, differences between

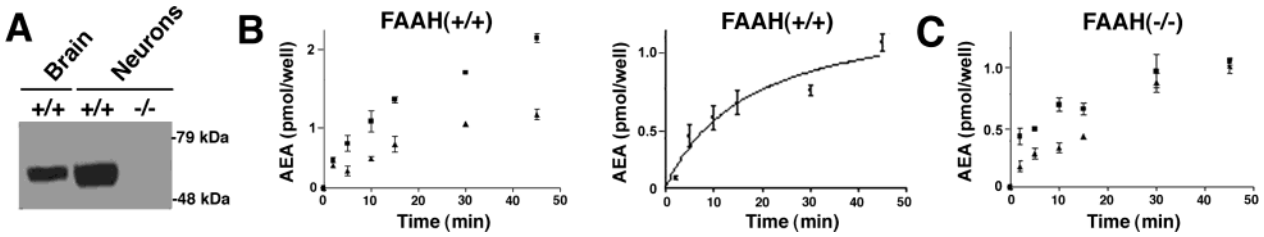

FIGURE 4: Time course of uptake of AEA in FAAH(+/+) and -(−/−) neurons. (A) Western blot analysis of FAAH expression in cortical neuronal cultures from FAAH(+/+) and -(−/−) mice. Brain homogenates from FAAH(+/+) mice were included as a positive control. FAAH immunoreactivity appears as a 63 kDa band, consistent with the predicted molecular mass of this protein (*8*). (B) Comparison of AEA uptake in FAAH(+/+) neurons (■) and plates without cells (▲) in the presence of 5% BSA (100 nM [^{3}H]AEA at 37 °C for 10 min) (left panel). Cell-specific uptake of AEA for FAAH(+/+) neurons after subtraction of noncellular uptake (right panel). (C) Comparison of AEA uptake in FAAH(−/−) neurons (■) and plates without cells (▲) in the presence of 5% BSA (100 nM [^{3}H]AEA at 37 °C for 10 min). Results are expressed as means ± SEM of at least two independent experiments performed in duplicate.

total and noncellular AEA uptake were detected over the first 20 min and were comparable, although lower in magnitude, to differences detected in FAAH(+/+) neurons (Figure 4C). However, at longer times, noncellular AEA uptake onto empty plates approached the same values as AEA uptake measured in the presence of FAAH(−/−) neurons, indicating that specific AEA uptake saturated at lower cellular concentrations of this lipid in FAAH(−/−) neurons than in FAAH(+/+) neurons. Consistent with this notion, FAAH(−/−) neurons accumulated less total AEA than FAAH(+/+) neurons at all time points that were examined (compare quantities of AEA in panels B and C of Figure 4). These data indicate that FAAH significantly contributes to AEA uptake, especially at extended incubation times. However, at earlier time points (e.g., 10 min), a specific component of AEA uptake could be measured in both FAAH(+/+) and -(−/−) neurons, and therefore, this FAAH-independent process was further investigated.

Inhibition of AEA Uptake by UCM707. Using a 10 min incubation, we assessed the inhibition of uptake of AEA into both FAAH(+/+) and FAAH(−/−) neurons in the presence of varying concentrations of the transport inhibitor UCM707 (Figure 1) (*18*). UCM707 was found to inhibit AEA uptake in both FAAH(+/+) and -(−/−) neurons (panels A and B of Figure 5, respectively) with comparable potencies [IC_{50}-(+/+) = 4 ± 1 μM; IC_{50}(−/−) = 3 ± 1 μM]. These data indicated that a UCM707-sensitive protein(s) distinct from FAAH contributed to AEA uptake. We next tested the role of the CB1 receptor, which is present at high concentrations in cortical neurons (5), in AEA uptake by assessing this process in the presence of the CB1 antagonist SR141716A (Figure 1). Notably, treatment of FAAH(+/+) and -(−/−) neurons with SR141716A (100 nM) reduced the uptake of AEA to a significant extent (∼20%) in both sets of cells (Figures 6B and 7B, respectively). Interestingly, however, in FAAH(−/−) neurons (Figure 7A,C) but not in FAAH-(+/+) neurons (Figure 6A,C), SR141716A was found to markedly alter the inhibition profile of UCM707, reducing both the potency and efficacy of this agent (IC_{50} values of 11 and 3 μM and maximal inhibition values of ∼50 and ∼80% in the presence and absence of SR141716A, respectively). These data suggest that both the FAAH- and CB1-dependent components of AEA uptake are sensitive to UCM707 and, once the contributions of these two proteins have been eliminated [e.g., in FAAH(−/−) neurons treated with SR141716A], the residual cellular uptake of AEA is

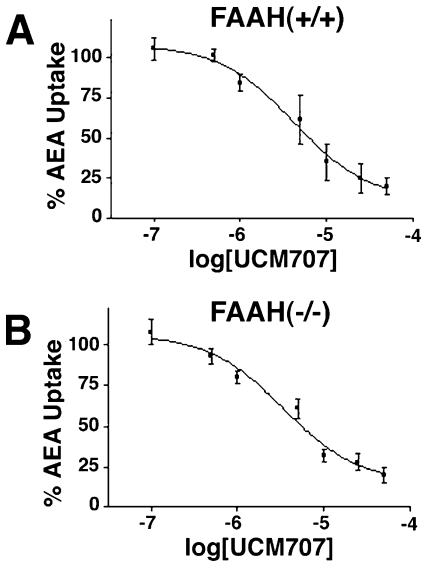

FIGURE 5: Inhibition of AEA accumulation by UCM707 in FAAH-(+/+) and -(−/−) neurons. FAAH(+/+) (A) and FAAH(−/−) (B) neurons were incubated with [^{3}H]AEA (100 nM) for 10 min in the presence of different concentrations of UCM707. Results represent specific AEA accumulation at 37 °C corrected for noncellular binding measured in parallel in plates without cells and are expressed as the percentage of control experiments carried out in the absence of UCM707, where 100% uptake corresponds to 0.85 and 0.55 pmol of AEA/well for FAAH(+/+) and -(−/−) neurons, respectively. Each data point corresponds to the mean ± SEM of at least two independent experiments performed in duplicate. The points were generated using GraphPad Prism, and the curves were obtained using the sigmoidal dose−response fit.

in large part mediated by simple diffusion (i.e., insensitive to inhibition by UCM707).

Relative Contribution of Known Proteins, Novel Proteins, and Diffusion to the Cellular Uptake of AEA. Collectively, the results described above indicate that the cellular uptake of AEA is a complex process that involves multiple proteins, as well as simple diffusion. The relative contributions made by each of these factors to AEA transport can be gleaned by directly comparing the absolute levels of AEA uptake into neurons under conditions in which specific proteins have been deleted or inhibited. As is shown in Figure 8, such a comparison reveals that AEA uptake is largely mediated by a combination of known proteins in the endocannabinoid sytem (FAAH and CB1 receptor) and simple diffusion, which together account for ∼70% of this process. Notably, however, the remaining component of AEA uptake is sensitive to

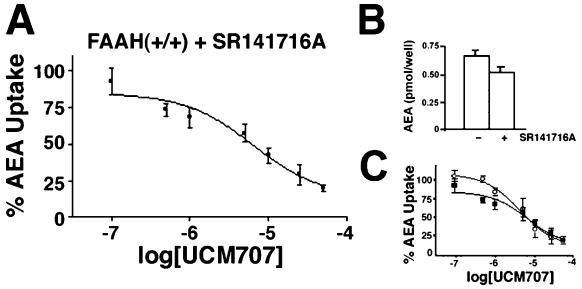

FIGURE 6: Inhibition of AEA uptake by UCM707 in FAAH(+/+) neurons in the presence of the CB1 antagonist SR141716A. (A) FAAH(+/+) neurons were preincubated for 10 min at 37 °C in the presence of 100 nM SR141716A and then incubated with AEA (100 nM) for 10 min in the presence of different concentrations of UCM707. Results represent specific AEA accumulation at 37 °C corrected for noncellular binding measured in parallel in plates without cells and are expressed as the percentage of control experiments carried out in the absence of UCM707, where 100% uptake corresponds to 0.52 pmol of AEA/well. Each data point corresponds to the mean ± SEM of at least two independent experiments performed in duplicate. The points were generated using GraphPad Prism, and the curves were obtained using the sigmoidal dose−response fit. (B) AEA uptake in FAAH(+/+) neurons in the absence or presence of 100 nM SR141716A. (C) Overlay of the inhibition of uptake of AEA by UCM707 in FAAH(+/+) neurons in the absence (○) or presence (■) of 100 nM SR141716A.

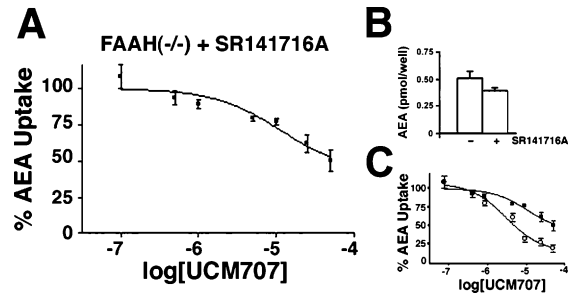

FIGURE 7: Inhibition of AEA uptake by UCM707 in FAAH(−/−) neurons in the presence of SR141716A. (A) FAAH(−/−) neurons were preincubated for 10 min at 37 °C in the presence of 100 nM SR141716A and then incubated with [^{3}H]AEA (100 nM) for 10 min in the presence of different concentrations of UCM707. Results represent specific AEA accumulation at 37 °C corrected for noncellular binding measured in parallel in plates without cells and are expressed as the percentage of control experiments carried out in the absence of UCM707, where 100% uptake corresponds to 0.40 pmol of AEA/well. Each data point corresponds to the mean ± SEM of at least two independent experiments performed in duplicate. The points were generated using GraphPad Prism, and the curves were obtained using the sigmoidal dose−response fit. (B) AEA uptake in FAAH(−/−) neurons in the absence or presence of 100 nM SR141716A. (C) Overlay of the inhibition of AEA uptake by UCM707 in FAAH(−/−) neurons in the absence (○) or presence (■) of 100 nM SR141716A.

UCM707, which suggests the existence of an additional UCM707-binding protein(s) (UCM707-BP) that also participates in the cellular transport of AEA.

DISCUSSION

Many experimental challenges must be addressed to define a role for potential lipid-binding and transporting proteins in the cellular uptake of hydrophobic molecules such as AEA. Indeed, even for well-studied lipid transport events, such as uptake of free fatty acid into cells, considerable debate still exists about whether proteins play a role in these processes

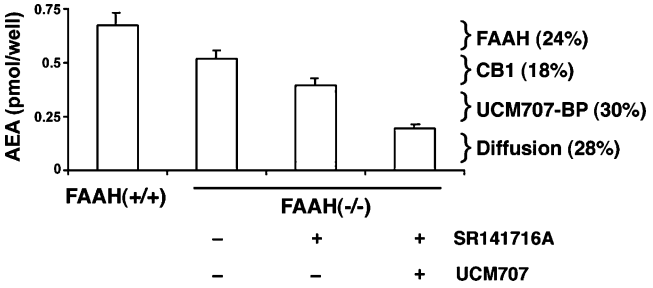

FIGURE 8: Relative contribution of individual proteins and diffusion to the cellular uptake of AEA. The absolute quantities of uptake of AEA into FAAH(+/+) neurons, FAAH(−/−) neurons, FAAH(−/−) neurons with SR141716A, and FAAH(−/−) neurons with SR141716A and UCM707 are compared. Values represent amounts of specific [^{3}H]AEA observed under each condition (total [^{3}H]AEA bound to plates with cells − [^{3}H]AEA bound to plates without cells). UCM707-BP is a putative UCM707-binding protein.

(*21*). Some complicating factors include (i) the nonspecific adsorption of lipids to plastic surfaces, such as culture plates, (ii) the protein-independent diffusion of lipids into and across cell membranes, and (iii) the presence of intracellular catabolic enzymes that create a driving force for the uptake of their lipid substrates. Our data indicate that, in the absence of a carrier protein, AEA binds culture plates lacking cells to an even greater extent than plates containing cells (Figure 2A). This adsorption is temperature-dependent (Figure 2A) and can be blocked by inhibitors of AEA uptake such as UCM707 in a dose-dependent manner (Figure 2B), demonstrating that traditional controls used to distinguish "specific" from "nonspecific" AEA transport (*5*, *11*) are not adequate in accounting for the noncellular component of uptake. Collectively, these results indicate that previous studies describing the neuronal uptake of AEA (*5*, *11*, *23*) may have been convoluted by the nonspecific binding of this lipid to culture plates, since this process exhibits many of the features (e.g., temperature dependence and pharmacological inhibition) that have been used to support the contention that AEA uptake is a protein-mediated process. We were able to significantly reduce the noncellular component of AEA uptake by including the lipid carrier protein BSA in the culture media, consistent with the broad use of this protein to improve the solubility and diffusion of lipids through aqueous media (*21*, *22*). We found that 5% BSA was sufficient for measuring a clear difference between the quantities of AEA taken up by plates containing and lacking neurons (Figure 3B).

After having established conditions under which the cellular uptake of AEA could be accurately measured, we compared this process in neurons from FAAH(+/+) and FAAH(−/−) mice to evaluate the contribution of FAAH to the AEA transport process. FAAH was found to make significant contributions to AEA transport, especially at later time points, where the cellular and noncellular uptake of AEA could not be distinguished in FAAH(−/−) neurons because lower absolute concentrations of AEA were taken up by these cells than by FAAH(+/+) neurons (Figure 4). These results are in agreement with previously reported studies that described an increased level of AEA transport in cells overexpressing FAAH and a reduced level of AEA transport in the presence of FAAH inhibitors (*13*−*15*). However, at early time points, we also observed a FAAH-independent component to AEA transport, indicating that the

initial cellular uptake of this lipid was not intrinsically linked to hydrolysis by FAAH. Consistent with this notion, synaptosomal preparations from FAAH(−/−) mice were recently shown to exhibit saturable (albeit greatly reduced) uptake of AEA (*24*).

Interestingly, this FAAH-independent component of AEA uptake was significantly reduced by the transport inhibitor UCM707 (*18*), suggesting that an additional protein(s) sensitive to this pharmacological agent participated in the transport of AEA. One protein that may contribute to the cellular uptake of AEA is its endogenous receptor, the CB1 receptor. Consistent with this notion, the CB1 antagonist SR141716A was found to reduce AEA uptake in both FAAH(+/+) and -(−/−) neurons (Figures 6B and 7B, respectively). Interestingly, however, while SR141716A had little effect on the inhibition of AEA uptake by UCM707 in FAAH(+/+) neurons (Figure 6C), this CB1 antagonist blocked a large portion of the UCM707-sensitive component of AEA transport into FAAH(−/−) neurons (Figure 7C). These data indicate that both FAAH and the CB1 receptor contribute to the cellular uptake of AEA and each of these components is sensitive to UCM707. UCM707's effect on the FAAH-specific portion of AEA uptake was initially somewhat surprising considering that this agent exhibits a relatively low potency for FAAH *in vitro* ($IC_{50} = 30\ \mu M$). However, these data may suggest that it is difficult to make direct comparisons between *in vitro* and *in situ* IC_{50} values, especially for lipophilic agents such as UCM707, which could accumulate to high concentrations in cells and, as a consequence, inhibit FAAH activity.

Once the FAAH- and CB1-dependent portions of AEA uptake, which combine to represent more than 40% of this process (Figure 8), are eliminated by, for example, treating FAAH(−/−) neurons with SR141716A, two distinct components remain that can be distinguished on the basis of their relative sensitivities to UCM707. Approximately 50% of the non-FAAH, non-CB1 portion of AEA uptake is UCM707-insensitive (Figure 8), suggesting that it likely represents the simple diffusion of AEA across cell membranes. However, the other half of this process is still inhibited by UCM707 (Figure 8), indicating that an additional UCM707-binding protein (UCM707-BP) distinct from FAAH and the CB1 receptor also contributes to AEA uptake. With regard to the potential identity of this unknown UCM707-BP, we hypothesize that it may represent a cytosolic lipid-binding protein that facilitates the transfer of AEA from CB1 receptors on the plasma membrane to intracellular membranes where FAAH is localized (*25*). In such a model, the protein-mediated uptake of AEA would constitute a concerted pathway from the site of action (CB1 receptor) to the site of degradation (FAAH). In general support of this idea, cytosolic lipid-binding proteins have been shown to play major roles in the intracellular transport of a variety of lipids between different membrane compartments, including fatty acids (*26*), cholesterol (*27*), and ceramide (*28*). Additionally, this model would also be consistent with recent findings showing that the cellular release of AEA is blocked by transport inhibitors (*24*, *29*), as an intracellular lipid-shuttling protein could promote the bidirectional movement of fatty acid amides into and out of neurons.

In summary, the studies described in this paper, which represent the first direct examination of the role that FAAH plays in regulating transport of AEA into neurons, provide compelling evidence that this enzyme makes significant contributions to AEA uptake. Our findings also suggest the involvement of additional proteins in this process, at least one of which is the CB1 receptor, while the other(s) remains to be identified. Overall, these results highlight the fact that the cellular uptake of AEA is a complex event mediated by several proteins, as well as by simple diffusion. Moreover, the net contribution made by any single protein (known or unknown) to the cellular uptake of AEA appears to be relatively modest, suggesting that the development of high-affinity antagonists of this multicomponent process may prove to be more challenging than originally anticipated.

ACKNOWLEDGMENT

We thank Prof. T. Bartfai, M. M. Bahrens, and J. Lucero for their help with neuronal cultures, Prof. M. Guzmán and G. Velasco for helpful discussions, and A. E. Speers and M. P. Patricelli for critical reading of the manuscript.

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BI049395F