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Membrane microdomains and metabolic pathways that define anandamide and 2-arachidonoyl glycerol biosynthesis and breakdown

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Abstract

Anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), endogenous ligands for the CB1 and CB2 cannabinoid receptors, are referred to as endocannabinoids because they mimic the actions of delta⁹-tetrahydrocannabinol (Δ^9 -THC), a plant-derived cannabinoid. The processes by which AEA and 2-AG are biosynthesized, released, taken up by cells and hydrolyzed have been of much interest as potential therapeutic targets. In this review we will discuss the progress that has been made to characterize the primary pathways for AEA and 2-AG formation and breakdown as well as the role that specialized membrane microdomains known as lipid rafts play in these processes. Furthermore we will review the recent advances made to track and detect AEA in biological matrices.

A list of key words

Cannabinoid; Anandamide; 2-Arachidonoyl glycerol; Delta⁹-tetrahydrocannabinol; Membrane Microdomain

Introduction

Delta-9-tetrahydrocannabinol (Δ^9 -THC) is the primary psychoactive ingredient of marijuana, a plant-derived cannabinoid (Childers and Breivogel, 1998). Δ^9 -THC produces a range of pharmacological effects in the central nervous system and in the periphery. Currently Δ^9 -THC and its analogs are being used as anti-emetic agents in the treatment of nausea and vomiting induced by radio- or chemotherapy and severe anorexia (wasting syndrome) in patients with AIDS. Furthermore, Δ^9 -THC may also be useful for the treatment of glaucoma, spasticity, and pain, however, the beneficial therapeutic effects of Δ^9 -THC are hampered by the psychotropic side effects of euphoria elicited by plant-derived cannabinoids (Watson et al., 2000). Therefore, a drug target system has to be exploited that will produce the positive therapeutic effects of Δ^9 -THC without the undesirable side effects.

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The pharmacologic properties of both plant-derived cannabinoids and endogenous cannabinoids (endocannabinoids) are similar. Since the discovery of endocannabinoids in the early 1990s, the actions of these substances have been rigorously studied. Endocannabinoids as well as Δ^9 -THC mediate their effects primarily via the G protein-coupled receptors (GPCR) type 1 cannabinoid (CB1) and type 2 cannabinoid (CB2) (Matsuda et al., 1990; Munro et al., 1993). Additionally, two orphan G-protein coupled non-CB1/CB2 receptors have been implicated to be a part of the endocannabinoid family, GPR55 and GPR119 (Brown, 2007). Anandamide (AEA) has also been shown to have agonist activity at the vanilloid receptor (VR1) that is a member of the transient receptor potential (TRP) superfamily of ion channels (Smart et al., 2000; Zygmunt et al., 1999).

AEA and 2-arachidonoylglycerol (2-AG) are the most studied members of the endocannabinoid family, although multiple pathways for endocannabinoid synthesis have been described, the prevalent pathway for *in vivo* biosynthesis has yet to be determined (Liu et al., 2006; Okamoto et al., 2004; Simon and Cravatt, 2006; Simon and Cravatt, 2008). The diversity of the signaling pathways in which AEA, 2-AG and other endocannabinoids partake, implies the significant roles that these molecules play in various physiological conditions. For example, endocannabinoids have been linked to retrograde signaling in various regions of the brain (Chevalleyre et al., 2006). Once released from the postsynaptic neuron, the endocannabinoid molecule travels in a retrograde fashion to transiently suppress the presynaptic neurotransmitter release through activation of the target cannabinoid receptors (Chevalleyre et al., 2006). Retrograde signaling modulated by endocannabinoids is critical for certain types of short-term and long-term synaptic plasticity at excitatory or inhibitory synapses and contributes to certain aspects of brain function including learning and memory (reviewed in Hashimoto et al., 2007). CB1 receptor-knockout mice have been shown to exhibit behavioral abnormalities and multiple defects in synaptic plasticity, supporting the notion that endocannabinoid signaling is involved in neural function (Kishimoto and Kano, 2006; Steiner et al., 1999). The endocannabinoid system has also been linked to a variety of neuropsychiatric and neurodegenerative disorders such as anxiety, prolonged stress, depression, psychosis, Alzheimer's, Parkinson's, and Huntington's Chorea disorders (reviewed in Bisogno and Di Marzo, 2007). Additionally, endocannabinoid signaling has been implicated in several physiological processes such as pain, nausea, reproduction, immune response, and cancer, indicating the diversity of biological targets that endocannabinoids modulate (Di Carlo and Izzo, 2003; Guzmán, 2003; Habayeb et al., 2002; Karsak et al., 2007; Parolaro et al., 2002; Pertwee, 2001; Tramèr et al., 2001). Characterization and pharmacologic manipulation of the endocannabinoid system is important because it may allow us to identify new drug targets and develop therapeutics for the treatment of many disorders without the undesirable side effects that plant-derived cannabinoids produce.

Biosynthesis of Endocannabinoids

Unlike the typical neurotransmitter molecules that are stored in and recycled into vesicles, endocannabinoids are synthesized and released "on demand." The processes that underlie the biosynthesis of endocannabinoids have been extensively studied for 2-AG and AEA. It is believed that 2-AG and AEA as well as their congeners are stored in the membrane as phospholipid precursors (Piomelli, 2003). The endocannabinoid 2-AG is synthesized from diacylglycerol (DAG) via the actions of sn1-specific DAG lipase in a calcium-dependent fashion (Bisogno et al., 2003), although PLC-independent mechanisms for 2-AG formation have also been suggested (Bisogno et al., 1999; Carrier et al., 2004). AEA is generally believed to be synthesized from a precursor, N-arachidonyl phosphatidylethanolamine (NAPE) in a calcium-dependent fashion by an enzyme NAPE phospholipase D (NAPE PLD) (Di Marzo et al., 1994; Okamoto et al., 2004). However, calcium-independent AEA biosynthesis has been described as well (Vellani et al., 2008). Two additional pathways have been suggested for the

biosynthesis of AEA, and these mechanisms appear to be cell-type specific: formation of phosphoAEA by phospholipase C (PLC) and subsequent dephosphorylation of phosphoAEA by the protein tyrosine phosphatase 22, PTPN22; and α/β hydrolase 4 (Abh4)-dependent NAPE hydrolysis followed by a phosphodiesterase cleavage step modulated by glycerophosphodiesterase 1 (GDE1).

Pathway 1 for AEA biosynthesis involves NAPE PLD—N-acyl ethanolamine (NAE) synthesis has been suggested to take place in two sequential steps (Fig. 1A). The first reaction, also considered to be the rate-limiting step, involves the transfer of a fatty acyl chain from the *sn*-1 position of a glycerolipid to phosphatidylethanolamine in a calcium-dependent fashion by an N-acyl transferase (NAT), yielding NAPE (Okamoto et al., 2007). Unsuccessful attempts have been made to clone NAT, however, Jin and colleagues (2007) discovered an enzyme that is capable of functioning as an N-acyl transferase, and called it lecithin-retinol acyltransferase-like protein (RLP-1) (Jin et al., 2007). The reaction catalyzed by RLP-1 yielded NAPE when phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were used as substrates (Jin et al., 2007). RLP-1, however, did not show selectivity between *sn*-1 and *sn*-2 positions of the acyl donor, was only modestly activated by calcium, and was predominantly expressed in the cytosolic fraction of the cell (Jin et al., 2007). NAT partially purified from brain tissue was specific for the *sn*-1 acyl group, highly calcium-dependent, and localized to the membrane fraction of the cell (Jin et al., 2007). Therefore, RLP-1 may function as a transferase in the process of AEA biosynthesis in the testis where RLP-1 is predominantly expressed, however a different NAT may be involved in the biosynthesis of AEA in the brain.

The second reaction in the formation of AEA is catalyzed by a calcium-dependent NAPE PLD and results in the formation of the NAE specific to its precursor. Okamoto and colleagues (2004) cloned a phospholipase D that specifically biosynthesized NAEs, including AEA (Okamoto et al., 2004). The involvement of NAPE PLD in the process of AEA biosynthesis remains unclear because the levels of polyunsaturated NAEs, including AEA remained unchanged in the brains of NAPE PLD knock-out mice (Leung et al., 2006). Also, the regional distribution of NAPE PLD does not always correlate with the distribution of the CB1 receptor and fatty acid amide hydrolase (FAAH), an enzyme that metabolizes AEA. NAPE PLD has been found to be highly expressed in the thalamus, the dentate gyrus, the cortex and the hippocampus, whereas the CB1 receptor and FAAH are predominantly expressed in the hippocampus, cortex, and cerebellum (Cristino et al., 2008; Egertová et al., 1998; Egertová et al., 2008; Morishita et al., 2005; Nyilas et al., 2008; Suarez et al., 2008). It appears that NAPE PLD may catalyze AEA formation *in vitro*, but additional enzymatic routes may prevail *in vivo*.

In addition, a lysoPLD enzyme, distinct from NAPE PLD, can utilize N-arachidonyl-lyso phosphatidylethanolamine as an active substrate for AEA formation (Sun et al., 2004). The involvement of lysoPLD in the processes of AEA biosynthesis *in vivo* remains unclear.

Pathway 2 for AEA biosynthesis involves PLC/PTPN22—An alternative route for AEA formation in mouse brain and RAW264.7 macrophages has been proposed to involve phospholipase C (PLC)-modulated hydrolysis of NAPE to generate a phosphoAEA intermediate, independent of NAPE PLD (Fig. 1B) (Liu et al., 2006). Treatment of RAW 264.7 macrophages with bacterial endotoxin lipopolysaccharide (LPS) upregulated the expression levels of a protein tyrosine phosphatase, PTPN22, which then dephosphorylated the phosphoAEA (Liu et al., 2006). Interestingly, the authors determined that treatment of RAW 264.7 macrophages with LPS caused a dramatic decrease in NAPE PLD gene expression, suggesting that AEA formation in this system does not involve NAPE PLD (Liu et al., 2006). The involvement of PTPN22 in the biosynthesis of AEA remains ambiguous because other phosphatases may be involved in the dephosphorylation of phosphoAEA as well.

Pathway 3 for AEA biosynthesis involves Abh4—An additional possible mechanism for the biosynthesis of AEA implicates double-deacylation of NAPE catalyzed by a serine hydrolase to form a glycerophospho-NAPE (GP-NAPE) molecule (Fig. 1C) (Simon and Cravatt, 2006). A new candidate for the selective hydrolysis of NAPE and lysoNAPE has been proposed to be the enzyme Abh4, a lysoNAPE lipase, isolated and identified by Simon and Cravatt (Simon and Cravatt, 2006). Activity-based proteomic analysis in concert with conventional protein chromatography characterized Abh4 as a B-type NAPE lipase that removes both O-acyl chains from NAPE to yield GP-NAE (Simon and Cravatt, 2006). Abh4 has been implicated in the catalysis of both O-deacylation steps in the generation of the GP-NAE intermediate (Simon and Cravatt, 2006). Overall, Abh4 possesses characteristics expected of an enzyme involved in the biosynthesis of AEA: expression in the CNS and testes, selectivity for NAPEs with distinct N-acyl chains, and preference for lysoNAPEs over other lysolipids (Simon and Cravatt, 2006). Once the GP-NAE intermediate is formed, a phosphodiesterase-modulated cleavage of the GP-NAE molecule takes place to generate a free NAE molecule and a glycerol-3-phosphate (Simon and Cravatt, 2006). Recently, Simon and Cravatt determined the identity of the enzyme that has robust GP-NAE phosphodiesterase activity—glycerophosphodiesterase GDE1, also known as MIR16 (Simon and Cravatt, 2008). The multistep pathway for the production of anandamide in the nervous system by the sequential actions of Abh4 and GDE1 may, in fact, turn out to be the prevalent pathway for AEA synthesis *in vivo*. In the future, genetic and pharmacologic approaches should be used to further characterize the roles of Abh4 and GDE1 in the biosynthesis of AEA.

The dopaminergic, cholinergic, and glutamatergic systems have also been implied in the biosynthesis of endocannabinoids. In 1999, Giuffrida and colleagues reported that the release of AEA was significantly increased after administration of D2-like dopamine receptor agonist quinpirole in the dorsal striatum (Giuffrida et al., 1999). Activation of muscarinic acetylcholine receptors with carbachol has been shown to trigger endocannabinoid synthesis and release in the hippocampus (Kim et al., 2002). Additionally, Varma and colleagues (2001) reported that the activation of metabotropic glutamate receptors in pyramidal cells may drive the release of endocannabinoids (Varma et al., 2001). Activation of both muscarinic acetylcholine and metabotropic glutamate receptors resulted in increases in depolarization-induced suppression of inhibition, confirming the role of endocannabinoids in retrograde signaling (Kim et al., 2002; Varma et al., 2001). In the future, the specific mechanisms that underlie endocannabinoid biosynthesis in response to activation of the dopaminergic, cholinergic and glutamatergic systems should be elucidated.

The role of lipid rafts in the biosynthesis of endocannabinoids

Whereas several pathways involved with endocannabinoid biosynthesis have been described, the involvement of specialized membrane microdomains in endocannabinoid production has been proposed. The specialized microdomains within the plasma membrane that are enriched in cholesterol, sphingolipids, plasmalogen ethanolamine, and arachidonic acid are referred to as lipid rafts (Brown and London, 2000; Pike et al., 2002). Caveolae are characterized by a similar lipid composition as lipid rafts and express a group of structural proteins known as caveolins that promote flask-shaped invaginations in the plasma membrane (Pike et al., 2002; Razani et al., 2002).

Lipid rafts and caveolae serve as important platforms for regulation of a variety of processes linked to the endocannabinoid system (McFarland and Barker, 2005). In particular, lipid rafts/caveolae have been implicated in the modulation of the CB1 receptor binding and signaling (Bari et al., 2005). Treatment of C₆ glioma cells with methyl- β -cyclodextrin (MCD), an agent that sequesters cholesterol and disrupts lipid rafts, results in an enhanced binding response of the CB1 receptor agonist CP 55940 to CB1 receptors and subsequent increase in G protein-

mediated signaling through adenylate cyclase and MAPK (Bari et al., 2005). The role of lipid rafts/caveolae has also been established in the modulation of AEA uptake (McFarland et al., 2008; McFarland et al., 2004). The pharmacologic disruption of the lipid raft microorganization results in an attenuation of AEA uptake (McFarland et al., 2008; McFarland et al., 2004). These and other data suggest that lipid raft/caveolae organization may be critical in the process of AEA uptake. Furthermore, the AEA metabolites arachidonic acid (AA) and ethanolamine become enriched in the lipid raft portion of the cell (McFarland et al., 2004). These metabolites, AA and ethanolamine, can then be utilized by the cell for recycling, synthesis, and release of new AEA molecules (McFarland et al., 2006). In other words, the phenomenon of recycling implies that intact AEA molecules are taken up by the cell, metabolized into AA and ethanolamine, and then these metabolites are efficiently re-used by the cell to form new intact AEA molecules.

The mechanism by which the biosynthesis of AEA is modulated within the lipid rafts remains unknown. Wettschureck and colleagues (2006) determined that endocannabinoid biosynthesis was impaired in the absence of G_q/G_{11} , G proteins that modulate various signaling events via lipid raft-dependent mechanisms (Sugawara et al., 2007; Wettschureck et al., 2006). Furthermore, we established that the cloned NAPE PLD may not be involved in the synthesis and release of AEA from the lipid rafts in RBL-2H3 cells because immunocytochemistry studies showed no overlap in the localization between NAPE PLD and flotillin-1, a marker for lipid rafts (Fig. 2). Therefore, other pathways may be involved in the biosynthesis of AEA in the lipid raft microdomains in RBL-2H3 cells. Rimmerman and colleagues (2008) reported that NAPE PLD was distributed among both the lipid raft and non-lipid raft fractions of the DRG X neuroblastoma F-11 cell line (Rimmerman et al., 2008). The finding that NAPE PLD did not localize with lipid raft markers in the cognate mast RBL-2H3 cells, and only a fraction of NAPE PLD localized to the lipid rafts in the neuroblastoma F-11 cell line may indicate that NAPE PLD has different distribution patterns that depend on the origin of the cell line. Rimmerman et. al. also discovered that the distribution of NAPE PLD and endogenous AEA was similar--in both lipid raft and non-lipid raft fractions (Rimmerman et al., 2008). The authors attributed the phenomenon of the similar distribution patterns of AEA and NAPE PLD to increased production of AEA in compartments that contain higher levels of the AEA synthetic enzyme (Rimmerman et al., 2008). It has not been established, however, that NAPE PLD is responsible for the formation of AEA in the F-11 cells. In fact, many studies report that NAPE PLD may not be involved in the biosynthesis of AEA *in vivo* (Leung et al., 2006; Simon and Cravatt, 2008).

The deuterated AA derived from exogenously-applied deuterated AEA co-localized mostly with non-lipid raft fractions (Rimmerman et al., 2008). In these experiments, Rimmerman and colleagues only determined the population of free AA molecules, and AA that had been re-esterified and incorporated into phospholipids or other fatty acid metabolites was not taken into account. In prior studies, McFarland and colleagues did not discriminate between the populations of free AA and its metabolites and observed an enrichment of tritium derived from AEA in the lipid raft fractions (McFarland et al., 2004). Examining the localization of free AA derived from AEA may be insufficient because most of the AA derived from AEA metabolism may be re-esterified into AA-containing lipids that have been trafficked to the membrane.

The biochemical machinery for the production of 2-AG is localized within lipid rafts suggesting that 2-AG synthesis via DAG occurs within these microdomains (Rimmerman et al., 2008). The finding that 2-AG and the synthetic machinery for the production of 2-AG, DAG lipase, were localized to the lipid raft portion of the cell is consistent with the results obtained in our lab. We discovered that exogenously applied AEA may be recycled by the RBL-2H3 cells to form not only new AEA molecules (as noted above), but also new 2-AG molecules (McFarland et al., 2006).

Cannabimimetic substances, related lipids, and novel cannabinoid targets

Besides AEA and 2-AG, other members of the endocannabinoid family have been discovered. Molecules such as N-arachidonyl dopamine (NADA), noladin ether (also called 2-arachidonl glycerol ether), oleamide, and virodhamine display affinity for the cannabinoid receptors, but have been studied much less in comparison to AEA and 2-AG (Hanus et al., 2001; Huang et al., 2002; Leggett et al., 2004; Porter et al., 2002).

There are also several examples in which the observed pharmacology is not consistent with the signaling of the classical cannabinoid CB1 and CB2 receptors. Other lipid mediators, despite the fact that they show no appreciable affinity for the CB1/CB2 cannabinoid receptors, display cannabimimetic effects and modulate pain, immune function, reproduction, and appetite (Tan et al., 2006). For example, oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), two naturally occurring acylethanolamides lack affinity for the cannabinoid CB1/CB2 receptors, and yet evoke effects consistent with cannabinoid pharmacology, such as stimulation of food intake and pain modulation, respectively (Lambert and Di Marzo, 1999; Lambert et al., 1999; Rodríguez de Fonseca et al., 2001). Accordingly, there has been a significant effort to discover the novel target(s) for cannabinoid ligands. Studies using genetic ablation of the CB1 and/or CB2 receptors in mice have confirmed the existence of additional non-CB1/CB2 receptor targets (reviewed in Brown, 2007). These targets include GPR119 and GPR55, orphan GPCRs that have been proposed to be novel receptors for fatty acid amides and endocannabinoids such as OEA and PEA, respectively (Fu et al., 2005; Lo Verme et al., 2005; Mackie and Stella, 2006; Overton et al., 2006). OEA endogenously targets GPR119 and PEA may activate GPR55 in addition to other targets, such as peroxisome proliferators activated receptors (PPARs) (Fu et al., 2005; Lo Verme et al., 2005; Mackie and Stella, 2006; Overton et al., 2006). However, further studies elucidating whether the GPR119 receptor is a true cannabinoid receptor should be conducted because OEA is not classified as a classical endocannabinoid.

Endocannabinoid lipidomics and mass spectrometry

The various endocannabinoids and related lipid mediators may function via overlapping signaling pathways and regulate the physiology of the classical cannabinoid system. In order to study the overlap between the signaling events that modulate the classical and the novel cannabinoid systems there is a need for accurate, precise, and timely detection approaches. Detection of the entire profile of endocannabinoid molecules produced *in vivo* is crucial for the characterization of the endocannabinoid system, and may allow us to identify other compounds that modulate the signaling of both the known endocannabinoids and the novel targets.

The sensitivity of endocannabinoid detection methods may limit the analysis of these lipids. Originally, various groups used thin layer chromatography (TLC) to detect endocannabinoid levels (Deutsch et al., 2001; Parrish and Nichols, 2006). The drawback of using the TLC method is that although the detection of AEA and 2-AG is achieved without difficulty, a profile of a range of endocannabinoids is not easily characterized and/or resolved. Detection of a variety of endocannabinoid molecules is necessary for the full characterization of the endocannabinoid system. In order to sufficiently characterize the signaling events that endocannabinoids modulate the entire endocannabinoid lipidome must be analyzed not just the two predominant species, AEA and 2-AG.

Advances in mass spectrometry (MS) allow for detection of many novel signaling lipids as well as simultaneous monitoring of the known members of the endocannabinoid family. For example, in 2002, Porter and colleagues identified a novel endocannabinoid, virodhamine, an AA molecule joined to ethanolamine by an ester linkage, in contrast to AEA that is joined by

the amide linkage (Porter et al., 2002). Virodhamine displays affinity for the CB1 cannabinoid receptor and acts as a partial agonist that possesses *in vivo* antagonist activity, whereas at the CB2 receptor, virodhamine displays full agonist activity (Porter et al., 2002). Virodhamine was discovered unexpectedly when Porter and colleagues were measuring tissue levels of AEA using Liquid Chromatographic Atmospheric Pressure Chemical Ionization Tandem Mass Spectrometry (LC-APCI-MS-MS), and an analyte with a different retention time but the same molecular weight as AEA was detected (Porter et al., 2002). Therefore, mass spectrometry may prove to be better suited for the detection of endocannabinoids and other related compounds than previously existing methods.

A sensitive and selective method for quantitation and detection of endocannabinoids involves a single or multiple reaction monitoring (SRM or MRM) modes on a triple-quadrupole mass spectrometer (Kingsley and Marnett, 2003; Kinglsey and Marnett, 2007; Richardson et al., 2007). The sensitivity is achieved on the triple-quadrupole mass spectrometer through monitoring of a single fragment species from a single precursor ion, an approach that allows for elimination of unnecessary background noise. Recently, Richardson and colleagues reported a quantitative, validated LC-MS-MS method using a triple quadrupole mass spectrometer for simultaneous detection of AEA, 2-AG, six other endocannabinoid species, and additional related compounds (Richardson et al., 2007). The limit of detection for AEA, oleoyl ethanolamide, palmitoyl ethanolamide, 2-linoleoyl glycerol, and noladin ether was reported to be 10 pmol/g, and for 2-AG 100 pmol/g, proving the LC-MS-MS technique to be extremely sensitive and suitable for the detection of species within the endocannabinoid family (Richardson et al., 2007).

In a different study, Hardison and colleagues reported that precise and reproducible quantification of endocannabinoids can be achieved using purification with C₁₈ solid-phase extraction prior to analysis with gas chromatography mass spectrometry (GC-MS) (Hardison et al., 2006). The limit of quantification for AEA was found to be 20 pmol/g (Hardison et al., 2006). Thus, it appears that the LC-MS-MS approach allows for more sensitive quantification of AEA. Furthermore, the LC-MS-MS technique does not require sample derivatization prior to analysis, whereas the GC-MS approach does. Also, a quantitative profile of endogenous cannabinoids has not yet been characterized by GC-MS. Both approaches, LC-MS-MS and GC-MS, however permit for quantitative and reproducible detection of AEA and 2-AG.

The limit of detection for endocannabinoids may potentially be improved on the LC-MS-MS instruments by exploiting silver cation coordination to the arachidonate backbone of these eicosanoid-like molecules (Kingsley and Marnett, 2003). The π electrons in the double bonds within the arachidonate backbone of neutral molecules such as AEA and 2-AG provide binding sites for the silver cations, resulting in a $[M+Ag]^+$ species that easily undergo conversion to the gas phase via an electrospray ionization (ESI) source. For example, Kingsley and Marnett reported a significant improvement in 2-AG detection as the $[M+Ag]^+$ species resulted in a more intense signal than any fragment of the non- Ag^+ species (Kingsley and Marnett, 2003). The authors reported that on average 13.4 nmol/g of 2-AG and 10.8 pmol/g of AEA were detected in mouse brain tissue, results consistent with previous reports (Kingsley and Marnett, 2003). The use of silver ion coordination facilitates SRM analysis for 2-AG. The ease of the analysis and specificity of the method make it ideal for endocannabinoid quantification in a range of experimental settings. In addition, the method should be readily adaptable to the analysis of other neutral lipids on other instruments including time-of-flight (TOF) mass spectrometers.

The recent advances in lipidomics and mass spectrometry analysis may allow for identification of novel endocannabinoids and related compounds that possess important biological signaling functions. Such approaches are critical to further our understanding of endocannabinoid

biology. Recently, Williams and colleagues (2007) devised and validated a robust, selective, and sensitive LC-MS-MS method for analyzing a variety of endocannabinoid molecules in a biological matrix (Williams et al., 2007). The authors expanded the endocannabinoid metabolome to include six different core fatty acid molecules and their corresponding glyceride and ethanolamide derivatives that varied in size and degrees of saturation (Williams et al., 2007). In particular, the core fatty acid molecules were represented by arachidonic acid (20:4), docosahexaenoic (22:6), eicosapentaenoic (20:5), eicosenoic (20:1), oleic (18:1), and palmitic (16:0) acid species among others (Williams et al., 2007).

The structural diversity represented by the species analyzed posed two main challenges in the development of the method—the suitable chromatographic separation and the appropriate solvent system. First, the hydrophobic nature of C8 or C18 columns would have been inadequate to allow for proper separation of the endocannabinoid metabolites. Therefore, the authors used a cyano column, which was able to provide the system with sufficient resolution (Williams et al., 2007). Secondly, it was necessary to determine the proper conditions for a mobile phase that would be capable of allowing for positive ionization of the ethanolamide and glycerol derivatives to take place and negative ionization of the free acids under the APCI conditions (Williams et al., 2007). The best-suited solvent system for the resolution of the related, yet structurally diverse members of the endocannabinoid metabolome proved to be a 10 mM ammonium acetate aqueous solution (pH 7.3) and methanol as the organic phase (Williams et al., 2007). The method established by Williams and colleagues provides a way to simultaneously detect eight metabolites that possess different structures and ionization efficiencies, thus, doubling the number of endocannabinoids that can be quantified simultaneously (Williams et al., 2007). The validity of the method was confirmed when rat frontal cortex tissue was analyzed for the endocannabinoid content (Williams et al., 2007).

As our knowledge of the endocannabinoid lipidome grows, it is crucial to further develop detection approaches such as MS. The MS technology may allow us to gain insight not only into the content but also into the distribution of endocannabinoids within the membrane microdomains of the cell (Rimmerman et al., 2008). The ability to identify endocannabinoids, precursors, and corresponding metabolites in specific intracellular compartments using MS can provide new opportunities to identify pathways involved with biosynthesis and metabolism. The levels and distribution of and interplay among all the endocannabinoid family members under diverse physiological conditions may aid in the development of novel therapeutics in the treatment of pathological conditions in which endocannabinoids are involved.

Uptake of endocannabinoids

The process by which cells transport AEA is crucial to understanding the inactivation of endocannabinoids because in order to be metabolized, AEA must first be trafficked to the cellular compartments in which FAAH is located (Cravatt et al., 1996). Two known isoforms of FAAH have been discovered: FAAH-1, specific for NAEs, and FAAH-2, specific for primary fatty acid amide substrates (Wei et al., 2006). For the purpose of this review, we will only focus on the FAAH-1 isoform because it is specific for AEA hydrolysis and refer to this enzyme as FAAH throughout the article.

To date, the identity of the protein(s) involved in the uptake and transport of AEA is unknown. Prior to describing the current models of AEA uptake it should be noted that the processes of AEA uptake may be intimately linked with the processes of AEA release from the cell, indicating that the biosynthetic machinery involved in the uptake process may also play a major role in endocannabinoid release (Adermark and Lovinger, 2007; Hillard et al., 1997; Ligresti et al., 2004; Ronesi et al., 2004). The uptake of AEA, however, has been characterized as a rapid, saturable, temperature-dependent, ion gradient- and ATP-independent process that can

be inhibited in a dose-dependent manner by AEA analogues or fatty acid amide derivatives (Beltramo et al., 1997; Bisogno et al., 1997; Di Marzo et al., 1994; McFarland and Barker, 2004). Based on the existing literature, there are four models of AEA uptake: 1) Carrier protein-mediated transport; 2) Facilitated FAAH-driven diffusion of AEA; 3) AEA sequestration in a saturable membrane compartment; 4) Caveolae-related endocytosis.

Pathway 1 for the uptake of AEA involves carrier protein-mediated transport—

The characteristics of AEA uptake suggest a carrier-mediated process. AEA uptake is rapid ($t_{1/2}=2.5$ min), temperature-dependent, saturable at 37°C, and selective (Di Marzo et al., 1994). Furthermore, Moore and colleagues identified a high-affinity, saturable anandamide transporter binding site using a potent, competitive small molecule inhibitor of anandamide transport and FAAH, LY2183240 (Alexander and Cravatt, 2006; Moore et al., 2005; Ortar et al., 2008). The model for carrier protein-mediated AEA uptake is consistent with other proposed models to describe transport for various lipophilic molecules (Kanai et al., 1995; Schaffer and Lodish, 1994).

Pathway 2 for the uptake of AEA involves facilitated FAAH-driven diffusion of AEA—

The role of FAAH in the uptake of AEA has been widely investigated. Various studies have reported that FAAH operates to maintain an inward concentration gradient of AEA to facilitate its uptake into the cell, thus, acting as a saturable component of the system (Day et al., 2001; Glaser et al., 2003). Even though FAAH may facilitate the transport of AEA, FAAH is not required for AEA transport to take place (Day et al., 2001; Fegley et al., 2004; Ligresti et al., 2004). Studies performed in FAAH knockout mice have confirmed that AEA uptake appears to be modulated by both FAAH-dependent and FAAH-independent mechanisms (Ortega-Gutiérrez et al., 2004) as temperature-sensitive AEA uptake occurs in preparations from FAAH knockout mice (Fegley et al., 2004; Ligresti et al., 2004). Thus, FAAH-driven AEA uptake may be a process specific to the particular system being investigated.

Pathway 3 for the uptake of AEA involves the sequestration of AEA into a saturable membrane compartment—

An intracellular compartment has been proposed to contain sequestered AEA, thus, resulting in two pools of AEA--free and sequestered (Hillard and Jarrahian, 2003). These intracellular compartments may act as reservoirs to contain AEA, and it is possible that compounds that inhibit intracellular AEA accumulation with no apparent activity to inhibit FAAH, may saturate these intracellular compartments and prevent AEA accumulation (Hillard and Jarrahian, 2003). This compartment, however, has yet to be identified by biochemical or analytical approaches.

Pathway 4 for the uptake of AEA involves caveolae-related endocytosis—

Caveolae-related endocytosis is a process that has been associated with lipid rafts and takes place independent of clathrin-mediated endocytosis (Parton, 2003). Studies by our lab indicate that AEA uptake takes place via caveolae-related endocytosis (McFarland et al., 2008; McFarland et al., 2004). Both AEA uptake and caveolae-related endocytosis share the characteristics described above. Furthermore, caveolae-related endocytosis meets the characteristics of carrier protein-mediated transport, facilitated FAAH-driven diffusion of AEA, and AEA sequestration in a saturable membrane compartment. Thus, caveolae-related endocytosis appears to encompass the criteria of the other models for AEA uptake.

Breakdown of endocannabinoids

In order to terminate endocannabinoid signaling, these molecules first have to be transported into the cell. The pharmacologic profiles of AEA and 2-AG are similar, including the events that lead to uptake and signal termination of these endocannabinoids (Lambert and Fowler, 2005). Following uptake, AEA is hydrolyzed by FAAH into AA and ethanolamine (Fig. 3),

whereas 2-AG may be hydrolyzed either by FAAH or a monoacylglycerol lipase (MGL) enzyme to yield AA and glycerol (Blankman et al., 2007; Cravatt and Lichtman, 2003; Deutsch and Chin, 1993; Di Marzo et al., 1998; Di Marzo and Maccarrone, 2008; Goparaju et al., 1998; Maccarrone et al., 2008; Muccioli et al., 2007). Experiments in tissue extracts indicate that potent FAAH inhibitors do not inhibit the hydrolysis of 2-AG suggesting that 2-AG inactivation may not utilize FAAH as a primary pathway for hydrolysis *in vivo* (Saario et al., 2004). Furthermore, observations in mice indicate that knockout of the FAAH gene does not affect 2-AG hydrolysis (Lichtman et al., 2002).

Additional pathways also exist for AEA breakdown as indicated by studies in which inhibition of cyclooxygenase-2 (COX-2), not FAAH, prolonged anandamide-modulated depolarization-induced suppression of inhibition (DSI) (Kim and Alger, 2004). 12- and 15- lipoxygenases (LOX), that oxygenate esterified polyunsaturated fatty acids, are able to metabolize anandamide (Ueda et al., 1995). Cytochrome P450 also has been shown to metabolize AEA into hydroxyeicosatetraenoic ethanolamide (Snider et al., 2007).

As mentioned above, the metabolites of AEA, AA and ethanolamine, are rapidly trafficked by the cell to the lipid raft region (McFarland et al., 2004). We hypothesized that NAPE PLD could be involved in the biosynthesis of AEA if it is localized in proximity to FAAH, a region of the cell where AA and ethanolamine are generated. In a study that was designed to determine the cellular location of FAAH in relation to NAPE PLD, we discovered that FAAH and green fluorescent protein (pEGFP)-tagged NAPE PLD expressed in neuronal-like CAD cells did not co-localize (Fig. 4). Therefore, we concluded that if NAPE PLD is involved in the biosynthesis of AEA in RBL-2H3 or CAD cells, this enzyme does not localize with known markers of lipid rafts or AEA metabolism—flotillin-1 and FAAH, respectively.

Concluding remarks

Much progress has been made in recent years to characterize the processes that underlie biosynthesis, uptake, and metabolism of AEA and 2-AG. A better understanding of the mechanisms by which endocannabinoid biosynthesis and uptake take place and the role that lipid rafts play in these processes will be necessary to further characterize endocannabinoid pharmacology in terms of regulation of the immune response, pain, nausea, and cancer. This is why it is important to discover other members of the endocannabinoid family and investigate the impact of novel endocannabinoid signaling on the existing endocannabinoids. New advances in the field of mass spectrometry may allow us not only to detect and quantify the existing endocannabinoid levels in biological tissue, but also characterize the profile of related compounds.

Methods and Materials

Cell Culture

RBL-2H3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal clone I and 5% bovine calf serum supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin. CAD cells were maintained in DMEM/F-12 (50/50) with 5% fetal clone I and 5% bovine calf serum supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin. Cells were maintained in a CO₂-containing (5%) humidified environment at 37° C.

Construction of the pEGFP-C1 NAPE PLD vector

A silent mutation removing the XbaI site in the NAPE PLD cDNA sequence was introduced at the position 364 from the beginning of the coding region. The sequence of pcDNA 3.1+ NAPE PLD (XbaI⁻) construct was verified by DNA sequencing. In order to generate pEGFP-

C1 NAPE PLD construct, the pEGFP-C1 plasmid (Clontech) and the pcDNA3.1+NAPE PLD (XbaI⁻) constructs were digested with XbaI and KpnI, and then the appropriate fragments were ligated together. This construct fused the pEGFP on the N-terminus of NAPE PLD, yielding pEGFP-C1 NAPE PLD.

Immunofluorescent localization of NAPE PLD and flotilin-1 in RBL-2H3 cells

RBL-2H3 cells were plated on coverslips in a 6-well plate that had been previously coated with poly-D-lysine. The next day, cells were fixed in a 4% paraformaldehyde solution for 30 min at room temperature. Cells were then washed twice with phosphate-buffered saline (PBS) for 15 min and twice for 5 min at room temperature. Next, cells were incubated in a permeabilization solution (5% fetal clone I, 5% bovine calf serum and 0.3 % Triton $\times$ 100) for 30 min at room temperature. Cells were washed again twice with PBS for 15 min and twice for 5 min. Primary antibodies directed against NAPE PLD (Cayman) and flotillin-1 (BD Biosciences) were prepared in PBS containing 5% fetal clone I and 5% bovine calf serum at dilutions of 1:1000 and 1:100, respectively, and incubated with the cells for 24 hours at 4°C. Cells were then washed with PBS five times for 5 min, and then the secondary antibodies were added, Alexa Fluor 488-conjugated goat anti-rabbit antibody at a dilution of 1:2000, and Alexa Fluor 568-conjugated goat-anti mouse antibody at a dilution of 1:1000. After incubation for 1 hour at room temperature, cells were washed five times with PBS. Coverslips were removed from the wells, mounted on slides using ProLong Antifade (Molecular Probes) and allowed to dry overnight. The cell images were acquired by oil immersion confocal microscopy at $\times$ 60 magnification with a Nikon Diaphot 300 microscope. The Bio-Rad MRC1024 confocal system was used with a krypton (488 nm)/argon (568 nm) laser, a 522–535-nm band-pass filter, and a 588-nm long-pass filter.

Immunofluorescent localization of NAPE PLD and FAAH in CAD cells

CAD cells were plated at 1.2×10^5 cells/well seeding density in a 6-well plate on coverslips, previously coated with poly-D-lysine. The cells were transfected with 8 μ g of pEGFP-C1 NAPE PLD or 8 μ g of pEGFP-C1 using Lipofectamine 2000 (Invitrogen). The cells were cultured at 37 °C for 24 hours with one change of culture medium at 4 hours. Cells were incubated with the primary polyclonal antibody, anti-FAAH (1:500) in a PBS solution containing 5% fetal clone I and 5% bovine calf serum for 24 hours at 4°C. Cells were then washed with PBS five times for 5 min and incubated with the secondary antibody conjugated to Alexa Fluor 568 (goat anti-rabbit, 1:1000) for 1 hour at room temperature. Cells were then washed five times with PBS, prepared and imaged as described above.

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A Pathway 1 for AEA biosynthesis involves NAPE PLD

B Pathway 2 for AEA biosynthesis involves PLC/PTPN22

C Pathway 3 for AEA biosynthesis involves Abh4

Figure 1. A schematic representation of the putative pathways implicated in the biosynthesis of AEA

The pathways are: NAPE PLD-modulated NAPE hydrolysis to yield AEA (A); PLC-modulated cleavage of NAPE, formation of a phosphoAEA intermediate and subsequent dephosphorylation by PTPN22 (B); Abh4-dependent NAPE hydrolysis to yield AEA (C).

Figure 2. NAPE PLD and flotillin-1 did not co-localize in RBL-2H3 cells

Confocal microscopy analysis of RBL-2H3 cells revealed the presence of (A) NAPE PLD localization (green; primary anti-NAPE PLD polyclonal antibody: 1:1000, secondary Alexa Fluor 488-conjugated goat anti-rabbit antibody: 1:2000) and (B) flotillin-1 localization in the lipid raft microdomains (red; primary anti-flotillin-1 monoclonal antibody: 1:100, secondary Alexa Fluor 568-conjugated goat anti-mouse antibody: 1:1000). The overlay of the red and green signal (C) revealed that the two proteins, NAPE PLD and flotillin-1 did not overlap in their cellular locations. The cell images were acquired by oil immersion confocal microscopy at $\times 60$ magnification with a Nikon Diaphot 300 microscope. The Bio-Rad MRC1024 confocal system was used with a krypton (488 nm)/argon (568 nm) laser, a 522–535-nm band-pass filter, and a 588-nm long-pass filter. Data are representative of three separate experiments.

Figure 3. A schematic representation of the AEA hydrolysis pathways

Anandamide can be metabolized by COX-2 to yield prostaglandin endoperoxide ethanolamide intermediates (PGE₂-EA); by 12-LOX and 15-LOX to result in 12-hydroperoxy-5,8,10,14-eicosatetraenoylethanolamide and 15-hydroperoxy-5,8,11,13-eicosatetraenoylethanolamide (HPETE-EA) molecules; by cytochrome P450 into hydroxyeicosatetraenoic ethanolamide molecules (HETE-EA), such as 20-HETE-EA or epoxyeicosatrienoic acids (EETs) such as 5,6-, 8,9-, 11,12-, and 14,15-EET-EA; or by FAAH to convert AEA to AA and ethanolamine.

Figure 4. FAAH and GFP-NAPE PLD did not co-localize in CAD cells

CAD cells were transfected with vector pEGFP-C1 (8 μ g) (A–D) or (pEGFP-C1 NAPE PLD (8 μ g) (E–H). A) pEGFP-C1 alone (green) revealed a diffuse signal. B) FAAH (red) localized in the perinuclear region of the cell (primary anti-FAAH polyclonal antibody: 1:500, secondary Alexa Fluor 568-conjugated goat anti-rabbit antibody: 1:1000). C) The overlay of the pEGFP-C1 (green) and FAAH (red) signal indicated that pEGFP-C1 did not localize with FAAH. D) bright-field image of the cells. E) pEGFP-C1 NAPE PLD (green) localized primarily in the nuclear area of the cell, consistent with localization in RBL-2H3 cells (Fig. 2). F) FAAH alone (red; primary anti-FAAH antibody: 1:500, secondary Alexa Fluor 568-conjugated goat anti-rabbit antibody: 1:1000). G) NAPE PLD (green) and FAAH (red) did not co-localize in CAD cells. H) Bright-field image of the cells. The cell images were acquired by oil immersion confocal microscopy at $\times 60$ magnification with a Nikon Diaphot 300 microscope. The Bio-Rad MRC1024 confocal system was used with a krypton (488 nm)/argon (568 nm) laser, a 522–535-nm band-pass filter, and a 588-nm long-pass filter. Data are representative of three separate experiments.