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Carrier-Mediated Uptake of the Endogenous Cannabinoid Anandamide in RBL-2H3 Cells[1]

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ABSTRACT

Anandamide (*N*-arachidonylethanolamide) is an endogenous cannabinoid that mimics the pharmacologic effects of Δ^9-tetrahydrocannabinol, the major bioactive substance in marijuana. Anandamide appears to be synthesized, released, and inactivated by mechanisms similar to those for other neurotransmitters. Of interest to the present studies are reports that anandamide undergoes carrier-mediated uptake into neuronal or glial cells after release, followed by rapid intracellular degradation by the intracellular fatty acid amidohydrolase. In addition to effects in the brain, anandamide has multiple effects in the periphery, particularly on cells of the immune system that express both a peripheral cannabinoid receptor and amidohydrolase enzyme. We have performed a detailed characterization of anandamide uptake in the cognate mast cell line RBL-2H3 to test the hypothesis that the uptake system in peripheral cells is also carrier-mediated and functionally similar to that observed in the central nervous system. RBL-2H3 cells exhibited robust, saturable transport of [^{3}H]anandamide that was both time- and temperature-sensitive. This transport activity was not dependent on extracellular ion gradients for uptake and was inhibited selectively by other fatty acid-derived molecules, anandamide congeners, and the psychoactive cannabinoids such as Δ^9-tetrahydrocannabinol. We conclude that anandamide transport in the RBL-2H3 cells is carrier-mediated, and uptake in peripheral cells is functionally and pharmacologically identical with that observed in neurons and astrocytes.

Anandamide (*N*-arachidonylethanolamide), 2-arachidonylglycerol (2-AG), and a family of fatty acid ethanolamides have been identified as endogenous cannabinoids (Devane et al., 1992; Stella et al., 1997). Biochemical and pharmacologic evidence indicates that these fatty acid-derived neuromodulators act via the cloned cannabinoid receptors (CB1 and CB2) to elicit similar behavioral and physiologic effects to the psychoactive cannabinoids like Δ^9-tetrahydrocannabinol (Δ^9-THC), thus supporting the assertion that anandamide is an endogenous cannabinoid (Crawley et al., 1993; Felder et al., 1993, 1995; Fride and Mechoulam, 1993; Smith et al., 1994). Although the precise physiologic role of the endogenous cannabinoids has not been fully elucidated, anandamide and 2-AG have been implicated in modulation of memory, cognition, blood pressure, pain, fever, and the immune system and as having potentially therapeutic effects in conditions such as convulsions, glaucoma, movement disorders, and multiple sclerosis (Hirst et al., 1998).

As putative neuromodulators, mechanisms must exist for the synthesis, release, and termination of endocannabinoid signaling. Piomelli and coworkers (Di Marzo et al., 1994; Cadas et al., 1997) have performed a series of elegant studies implicating a calcium-dependent phosphodiesterase-mediated cleavage of a membrane phospholipid precursor, *N*-arachidonoyl-phosphatidylethanolamine, as the major route of fatty acid amide biosynthesis. Once formed and released, anandamide is rapidly transported into neurons and astrocytes for subsequent hydrolytic degradation to ethanolamine and arachidonic acid (Deutsch and Chin, 1993; Di Marzo et al., 1994; Cravatt et al., 1996; Beltramo et al., 1997; Hillard et al., 1997). The fatty acid amidohydrolase (FAAH) responsible for anandamide metabolism has been cloned, and functional studies reveal that 2-AG and the sleep-inducing lipid, oleamide, also can serve as substrates for this enzyme (Cravatt et al., 1996; Goparaju et al., 1998). Thus, both synthesis and catalysis pathways exist for anandamide in the central nervous system. However, for degradation to occur, anandamide first must be transported into cells possessing the FAAH activity, making the uptake process a critical and potentially rate-limiting step in the metabolism of anandamide.

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ABBREVIATIONS: 2-AG, 2-arachidonylglycerol; THC, tetrahydrocannabinol; 11-OH-Δ^9-THC, 11-hydroxy-*nor*-Δ^9-THC; FAAH, fatty acid amidohydrolase; AM404, *N*-(4-hydroxyphenyl)-arachidonamide; KRH, Krebs-Ringer-HEPES; PMSF, phenylmethylsulfonyl fluoride; ATFK, arachidonyl trifluoromethyl ketone; MAFP, methyl arachidonyl fluorophosphonate.

Uptake of anandamide has been demonstrated in multiple central nervous system-derived cell lines (Deutsch and Chin, 1993; Piomelli et al., 1999) as well as in primary cultures of cerebellar granule cells (Hillard et al., 1997) and striatal neurons and astrocytes (Di Marzo et al., 1994; Beltramo et al., 1997). Despite the apparent lipophilicity and membrane permeability of anandamide and other fatty acid-derived compounds, the rapid and efficient clearance of fatty acids and related compounds is dependent on carrier-mediated processes (Kanai et al., 1995; Hirsch et al., 1998). Characterization of anandamide transport in central nervous system cell types supports the hypothesis that this process is carrier-mediated. Uptake is: 1) rapid ($t_{1/2}$ = 2.5 min), 2) temperature-dependent, 3) saturable with high affinity at 37°C, and 4) inhibited selectively in a concentration-dependent fashion by low micromolar concentrations of nonisotopic anandamide and not by the structurally related *N*-arachidoylethanolamide, *N*-stearoylethanolamide, and *N*-linolenoylethanolamide (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997). Furthermore, Beltramo et al. (1997) have reported the development of a selective anandamide transport inhibitor, AM404 [*N*-(4-hydroxyphenyl)-arachidonamide], that can be used as a pharmacologic tool in the study of anandamide uptake.

Whereas considerable efforts have focused on defining endogenous cannabinoid signaling in the central nervous system, anandamide and related compounds also may play a significant role in modulating physiologic processes in the periphery, particularly within the immune system (Klein et al., 1998). To that end, anandamide transport was identified in the cognate mast cell line RBL-2H3 (Bisogno et al., 1997); however, little characterization of anandamide uptake in this peripheral cell type has been reported. RBL-2H3 cells are an immortalized basophilic leukemia cell line that is used as a model for immune cell function and that also possesses the biochemical components required for endocannabinoid synthesis, receptor signaling, uptake, and metabolism (Facci et al., 1995; Bisogno et al., 1997). To address the question of whether anandamide uptake in the central nervous system and periphery is mediated by a similar carrier-mediated process, we have performed a detailed characterization of anandamide uptake in RBL-2H3 cells, representing the first such analysis of anandamide uptake in a peripherally derived cell type. Our studies demonstrate many similarities between peripheral and central anandamide uptake mechanisms, including similar transport kinetics, a lack of dependence on ionic gradients, and similar pharmacologic sensitivity, suggesting correspondence in the identity of the protein(s) involved with mediating anandamide transport in these two tissues.

Experimental Procedures

[^{3}H]Anandamide Uptake Assays. RBL-2H3 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum supplemented with 2 mM glutamine and 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 environment. Uptake assays were carried out in 24-well culture dishes. Cells (2×10^5 cells/well) were washed once with Krebs-Ringer-HEPES (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM HEPES, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, pH 7.4) and preincubated in KRH buffer at 37°C for 10 min. When potential uptake inhibitors were used, they were added 10 min before the addition of [^{3}H]anandamide. [^{3}H]Anandamide (1 nM) was added to the buffer, and uptake at 37°C was allowed for 5 or 10 min. Saturation kinetics were determined using increasing concentrations of [^{3}H]anandamide with the specific activity diluted to $\sim 5.0 \times 10^{-3}$ Ci/mmol with unlabeled anandamide. Transport was terminated by three washes with KRH buffer containing 1% BSA. Nonspecific uptake was determined by the addition of 100 μM (*R*)-2-methanandamide or AM404. Addition of liquid scintillant to the wells and subsequent overnight incubation solubilized the cells for direct counting using a Wallac MicroBeta (Gaithersburg, MD) or Packard TopCount scintillation plate analyzer (Meriden, CT) to determine accumulated [^{3}H]anandamide. Mean nonspecific uptake was subtracted from total uptake to yield specific anandamide uptake. Substrate K_m and antagonist IC_{50} values were derived by nonlinear least-square fits with Kaleidagraph (Synergy Software, Reading, PA) or GraphPad Prism v.2.01 (GraphPad Software, Inc., San Diego, CA) using either the Hill equation for a rectangular hyperbola or the four-parameter logistic equation with necessary adjustments of IC_{50} values for substrate concentration to determine apparent K_i values (Cheng and Prusoff, 1973). Experiments were performed in duplicate or triplicate and repeated in two to three separate assays. Statistical comparisons of V_{max}, K_m, and K_i values were performed using two-tailed *t* tests for V_{max} and K_m values or one-way ANOVA with Dunnett's post test for antagonist K_i values (GraphPad Prism v. 2.01).

The Na^+ dependence of [^{3}H]anandamide uptake was assessed in KRH buffer using isotonic replacement of NaCl with choline chloride, whereas the Cl^- dependence was determined in KRH buffer with Cl^- salts replaced by sodium gluconate, potassium gluconate, and calcium nitrate at molarities equivalent to those in regular KRH buffer. For inactivation experiments, cells were incubated at 37°C for 30 min in the presence of *N*-ethylmaleimide (500 mM), phenoxybenzamine (100 mM), or Pronase (1.0 mg/ml; Boehringer Mannheim, Indianapolis, IN) before initiating [^{3}H]anandamide or [^{3}H]serotonin uptake assays. [^{3}H]Serotonin transport assays (Barker et al., 1998) were performed as control experiments to verify effectiveness of inactivating reagents.

FAAH Enzymatic Activity Assay. Assays were performed by a modification of the previously published method (Omeir et al., 1995). Briefly, RBL-2H3 cells were incubated with 5 nM anandamide [ethanolamine 1-^{3}H] for various times in the presence or absence of 500 nM methyl arachidonyl fluorophosphonate (MAFP) or 100 mM AM404. Reactions were terminated by three washes in ice-cold KRH buffer containing 1% BSA. Cells were solubilized immediately in 1% Triton X-100 followed by extraction in two volumes of chloroform/methanol (1:1, v/v). Production of [^{3}H]ethanolamine was determined by liquid scintillation counting of the aqueous phase. Nondegraded anandamide [ethanolamine 1-^{3}H] was assessed by liquid scintillation counting of the organic phase. The integrity of the anandamide [ethanolamine 1-^{3}H] in the organic phase was determined by thin-layer chromatography (TLC) using silica gel sheets (Z12,278-5; Aldrich, Milwaukee, WI) developed in the organic layer of ethyl acetate/hexanes/acetic acid/water (100:50:20:100, v/v/v/v). A single radioactive product, R_f, 0.78–0.80, was identified using a Berthold Tracemaster 40 Automatic TLC-Linear analyzer (Berthold Systems Inc., Pittsburgh, PA).

Materials. Dulbecco's modified Eagle's medium was purchased from Fisher Scientific (Pittsburgh, PA), fetal bovine serum from Hyclone (Logan, UT), and RBL-2H3 cells from the American Type Culture Collection (Manassas, VA). Trypsin, glutamine, penicillin, and streptomycin were obtained from Life Technologies (Grand Island, NY), and cell culture plates from Falcon/Becton-Dickinson Labware (Mountain View, CA) and Packard (Meriden, CT). [^{3}H]anandamide (223.00 Ci/mmol) for uptake assays was purchased from New England Nuclear (Boston, MA) or anandamide [ethanolamine 1-^{3}H] (20 Ci/mmol) from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Arachidonyl trifluoromethyl ketone (ATFK), (*R*)-1- and (*R*)-2-methanandamide, palmitoyl ethanolamide, and MAFP were obtained from Cayman Chemical, Inc. (Ann Arbor, MI),

and cannabinoids, anandamide, 2-AG, AM404, and arachidonic acid were purchased from RBI-Sigma Aldrich (Natick, MA). Ecoscint H and Optiphase SuperMix scintillation fluor was obtained from National Diagnostics (Atlanta, GA) and Wallac (Gaithersburg, MD), respectively. SR141716A and HU-210 were kindly provided by Dr. Emanuel Onaivi (Vanderbilt University, Nashville, TN). All other drugs and materials were obtained from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were of the highest grade possible.

Results

[^{3}H]Anandamide Uptake in RBL-2H3 Cells. Previous characterizations of anandamide uptake in brain-derived cells indicate that this process meets several criteria for carrier-mediated transport (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997). These properties include time- and temperature-dependence, saturability, and selectivity. Anandamide uptake in RBL-2H3 cells was also time-dependent ($t_{1/2}$ = 3.2 ± 1.3 min) (Fig. 1A), saturable (Fig. 1B), and temperature-dependent because uptake at 0 to 4°C was reduced to levels observed in the presence of 100 μM (*R*)-1-methanandamide (data not shown). The apparent K_m and V_{max} values of this transport process were 11.4 ± 2.3 μM and 17.5 ± 2.1 × 10^{-17} mol/min/cell, respectively.

Active transport systems associated with uptake of many neurotransmitters have been identified as ion-coupled co-transport processes (Nelson, 1998). In contrast, anandamide uptake in RBL-2H3 cells did not demonstrate dependence on Na^+, Cl^-, or H^+ (data not shown). This lack of ion dependence is consistent with a predicted hydrophobic permeation pathway required for such lipophilic substrates. Furthermore, treatment of intact RBL-2H3 cells with the alkylating agent *N*-ethylmaleimide (500 μM) or phenoxybenzamine (100 μM) did not alter anandamide transport (data not shown). Proteolytic digestion with Pronase (1 mg/ml) also had no effect on anandamide uptake (data not shown). The lack of sensitivity of the anandamide carrier to these inactivating reagents or proteolytic digestion is consistent with the major functional domains of the carrier protein being embedded in the hydrophobic environment of the plasma membrane. Future experiments using more lipophilic inactivating reagents may be useful in revealing additional structural information about the anandamide transporter.

Pharmacologic Profile of Anandamide Transport in RBL-2H3 Cells. Many of the structural relatives of anandamide are not commercially available in radiolabeled forms, hindering the ability to directly assess potential substrate selectivity of the anandamide transporter in RBL-2H3 cells. To determine the requirements for recognition by the anandamide transporter, we examined the ability of various fatty acid-derived compounds to inhibit [^{3}H]anandamide uptake. The other major endocannabinoid, 2-AG, inhibited anandamide transport as did the related fatty acid amide, oleoylethanolamide (Fig. 2A). Interestingly, palmitoylethanolamide (Fig. 2A) and the anandamide precursor ethanolamine (1 mM; data not shown) had no effect on anandamide uptake. The long-chain fatty acid, arachidonic acid, which is the structural precursor to anandamide, also elicited a dose-dependent decrease in anandamide uptake as did the other long-chain fatty acid, oleic acid (data not shown). In contrast, the related saturated fatty acid, stearic acid (1 mM), and the short-chain fatty acid, maleic acid (1 mM), did not inhibit

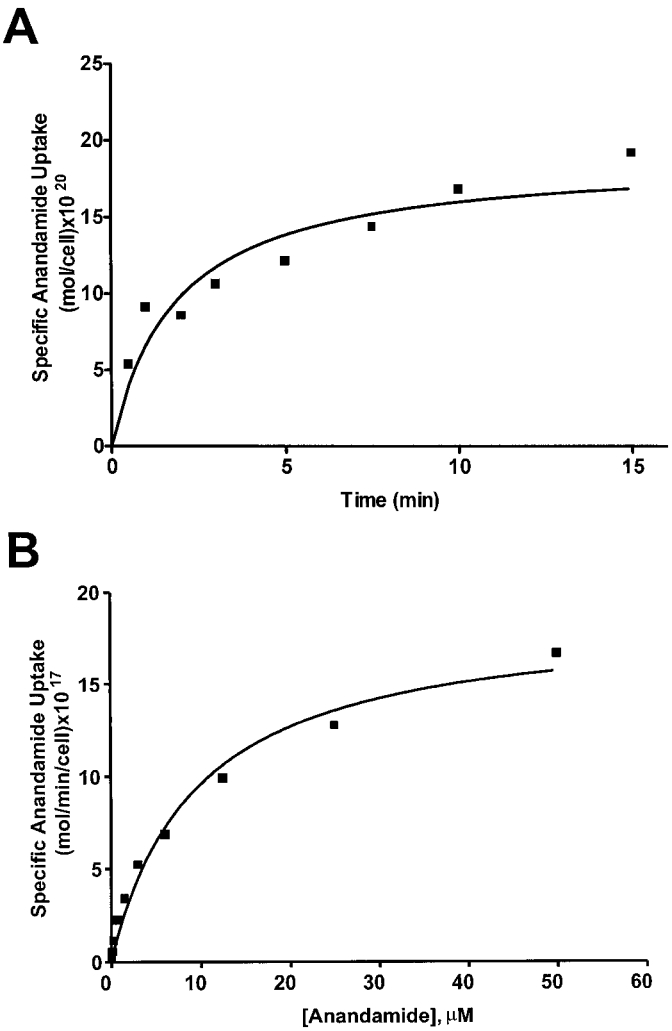

Fig. 1. Kinetic analysis of anandamide transport in RBL-2H3 cells. A, time dependence of anandamide uptake. RBL-2H3 cells were incubated at 37°C with [^{3}H]anandamide for the times indicated as described in *Experimental Procedures*. Nonspecific uptake was determined in the presence of 100 μM AM404. Data plotted represent means of triplicate determinations and are representative of three separate experiments. B, saturation kinetics of anandamide uptake. RBL-2H3 cells were incubated with increasing concentrations of [^{3}H]anandamide for 10 min at 37°C. Nonspecific uptake was determined in the presence of 100 μM (*R*)-2-methanandamide. The K_m and V_{max} values were 11.4 ± 2.3 μM and 17.5 ± 2.1 × 10^{-17} mol/min/cell, respectively. Data plotted represent means of duplicate determinations and are representative of six separate experiments.

anandamide transport (data not shown). These data confirm that anandamide transport in RBL-2H3 cells demonstrates selectivity. Furthermore, sensitivity to inhibition by long-chain fatty acids may suggest potential similarities between the anandamide transporter and proteins that mediate uptake of long-chain fatty acids (Abumrad et al., 1993; Isola et al., 1995; Berk et al., 1996; Hirsch et al., 1998).

Presently, the only selective anandamide transport inhibitor that has been reported is AM404, which potently inhibited anandamide transport in RBL-2H3 cells (Fig. 2B) with an apparent K_i value of 14.0 ± 2.5 μM, a value that is relatively similar to those previously reported for this compound (Beltramo et al., 1997; Piomelli et al., 1999). Two derivatives of anandamide, (*R*)-2-methanandamide [(*R*)-(−)-arachidonyl-2′-hydroxy-1′-propylamide] and (*R*)-1-meth-

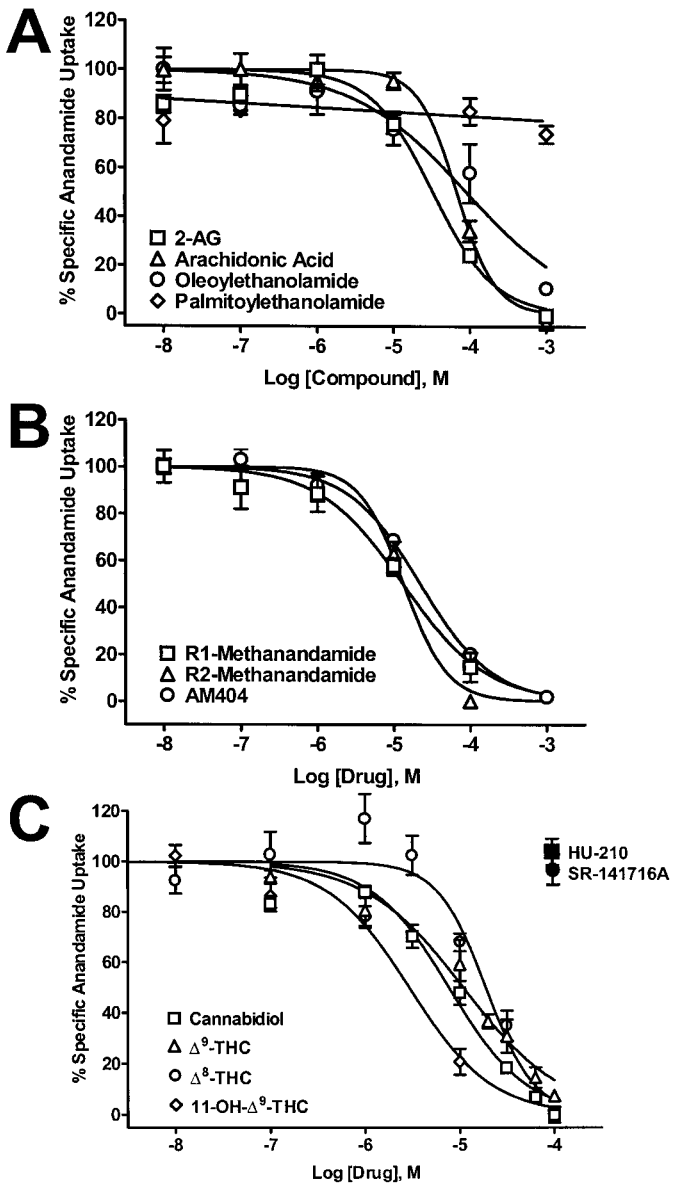

Fig. 2. Distinct pharmacologic sensitivity of anandamide transport. A, anandamide uptake inhibition by 2-AG and related fatty acid amides. B, anandamide uptake inhibition by anandamide derivatives (*R*)-1-methanandamide, (*R*)-2-methanandamide, and AM404. C, anandamide inhibition by Δ^9-THC and related cannabinoids. All of the above [^{3}H]anandamide uptake assays were performed on RBL-2H3 cells as described in *Experimental Procedures*. Data were plotted as percentage of specific anandamide uptake. All data plotted represent means ± S.E. (except for 11-OH-Δ^9-THC which is plotted as means ± S.D.) of triplicate determinations and are representative of two to four separate experiments. Nonspecific uptake was determined either in the presence of 100 μM (*R*)-2-methanandamide or 100 μM AM404 as described in *Experimental Procedures*. Summary data for apparent K_i values: 2-AG, 26.9 ± 6.9 μM; arachidonic acid, 56.0 ± 1.0 μM*; oleoylethanolamide, 55.7 ± 21.0 μM*; palmitoylethanolamide, 100 μM; (*R*)-1-methanandamide, 24.5 ± 9.0 μM; (*R*)-2-methanandamide, 12.6 ± 5.2 μM; AM404, 14.0 ± 2.5 μM; cannabidiol, 11.4 ± 0.1 μM; Δ^9-THC, 16.5 ± 0.1 μM; Δ^8-THC, 14.8 ± 3.0 μM; 11-OH-Δ^9-THC, 2.3 ± 1.4 μM. *$P < .01$, one-way ANOVA with Dunnett's post test using AM404 as the reference compound.

anandamide [(*R*)-(+)-arachidonyl-1′-hydroxy-2′-propylamide], also demonstrated transport blocking activity. (*R*)-2 methanandamide appeared more potent than (*R*)-1-methanandamide (K_i value of 12.6 versus 24.5 μM, respectively), although this difference was not statistically significant (Fig. 2B). Finally, a previous report of anandamide transport indicated that plant-derived cannabinoids, such as Δ^9-THC, may possess anandamide uptake blocking activity. We found that Δ^9-THC, Δ^8-THC, 11-hydroxy-*nor*-Δ^9-THC (11-OH-Δ^9-THC), and cannabidiol all potently inhibited anandamide transport with potencies comparable to AM404 (Fig. 2C). The potent cannabinoid receptor agonist HU-210 (100 μM) and the cannabinoid receptor antagonist SR141716A (100 μM) did not inhibit anandamide transport, suggesting a lack of direct cannabinoid receptor involvement with the uptake process (Fig. 2C). These results reveal a clear pharmacologic profile for anandamide transport and add further support to the notion that this transport process is carrier-mediated.

Pharmacologic sensitivities can provide clues to the identity of a protein involved in a specific biologic function. Thus, we tested inhibitors of many known transport processes, seeking to obtain pharmacologic evidence for the molecular identity of the carrier involved with anandamide uptake. The transport processes and inhibitors evaluated as potential anandamide transport inhibitors included: 1) the organic anion transporter family: bromosulfophthalein (100 μM), taurocholate (100 μM), bromocresol green (100 μM), and prostaglandin E2 (10 μM); 2) the Na^+-dependent biogenic amine transporters: cocaine (100 μM) and citalopram (100 μM); 3) P-glycoprotein: verapamil (100 μM); and 4) ion transport systems: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS; 200 μM), probenecid (200 μM), oubain (1 mM), digoxin (100 μM), and cortisol (100 μM). None of these various transport inhibitors altered anandamide uptake in the RBL-2H3 cells (data not shown), suggesting that anandamide transport in these cells is mediated by a yet unidentified transport protein.

FAAH Inhibitors Reduce Anandamide Uptake. The enzyme responsible for anandamide metabolism, FAAH, demonstrates broad substrate recognition, catalyzing the breakdown of the sleep-inducing lipid, oleamide, as well as 2-AG (Cravatt et al., 1996; Goparaju et al., 1998). FAAH possesses a single putative transmembrane domain and an intracellular catalytic domain (Cravatt et al., 1996). Furthermore, FAAH activity has been reported in RBL-2H3 cells (Bisogno et al., 1997). Could FAAH-mediated anandamide metabolism play a role in anandamide uptake? The FAAH inhibitors phenylmethylsulfonyl fluoride (PMSF), ATFK, and MAFP demonstrated inhibitory action on anandamide uptake (Fig. 3A). Although ATFK completely inhibited anandamide transport at high concentrations (100 μM), MAFP and PMSF showed only partial inhibition (~50%) at both 10 and 100 μM. The lack of complete inhibition by MAFP, the most potent FAAH inhibitor available (K_i value for FAAH inhibition, 1–3 nM), suggests that FAAH alone does not directly mediate anandamide transport (De Petrocellis et al., 1997).

To determine the extent of anandamide metabolism following uptake, FAAH assays were performed on the RBL-2H3 cells under conditions comparable to our transport assays. We observed that the majority of anandamide taken up into the cells was rapidly broken down. At 2 and 10 min of uptake, 74 and 68%, respectively, of the transported anandamide had been metabolized (Fig. 3B). The presence of 500 nM MAFP totally inhibited the intracellular breakdown of anandamide but also reduced specific uptake by ~25% (Fig. 3C). These data indicate that even at the 10 min uptake time points ~30% of the accumulated tritium represents intact anand-

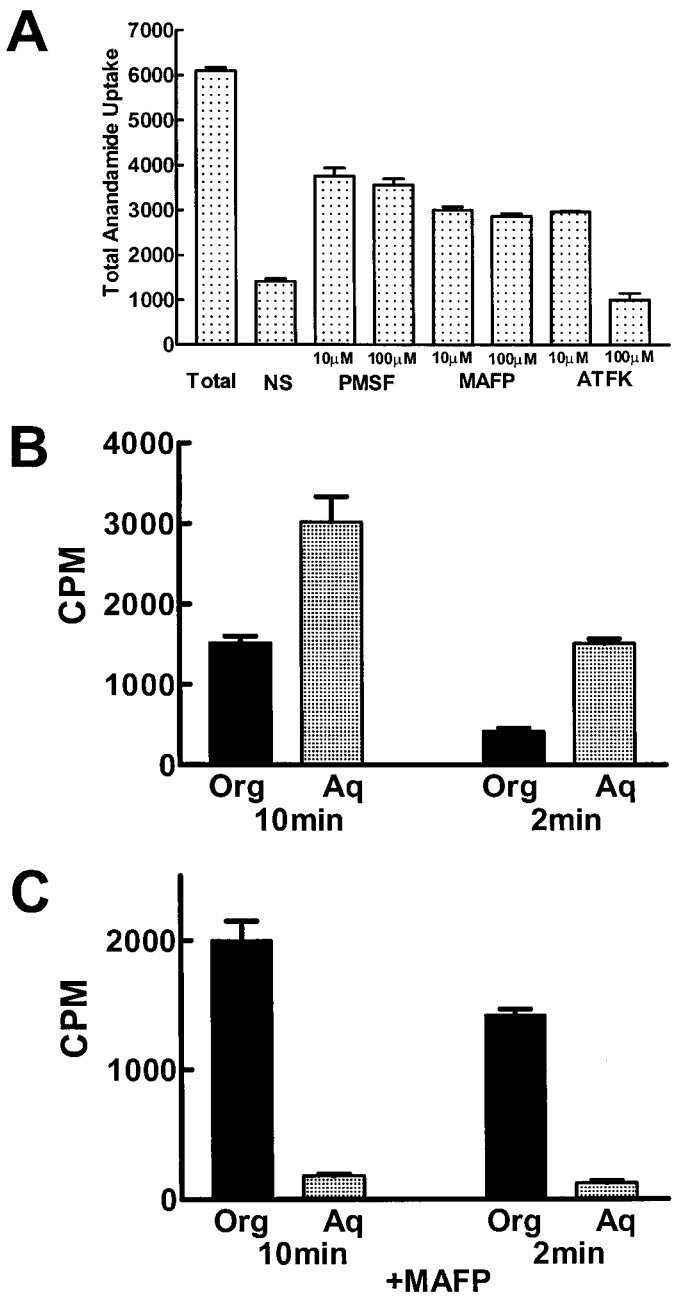

Fig. 3. Role of FAAH in anandamide uptake. A, sensitivity of anandamide uptake to FAAH inhibitors. Anandamide uptake was performed on RBL-2H3 cells as described in *Experimental Procedures*. Cells were treated with 10- and 100-μM concentrations of PMSF, MAFP, and ATFK 10 min before addition of 1 nM [^{3}H]anandamide. Nonspecific (NS) uptake was determined in the presence of 100 μM AM404. Data were plotted as total anandamide uptake and are the means ± S.E. of triplicate determinations and are representative of three separate experiments. B, metabolism of anandamide in RBL-2H3 cells. RBL-2H3 cells were incubated with 5 nM anandamide[ethanolamine 1-^{3}H] for indicated times, and extent of degradation by FAAH was determined as described in *Experimental Procedures*. Org = cpm in organic phase, representing intact, nondegraded anandamide. Aq = cpm in aqueous phase, representing [^{3}H]ethanolamine or metabolized anandamide. Nonspecific uptake was determined in the presence of 100 μM AM404 and subtracted from total uptake values for both phases to obtain cpm of specific anandamide uptake. Data plotted are the means ± S.E. of triplicate determinations and are representative of three separate experiments. Summary data of anandamide degradation: 10 min, 68.0 ± 1.5% metabolized; 2 min, 74.3 ± 3.7% metabolized. C, inhibition of FAAH by MAFP. The presence of 500 nM MAFP reduced the cpm in the aqueous phase (metabolized anandamide) to background levels.

amide, providing further evidence that FAAH alone is not responsible for anandamide uptake. If the anandamide transporter is a facilitative carrier, then FAAH activity could represent a mechanism for maintaining the anandamide concentration gradient needed to promote uptake. Therefore, the ability of FAAH inhibitors to partially inhibit anandamide transport may result from increased intracellular accumulation of intact anandamide that reduces the transmembrane anandamide gradient and overall transport rate. However, using the above data on anandamide metabolism in RBL-2H3 cells, we have estimated, using a predicted cytoplasmic volume of 0.5 to 4.0 pl, that intracellular concentrations of intact anandamide may exceed 100 nM. This concentration would be far greater than the extracellular anandamide concentration (5 nM) used in these assays and would suggest that additional mechanisms for intracellular accumulation of anandamide may exist. Intracellular membrane pools or binding proteins could serve in such a capacity, thereby working in concert with FAAH to maintain the needed inward concentration gradient of free anandamide.

Mechanism of Transport Inhibition by AM404 and Δ^9-THC. To determine how Δ^9-THC and AM404 inhibit anandamide uptake, we performed [^{3}H]anandamide transport saturation experiments in the presence or absence of the proposed transport antagonist. In these experiments, Δ^9-THC (10 μM) produced an increase in the transport K_m value from 10 to 29 μM with no alteration in the V_{max} value, which is consistent with the cannabinoids being competitive antagonists of the anandamide transport system (Fig. 4A). Likewise, increasing concentrations of AM404 (1 or 3 μM) increased transport K_m values from 12 to 88 μM, thus suggesting a competitive-type inhibition on anandamide uptake in RBL-2H3 cells (Fig. 4B). However, 3 μM AM404 treatment also induced a 2-fold increase in the transport V_{max} value ($24.2 \pm 1.8 \times 10^{-17}$ versus $57.7 \pm 3.0 \times 10^{-17}$ mol/min/cell) (Fig. 4B), an effect that is not predicted for a simple competitive inhibitor or, for that matter, any pharmacologic antagonist of a transport system.

Discussion

Whereas anandamide transport activity has been previously reported in RBL-2H3 cells (Bisogno et al., 1997) and characterized in various cell types of the central nervous system (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997; Piomelli et al., 1999), our data represent the first detailed functional and pharmacologic characterization of anandamide transport in a peripheral cell type and support the hypothesis that anandamide transport is carrier-mediated. Similar to studies examining anandamide uptake in central nervous system-derived cells, we observed saturable anandamide uptake that was both time- and temperature-dependent. Interestingly, the K_m and V_{max} values for anandamide transport observed in RBL-2H3 cells are similar to the kinetics of anandamide uptake reported for primary cultures of cerebellar granule cells (Hillard et al., 1997). These kinetic parameter values are in contrast to those from primary cultures of striatal neurons and astrocytes (Beltramo et al., 1997) and CCF-STTG1 astrocytoma cells (Piomelli et al., 1999) that reportedly exhibit a higher-affinity process with a K_m value near or below 1 μM. These latter experiments appear to have relied on Lineweaver-Burk transformations of

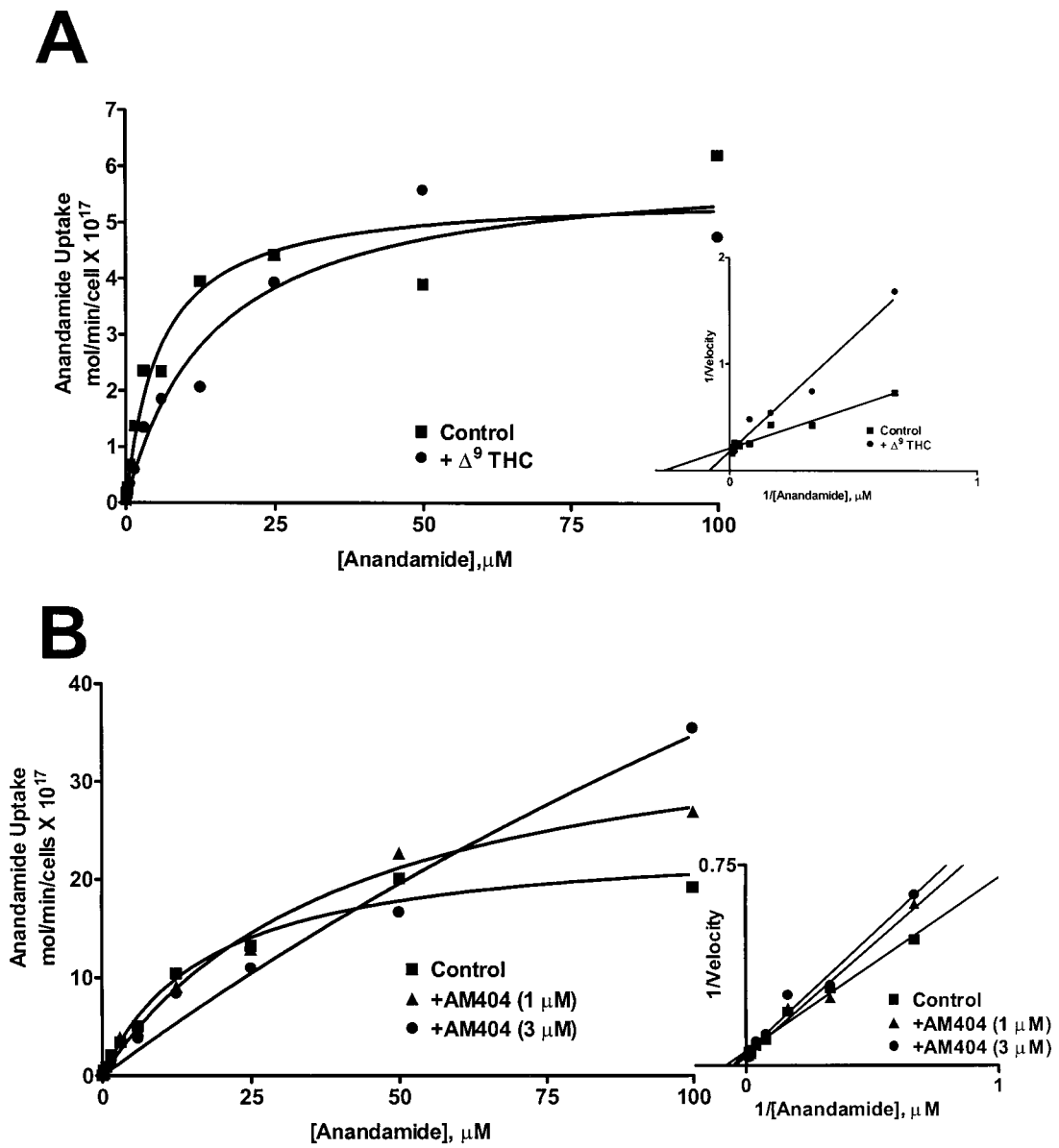

Fig. 4. Competitive inhibition of anandamide uptake by Δ^9-THC and AM404. RBL-2H3 cells were incubated with increasing concentrations of [^{3}H]anandamide for 10 min at 37°C in the presence or absence of 10 μM Δ^9-THC (A) or 1 or 3 μM AM404 (B). Nonspecific uptake was determined in the presence of 100 μM AM404 (A) or 100 μM (*R*)-2-methanandamide (B). Data plotted are means of duplicate determinations and are representative of three separate experiments. Insets, Lineweaver-Burke plots of saturation curves shown above. Summary data for K_m and V_{max} values were: A, control, 10.1 ± 4.0 μM and 5.5 ± 0.5 × 10^{-17} mol/min/cell; +Δ^9-THC, 28.6 ± 9.6 μM and 6.1 ± 0.6 × 10^{-17} mol/min/cell; B, control, 12.3 ± 1.1 μM and 24.2 ± 1.8 × 10^{-17} mol/min/cell; +1 μM AM404, 40.3 ± 17.0 μM and 32.2 ± 5.9 × 10^{-17} mol/min/cell; +3 μM AM404, 88.3 ± 16.3 μM* and 57.7 ± 3.0 × 10^{-17} mol/min/cell*. *$P < .05$ compared with control value.

nonsaturating concentrations of [^{3}H]anandamide to derive kinetic parameters, which might have led to an underestimation of kinetic values. Our values are consistent with Hillard et al. (1997) and suggest that transport kinetics are similar in all of these multiple cell types. Despite potential differences in K_m values, anandamide transport in RBL-2H3 cells was sensitive to inhibition by the transport blocker AM404, suggesting that the uptake process in these cells is similar to that observed in the central nervous system. The only distinction that we have observed between anandamide uptake in peripheral and central nervous system cell types is our data indicating sensitivity to arachidonic acid. We believe the molecular identification of the protein involved with anandamide transport in both the periphery and central nervous system will provide insight into this functional difference.

Considerable debate has persisted regarding protein-mediated uptake of highly lipophilic compounds such as the long-chain fatty acids and related molecules (Kamp and Hamilton, 1993; Hamilton, 1998). The molecular cloning of proteins that when heterologously expressed in mammalian cells promote uptake of these compounds would strongly suggest that uptake of lipophilic substrates can be facilitated by carrier-mediated mechanisms. For the long-chain fatty acids, at least three classes of proteins have been identified that promote uptake of oleic and arachidonic acids: the fatty acid translocase (Abumrad et al., 1993), a plasma membrane fatty-acid-binding protein (Isola et al., 1995), and the fatty acid

transport proteins (Schaffer and Lodish, 1994; Hirsch et al., 1998). In addition to uptake of fatty acids, transporters have been identified for mevalonate (Garcia et al., 1994) as well as prostaglandins (Kanai et al., 1995). What type of transport protein might mediate uptake of anandamide and the other endocannabinoids? Classically, there are two major types of transport proteins for physiologic substances: 1) active cotransport systems that rely on either cellular ATP or ion gradients to drive substrate translocation or 2) facilitative carriers that depend more on substrate concentration gradients to promote transport (Stein, 1986). Transporters for most neurotransmitters, such as glutamate and the biogenic amines, are Na^+-dependent and thus fall into the former classification (Nelson et al., 1998). Some glucose transporters are also Na^+-dependent, whereas others, including those that are insulin stimulated, are facilitated-diffusion carriers with glucose transported down its own concentration gradient (Hediger et al., 1987; Takata et al., 1993). Anandamide transport is not coupled to ion (Na^+, Cl^-, or H^+) gradients (present data; Beltramo et al., 1997; Hillard et al., 1997) or to ATP (Hillard et al., 1997), thus suggesting that the anandamide transporter is most likely a facilitative carrier. As suggested by previously reported data (Hillard et al., 1997), such a transport protein could, in fact, function as a bidirectional carrier, participating not only in anandamide uptake but also anandamide release. To explore this possibility, more detailed mechanistic studies should be performed to determine the functional symmetry of the anandamide transport system.

The distinct pharmacologic sensitivity of the anandamide transporter in RBL-2H3 cells provides the basis for future studies aimed at developing potent and selective transport inhibitors. The panel of compounds that we identified as having transport blocking activity display a range of potencies, suggesting some distinctions in the molecular recognition of these drugs. For example, the endocannabinoids displayed a rank order of potency consisting of 2-AG > oleoylethanolamide > palmitoylethanolamide. These data might suggest that some, but not all, endogenous cannabinoids are substrates for the anandamide transporter. Indeed, Piomelli et al. (1999) have reported a detailed characterization of structural determinants required for recognition by this uptake system. These structure-activity studies, using a set of radiolabeled compounds, revealed that [^{3}H]AM404 and [^{3}H]2-AG are transported into CCF-STTG1 astrocytoma cells with transport kinetics similar to those for anandamide; however, [^{3}H]oleoylethanolamide and [^{3}H]palmitoylethanolamide were not readily transported into these cells (Piomelli et al., 1999). The fact that AM404 is also a substrate for the anandamide transporter indicates that AM404 most likely acts as a competitive substrate to produce an inhibitory effect. Consistent with AM404 being a competitive inhibitor of anandamide uptake, we observed an increase in the anandamide transport K_m value in the presence of AM404. In addition to the effect on the K_m value, we also observed that AM404 produced a 2-fold increase in transport V_{max}. The observed increase in the V_{max} value could result from regulatory effects of AM404 directly on the transporter or allosteric effects of elevated extracellular anandamide concentrations on the transporter and/or putative associated proteins.

Many of the pharmacologic and psychotropic effects associated with the marijuana-derived cannabinoids such as Δ^9-THC are presumed to be the result of activation of CB1 receptors (Howlett, 1995). Interestingly, we observed that many of the cannabinoids, including Δ^9-THC, Δ^8-THC, and cannabidiol, also possess anandamide transport blocking activity. Pharmacologic studies suggest that Δ^9-THC is a simple competitive inhibitor of uptake, although the potency for transport inhibition is approximately 100-fold less than the potency required for activation of cannabinoid receptors. In addition, cannabidiol is a nonpsychotropic cannabinoid and is essentially devoid of agonist activity at the CB1 receptor, yet this compound is as potent as AM404 for anandamide transport inhibition. If a cannabinoid were to block anandamide uptake, thereby enhancing endogenous cannabimimetic activity, this would be a novel mechanism by which the Δ^9-THC derivatives exert their pharmacologic actions in addition to interactions with the cannabinoid receptors. The transport inhibitor AM404 potentiates the effects of anandamide both in vitro and in vivo (Beltramo et al., 1997; Calignano et al., 1997). The pharmacologic significance of cannabinoid-mediated transport blockade should be investigated in future studies seeking to determine whether cannabidiol, which is inactive at the CB1 receptor, can also prolong the effects of anandamide in a manner similar to AM404.

The identification of anandamide uptake in RBL-2H3 cells represents the third component of endocannabinoid signaling characterized in these cells. In addition to the uptake process, a CB2-like receptor (Facci et al., 1995) and the FAAH activity responsible for anandamide metabolism (Bisogno et al., 1997) have been demonstrated in RBL-2H3 cells. RBL-2H3 cells are a cognate mast cell line and thus represent a model system for studying inflammatory processes and, in particular, cannabinoid-mediated modulation of hypersensitivity and inflammatory reactions. Cannabinoids have long been recognized as having immunomodulatory activity, including effects on T-cell proliferation, NK cell cytolysis, macrophage-mediated tumorcidal activity, and mast cell activation (Klein et al., 1998). The fatty acid amide, palmitoylethanolamide, appears to have anti-inflammatory properties that are mediated via activation of the CB2 receptor (Facci et al., 1995; Skaper et al., 1996). By comparison, anandamide lacks intrinsic activity at the mast cell CB2 receptor, thus failing to prevent mast cell degranulation; however, anandamide is capable of antagonizing the anti-inflammatory effects of palmitoylethanolamide (Facci et al., 1995). Our data indicate that palmitoylethanolamide is not recognized by the anandamide transport system, thus a separate distinct transporter may exist for this fatty acid amide. Certain pathologic conditions, such as inflammation and ischemia, may be capable of regulating the uptake system for palmitoylethanolamide because these disorders appear to increase tissue accumulation of palmitoylethanolamide and other free *N*-acylamides (Natarajan et al., 1982). Identification of a specific uptake system for palmitoylethanolamide would be of great clinical interest as a putative pharmacologic target for the treatment of inflammatory diseases, whereby transport blockade would result in increases in extracellular palmitoylethanolamide and augmentation of the anti-inflammatory effects of the compound. If anandamide does not activate the CB2 receptor found in RBL-2H3 cells and these cells lack CB1 receptors (Facci et al., 1995), then why would RBL-2H3 cells demonstrate robust anandamide transport activity? Because anandamide is capable of blocking the anti-

inflammatory actions of palmitoylethanolamide, perhaps the anandamide transporter in RBL-2H3 cells serves as a primary mechanism for regulating the antagonistic action of anandamide at the CB2 receptor, providing for precise regulation of the fatty acid amide modulation of mast cell activation.

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