HEP (2005) 168:187–207
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Modulators of Endocannabinoid Enzymic Hydrolysis and Membrane Transport

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Abstract Tissue concentrations of the endocannabinoids *N*-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) are regulated by both synthesis and inactivation. The purpose of this review is to compile available data regarding three inactivation processes: fatty acid amide hydrolase, monoacylglycerol lipase, and cellular membrane transport. In particular, we have focused on mechanisms by which these processes are modulated. We describe the in vitro and in vivo effects of inhibitors of these processes as well as available evidence regarding their modulation by other factors.

Keywords Fatty acid amide hydrolase · Monoacylglycerol lipase · Transporter · Carrier · Anandamide · 2-Arachidonoylglycerol · *N*-Arachidonoylethanolamine

1 Introduction

It is becoming clear that like most neuromodulatory molecules, the effective concentrations of the endocannabinoids (eCBs) *N*-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) are regulated by both synthesis and catabolism (Di Marzo, this volume). Catabolism of both AEA and 2-AG occurs via hydrolysis to arachidonic acid, and ethanolamine and glycerol, respectively. Hydrolysis of AEA is mediated primarily via fatty acid amide hydrolase (FAAH) (Cravatt et al. 2001). 2-AG is also a substrate for FAAH (Goparaju et al. 1998), but monoacylglycerol lipase (MGL) likely plays a more important role in its hydrolysis in vivo (Cravatt and Lichtman 2002). Both of these catabolic enzymes are localized intracellularly (Tsou et al. 1998; Dinh et al. 2002). This compartmentalization of the catabolic enzymes begs the question of whether a mechanism exists by which the eCBs move from the extracellular environment where they are functional signaling molecules into the intracellular environment where they are degraded. Functional studies support the possibility that a transmembrane carrier protein can transport AEA (Hillard and Jarrahian 2003), and perhaps 2-AG (Beltramo and Piomelli 2000; Bisogno et al. 2001), from one side of the plasma membrane to the other. This putative carrier has been suggested to function as an inactivation mechanism, since it would remove the eCBs from extracellular space, effectively sequestering the ligands away from their CB_1 cannabinoid receptor target. Since the putative carrier has the characteristics of a facilitated diffusion process and can also transport AEA from inside to outside (Hillard et al. 1997), it could also play a role in the release of newly synthesized AEA. Indeed, intracellular administration of uptake inhibitors blocks eCB-dependent activation of the CB_1 receptor in striatal slices (Ronesi et al. 2004).

In light of the widespread role of the eCB/CB_1 receptor signaling system in the regulation of CNS function, it is a near certainty that drugs acting on one or more of the three eCB inactivation processes characterized to date (i.e., FAAH, MGL, and cellular uptake) will be useful therapeutic agents in the future. Of the three processes, FAAH is the best characterized, and inhibitor development is the most mature. MGL has been cloned (Karlsson et al. 1997; Dinh et al. 2002), which will allow for clear identification of its role in 2-AG inactivation as well as facilitate inhibitor development. The cellular uptake process is the least characterized of the three at this point. The molecular identities of the proteins involved are not known, with the exception of data suggesting that FAAH can drive cellular uptake in some cell types (Glaser et al. 2003). In spite of the lack of molecular information, inhibitors of the uptake process have been developed and are discussed in this chapter.

2 Fatty Acid Amide Hydrolase

2.1 Characteristics of FAAH

FAAH is an integral-membrane serine hydrolase found in intracellular compartments (predominantly in microsomal fractions) of various cell types in the central nervous system and the periphery. FAAH is widely expressed in the brain, particularly in the neocortex, hippocampal formation, amygdala, and cerebellum (Herkenham et al. 1990; Giang and Cravatt 1997; Thomas et al. 1997; Yazulla et al. 1999). In the periphery, FAAH activity has been reported in the lung, liver, kidney, blood vessels, blood cells, and gastrointestinal tract, as well as the reproductive tract (Deutsch and Chin 1993; Desarnaud et al. 1995; Bisogno et al. 1997a; Giang and Cravatt 1997; Pratt et al. 1998; Maccarrone et al. 2001b).

The FAAH cDNA has been cloned from several mammalian species, and a functional homolog of the mammalian FAAH has also been reported in the plant *Arabidopsis thaliana* (Cravatt et al. 1996; Giang and Cravatt 1997; Shrestha et al. 2003). The rat and mouse FAAH sequences share 91% identity, while the human FAAH shares over 80% sequence identity with rat and mouse FAAHs. Given that human and rodent FAAHs have been shown to display broadly similar substrate selectivity and inhibitor sensitivity profiles (Giang and Cravatt 1997), FAAH activities detected in animal model systems are likely to be relevant to humans.

FAAH belongs to a class of hydrolytic enzymes called the “amidase signature family,” which are defined by a conserved serine- and glycine-rich “amidase signature sequence” of approximately 130 amino acids (Cravatt et al. 1996). Its optimal pH is 8 to 9. Site-directed mutagenesis studies and structural determination of FAAH have indicated that the conserved residues Ser-241, Ser-217, Ser-218, Lys-142, and Arg-243 within the signature sequence of FAAH are essential for its catalytic activity (Patricelli and Cravatt 1999; Patricelli et al. 1999; Patricelli and Cravatt 2000; Bracey et al. 2002; McKinney and Cravatt 2003). Ser-241, Ser-217, and Lys-142 are hypothesized to form a catalytic triad. The carbonyl group of AEA or another substrate is believed to react with the hydroxyl group of Ser-241 (the catalytic nucleophile) of FAAH, forming an oxyanion tetrahedral intermediate (the “transition-state”), followed by protonation, facilitated by Ser-217 and Lys-142, of the substrate-leaving group. It has been hypothesized that an almost simultaneous occurrence of the oxyanion formation and subsequent protonation contributes to the unusual ability of FAAH to hydrolyze amides and esters at equivalent rates (McKinney and Cravatt 2003). Interestingly, FAAH with mutated Arg-243, but not the other four critical residues, has differentially reduced amidase over esterase activity (Patricelli and Cravatt 2000), indicating potential separation of the amidase and esterase activities of FAAH.

2.2
Substrate Specificity of FAAH

FAAH hydrolyzes a broad spectrum of long, unsaturated acyl chain amides and esters. Both AEA and 2-AG are hydrolyzed by FAAH at similar concentrations (K_m 3–12 μM for both AEA and 2-AG; Hillard et al. 1995; Goparaju et al. 1998). There is some evidence that other putative eCBs, arachidonoyldopamine and virodhamine, are substrates of FAAH (Bisogno et al. 2000; Huang et al. 2002; Porter et al. 2002). FAAH also hydrolyzes the sleep-inducing factor oleamide, a fatty acid amide, to its corresponding acid (Cravatt et al. 1996), as well as other biologically active fatty acid ethanolamides, including *N*-oleoylethanolamine (a satiety factor), *N*-palmitoylethanolamine (PEA; an anti-inflammatory and analgesic agent), and the lipoamino acid *N*-arachidonoylglycine (a potential analgesic agent) (Cravatt et al. 1996; Huang et al. 2001; Rodriguez de Fonseca et al. 2001; see also Ueda et al. 2000 for review).

2.3
Mechanisms of FAAH Regulation

Expression of FAAH is up-regulated by progesterone and leptin and down-regulated by estrogen and glucocorticoids (Maccarrone et al. 2001a, 2003b; Waleh et al. 2002). Changes in FAAH protein concentrations are paralleled by changes in mRNA levels, consistent with transcription regulation by these factors. Although steroid hormone-response elements have been described in the promoter region of the FAAH gene in rodents and human, the precise mechanisms by which progesterone, estrogen, and glucocorticoids regulate FAAH transcription remain unclear (Maccarrone et al. 2001a, 200, 2003b; Puffenbarger et al. 2001; Waleh et al. 2002). Regulation is tissue- and species-specific; FAAH expression is decreased in mouse uterus, but increased in rat uterus, in response to sex hormones (Maccarrone et al. 2000b; Xiao et al. 2002). The FAAH promoter region also contains a cyclic adenosine monophosphate (cAMP)-response element-like site, which is a transcriptional target of signal transducer and activator of transcription (STAT)3. It has been shown that activation of leptin receptors, probably via activation of STAT3, increases FAAH gene transcription and translation (Maccarrone et al. 2003a).

FAAH contains a type II polyproline sequence that binds Src homology 3 (SH3)-containing proteins. Given that SH3 domains are found in many signal transduction proteins, including phospholipase Cγ and phosphoinositol-3-kinase, and cytoskeletal proteins, these proteins could potentially regulate the activity and subcellular localization of FAAH (Kuriyan and Cowburn 1997; Arreaza and Deutsch 1999). Indeed, ablation of the SH3-binding domain results in loss of enzymatic activity (Arreaza and Deutsch 1999).

AEA hydrolysis by FAAH in vitro is not affected by calcium (Hillard et al. 1995; Maurelli et al. 1995). Interestingly, however, lipoxygenase products appear to inhibit FAAH activity such that inhibition of 5-lipoxygenase enhances AEA hydrolysis in mast cells (Maccarrone et al. 2000c) and neuroblastoma cells (Mac-

carrone et al. 2000a). Inhibition of FAAH activity in cultured endothelial cells by estrogen seems to require 15-lipoxygenase (Maccarrone et al. 2002). Maccarrone et al. (2004) have recently reported that a yet-to-be-characterized soluble lipid, which is released from blastocytes, increases FAAH activity without affecting its expression.

2.4 FAAH Inhibitors

The characterization of FAAH activity and its role in eCB signaling has been enabled by the development of effective FAAH inhibitors, with diverse structures and affinities for the enzyme (Table 1). Most of the inhibitors target the catalytic site of FAAH and thereby prevent the interaction of the enzyme and its substrates. The first identified inhibitor of FAAH was phenylmethylsulfonyl fluoride (PMSF) an agent widely used to inhibit serine proteases (Deutsch and Chin 1993). PMSF inhibits FAAH irreversibly via sulfonation of serine residues (Hillard et al. 1995; Ueda et al. 1995; Deutsch et al. 1997b). It is commonly included in CB_1 receptor ligand binding studies to inhibit FAAH-mediated catabolism of AEA. Analogs of PMSF with fatty acyl substitutions, such as palmitylsulfonyl fluoride (AM374) and stearylsulfonyl fluoride (AM381) also covalently modify serine residues in FAAH with nanomolar IC_{50} values (Lang et al. 1996; Deutsch et al. 1997b). These acyl sulfonyl fluorides display reasonable separation between FAAH inhibition and CB_1 receptor binding, especially for those with a longer saturated alkyl chain (K_i for CB_1 receptor, AM374:520 nM; AM381:19 μM; Deutsch et al. 1997b).

Another series was derived from inhibitors of phospholipase A_2 (PLA_2) and exploits the preference of FAAH for substrates with long, unsaturated acyl chains. Arachidonoyltrifluoromethylketone (ATFMK) is a reversible inhibitor of AEA hydrolysis at low micromolar range (Maurelli et al. 1995; Ueda et al. 1995; Beltramo et al. 1997a; Deutsch et al. 1997a), probably by forming a stabilized adduct of the trifluoromethylketone and an active-site serine residue (the so-called "transition-state" enzyme inhibitor). However, ATFMK is also a slow- and tight-binding inhibitor of cytosolic PLA_2 with an IC_{50} of 2–15 μM (Street et al. 1993; Riendeau et al. 1994) and it binds to CB_1 receptors in the same concentration range that inhibits AEA degradation (Koutek et al. 1994; Deutsch et al. 1997b). ATFMK also inhibits MGL (see Sect. 3.6). Methyl arachidonoyl fluorophosphonate (MAFP) is another inhibitor of arachidonoyl-selective PLA_2 (Street et al. 1993; Lio et al. 1996). It also interacts with CB_1 receptors in an irreversible manner (Deutsch et al. 1997a; Fernando and Pertwee 1997). The X-ray structure of FAAH crystallized with MAFP has shed light on FAAH substrate recognition and position in the lipid bilayer (Bracey et al. 2002).

Diazomethylarachidonoylketone (DAK) (De Petrocellis et al. 1997; Edgemond et al. 1998) also inhibits FAAH; its carbonyl carbon is likely to bind to an active site serine, whereas the diazomethyl carbon reacts with the imidazolium residue of a histidine, resulting in a very stable complex. In line with this model, three histidine residues are conserved in rodent and human FAAHs (Giang and Cravatt

Table 1. Inhibitors of FAAH activity

Inhibitor	Tissue	IC_{50} (nM)	Reference	Remarks
PMSF	Rat brain homogenates	290	Deutsch et al. 1997b	Irreversible. Unstable in aqueous solution ($t_{1/2}$ <60 min, pH 7)
	Rat forebrain membranes	13,000	Hillard et al. 1995	
	Solubilized from porcine brain	~15,000	Ueda et al. 1995	
AM374	Rat brain homogenates	13	Deutsch et al. 1997b	Irreversible; binds to CB_1 receptors (IC_{50} 520 nM; Deutsch et al. 1997b)
	Brain microsomes	50	Lang et al. 1996	
ATFMK	N18TG2 cells	700	Deutsch et al. 1997a	Reversible; inhibits $cPLA_2$ (IC_{50} 2–15 μM; Street et al. 1993); binds to CB_1 receptor (IC_{50} 0.65–2.5 μM, Koutek et al. 1994)
	Rat brain homogenate	900	Deutsch et al. 1997b	
	Solubilized porcine brain FAAH	1,000	Ueda et al. 1995	
	Mouse neuroblastoma N18TG2 cells	3,000	Maurelli et al. 1995	
	Rat brain microsomes	4,000	Beltramo et al. 1997a	
MAFP	Solubilized from N18TG2 cells, RBL-1 cells, and porcine brain RBL-1 cells, and porcine brain	1–3	De Petrocellis et al. 1997	Irreversible. Also inhibits $cPLA_2$ (Lio et al. 1996). Binds irreversibly to CB_1 receptor IC_{50} 20 nM; Deutsch et al. 1997a
	Rat brain homogenates	2.5	Deutsch et al. 1997a	
	N18TG2 cells	20	Deutsch et al. 1997a	
DAK	Rat brain membranes	500	Edgemond et al. 1998	Irreversible; binds to CB_1 receptors (IC_{50} 1.3 μM; Edgemond et al. 1998)
	Solubilized from N18TG2 cells, RBL-1 cells, and porcine brain	2,000–6,000	De Petrocellis et al. 1997	Reversible
AA-5-HT	RBL-2H3 cells	560	Bisogno et al. 1998	Reversible; inactive at $cPLA_2$, CB_1, or CB_2 receptors
	N18TG2 cells	1,200	Bisogno et al. 1998	
URB597	Rat cortical neurons	0.5	Kathuria et al. 2003	Irreversible; active in vivo at 0.3 mg/kg
	Rat brain membranes	4.6	Kathuria et al. 2003	

AA-5-HT, arachidonoylserotonin; AM374, palmitylsulfonylfluoride; ATFMK, arachidonyltrifluoromethylketone; DAK, diazomethylarachidonylketone; MAFP, methylarachidonylfluorophosphonate; PMSF, phenylmethylsulfonyl fluoride.

1997). Structural alignment of the residues are crucial for the irreversible action of DAK, as indicated by the observation that DAK inhibition of FAAH in detergent-solubilized preparation, but not that in native membranes, is reversed after anion exchange chromatography of the proteins (De Petrocellis et al. 1997; Edgemond et al. 1998). It is unclear if DAK also inhibits PLA_2, but it binds to neuronal CB_1 receptors at concentrations similar to those producing FAAH inhibition (K_i 1.3 μM; Edgemond et al. 1998).

While the inclusion of arachidonic acyl groups in the inhibitors results in high affinity for FAAH, these inhibitors also bind to other arachidonate-binding proteins, such as PLA_2 and the CB_1 receptor. An exception to this is arachidonoylserotonin (AA-5-HT), which is a tight-binding but reversible inhibitor of FAAH that is devoid of activity at CB_1 receptors and $cPLA_2$ (Bisogno et al. 1998). More recently, Boger et al. (2000) reported a new class of α-keto heterocyclic inhibitors of FAAH by combining several features—an optimal fatty acid chain length (C8-C12), *cis*-double bond at the corresponding arachidonoyl location and an α-keto oxazolopyridine ring with a weakly basic nitrogen. These compounds inhibit FAAH reversibly at a picomolar or low nanomolar range, including $\Delta^{9,10}$-octadecynoyl-α-keto-oxazolopyridine, which exhibits a K_i of 140 pM (Boger et al. 2000). However, its pharmacological profile and specificity for FAAH remain to be determined.

Another series of irreversible inhibitors—alkylcarbamic acid aryl esters, with apparent specificity for FAAH—has also been reported (Kathuria et al. 2003; Tarzia et al. 2003). These inhibitors, which do not bind to CB_1 or CB_2 receptors or inhibit MGL or AEA cellular uptake, act by carbamoylation of the active site serine residue. The most potent of the series is URB597 (Kathuria et al. 2003). Of added significance is that these analogs, although difficult to emulsify, are also active as inhibitors of FAAH in vivo, resulting in an elevation of brain AEA content of approximately threefold at a dose of 0.3 mg/kg without an effect on the content of 2-AG (Kathuria et al. 2003).

Several endogenous fatty acid derivatives can inhibit FAAH-mediated catabolism of AEA by virtue of the fact that they function as alternative substrates. For example, *N*-arachidonoylglycine does not bind to CB_1 or CB_2 receptors but, as a substrate of FAAH, can decrease AEA catabolism (Huang et al. 2001; Burstein et al. 2002; Grazia Cascio et al. 2004). FAAH inhibition and the subsequent increases in concentrations of AEA and/or PEA mediate the analgesic effect of *N*-arachidonoylglycine. Oleamide has also been suggested to induce sleep, at least in part, by competing with AEA for FAAH (Mechoulam et al. 1997; Mendelson and Basile 1999), although recent studies have cast doubt over this mechanism (Fedorova et al. 2001; Lichtman et al. 2002; Leggett et al. 2004).

Understanding the effects of endogenous or pharmacological inhibition of FAAH is important for the elucidation of the biological activity of fatty acid-derived substances and the investigation of the therapeutic potentials of selective FAAH inhibitors (Gaetani et al. 2003). Modulation of FAAH activity could play a role in the mechanism of currently used drugs. For example, propofol (2,6-diisopropyl phenol), an intravenous anesthetic that is frequently used for both induction and maintenance of general anesthesia, inhibits FAAH, elevates brain AEA content, and is dependent upon activation of CB_1 receptors for its effect after i.p. admin-

istration (Patel et al. 2003). Similarly, the non-steroidal anti-inflammatory drugs indomethacin, ibuprofen, and suprofen have been suggested to inhibit FAAH as well as cyclooxygenase (Fowler et al. 1997a,b). While these compounds are not very potent inhibitors of FAAH (IC_{50} values of 10^{-5}–10^{-4} M), high doses of ibuprofen (400 mg) used by patients with rheumatoid arthritis result in peak plasma concentrations in the range 110 to 150 μM (Karttunen et al. 1990). In addition, FAAH is most sensitive to inhibition by these compounds at acidic pH (Fowler et al. 1997a; Holt et al. 2001; Fowler et al. 2003), which might occur in certain inflammatory conditions including rheumatoid arthritis.

3 Monoacylglycerol Lipase

3.1 Biochemical and Molecular Characteristics of MGL

MGL was first purified and characterized from rat adipose tissue (Tornqvist and Belfrage 1976). This enzyme has a molecular weight of approximately 33 kDa and a pI of 7.2. The purified protein was shown to hydrolyze 1(3)- and 2-monoacylglycerols at equal rates but to have no hydrolytic activity against triacylglycerols or diacylglycerols. Enzymatic activity is inhibited by micromolar concentrations of diisopropylfluorophosphate (DFP), indicating that the enzyme active site contains a reactive serine, and by mercurials, indicating the presence of essential sulfhydryl groups. Cloning of MGL from mouse adipose tissue confirmed and extended these biochemical studies (Karlsson et al. 1997). MGL is a serine hydrolase with a $GXS_{122}XG$ consensus sequence; the other members of the catalytic triad are Asp-239 and His-269. MGL has a ubiquitous tissue distribution, including brain, heart, and spleen. However, Western blot analyses of different mouse tissues reveal protein size differences (Karlsson et al. 2001). In particular, mouse brain MGL exhibits two immunoreactive bands, one migrating at the same molecular weight as the adipose enzyme and another with a slightly larger size. The same doublet has been observed in rat brain tissue (Dinh et al. 2002). The differences in migration on Western blot of MGL-like immunoreactive proteins could be evidence of MGL splice variants, isoforms, or post-translational processing. Interestingly, neuronal nuclei from rabbit cerebral cortex express a 1-monoacylglycerol lipase that has not been well characterized (Baker and Chang 2000). Human (Karlsson et al. 2001; Ho et al. 2002) and rat brain (Dinh et al. 2002) MGL have also been cloned; these two sequences are highly homologous to the mouse adipose clone.

3.2 Brain MGL

Within the brain, the distribution of MGL mRNA is ubiquitous but the expression levels are variable (Dinh et al. 2002). Regions with high transcript expression include cerebellum, cortex, and hippocampus, while low transcript levels are found

in the brain stem and hypothalamus. Protein distribution within the hippocampus is consistent with the presence of MGL in axon terminals; no protein is detected in hippocampal principal cells. This distribution agrees with earlier work in which MGL activity was found to be enriched in synaptosomes (Vyvoda and Rowe 1973) and synaptoneurosomes (Farooqui and Horrocks 1997) and contrasts with the distribution of FAAH, which is found predominately within hippocampal pyramidal cell bodies and is absent from presynaptic profiles (Tsou et al. 1998).

3.3 Subcellular Distribution of MGL

The subcellular distribution of MGL has been studied in several tissues and cell types using the distribution of enzymatic activity as an assay. In many tissues and cells, including brain and adipocytes, MGL activity is nearly equivalent in cytosolic and particulate fractions (Sakurada and Noma 1981; Bisogno et al. 1997b; Di Marzo et al. 1999; Goparaju et al. 1999). However, in pancreatic islet cells (Konrad et al. 1994) and erythrocytes (Somma-Delpero et al. 1995), the majority of MGL activity is associated with plasma membrane-enriched fractions and very little activity is seen in the cytosolic fractions. These data suggest that MGL can be associated with membranes but that it is not an intrinsic membrane protein. This conclusion agrees with the lack of obvious transmembrane domains in the MGL amino acid sequence.

A few studies have examined the question of whether different subcellular pools of MGL are kinetically similar. In rat adipocytes, the particulate and cytosolic enzymes are essentially identical with respect to pH dependence and substrate and inhibitor profiles (Sakurada and Noma 1981). Similar inhibitor profiles are also seen in cytosolic and membrane fractions from porcine brain (Goparaju et al. 1999). However, cytosolic MGL activity is reduced by 50% in adipocytes isolated from 24-h fasted rats without any change in membrane MGL activity (Sakurada and Noma 1981). Similarly, treatment of rat macrophages and platelets with lipopolysaccharide results in inhibition of MGL activity in particulate fractions but has no effect on cytosolic enzymatic activity (Di Marzo et al. 1999). The available data suggest that MGL of different subcellular compartments is likely the same enzyme isoform but that the location of the enzyme results in differential regulation by cellular processes.

A study comparing the subcellular distribution of MGL activity between resting and activated human neutrophils suggests that MGL can translocate from one subcellular compartment to another in response to cellular changes (Balsinde et al. 1991). In this study, MGL activity in resting neutrophils was localized primarily in gelatinase-containing granules, but upon activation by A23187, a dramatic shift in activity to the plasma membrane occurred. Interestingly, the enzyme associated with the plasma membrane exhibited an increase in $V_{\max}$ for the hydrolysis of 2-AG, suggesting there is a greater pool of substrate available at the plasma membrane.

3.4 Substrate Specificity of MGL

MGL can hydrolyze both 1(3)- and 2-monoacylglycerols and has very little ability to hydrolyze triacylglycerols or diacylglycerols (Tornqvist and Belfrage 1976; Di Marzo et al. 1999; Goparaju et al. 1999). MGL hydrolyzes fatty acyl esters but not fatty acyl amides or ethers. In particular, neither AEA (Bisogno et al. 1997b; Goparaju et al. 1999; Dinh et al. 2002; Saario et al. 2004) nor noladin ether (Saario et al. 2004) are hydrolyzed by MGL. Interestingly, there is one report that AEA (at a concentration of 100 μM) inhibits MGL activity by 77% in macrophage membranes (Di Marzo et al. 1999). The enzyme prefers but does not require a glycerol head group as MGL purified from erythrocytes hydrolyzes oleoylethanol at a rate about 50% of the oleic ester of glycerol (Somma-Delpero et al. 1995) and the ester virodhamine is hydrolyzed to arachidonic acid at a rate about twofold lower than that of 2-AG (Saario et al. 2004).

The glycerol esters of arachidonic acid, oleic acid, and palmitic acid are all hydrolyzed by MGL (Vyvoda and Rowe 1973; Dinh et al. 2002; Saario et al. 2004). Only a few studies have compared the rates of hydrolysis of various monoacylesters. In macrophages the 1(3)-monoglycerols of arachidonic acid, γ-linolenoyl and linolenoyl acid were hydrolyzed at a higher specific activity than the palmitic acid analog (Di Marzo et al. 1999). In another study, the 1(3)-monoglycerol of arachidonic acid was hydrolyzed at a higher rate than the corresponding oleic acid ester (Goparaju et al. 1999). However, none of these differences is large, and when K_m values are compared across studies the values are very similar, in spite of the fact that different substrates and tissue sources were used (Table 2). These data suggest that any possible isoforms of MGL differ little in their affinity for monoacylglycerols and that there is little selectivity for the acyl substituents of the substrates, at least among long chain fatty acid esters.

Table 2. K_m values for monoacylglycerol hydrolysis by MGL-like enzymatic activities

Tissue	Substrate	K_m (μM)	Reference
Rat adipocyte membrane	1(3)- and 2-oleoylglycerol	210	Sakurada and Noma 1981
Rat adipocyte cytosol	1(3)- and 2-oleoylglycerol	370	Sakurada and Noma 1981
Human erythrocyte membranes	1(3)-Oleoylglycerol	270	Somma-Delpero et al. 1995
Human erythrocyte membranes	2-Oleoylglycerol	490	Somma-Delpero et al. 1995
Rat pancreatic islet homogenates	2-Arachidonoylglycerol	0.14	Konrad et al. 1994
J774 macrophage membranes	2-Arachidonoylglycerol	110	Di Marzo et al. 1999
J774 macrophage membranes	2-Palmitoylglycerol	170	Di Marzo et al. 1999
Purified rat adipose enzyme in detergent	1(3)- or 2-oleoylglycerol	200	Tornqvist and Belfrage 1976
Human neutrophil supernatants	2-Arachidonoylglycerol	34	Balsinde et al. 1991

3.5 Regulation of MGL Activity

MGL activity in vitro is not affected by the addition of calcium to the incubation buffer (Sakurada and Noma 1981; Balsinde et al. 1991; Konrad et al. 1994). However, MGL activity could be inhibited by calcium, as two studies have reported an increase in activity following the addition of ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′,-tetraacetic acid (EGTA) to the assay buffer (Sakurada and Noma 1981; Witting et al. 2004). Very high concentrations of sodium (i.e., 1 M) are inhibitory (Sakurada and Noma 1981) as is zinc (Tornqvist and Belfrage 1976).

Although calcium does not appear to have a direct effect on MGL activity, activation of *N*-methyl-D-aspartate (NMDA) receptors in spinal cord neurons in

Table 3. Inhibitors of MGL activity

Inhibitor	Tissue	IC_{50} or % inhibition (concentration)	Reference
DFP	Purified from adipocyte	100% (10 μM)	Tornqvist and Belfrage 1976
	Rat adipocyte cytosol	81% (2 mM)	Sakurada and Noma 1981
	Rat adipocyte membrane	89% (2 mM)	Sakurada and Noma 1981
	Purified enzyme in CHAPS	88% (5 mM)	Somma-Delpero et al. 1995
PMSF	Purified enzyme in CHAPS	43% (0.5 mM)	Somma-Delpero et al. 1995
	N18 cell cytosol	30% (0.5 mM)	Bisogno et al. 1997b
	N18 cell microsomes	47% (0.5 mM)	Bisogno et al. 1997b
	Platelet membranes	1% (0.1 mM)	Di Marzo et al. 1999
	Macrophage membranes	21% (0.1 mM)	Di Marzo et al. 1999
	Rat cerebellar membranes	155 μM	Saario et al. 2004
MAFP	Porcine brain cytosol	2 nM	Goparaju et al. 1999
	Platelet membranes	0 (50 nM)	Di Marzo et al. 1999
	Macrophage membranes	21% (50 nM)	Di Marzo et al. 1999
	Rat brain cytosol	800 nM	Dinh et al. 2002
	Rat cerebellar membranes	2 nM	Saario et al. 2004
ATFMK	N18 cell cytosol	11% (0.5 mM)	Bisogno et al. 1997b
	N18 cell microsomes	23% (0.5 mM)	Bisogno et al. 1997b
	Porcine brain cytosol	30 μM	Goparaju et al. 1999
	Platelet membranes	7.5% (10 μM)	Di Marzo et al. 1999
	Macrophage membranes	89% (10 μM)	Di Marzo et al. 1999
	Rat brain cytosol	2.5 μM	Dinh et al. 2002
	Rat cerebellar membranes	66 μM	Saario et al. 2004
HDSF	Rat brain cytosol	6.2 μM	Dinh et al. 2002
	Rat cerebellar membranes	241 nM	Saario et al. 2004
URB597	Rat brain cytosol	0 (30 μM)	Kathuria et al. 2003
	Rat cerebellar membranes	30% (1 mM)	Saario et al. 2004

ATFMK, arachidonyltrifluoromethylketone; DFP, diisopropylfluorophosphate; HDSF, hexadecylsulfonyl fluoride; MAFP, methylarachidonylfluorophosphonate; PMSF, phenylmethylsulfonyl fluoride.

culture (Farooqui et al. 1993) and in synaptoneurosomes prepared from young rat brain (Farooqui and Horrocks 1997) results in a very significant activation of MGL activity. While the mechanism of this activation is not known, the time course of activation following NMDA or glutamate treatment is short (onset at 6 min in cells). Since MGL is not activated by calcium directly, it is possible that the regulation involves phosphorylation or another post-translational modification. Interestingly, a recent study by Di Marzo and colleagues revealed that MGL activity in the striatum but not the hippocampus was reduced in rat brain harvested during the dark phase (i.e., active phase) compared to the light phase of the day (Valenti et al. 2004).

3.6 MGL Inhibitors

Since MGL is a serine hydrolase, its sensitivity to many of the available serine hydrolase inhibitors has been explored (Table 3). The results support the hypothesis that MGL can be inhibited by compounds that interact with its reactive serine. On the other hand, the potencies of the inhibitors are quite variable; in some cases, this likely reflects differences in assay methodology (i.e., substrate concentration, pH, form of the enzyme). However, in a few cases, the same assay conditions revealed very different inhibitory potencies (e.g., compare the platelet and macrophage membrane studies by Di Marzo et al. 1999). In any event, studies of these compounds are not likely to yield selective inhibitors of MGL. All of these compounds are inhibitors of FAAH (see above) and many are also inhibitors of PLA_2, diacylglycerol lipase, and acetylcholine esterase, among other hydrolases. By analogy to the development of the URB series of FAAH inhibitors (Kathuria et al. 2003), it is likely that selective inhibitors of MGL will come from other synthetic avenues.

4 Endocannabinoid Transmembrane Movement

4.1 Introduction

While the molecular identities of the proteins involved are not yet understood, it is clear that neurons and other cell types accumulate AEA intracellularly (Hillard and Jarrahian 2003). There are several characteristics of endocannabinoid transmembrane movement that are well supported by data obtained in multiple laboratories. To summarize, the accumulation of AEA by cells does not require sodium or ATP and is moderately temperature dependent. The accumulation exhibits saturation in the micromolar range and is inhibitable by a variety of structural analogs of AEA, suggesting that AEA accumulation involves its interaction with a saturable cellular component. Some data are consistent with the component being a plasma membrane transporter (see for example Hillard and Jarrahian 2000; Ronesi et al. 2004) while other data indicate that, in some cells, the accumulation is driven by

FAAH-mediated catabolism (Deutsch et al. 2001; Glaser et al. 2003). Regardless of the mechanisms involved, inhibitors of the accumulation process have been developed that will help to shed light on the fundamental processes involved in the accumulation as well as the importance of this process in the biological activity of the eCBs.

4.2
AEA Uptake Inhibitors

Many analogs of AEA have been tested as inhibitors of the AEA uptake process (Table 4). The reader is referred to comprehensive papers that include most of the structure–activity profiles of the first generation of inhibitors (Piomelli et al. 1999; Jarrahian et al. 2000; Di Marzo et al. 2002). Of these analogs, the best studied has been AM404 (*N*-(4-hydroxybenzyl)arachidonoylamine), which inhibits AEA uptake into neurons and other cell types with IC_{50} values in the low micromolar range and potentiates the effects of exogenously administered AEA in vivo (Beltramo et al. 1997b). However, AM404 is not an ideal inhibitor because it also inhibits FAAH (Jarrahian et al. 2000) and activates TPRV1 vanilloid receptors (De Petrocellis et al. 2000) at similar concentrations. VDM 11 (*N*-(4-hydroxy-2-methylphenyl)arachidonoylamine) has also been used as an uptake inhibitor in vitro (De Petrocellis et al. 2000) and in vivo (Gubellini et al. 2002). While VDM 11 has the advantage over AM404 of much lower affinity for the TRPV1 receptors, it inhibits FAAH-mediated hydrolysis of AEA at the same concentration range (Fowler et al. 2004).

Another series of analogs of arachidonic acid with furyl substitutions in the head group have been tested (Lopez-Rodriguez et al. 2001). Of this series, UCM707 (*N*-(3-furylmethyl)arachidonoylamine) has the highest affinity for the transporter and exhibits low binding affinities for the CB_1 and TPRV1 receptors. Interestingly, UCM707 has relatively high affinity for the CB_2 receptor (67 nM). UCM707 is hydrolyzed by FAAH with an IC_{50} of 30 μM (Lopez-Rodriguez et al. 2003; Fowler et al. 2004), which makes it metabolically unstable and, although it does not inhibit FAAH as potently as AM404, this feature of the molecule could be responsible for its ability to inhibit uptake in some cell types (Fowler et al. 2004).

Another series of inhibitors, OMDM-1, -2, -3, and-4, like AM404, are fatty acid amides with aromatic head groups but the acyl chain has been changed to oleoyl (18:1) (Ortar et al. 2003). Two members of the series, OMDM-1 and OMDM-2 (*R*- and *S*-1′-4-hydroxybenzyl derivatives of *N*-oleoylethanolamine, respectively) exhibit affinity for inhibition of AEA uptake similar to AM404 in RBL-2H3 cells and are resistant to FAAH. However, both compounds have a small but measurable effect on TPRV1 receptor activity in the same concentration range. The inhibitory potencies and efficacies of these two compounds as uptake inhibitors appear to be cell-specific, having greater potency and, for OMDM-2, greater efficacy in RBL2H3 cells than C6 glioma cells (Fowler et al. 2004). Their affinities for inhibition of uptake in primary neurons have not been determined. In vivo studies of these compounds have been carried out, and they exhibit anti-spasticity efficacy in a mouse model of

Table 4. Inhibitors of AEA cellular uptake

Inhibitor	Cell	IC_{50} (μM)	Reference(s)	Remarks
AM404	Primary neurons	4–5	Beltramo et al. 1997b	Inhibits FAAH, VR1 agonist
VDM 11	RBL-2H3 and C6 glioma	10–11	De Petrocellis et al. 2000	Inhibits FAAH, little effect on VR1
	PC-3 cells	11	Ruiz-Llorente et al. 2004	
UCM-707	U937 cells	0.8	Lopez-Rodriguez et al. 2001	Moderate inhibition of FAAH, little effect on VR1, binds CB_2 receptors
	RBL-2H3	25	Fowler et al. 2004	
	C6 glioma	41 (max. 50% inhibition)	Fowler et al. 2004	
	PC-3 cells	25% inhibition at 100 μM	Ruiz-Llorente et al. 2004	
UMC-119	PC-3 cells	11	Ruiz-Llorente et al. 2004	
OMDM-1	RBL-2H3	2.4	Ortar et al. 2003; Fowler et al. 2004	K_i: CB_1—12 μM; FAAH>50 μM; VR1>10 μM
	C6 glioma	>20	Fowler et al. 2004	
OMDM-2	RBL-2H3	3	Ortar et al. 2003	Ki: CB_1—5 μM; FAAH>50 μM; VR1—10 μM
	RBL-2H3	3	Fowler et al. 2004	
	C6 glioma	17 (max. 50% inhibition)	Fowler et al. 2004	
AM1172	Cortical neurons	2	Fegley et al. 2004	No inhibition of FAAH at 10 μM; moderate affinity for CB_1 and CB_2; no effect at VR1

Please refer to text for the chemical names of the inhibitors.

multiple sclerosis alone and can produce potentiation of exogenously administered AEA (de Lago et al. 2004). However, the role of the CB_1 receptor in the effects was not determined.

The "reverse" of AM404, i.e., *N*-arachidonoyl-4-hydroxybenzamide (also called AM1172) has been synthesized and studied (Fegley et al. 2004). This compound is not a substrate for FAAH and does not inhibit the hydrolysis of AEA at concentrations up to 10 μM but is equivalent to AM404 in its ability to inhibit AEA uptake into primary cortical neurons. Interestingly, however, this analog has a moderate affinity for both CB_1 and CB_2 receptors and behaves as a partial agonist in biochemical assays of receptor activation.

5 Summary

It is easy to argue from the current eCB literature that pharmacological manipulations of eCB inactivation will be important for human health. It is also important that selective inhibitors of each of the inactivation processes be designed so that mechanistically interpretable studies of these processes can be accomplished. Although significant progress has been made in the development of these agents, it is clear that more selective inhibitors are needed.

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