A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide.*

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SUMMARY

The mechanisms responsible for the uptake and cellular processing of the endogenous cannabinoid anandamide are not well understood. We propose that anandamide uptake may occur via a caveolae/lipid raft-related endocytic process in RBL-2H3 cells. Inhibitors of caveolar-related (clathrin-independent) endocytosis reduced anandamide transport by approximately 50% as compared to control. Fluorescein derived from fluorescently labeled anandamide colocalized with protein markers of caveolae at early time points following transport. In the current study, we have also identified a yet unrecognized process involved with trafficking events affecting anandamide following its uptake. Following uptake of [3H]anandamide by RBL-2H3 cells, we found an accumulation of tritium in the caveolin-rich membranes. Both inhibitors of anandamide uptake and metabolism blocked the observed enrichment of tritium in the caveolin-rich membranes. Mass spectrometry of subcellular membrane fractions revealed that the tritium accumulation observed in the caveolin-rich membrane fraction was not representative of intact anandamide suggesting that following metabolism by the enzyme fatty acid amide hydrolase, anandamide metabolites are rapidly enriched in caveolae. Furthermore, Hela cells, which do not express high levels of fatty acid amide hydrolase, only showed an accumulation of tritium in the caveolin-rich membrane fraction when transfected with fatty acid amide hydrolase cDNA. Western blot analysis and immunocytochemistry from RBL-2H3 cells revealed that fatty acid amide hydrolase is localized in intracellular compartments distinct from caveolin-1 localization. Together, these data suggest that following uptake via caveolae/lipid raft-related endocytosis, anandamide is rapidly metabolized by fatty acid amide hydrolase with the metabolites efficiently recycled to caveolin-rich membrane domains.
INTRODUCTION

The endocannabinoid anandamide (AEA), a long-chain fatty acid amide, was first shown to be an agonist of the brain and peripheral cannabinoid receptors (CB1 and CB2 respectively) in the early 1990’s (1,2). AEA has received much attention in the last decade due to its ability to mimic the effects of the plant-derived cannabinoids such as Δ⁹-tetrahydrocannabinol, the major active component of marijuana (3,4). The mechanisms responsible for the biosynthesis and release of AEA as well as the termination of endocannabinoid signaling have not been fully defined (4). A better understanding of the process by which endocannabinoid signaling is regulated may identify novel drug targets for regulating the neuromodulatory actions of AEA and provide benefits in the treatment of pain, appetite loss, nausea, asthma, autoimmune disease, arthritis, fever, and glaucoma (4).

To terminate endocannabinoid signaling, AEA undergoes a carrier-mediated uptake process that is rapid, temperature-dependant, saturable at 37°C, inhibited by select fatty acid amide derivatives or cannabinoids in a concentration-dependant fashion, and independent of ion gradients or adenosine triphosphate (ATP hydrolysis) (5-7). The actual protein(s) involved with AEA uptake have not been completely identified with much debate regarding the existence of a putative AEA transporter (8,9). Even less is understood about the cellular processing and cellular fate of AEA following uptake. Fatty acid amide hydrolase (FAAH) has been shown to carry out the enzymatic hydrolysis of AEA (3,10,11). We and others have suggested that FAAH plays a critical role in maintaining the inward concentration gradient needed for uptake of AEA (12,13). However AEA uptake occurs in cells that do not express FAAH, indicating that mechanisms other than hydrolysis by FAAH are involved with AEA transport (6). In cells that do express FAAH, FAAH is localized to intracellular membrane compartments suggesting that
an efficient means of trafficking to the intracellular compartments containing FAAH exists for rapid metabolism of AEA to take place (8,10,14,15).

The concept of organized trafficking of lipids is not well defined, and the issues surrounding the concept remain somewhat controversial (16). Lipid rafts are specialized plasma membrane microdomains that are enriched in cholesterol, sphingolipids, arachidonic acid, and plasmenylethanolamine (17,18). Related domains, caveolae, are non-clathrin coated flask-shaped invaginations in the plasma membrane that have been identified by electron microscopy and other biochemical techniques (18,19). The lipid composition of caveolae is similar to that of lipid rafts (18-20). The existence of caveolae is well substantiated, and they have been implicated in serving many functions including the organization of key signaling proteins, cholesterol transport, endocytosis, and potocytosis (21).

The present studies from our laboratory have shown that the cellular accumulation of AEA is possibly linked to a caveolae-related process. Disruption of caveolae/lipid rafts by cholesterol depletion as well as treatment with agents known to inhibit caveolae-related endocytic processes were able to reduce [3H]AEA uptake in RBL-2H3 cells. Furthermore, fluorescence derived from a fluorescently labeled AEA analog was colocalized with markers for caveolae following uptake.

We next determined how the intracellular processing of AEA might take place following uptake by utilizing immunocytochemistry studies, biochemical analysis of subcellular fractions, and molecular approaches. Our data indicate that the metabolites of AEA accumulate in the caveolin-1 rich membrane fraction obtained from sucrose gradient centrifugation. This phenomenon was not observed in cells lacking detectable FAAH. Immunocytochemistry studies in the RBL-2H3 cells confirmed that FAAH is localized to intracellular membrane compartments.
We propose that AEA is rapidly trafficked to intracellular compartments where it is metabolized by FAAH, and that following hydrolysis, AEA’s metabolites undergo recycling to the plasma membrane where they are enriched in domains containing caveolin-1. These studies suggest a new model for AEA uptake that includes endocytic processes and furthermore, implicate specialized membrane microdomains in the uptake as well as possible recycling of endocannabinoids.
EXPERIMENTAL PROCEDURES

Cell culture. RBL-2H3 cells and Hela cells were maintained in Dulbecco’s Modified Eagle’s Medium with 10% fetal bovine serum supplemented with penicillin, streptomycin, and L-glutamine. Cells were grown in a humidified environment containing 5% CO₂ and held at the constant temperature of 37 °C.

[^3H]AEA uptake following endocytic inhibitor treatments. RBL-2H3 cells were plated at 2x10⁵ cells/well in a 24-well culture plate. Twenty-four hours later, cells were washed with Krebs-Ringers-Hepes (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 10 mM Hepes, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, pH 7.4) and incubated for two hours in OptiMEM (Gibco) containing genistein (200 µM) or for 30 minutes in KRH containing either nystatin (25 µg/mL)/progesterone (10 µg/mL), N-ethylmaleimide (NEM) (500 µM), or chlorpromazine (28 µM). Cells were also treated with potassium-free KRH for 30 minutes. Control cells were incubated for 30 minutes in 1X KRH. 100 µM AM404 was added to the indicated groups for the final 10 minutes of each incubation. Following the incubations, 1 nM[^3H]AEA was added to all samples and allowed to incubate for 5 minutes at 37 °C. Cells were then washed 3 times with 1X KRH and Microscint-20 was added to each well. The amount of[^3H] present was determined using a Packard TopCount Microplate Scintillation and Luminescence Counter.

Immunofluorescent localization of SKM4-45-1. The AEA analog SKM 4-45-1 was synthesized as described by Muthian et al. (22). 1.5 X 10⁵ RBL-2H3 cells were plated in a 6-well cell culture plate containing a cover slip that had been poly-D-lysine coated overnight. The next day the cells were treated with 100 nM AEA analog SKM 4-45-1 for 30 seconds at 37 °C.
and then immediately fixed for 30 minutes at room temperature using ice cold 4% paraformaldehyde in phosphate buffered saline (PBS). Cells were washed 4 times in PBS. Primary antibodies directed against caveolin-1 (RDI), flotillin-1 (Transduction Laboratories), and transferrin receptor (TFR) (PharMingen) were prepared in 0.3% Triton-X 100 and 0.5% BSA/PBS at dilutions of 1:2000 and added to the appropriate wells. Cells were incubated for 24 hours at 4 °C. The cells were then washed three times with PBS, and the secondary antibodies were added. The Alexa Fluor 568 conjugated goat anti-mouse antibody (Molecular Probes) was added 1:1500. After incubating 1 hour, cells were washed six times with PBS. Cover slips were removed from the wells and mounted to slides using ProLong Antifade and allowed to dry overnight. Cells were imaged using oil immersion confocal microscopy at 60X magnification with a Nikon Diaphot 300 microscope (Tokyo, Japan). The confocal system used was a BioRad MRC1024 (Hemel Hempstead, England) with Krypton (488) Argon (568) laser, 522-35 nm band pass filter and a 588 nm long pass filter. Images were produced as an accumulation of 3 scans.

**Subcellular fractionation of [³H]AEA treated cells.** Cells were grown to be approximately 85% confluent in 150 mm culture plates and washed once with 1X KRH. Cells were then incubated for 10 minutes at 37 °C in either 100 µM AM404, 500 nM methylarachidonylfluorophosphonate (MAFP), or 1X KRH. [³H]AEA was then added to each culture dish to yield a final concentration of 1 nM, and culture plates were incubated for 5 minutes at 37 °C. Culture plates were washed three times with ice-cold KRH.

On ice, treated cells were washed in cold KRH and exposed to 2.5 ml ice-cold lysis buffer (0.5% Triton X-100, 50 mM Tris-HCL, 150 mM NaCl, 5 mM EDTA) for 20 minutes. On ice, cell lysates were then mixed with 2.5 ml 80% sucrose buffer, and overlaid with 4 ml 30%
sucrose buffer followed by 2 ml 5% sucrose buffer in a Beckman centrifuge tube (model # 344059) (80%, 30%, and 5% sucrose solutions were made in lysis buffer minus detergent). The sucrose gradients were centrifuged at 200,000 × g for 18 hours at 4 °C. 1 ml fractions were collected, with the detergent-insoluble caveolin-rich component appearing as a milky-white band in approximately the second to third fraction from the top of the gradient. Eleven fractions total were collected from the gradient. To determine the tritium content of fractions, 50 µl of each fraction was added to individual wells of a Packard 24-well, flat bottom, white polystyrene tissue culture microplate. Microscint-20 was added to each well, and tritium determined using a Packard TopCount Microplate Scintillation and Luminescence Counter. Protein content of individual fractions was determined using the Bradford protein assay reagent (Bio-Rad) and used to normalize fractions for protein levels. In subcellular fractionation experiments performed on Hela cells, cells were transiently transfected in 150 mm culture plates with either rat FAAH cDNA/pBluescript II SK- (generous gift from Dr. Benjamin Cravatt, Scripps Research Institute) or vector only using the vaccinia virus T7 expression system (23,24).

**Western blot analysis.** Fractions were prepared for gel electrophoresis by adding a 1:1 volume of Laemmli buffer (5% bromophenol blue, 5% β-mercaptoethanol, 62.5 mM Tris-HCL, 20% glycerol, and 2% SDS). Samples were electrophoresed for 50 minutes at 145 V using the Mini-Protean 3 system (Bio-Rad) after being loaded onto a 10% SDS-polyacrylamide gel electrophoresis Tris-HCL gel. Only fractions one through nine were analyzed as previous Western blot analysis revealed that subcellular fractions ten and eleven taken from RBL-2H3 cells contained no significant levels of the proteins of interest (data not shown). Proteins were then transferred to a polyvinylidene difluoride membrane using the Bio-Rad Mini Trans-Blot...
system. The membrane was blocked for 24 hours in phosphate-buffered saline containing 0.1% Tween-20 and 5% dry milk at 4 °C. The presence of FAAH, caveolin-1, flotillin-1, transferrin receptor (TFR), and Bip/GRP78 was detected using α-FAAH rabbit polyclonal 1° antibody (epitope: VGYETDNYTMPSPAMR), α-caveolin-1 rabbit polyclonal 1° antibody (Santa Cruz), α-flotillin-1 mouse monoclonal 1° antibody (BD Transduction Laboratories), α-TFR mouse monoclonal 1° antibody (PharMingen), α-BiP/GRP78 mouse monoclonal 1° antibody (BD Transduction Laboratories) respectively, followed by either horseradish peroxidase-labeled goat-anti-rabbit 2° antibody (Bio-Rad) or horseradish peroxidase-labeled goat anti-mouse 2° antibody and enhanced chemiluminescence (ECL) detection reagents. Membranes were then exposed to X-ray film.

Immunofluorescent localization of FAAH. RBL-2H3 cells were plated in a 6-well cell culture plate containing a cover slip that had been poly-D-lysine coated overnight. The next day the cells were washed two times in phosphate buffered saline (PBS) and then fixed for 30 minutes at room temperature using 4% paraformaldehyde. Cells were washed 4 times in PBS. Primary antibodies directed against FAAH and Caveolin-1 were prepared in 0.3% Triton-X 100/PBS at dilutions of 1:3000 and 1:2000 respectively and added to the appropriate wells. Cells were incubated for 48 hours at 4° C. The cells were then washed six times with PBS, and the secondary antibodies were added. The Alexa Flour 488 conjugated donkey anti-rabbit IgG antibody (Molecular Probes) was added 1:4000, and the Alexa Flour 568 conjugated goat anti-mouse antibody (Molecular Probes) was added 1:3000. After incubating 1 hour, cells were washed six times with PBS. Cover slips were removed from the wells and mounted to slides using ProLong Antifade and allowed to dry overnight. Cells were imaged using oil immersion
confocal microscopy at 60X magnification with a Nikon Diaphot 300 microscope (Tokyo, Japan). The confocal system used was a BioRad MRC1024 (Hemel Hempstead, England) with Krypton (488) Argon (568) laser, 522-35 band pass filter and a 588 long pass filter.

**Determination of endogenous cannabinoids using LC/MS/MS.** A quantitative bioanalytical method utilizing liquid chromatography (LC) tandem mass spectrometry (MS/MS) was used to measure AEA in membrane fractions (25). Following liquid extraction with organic solvent and further purification by solid phase extraction, samples were separated using a Phenomenex Prodigy ODS-3 column with acetonitrile:water (50:50) as the mobile phase at a flow rate of 0.2 mL/minute. Quantification of AEA was accomplished by using $m/z$ 348 ([M+H]$^+$) as a precursor ion and $m/z$ 62 as a product ion in a selected reaction monitoring mode using [2H8]anandamide as an internal standard. The lower limit of quantification for AEA was 25 pg/mL.
RESULTS

Effect of Endocytic Inhibitors on AEA Uptake- Pretreatment of cells with nystatin and progesterone disrupts synthesis of cholesterol and cholesterol transport to the membrane thereby disrupting caveolae (26). To assess the effects of cholesterol depletion and the disruption of caveolae on AEA uptake, RBL-2H3 cells were pretreated with nystatin and progesterone, and [3H]AEA uptake assays were performed (Fig. 1A). Nystatin and progesterone pretreatment reduced the specific uptake of AEA in RBL-2H3 cells by approximately 50%. Genistein and N-ethylmaleimide (NEM) are both known to inhibit caveolae-related endocytosis (16,27,28). We pretreated RBL-2H3 cells with genistein or NEM and then, as with the nystatin and progesterone treatment, assessed [3H]AEA transport. Both genistein and NEM were found to reduce AEA uptake similar to the nystatin/progesterone treatment (Fig. 1A). Previous studies have suggested that a 20 ºC temperature block may interfere with caveolae-related trafficking from the plasma membrane to intracellular compartments (29,30). When uptake assays were performed at 18 ºC, the specific uptake of AEA was again reduced by approximately 50 % (Fig. 1A). Treatment of cells with either chlorpromazine or potassium-free buffer, which are known to inhibit clathrin-dependent endocytosis, had no effect on the uptake of AEA by RBL-2H3 cells (Fig. 1B).

Localization of the fluorescent AEA derivative SKM4-45-1- The fluorescent AEA derivative SKM4-45-1 has been shown to undergo carrier mediated uptake presumably via the same process that transports AEA. Once taken up into cells, SKM4-45-1 undergoes rapid cleavage by nonspecific esterases to release fluorescein (22). RBL-2H3 cells were treated with SKM4-45-1 and uptake was allowed to take place for a period of 30 seconds. This early timepoint was chosen to provide an indication for the initial entry point of AEA into cells. After fixing SKM4-45-1 treated cells, flotillin-1, caveolin-1, or TFR were immunofluorescently...
labeled and the cells were analyzed by confocal microscopy. Fluorescein derived from SKM4-45-1 appeared to be colocalized with flotillin-1 (Fig. 2A) and caveolin-1 (Fig. 2B), markers for caveolae, when cells were fixed 30 seconds after treatment with SKM4-45-1. Fluorescein did not colocalize with the transferrin receptor (TFR), which is a marker for clathrin-coated pits (Fig. 2C). These data directly implicate that the site of SKM4-45-1, and thus AEA entry into the cell may be at caveolae/lipid raft domains and support the hypothesis that AEA uptake occurs via a caveolae-related endocytic process.

**Accumulation of Tritium in the Caveolin-rich Membrane Fraction of RBL-2H3 Cells Following the Uptake of AEA** Subcellular fractionation of RBL-2H3 cells was performed by sucrose gradient centrifugation (18). A milky-white band representing the detergent-insoluble caveolin-rich membrane fraction typically appeared in the second fraction taken from the top of the gradient. Western blot analysis of individual fractions confirmed an enrichment of caveolin-1 in fraction 2 (Fig. 3) suggesting that we had successfully isolated caveolin-rich membranes. Large amounts of the protein flotillin-1 are also found in caveolae/lipid rafts (31). To further verify the isolation of caveolae-related membranes, we probed for flotillin-1 and were able to observe the expected enrichment in fraction 2 (Fig. 3). We also verified that both the endoplasmic reticulum marker BiP/GRP78 and the transferrin receptor (TFR) were excluded from the caveolin-1 rich membrane fraction. (Fig. 3).

Based upon the endocytic inhibitor data above, we speculated that AEA may accumulate in cells via a caveolae-related process. Thus, we determined whether AEA or its metabolites could be isolated in the caveolin-1 rich cellular fractions after uptake. Subcellular fractionation of RBL-2H3 cells was performed by sucrose gradient centrifugation following the uptake of $[^3\text{H}]$AEA (AEA was labeled on the arachidonic acid backbone of the molecule). We quantified
the amount of tritium present in each fraction collected from the gradient and normalized for the protein concentration of each fraction. Tritium profiles revealed a 20-fold enrichment of tritium in the caveolin-rich membrane fraction as compared to AM404 treated cells (Fig. 4A). The anandamide uptake inhibitor AM404 and the FAAH inhibitor methylarachidonylfluorophosphonate (MAFP) were both found to inhibit the enrichment of tritium in the caveolin-rich membrane fraction. RBL-2H3 cells pretreated with [3H]AEA labeled on the ethanolamine portion of the molecule also displayed a similar tritium profile to that observed in Fig. 4A (data not shown).

RBL-2H3 cells are known to natively express the serotonin transporter and exhibit robust serotonin uptake (32). Following uptake, in RBL-2H3 cells, serotonin should be sequestered in secretory granules and would not be expected to appear in caveolin-containing microdomains. As a control experiment, we performed fractionation experiments on RBL-2H3 cells pretreated with [3H]serotonin to exclude the possibility that the observed tritium accumulation in the caveolin-rich membrane fraction was a non-specific general phenomenon. We observed no accumulation of serotonin-associated tritium in the caveolin-1 rich membrane fraction when RBL-2H3 cells were treated with [3H]serotonin (Fig. 4B). Cells treated with [3H]serotonin demonstrated an accumulation of tritium that appeared in more dense fractions from the gradient.

To determine if the tritium accumulation observed in the caveolin-1 rich membrane fraction represented intact AEA, we performed mass spectrometry on samples collected from the fractionation of RBL-2H3 cells that had undergone uptake of AEA at 37 °C. RBL-2H3 cells were treated with unlabeled AEA at a concentration equal to [3H]AEA used in Fig. 3 above. Membrane fractions were isolated and the AEA content in each fraction was determined using liquid chromatography tandem mass spectrometry (LC/MS/MS) (Fig. 5). To verify successful
isolation of caveolin-rich membranes, these gradient fractions were also analyzed by Western blot analysis in parallel experiments (data not shown). In the non-AM404 treated cells (representing total uptake), we observed only small amounts of intact AEA in the caveolin-rich membranes and the other fractions (Fig. 5). These data indicate that the tritium observed in the caveolin-rich membranes (Fig. 4A (-)AM404) was most likely arachidonate derived from AEA metabolism that has been targeted to the caveolae, and not intact AEA. Those fractions isolated from cells treated with AM404, which inhibits both specific uptake and metabolism by FAAH, contained intact AEA at levels that paralleled the observed tritium profile prior to normalization (Fig. 5). Since the tritium profiles in membranes from cells treated with [3H]AEA labeled on the ethanolamine moiety displayed a similar profile to that observed for the arachidonate-labeled AEA (data not shown), both arachidonate and ethanolamine may be enriched in caveolin-rich membranes following the metabolism of AEA.

 Trafficking from intracellular compartments to the plasma membrane is inhibited at 18 °C (32). Cellular trafficking of the metabolites of AEA to the caveolae/lipid raft domains of the plasma membrane following hydrolysis by FAAH should be inhibited if uptake (prior to fractionation) is performed at 18 °C. Although specific uptake was reduced at 18 °C (Fig. 1), approximately 50% of uptake does remain. RBL-2H3 cells were treated with [3H]AEA at 18°C and then fractionated by sucrose gradient centrifugation. There was no significant difference in the enrichment of tritium found in the caveolin-1 rich membrane fraction of control and AM404-treated cells from 18°C uptake experiments (Fig. 6). This result suggests that cellular trafficking machinery involved with export to the plasma membrane is involved in the enrichment of AEA metabolites in caveolin-rich domains.
Requirement of FAAH for Accumulation of Tritium in Caveolin-rich Membrane Fractions- Caveolin-rich membrane fractions obtained from RBL-2H3 cells following the uptake of $[^3H]$AEA showed no accumulation of tritium in the presence of the FAAH inhibitor MAFP (Fig. 4A). We predicted that FAAH activity is necessary for the enrichment of AEA metabolites in the caveolin-1 rich membrane fractions. To test this hypothesis, Hela cells, which lack detectable FAAH activity (13), were transiently transfected with either FAAH cDNA/pBluescript II SK$^-$ or vector using the vaccinia virus T7 expression system. An enrichment of tritium in the caveolin-1 rich membrane fraction following exposure to $[^3H]$AEA was not observed in Hela cells transiently transfected with vector only. However, Hela cells transfected with the FAAH cDNA revealed an accumulation of tritium in the caveolin-1 rich membrane fraction similar RBL-2H3 cells (Fig. 7). This finding suggests that AEA metabolism must occur for the enrichment of tritium in caveolin-1 rich membrane fractions. Interestingly, both FAAH- and vector-transfected Hela cells revealed a peak of tritium in fraction 5. This peak was greater in the FAAH-transfected cells most likely because more AEA was accumulated in the cells due to the expression of FAAH. Since this peak is observed in vector-transfected cells, we believe this peak represents intact $[^3H]$AEA, and this fraction may contain remnants of an intermediate compartment involved with AEA transport and trafficking.

Exclusion of FAAH from Caveolin-rich Membrane Fractions- Previous studies have shown FAAH to be located in intracellular membrane compartments (8,10). Taking this into consideration, we next determined whether FAAH was present in caveolae, or caveolin-1 rich membrane fractions, thereby indicating whether metabolism of AEA was occurring in the caveolin-rich membrane domains. Western blot analysis of FAAH confirmed that FAAH was not localized to the caveolin-1 rich membrane fraction of RBL-2H3 cells in which an enrichment
of AEA metabolites was observed (Fig. 3). Bands representative of FAAH were observed in fractions 7 through 9. No band representing FAAH was detectable in fraction number 2 (Fig. 3).

To further investigate the localization of FAAH, we performed immunocytochemistry studies. We labeled both FAAH and caveolin-1 in RBL-2H3 cells, and then imaged the cells using confocal microscopy. The overlay of FAAH-labeled (green) cells and caveolin-1-labeled (red) cells revealed that FAAH did not appear to be colocalized with caveolin-1 (Fig. 8), and therefore, we conclude that FAAH is most likely excluded from caveolae and that AEA metabolism occurs in non-caveolin containing intracellular compartments.
DISCUSSION

The accumulation of AEA in cells has been shown to be temperature-dependant, saturable at 37°C, and inhibited by select fatty acid amide derivatives (5-7). The presence of an AEA transporter has been proposed based on these characteristics. However, there has been no success in attempts to identify the putative AEA transporter. It has also been proposed that AEA uptake is the result of simple diffusion, and that the necessary concentration gradient for diffusion is driven by the temperature-dependent hydrolysis of AEA by FAAH (8). Even if AEA enters the plasma membrane via diffusion, being a hydrophobic molecule it should not readily enter the hydrophilic cytoplasm of the cell. The presence of potential binding proteins and the potential for AEA to associate with various intracellular membranes further complicate simple diffusion of AEA. We suggest that in order for AEA to be rapidly metabolized by FAAH, intracellular AEA would need to be efficiently trafficked to the intracellular domains containing FAAH.

There is emerging evidence for the tight control of lipid trafficking to intracellular compartments via endocytosis (16,34). Endocytic processes meet the criteria for the cellular uptake of AEA that have been used to imply the presence of an actual transporter protein. There are two endocytic pathways that have been identified: clathrin-dependent and clathrin-independent (associated with caveolae and lipid rafts) (35). Both types of endocytosis follow similar cellular pathways. Internalized vesicles are sorted to the early endosomes. Vesicles can then be sent to recycling endosomes for trafficking back to the plasma membrane, late endosomes or lysosomes for degradation, or to the Golgi network and endoplasmic reticulum for further processing. The notion that AEA uptake occurs via an endocytic process does not exclude the possibility that there is an AEA carrier protein. For example, transferrin is taken up
by the TFR which is a marker for clathrin-dependent endocytosis (35). Proteins such as albumin, fatty acid binding protein, and caveolin-1 have been implicated to serve as shuttles in the intracellular trafficking of long-chain fatty acids to various subcellular organelles (36). Interestingly, caveolin-1, the marker for caveolae, has been shown to display properties of a fatty acid binding protein (36).

We explored the possibility that the uptake of AEA may take place via a caveolae-related endocytic process. Treatment of cells with nystatin and progesterone inhibits cholesterol synthesis and transport to the membrane thereby disrupting caveolae/lipid rafts (26). Our data show that nystatin and progesterone pretreatment of RBL-2H3 cells reduced the specific uptake of AEA by approximately 50%, suggesting that a caveolae/lipid raft-related process may be involved. However, cholesterol depletion could possibly interfere with clathrin-dependent endocytic processes as well (26). We were able to exclude the possibility that clathrin-dependent processes play a role in AEA uptake by treatment of RBL-2H3 cells with either chlorpromazine or potassium-free buffer, treatments that inhibit clathrin-dependent endocytic processes. Neither treatment had any effect on AEA uptake. The tyrosine kinase inhibitor genistein and NEM have both been shown to inhibit caveolae-related endocytic processes (16, 27, 28). Our results show that pretreatment of RBL-2H3 cells with these compounds reduced AEA transport. Each of these drug treatments may have multiple non-specific effects on our cells. However, we have shown similar results with multiple inhibitors that each inhibit endocytosis by different mechanisms. Taken together, our data implicate a caveolae/lipid raft-related endocytic process as being involved with AEA uptake in RBL-2H3 cells. The observation that fluorescein derived from the AEA derivative SKM4-45-1 colocalizes with markers for caveolae at early time points following uptake further supports this hypothesis.
The peak of tritium that was observed in the caveolin-1 rich membrane fraction following the uptake of \( [^{3}\text{H}]\text{AEA} \) was not representative of intact AEA. This fact was confirmed by mass spectrometry. Note that the subcellular fractions taken from cells treated with the AEA uptake inhibitor AM404 prior to AEA treatment showed significantly higher levels of intact AEA than those fractions collected from control (total uptake) cells. This result was not surprising because the 100 \( \mu \text{M} \) AM404 used in these experiments would also act as an inhibitor of FAAH preventing the metabolism of any AEA that is accumulated in the cell. AM404 could also cause an increase in the levels of intact AEA observed in the caveolin-rich membrane fraction by preventing the internalization of AEA that has partitioned into the caveolae or lipid raft domains. The RBL-2H3 fractionation experiments presented in this study would require FAAH or other enzymatic activity to observe the enrichment of tritium (representing AEA metabolites) in the caveolin-rich membrane fraction. The necessity of FAAH activity for this phenomenon was confirmed by our studies in which we transiently transfected Hela cells, which express no detectable levels of FAAH, with FAAH cDNA. Without FAAH, AEA metabolites should not be produced, and thus, we would not expect to see an enrichment of tritium in the caveolin-rich membrane fraction. Our data confirm this hypothesis as only those Hela cells transfected with FAAH displayed a tritium profile similar to that of RBL-2H3 cells with an accumulation of tritium found in the caveolin-rich fraction.

Our results support the notion that following metabolism, the AEA metabolites are rapidly trafficked to caveolin-containing membrane microdomains. Prior to the enrichment in caveolin-rich membrane, the AEA metabolites (arachidonic acid and ethanolamine) are most likely incorporated into phospholipids or re-esterified. It would be advantageous for the cell to enrich AEA metabolites in a domain such as caveolae because they may also serve as precursors
for the synthesis of new AEA. The widely accepted biosynthetic mechanism for AEA centers around the synthesis and release from a membrane precursor N-arachidonylphosphatidylethanolamine (NAPE) (37). NAPE is formed by the transfer of arachidonate from the sn-1 position of a phospholipid to the primary amine of phosphatidylethanolamine (37). This process is catalyzed by a Ca\textsuperscript{2+}-dependent N-acyltransferase (5,37,38). The formation and release of AEA occurs via a phosphodiesterase-mediated cleavage of NAPE (5,37). The enzymatic cleavage of NAPE takes place in a Ca\textsuperscript{2+}-dependent manner and appears to display properties similar to the activity of phospholipase D (PLD) (37). A novel PLD was isolated from rat heart and brain by Petersen and Hansen (1999) and it was suggested that this enzyme might be specific for the production of N-acylethanolamides (39). Okamoto et al. (2004) recently reported the molecular characterization of a PLD isolated from rat heart that is specific for the production of AEA and other N-acylethanolamides (40). Interestingly, PLD activity has been shown to be enriched in caveolin-rich membranes (41). The biosynthesis of the hydrophobic amide signaling molecule ceramide appears to be compartmentalized to caveolae establishing a precedence for the localized production of lipid signaling molecules in caveolae (42). Could the metabolites of AEA be available for incorporation into newly synthesized AEA? Certainly, the recycling of re-esterified arachidonic acid could have functions other than to form AEA. This arachidonic acid could be involved with receptor-stimulated arachidonic acid release or the conversion of arachidonic acid by phospholipase A2 into other eicosanoid signaling molecules such as the prostaglandins. Interestingly, analysis of lipid raft composition revealed that arachidonic acid was found mainly in arachidonic acid-containing plasmenylethanolamines that are the major source of released arachidonic acid (18). Under basal conditions, low levels of NAPE were observed in lipid rafts,
but this finding would be expected as NAPE has been reported to be formed only in response to elevation of intracellular Ca\(^{2+}\), and thus, would be found at very low levels under basal conditions (18,37,43). We propose that caveolae could serve the function of regulating the membrane organization of both phospholipid precursors of AEA as well as restricting the spatial distribution of the enzymes involved with synthesis and release.

Having established that AEA’s metabolites are enriched in caveolae following hydrolysis by FAAH, it was next necessary to address the question of where AEA metabolism occurs. The presence of AEA metabolites in caveolin-rich membranes could suggest that FAAH may be localized to caveolae. However, previous studies have reported FAAH to be found in intracellular membranes and not the plasma membrane making it unlikely that FAAH would be present in caveolae (8,15). Both Western blot and immunocytochemistry in the RBL-2H3 cells confirmed that FAAH localization did not correspond with caveolin-1. Thus, following uptake, AEA would be targeted to the intracellular domains containing FAAH. This process remains unclear; however, our data could be interpreted as implicating a caveolae-related trafficking process. There is also the possibility that FAAH is trafficked to the domains containing AEA, but we have observed no evidence of AEA-stimulated FAAH redistribution (data not shown). The findings of this study suggest that the uptake of AEA takes place via a caveolae-related endocytic process. Although caveolin-1 is not found in neurons, caveolin-1 is found in brain tissue (possibly glia) (44). Detergent-insoluble membranes lacking caveolin-1 are found in brain tissue (44). These membranes, characterized as lipid rafts, are capable of endocytosis in the absence of caveolin-1, and therefore, could be involved in endocannabinoid uptake and signaling in the brain (45).
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FOOTNOTES

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1The abbreviations used are: AEA, anandamide; ATP, adenosine triphosphate; FAAH, fatty acid amide hydrolase; KRH, Krebs-Ringers-Hepes; NEM, N-ethylmaleimide; MAFP, methylarachidonylfluorophosphonate; TFR, transferrin receptor; ECL, enhanced chemiluminescence; PBS, phosphate buffered saline; NAPE, N-arachidonylphosphatidylethanolamine; PLD, phospholipase D.
FIGURE LEGENDS

Fig. 1. Effect of endocytic inhibitors on AEA uptake. RBL-2H3 cells were pre-treated for 30 min with inhibitors of (A) caveolae-related (clathrin-independent) endocytosis: 25 µg/ml nystatin and 10 µg/ml progesterone; genistein 200 µM; N-ethylmaleimide 500 µM; or 18°C temperature block or (B) inhibitors of clathrin-dependent endocytosis: potassium-free buffer or chlorpromazine 28 µM. Assays were performed in 1X KRH at 37 °C in the presence or absence of AM404 100 µM to define non-specific transport. Transport was determined for 5 min with 1 nM [³H]AEA as described in experimental procedures. Statistical analysis was performed using one-way ANOVA followed by Dunnett’s multiple comparison test. *p < 0.001. Data represent mean ± SEM of 3 separate experiments.

Fig. 2. Localization of the fluorescent AEA derivative SKM4-45-1. Confocal microscopy analysis of RBL-2H3 cells following 30 second uptake of the AEA derivative SKM4-45-1 suggests that AEA may be colocalized with markers for caveolae in the initial phase of uptake. SKM4-45-1 treated cells were co-labeled with various cellular markers and accumulation images were collected as described in Experimental Procedures. Fluorescein (green) derived from SKM4-45-1 colocalized with flotillin-1 (red) (A) and caveolin-1 (red) (B). Fluorescein derived from SKM4-45-1 did not appear to colocalize with transferrin receptor (red) (C). Data are representative of 3 separate experiments.

Fig. 3. Isolation of caveolin-rich membranes from RBL-2H3 cells. Western blots were performed on fractions obtained from sucrose gradient centrifugation as described in
Experimental Procedures. Bands representing both caveolin-1 and flotillin-1 were enriched in fraction number 2. BiP/GRP78 (a marker for ER), the transferrin receptor (TFR), and FAAH were all excluded from the caveolin-1 rich membrane fraction. Data are representative of 5 separate experiments for caveolin-1 and FAAH and 2 separate experiments for flotillin-1, BiP/GRP78, and TFR.

Fig. 4. Enrichment of AEA-derived tritium in the caveolin-rich membranes of RBL-2H3 cells. (A) RBL-2H3 cells treated with [3H]AEA and subjected to sucrose gradient fractionation showed a marked increase in tritium in the caveolin-1 rich membrane fraction (fraction 2) (▲). The results of [3H]AEA treatment in the presence of 100 µM AM404 (■) or 250 nM MAFP (▼) followed by fractionation are also shown. The level of accumulated tritium was normalized to protein concentration for each fraction as described in Experimental Procedures. Data are representative of 4 separate experiments. (B) RBL-2H3 cells treated with [3H]serotonin (20 nM for 10 minutes at 37 °C) either in the presence (■) or absence (▲) of 10 µM fluoxetine, a serotonin transport inhibitor, and subjected to sucrose gradient fractionation showed no accumulation of tritium in the caveolin-1 rich membrane fraction. Data are representative of 3 separate experiments.

Fig. 5. Quantitation of AEA in fractions from sucrose gradient centrifugation by LC/MS/MS. AEA levels in membrane fractions of RBL-2H3 cells that had undergone AEA uptake for 5 minutes with and without AM404 100 µM were determined by LC/MS/MS as described in Experimental Procedures. The levels of intact AEA for each fraction are shown as pmol/fraction. Data represent mean ± SD of 2 separate experiments.
Fig. 6. Tritium in caveolin-rich membrane fractions after AEA uptake at 18°C. RBL-2H3 cells treated with [³H]AEA and subjected to sucrose gradient fractionation showed no significant increase in tritium in the caveolin-1 rich membrane fraction (fraction 2) either in the presence or absence of 100 µM AM404. Data represent mean ± SEM of 3 separate experiments.

Fig. 7. Determination of anandamide-derived tritium in subcellular fractions from FAAH-transfected Hela cells. Hela cells were transiently transfected with either FAAH cDNA/pBluescript II SK- (■) or pBluescript II SK- only (▲) and treated with [³H]AEA, followed by sucrose gradient fractionation as described in Experimental Procedures. The tritium levels of each fraction were normalized to protein concentration. Data are representative of 3 separate experiments. Inset: Western blot analysis of sucrose gradient fractions 1 through 9 obtained from Hela cells. Caveolin-1 is labeled with rabbit anti-caveolin-1 (Santa Cruz) followed by goat anti-rabbit IgG conjugated to HRP (Bio Rad). Membranes were imaged using ECL detection reagents (Amersham).

Fig. 8. Immunocytochemical localization of FAAH and caveolin-1. Confocal microscopy analysis revealed that FAAH was excluded from the caveolin-1 containing compartments of RBL-2H3 cells. (A) FAAH labeling was achieved with rabbit anti-FAAH followed by donkey anti-rabbit IgG conjugated to (Green) Alexa Flour 488 (Molecular Probes). (B) Caveolin-1 was labeled with mouse anti-caveolin-1 (RDI) followed by goat anti-Mouse IgG conjugated to (Red) Alexa Flour 568 (Molecular Probes). (C) Overlay of FAAH and caveolin-1 showing distinct localization of the two proteins. (D) Bright field image of cells.
Figure 1.

A

% of Control Uptake

Nystatin/Prog | Genistein | NEM | 18 °C

B

% of Control Uptake

K⁺-free | CPZ
Figure 2.

A

SKM flotillin 1 SKM & flotillin 1

B

SKM caveolin 1 SKM & caveolin 1

C

SKM TFR SKM & TFR
Figure 3.

| Fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|----------|---|---|---|---|---|---|---|---|---|
| Caveolin-1 | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] |
| Flotillin-1 | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] |
| TFR | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] |
| BiP/GRP78 | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] |
| FAAH | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] | [Image] |
Figure 5.
Figure 8.
A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide
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