RNA Interference-Mediated Knockdown of Dynamin 2 Reduces Endocannabinoid Uptake into Neuronal dCAD Cells


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ABSTRACT

The precise mechanism by which the cellular uptake of the endocannabinoid anandamide (AEA) occurs has been the source of much debate. In the current study, we show that neuronal differentiated CAD (dCAD) cells accumulate anandamide by a process that is inhibited in a dose-dependent manner by N-(4-hydroxyphenyl)arachidonamide (AM404). We also show that dCAD cells express functional fatty acid amide hydrolase, the enzyme primarily responsible for anandamide metabolism. Previous data from our laboratory indicated that anandamide uptake occurs by a caveolae-related endocytic mechanism in RBL-2H3 cells. In the current study, we show that anandamide uptake by dCAD cells may also occur by an endocytic process that is associated with detergent-resistant membrane microdomains or lipid rafts. Nystatin and progesterone pretreatment of dCAD cells significantly inhibited anandamide accumulation. Furthermore, RNA interference (RNAi)-mediated knockdown of dynamin 2, a protein involved in endocytosis, blocked the internalization of the fluorescently labeled anandamide analog SKM 4-45-1 ([3H]-[9-hydroxy-5Z,11Z,14Z-eicosatetraenyl]laminooethyl ester carbamic acid). RNAi-mediated knockdown of the β2 subunit of the clathrin-associated activator protein 2 complex had no effect on SKM 4-45-1 internalization. We were surprised to find that dynamin 2 knockdown in dCAD cells did not affect [3H]AEA uptake. However, dynamin 2 knockdown caused a significant increase in the overall levels of intact [3H]AEA associated with the cells, suggesting that trafficking of [3H]AEA to FAAH had been disrupted. This finding may be the result of an accumulation of the anandamide carrier protein in detergent-resistant membranes after dynamin 2 knockdown. Our studies provide evidence that the cellular uptake of anandamide may occur by a dynamin 2-dependent, caveolae-related endocytic process in dCAD cells.
differentiation of CAD cells is an observed change in the expression and function of several neuronal proteins. For example, dCAD cells express higher levels of tyrosine hydroxylase (Lazaroff et al., 1998) and somatostatin receptor subtype 2a (Hashemi et al., 2003), exhibit a decrease in sodium current accompanied by an increase in potassium current (Wang and Oxford, 2000), and display alterations in cyclic AMP-mediated signaling (Hashemi et al., 2003). Despite reported differences in their cellular signaling and protein expression profiles, both cycling CAD (cCAD) and dCAD cells express neuron-specific proteins and display the biochemical characteristics of neuronal cells (Qi et al., 1997).

In RBL-2H3 cells, AEA transport occurs via a clathrin-independent, or caveolae-related endocytic process, substantiated by studies using pharmacological inhibitors of endocytosis (McFarland et al., 2004). We tested the hypothesis that the disruption of lipid rafts/detergent-resistant membranes in dCAD cells would also decrease the cellular accumulation of AEA to confirm that endocytosis may play a role in AEA uptake in neuronal cells. To avoid the potential problems associated with biochemical disruption of lipid rafts, we examined AEA uptake in dCAD cells after treatment with short interfering RNA (siRNA) to knockdown expression of the β2 subunit of the AP2 complex, a protein necessary for clathrin-mediated endocytosis (Huang et al., 2004), as well as dynamin 2 (Dyn 2), a protein involved in both clathrin-mediated and clathrin-independent endocytic processes (Altschuler et al., 1998; Maxfield and McGraw, 2004). The results of our studies are consistent with a role for endocytosis in the neuronal uptake of endocannabinoids.

Materials and Methods

Cell Culture. cCAD cells were maintained in a 1:1 ratio of Ham's F-12/Dulbecco's modified Eagle's medium with 5% bovine calf serum and 5% fetal calf clone 1 supplemented with 1% penicillin/streptomycin, 5% CO2 and held at the constant temperature of 37°C. cCAD cells were maintained in serum-free medium for 36 h before experiments to generate dCAD cells.

[3H]AEA Uptake and SKM 4-45-1 Internalization Assays. CAD cells were plated at approximately 50,000 cells per well in 24-well culture dishes and then allowed to differentiate in serum-free media for 36 h. The resulting dCAD cells were then transfected with siRNA oligonucleotides as described above. After the transfection period, media were removed and 1 nM [3H]AEA (labeled on the ethanolamine portion of the molecule and not the arachidonate backbone; therefore, as FAAH activity increased so did the level of radioactivity in the aqueous phase). Membrane cholesterol was extracted by a 10 min incubation with medium containing 4% methyl-β-cyclodextrin. Media was collected, cells were washed three times with PBS, and proteins were extracted. Protein concentrations were determined using the bicinchoninic acid assay. [3H]Cholesterol extracted into the medium was quantified by scintillation counting and normalized to total protein values.

FAAH Activity Assays. FAAH activity was determined by a modification of a previously published method (Day et al., 2001). In brief, CAD cells were homogenized in buffer containing 20 mM Tris-HCl, 1 mM EDTA, 0.7 μg/ml pepstatin A, and 0.5 μg/ml leupeptin. Cell homogenates were then incubated with 5 nM [3H]AAEA [ethanolamine 1-3H] (ARC, St. Louis, MO) in the presence or absence of 500 nM methyl arachidonyl fluorophosphate (MAFP) with a reaction volume of 250 μl. After the incubation, the reaction was terminated by adding 500 μl of 1:1 chloroform/methanol (2× assay volume) to each tube. Samples were vortexed for 30 s and centrifuged at 3900g for 1 min to separate the aqueous and organic phases. Then, 100-μl aliquots were collected from both the aqueous and organic phases, and radioactivity was quantified using a TopCount Microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences). The [3H]AAEA used in these experiments was labeled on the ethanolamine portion of the molecule and not the arachidonate backbone; therefore, as FAAH activity increased so did the level of radioactivity in the aqueous phase.

Western Blot Analysis. Western blot analysis was performed as described previously (McFarland et al., 2004). The presence of FAAH, dynamin 2, and the β2 subunit of the AP2 complex was detected using rabbit polyclonal anti-FAAH, rabbit polyclonal anti-dynamin 2 (Abcam Inc., Cambridge, MA), and mouse monoclonal anti-β2 subunit (Novus Biologicals, Inc., Littleton, CO) primary antibodies, respectively, followed by an incubation with horseradish peroxidase-labeled goat anti-rabbit or goat anti-mouse secondary antibodies, followed by an incubation with horseradish peroxidase-labeled goat anti-rabbit or goat anti-mouse secondary antibodies (Bio-Rad Laboratories, Hercules, CA), and enhanced chemiluminescence detection reagents. Membranes were then exposed to X-ray film.

RNAi Transfections. CAD cells were transfected with siRNA oligonucleotides (Stealth RNAi; Invitrogen, Carlsbad, CA) directed against either Dyn2 or the β2 subunit of the AP2 complex mRNA using the Lipofectamine 2000 (Invitrogen) RNAi transfection protocol for mammalian cells provided by Invitrogen (16 pmol of each siRNA oligo was added for every 1 μl of Lipofectamine 2000 used). For later experiments, the siLentFect Lipid Reagent was used following the manufacturer’s protocol (Bio-Rad Laboratories). Protein knockdown was achieved by cotransfection of two different siRNA oligonucleotides targeted at each individual protein. The siRNA sequences were as follows: Dyn 2: NM_007871_stealth_1410, GUG-GACCUGGUUAUCCAGGAGCUAA, and NM_007871_stealth_2009, GGCAGAGAAUGAGGAUGGACCAAA, β2AP2: NM_027915_
Endocannabinoid Uptake Properties of dCAD and cCAD Cells. Differentiated and undifferentiated CAD (dCAD) and cCAD, respectively) cells displayed robust uptake of radio-labeled anandamide. The accumulation of AEA by CAD cells was inhibited by the AEA uptake inhibitor AM404, with a \( K_i \) value in the low micromolar to high nanomolar range (Fig. 1A). SKM 4-45-1 is a fluorescent analog of AEA (Muthian et al., 2000), we expected that this treatment would decrease the specific uptake of AEA by dCAD cells. Indeed, the disruption of detergent-resistant membrane microdomains in dCAD cells resulted in an ~50% decrease in the specific uptake of \([3H]\)AEA (Fig. 3). Control experiments confirmed that nystatin and progesterone treat-
ment reduced membrane cholesterol by approximately 50%, consistent with the effect on \( ^{[3]}H \)AEA uptake (Fig. 3, inset).

**RNAi-Mediated Knockdown of Endocytic Machinery.** The studies described above with cholesterol depletion, along with our previous work (McFarland et al., 2004), suggested that AEA uptake occurs via a caveolae-related endocytic process. The nonspecific nature of the cholesterol depletion treatments limits interpretation of our results.

Therefore, we assessed the role of clathrin-independent versus clathrin-dependent endocytosis of AEA in dCAD cells using molecular inhibitors. Specifically, we used an RNAi approach to knockdown expression of either the \( \beta_2 \) subunit of the AP2 complex or dynamin 2. Knockdown of the \( \beta_2 \) subunit of the AP2 complex should only inhibit clathrin-mediated endocytosis and have no effect on clathrin-independent, or caveolae-related, endocytic processes (Johannes and Lamaze, 2002; Huang et al., 2004). Dynamin 2, however, is a GTPase that has been shown to play a role in both clathrin-mediated and clathrin-independent endocytosis (Altschuler et al., 1998; Maxfield and McGraw, 2004). After transfection with siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen), the cells were incubated for 72 h to allow for the degradation of resident protein. Western blot analysis confirmed that we had successfully reduced the expression of both proteins by 70 to 80% (Fig. 4). Control transfection experiments using green fluorescent protein (pEGFP-N1; Clontech, Mountain View, CA) confirmed transfection efficiency in CAD cells to be greater than 80% (data not shown), consistent with our observed knockdown effects.

To verify the effects of both \( \beta_2 \) subunit and dynamin 2 knockdown, we used the Alexa Fluor 488-conjugated probes transferrin (488-Tf) and the B subunit of cholera toxin (488-CT), which are endocytosed by clathrin-dependent and lipid raft/caveolae-related endocytic processes, respectively. As was expected, after a 10-min treatment with either probe at 37°C, RNAi knockdown of the \( \beta_2 \) subunit of the AP2 complex significantly reduced the internalization of 488-Tf (Fig. 5) but had no effect on 488-CT endocytosis in dCAD cells compared with control (Fig. 6). In the case of dynamin 2, RNAi knockdown inhibited internalization of both 488-Tf (Fig. 5) and 488-CT (Fig. 6) in dCAD cells. These control experiments confirm that by knocking down expression of the \( \beta_2 \) subunit of the AP2 complex we are only inhibiting clathrin-mediated endocytosis (i.e., 488-Tf internalization), whereas knockdown of dynamin 2 inhibits both clathrin-dependent endocytosis as well as a lipid raft-related endocytic process that is clathrin-independent.

**AEA Uptake after RNAi Transfection.** Our results above showing AM404 inhibition of both \( ^{[3]}H \)AEA and SKM 4-45-1 internalization (Fig. 1) suggest that both compounds are transported by a common process in the CAD cells. Because the fluorescence associated with SKM 4-45-1 is only observed after internalization of the compound, it is a true marker of actual uptake into the cell (Muthian et al., 2000). To assess the effects that RNAi knockdown of endocytic machinery might have on
AEA uptake, cells were treated with 25 μM SKM 4-45-1 at 37°C for 10 min after knockdown of the expression of either the β2 subunit of the AP2 complex or dynamin 2. Compared with control, RNAi knockdown of the β2 subunit of the AP2 complex caused no observable change in the internalization of SKM 4-45-1 (Fig. 7). However, knockdown of dynamin 2 expression abolished the SKM 4-45-1 fluorescence accumulation in dCAD cells (Fig. 7).

In addition to examining SKM 4-45-1 accumulation, we investigated the effects of RNAi-mediated knockdown of both the β2 subunit of the AP2 complex and dynamin 2 on the specific uptake of [3H]AEA. We were surprised to find that neither the β2 subunit of the AP2 complex nor dynamin 2 knockdown displayed any significant effect on the specific uptake of [3H]AEA (Fig. 8). To explain the potentially conflicting result between [3H]AEA and SKM 4-45-1 internalization, we hypothesized that dynamin 2 siRNA-transfected dCAD cells might be accumulating more [3H]AEA at the plasma membrane instead of internalizing the AEA. If this were true, then FAAH would be unable to metabolize the [3H]AEA that has been retained at the plasma membrane. Such an effect would be revealed by an increase in intact [3H]AEA in the dynamin 2 siRNA-transfected cells after a 5-min uptake assay.

dCAD cells transfected with either mock or dynamin 2 siRNA were treated with [3H]AEA for 5 min in the presence or absence of AM404. After [3H]AEA treatment, each treat-
samples of each treatment group, and the levels of intact \[^3H\]AEA were quantified by scintillation counting. TLC analysis revealed that the specific levels of intact \[^3H\]AEA were approximately 2-fold higher in dCAD cells in which dynamin 2 had been knocked down compared with mock-transfected dCAD cells (Fig. 9A).

One explanation for the above-mentioned results could be that the knockdown of dynamin 2 altered FAAH activity. Examination of FAAH protein by Western blot analysis (Fig. 9B) and FAAH activity (Fig. 9C) after dynamin 2 knockdown indicated that the dynamin 2 knockdown had no detectable effect on FAAH. The lack of effect on FAAH and actin levels also confirms the specificity of our dynamin 2 siRNA (Fig. 9B).

**Discussion**

dCAD cells represent a biochemically and morphologically neuronal cell line that rapidly accumulates the endocannabinoid AEA and displays robust FAAH activity. Thus, dCAD cells may present a novel system useful in the study of endocannabinoid biosynthesis and inactivation.

Pharmacological inhibition of endocytosis in RBL-2H3 cells suggests that an endocytic process is involved in AEA uptake and that this process is clathrin-independent, potentially involving detergent-resistant membrane microdomains such as lipid rafts (McFarland et al., 2004). Our present results confirm these findings in the neuronal dCAD cell line as well. Pretreatment with nystatin/progesterone to disrupt detergent-resistant membrane microdomains significantly reduced the specific uptake of \[^3H\]AEA by dCAD cells by \(-50\%\). An alternative explanation for our results with nystatin/progesterone could be that the treatment is generally toxic to the cells. Because a major factor determining nonspecific uptake relates to cell number, it is unlikely that the brief treatments caused a decrease in cell viability because nonspecific uptake of AEA was unaltered by the treatment (data not shown). We recognize that there is a lack of specificity associated with the chemical disruption of lipid rafts. Thus, a means of specifically disrupting endocytic processes without causing the potential nonspecific effects associated with the chemical disruption of lipid rafts was needed to assess the role that endocytosis may play in the cellular uptake of AEA.

The β2 subunit of the AP2 complex allows for the association of this complex with clathrin and is necessary for clathrin-mediated endocytosis to occur (Huang et al., 2004). Transferrin is internalized by a clathrin-mediated endocytic process (Johannes and Lamaze, 2002). As expected, RNAi knockdown of the β2 subunit of the AP2 complex inhibited transferrin internalization in dCAD cells but did not disrupt

![Fig. 8. \[^3H\]AEA uptake by dCAD cells after siRNA transfection. Seventy-two hours after transfection with mock, dynamin 2, or β2 subunit siRNA, \[^3H\]AEA uptake assays were performed in 1× KRH at 37°C as described under Materials and Methods. AM404 (100 μM) was used to define nonspecific transport. Data represent mean ± S.E.M. for three separate experiments performed in triplicate. The cpm values for mock cells were total = 2887 ± 136 and nonspecific = 1125 ± 128. The cpm values for dynamin 2 knockdown cells were total = 2973 ± 89 and nonspecific = 905 ± 83. The cpm values for β2 subunit knockdown cells were total = 2638 ± 104 and nonspecific = 777 ± 27.](image_url)

![Fig. 9. Metabolism of \[^3H\]AEA in dynamin 2 siRNA-transfected dCAD cells. A, 72 h after transfection with either mock or dynamin 2 siRNA, dCAD cells were treated with \[^3H\]AEA for 5 min and then lysed. Cellular lipids were extracted from the cell lysates using chloroform/methanol and analyzed by TLC for intact \[^3H\]AEA. Nonspecific uptake was determined using 100 μM AM404. The graph represents data from three separate experiments. Statistical analysis was performed using a one-sample t test comparing with the value of 100 (mock control). *, p < 0.05. The cpm values from the TLC analysis for mock cells were total = 2986 ± 795 and nonspecific = 2243 ± 407. The cpm values from the TLC analysis for dynamin 2 knockdown cells were total = 3257 ± 545 and nonspecific = 1783 ± 198. B, FAAH expression after RNAi-mediated knockdown of dynamin 2. Knockdown of dynamin 2 in cCAD cells was performed as described under Materials and Methods except siLentFect Lipid Reagent (Bio-Rad) was used as the transfection reagent for 48 h with 10 nM siRNA oligo. Western blots were performed using the polyclonal dynamin 2 antibody, monoclonal FAAH antibody (1:1000; Abnova Corporation, Taipei City, Taiwan), monoclonal actin antibody (1:2000; Sigma-Aldrich, St. Louis, MO). C, FAAH activity after RNAi-mediated knockdown of dynamin 2. FAAH activity assays were performed on whole cell lysates as described under Materials and Methods. Data shown represent means ± S.D. from two experiments performed in quadruplicate.](image_url)
the cellular accumulation of the cholera toxin B subunit or SKM 4-45-1, the fluorescently tagged analog of AEA. The cholera toxin B subunit is known to be internalized via a clathrin-independent (lipid raft-related) endocytic process (Johannes and Lamaze, 2002).

Whereas its role in neurons is not completely clear, the small GTPase dynamin 2 is probably involved in both clathrin-dependent and clathrin-independent endocytic recycling (Altschuler et al., 1998; Maxfield and McGraw, 2004). Thus, both transferrin and cholera toxin should require dynamin 2 expression for internalization to occur. RNAi knockdown of dynamin 2 significantly reduced both transferrin and cholera toxin accumulation by dCAD cells. Furthermore, dynamin 2 knockdown in dCAD cells inhibited the cellular internalization of SKM 4-45-1. Because the fluorescence associated with SKM 4-45-1 is only observed after internalization of the compound, it can be used as a marker for actual uptake (Muthian et al., 2000). One caveat of using only [3H]AEA uptake is that conventional uptake assays do not differentiate between the tritium bound to the cells or membranes and the AEA that is actually transported into the cells. Taken together, our data suggest that actual internalization of AEA by dCAD cells occurs via a clathrin-independent endocytic process. Furthermore, dynamin 2 seems to play a role in the cellular uptake of AEA by the neuronal dCAD cells. Internalization of the B subunit of cholera toxin occurs by a lipid raft-mediated endocytic process (Johannes and Lamaze, 2002). Dynamin 2 knockdown significantly reduced 488-CT internalization in dCAD cells consistent with this dynamin 2-dependent, clathrin-independent endocytic process involving detergent-resistant membrane microdomains. An alternative approach to the knockdown of dynamin 2 or the β subunit of the AP2 complex could be to use siRNA targeted to clathrin itself. We were concerned about potential membrane effects reported with clathrin knockdowns and as such, we opted to focus on proteins downstream of clathrin in the endocytic pathways (Hinrichsen et al., 2003, 2006).

Dynamin 2 knockdown displayed a profound inhibitory effect on SKM 4-45-1 internalization in dCAD cells, and yet it did not have any observable effect on the specific uptake of [3H]AEA. This discrepancy may be reconciled with the observation that [3H]AEA uptake assays cannot discriminate between [3H]AEA that has actually been internalized and [3H]AEA that is binding to a target on the plasma membrane. Interestingly, a study conducted by Schmid and colleagues showed that expression of dominant-negative mutants of dynamin proteins inhibited endocytic processes but had no effect on endosomal recycling of membrane components to the cell surface (Altschuler et al., 1998). dCAD cells that have lost functioning dynamin 2 protein during the 72-h incubation after transfection with siRNA may be accumulating the AEA carrier in the detergent-resistant membrane microdomains. Precedence for such an effect comes from studies showing that expression of dominant-negative dynamin 2 mutant causes an accumulation of transferrin receptor at the cell surface (Altschuler et al., 1998). A similar phenomenon could be occurring with the putative AEA carrier after dynamin 2 knockdown. The accumulation of detergent-resistant membrane components at the cell surface with dynamin 2 knockdown could create a scenario in which dCAD cells, although they are not internalizing [3H]AEA, are accumulating [3H]AEA at the membrane. This effect would be due to more [3H]AEA being able to associate with the plasma membrane that is now enriched with the putative carrier protein. This experimental limitation is not an issue in the case of SKM 4-45-1 internalization assays. Fluorescence from SKM 4-45-1 is not observed until after the compound is transported into the cell, and the fluorescent component of the molecule is liberated by esterases in the cell (Muthian et al., 2000). Thus, excess SKM 4-45-1 bound only at the membrane is not detectable.

We propose that knockdown of dynamin 2 in dCAD cells causes an accumulation of the AEA carrier in detergent-resistant membrane domains at the cell surface. There is no observable difference between the [3H]AEA uptake in mock siRNA-transfected and dynamin 2 siRNA-transfected dCAD cells because more [3H]AEA is able to associate with the plasma membrane carrier in the dynamin 2 siRNA-transfected cells. If this is true, then the majority of the radioactivity obtained from the dynamin 2 siRNA-transfected cells should be represented by intact [3H]AEA. This is because AEA that is only associating with the cell surface and is not internalized will not be available to FAAH for metabolism. Indeed, TLC analysis of lipid extracts from dynamin 2 siRNA-transfected dCAD cells that were treated with [3H]AEA for 5 min revealed levels of intact [3H]AEA that were 2-fold greater than the levels of [3H]AEA extracted from mock siRNA-transfected dCAD cells treated in the same manner. Furthermore, our results indicate that dynamin 2 knockdown does not alter FAAH protein or activity. These data support the hypothesis that dynamin 2 siRNA-transfected dCAD cells are able to accumulate more [3H]AEA on their cell surface than mock-transfected dCAD cells due to an enrichment of the putative AEA carrier in the microdomains. If this is the case, then [3H]AEA uptake assays may not be an ideal method for characterizing AEA internalization after the knockdown of proteins, such as dynamin 2, that are important in recycling events that maintain the composition of the plasma membrane. One alternative explanation for our results involves a possible role for the CB1 cannabinoid receptor in the uptake process. CB1 receptors have been shown to signal through lipid raft domains (Bari et al., 2005); thus, binding to CB1 receptors and subsequent internalization would be one possible mechanism for AEA uptake.

Unlike RBL-2H3 cells, we do not expect that dCAD cells contain caveolin-1, but we have preliminary evidence that dCAD cells express caveolin-3 (data not shown). Thus, caveo- lae-related endocytic processes may still be present in neurons and are dependent on intact detergent-resistant membrane microdomains. In summary, our data, combined with previous experiments in RBL-2H3 cells (McFarland et al., 2004), offer evidence that endocannabinoids are internalized by a lipid raft- or caveola-related endocytic process. We speculate that the yet unidentified anandamide transporter will in fact be enriched in lipid rafts and functionally participate in endocytosis.

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References


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