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Monoglyceride lipase-like enzymatic activity is responsible for hydrolysis of 2-arachidonoylglycerol in rat cerebellar membranes

Susanna M. Saario[a,*], Juha R. Savinainen[b], Jarmo T. Laitinen[b], Tomi Järvinen[a], Riku Niemi[a]

[a]*Department of Pharmaceutical Chemistry, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland*
[b]*Department of Physiology, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland*

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Abstract

2-Arachidonoylglycerol (2-AG) is an endogenous cannabinoid that binds to CB1 and CB2 cannabinoid receptors, inducing cannabimimetic effects. However, the cannabimimetic effects of 2-AG are weak *in vivo* due to its rapid enzymatic hydrolysis. The enzymatic hydrolysis of 2-AG has been proposed to mainly occur by monoglyceride lipase (monoacylglycerol lipase). Fatty acid amide hydrolase (FAAH), the enzyme responsible for the hydrolysis of *N*-arachidonoylethanolamide (AEA), is also able to hydrolyse 2-AG. In the present study, we investigated the hydrolysis of endocannabinoids in rat cerebellar membranes and observed that enzymatic activity towards 2-AG was 50-fold higher than that towards AEA. Furthermore, various inhibitors for 2-AG hydrolase activity were studied in rat cerebellar membranes. 2-AG hydrolysis was inhibited by methyl arachidonylfluorophosphonate, hexadecylsulphonyl fluoride and phenylmethylsulphonyl fluoride with IC_{50} values of 2.2 nM, 241 nM and 155 μM, respectively. Potent FAAH inhibitors, such as OL-53 and URB597, did not inhibit the hydrolysis of 2-AG, suggesting that 2-AG is inactivated in rat cerebellar membranes by an enzyme distinct of FAAH. The observation that the hydrolysis of 1(3)-AG and 2-AG occurred at equal rates supports the role of MGL in 2-AG inactivation. This enzyme assay provides a useful method for future inhibition studies of 2-AG degrading enzyme(s) in brain membrane preparation having considerably higher MGL-like activity when compared to FAAH activity.

Keywords: Cannabinoid; 2-Arachidonoylglycerol; Monoglyceride lipase; Monoacylglycerol lipase; Fatty acid amide hydrolase; Inhibition

1. Introduction

N-Arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) are presently considered to be the most important endogenous ligands for central cannabinoid receptor (CB1) and peripheral cannabinoid receptor (CB2) [1–3]. The endocannabinoids are produced by neurons ''on demand,'' act near the site of their synthesis and, as is typical for neuromodulators, they are effectively metabolized to ensure rapid signal inactivation [1–3]. AEA has remained in the spotlight of endocannabinoid research, although increasing number of observations support an even more important role for 2-AG in the endocannabinoid system. 2-AG is present in the rat brain in amounts 170–1000 times greater than AEA [3,4], and 2-AG acts as a potent and full-efficacy agonist at both CB1 and CB2 receptors [4–8]. In contrast, AEA is clearly less potent and acts only as a partial agonist at the CB receptors [3,7,8]. The biological inactivation of AEA is generally believed to occur via cellular uptake through facilitated diffusion [9–11], followed by intracellular enzymatic hydrolysis to arachidonic acid and ethanolamine by the membrane-bound fatty acid amide hydrolase (FAAH) [9,12–14]. However, the existence of any AEA transporter has been questioned [15]. FAAH has a broad substrate specificity, and it is also able to hydrolyse other fatty ethanolamides and fatty esters such as 2-AG [16]. In fact,

* Corresponding author. Tel.: +358-17-163-713; fax: +358-17-162-456.
E-mail address: susanna.saario@uku.fi (S.M. Saario).

Abbreviations: AA-5-HT, arachidonoyl serotonin; AEA, *N*-arachidonoylethanolamide; 2-AG, 2-arachidonoylglycerol; AM404, *N*-(4-hydroxyphenyl)-arachidonylamide; ATFMK, arachidonoyl trifluoromethylketone; BSA, bovine serum albumin; BTNP, (*E*)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FAAH, fatty acid amide hydrolase; HDSF, hexadecylsulphonyl fluoride; MAFP, methyl arachidonylfluorophosphonate; MGL, monoglyceride lipase; PMSF, phenylmethylsulphonyl fluoride.

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the specific activity of FAAH for 2-AG has been reported to be even higher when compared to AEA [17–19]. In addition, it is well known that also other enzymes such as lipases and esterases are able to hydrolyse 2-AG [20,21]. Recent evidence contradicts the physiological role of FAAH in 2-AG inactivation. Goparaju *et al*. [22] reported that the 2-AG hydrolase activity of porcine brain can be separated from FAAH. Beltramo and Piomelli [23] demonstrated that a known FAAH inhibitor does not inhibit 2-AG hydrolysis in human astrocytoma cells, indicating that 2-AG hydrolysis may depend on another hydrolase for inactivation. Furthermore, 2-AG hydrolysis is preserved in FAAH-deficient mice, which cannot hydrolyse AEA [24]. The most plausible enzyme responsible for 2-AG hydrolysis *in vivo* remains monoglyceride lipase (MGL, EC 3.1.1.23). This enzyme was reported over 20 years ago to catalyze the hydrolysis of monoacylglycerols that contain an arachidonoyl group in the *sn*-2 position [25]. MGL is a serine hydrolase that specifically hydrolyses 2- and 1(3)-ester bonds of monoglycerides to fatty acid and glycerol [20], preferring the hydrolysis of *sn*-2-arachidonoylglycerol to *sn*-2-oleoyl- [25,26] or *sn*-2-palmitoylglycerol [27]. Additionally, a recent report by Dinh *et al*. [28] supports the observation that 2-AG is mainly hydrolyzed by MGL in intact neurons. However, the relative importance of these degradative pathways in 2-AG inactivation remains elusive.

Inhibitors of endocannabinoid hydrolases could offer a rational therapeutic approach in treating certain disease states, where higher endocannabinoid activity would be beneficial. An advantage of such enzyme inhibition over direct cannabinoid agonists could result in higher selectivity, as it would increase activity of endocannabinoid system only at sites where on-going production of endocannabinoids is taking place.

In our previous CB1 receptor activation studies (Savinainen *et al*. [7]), we reported that rat cerebellar membrane preparation was able to hydrolyse 2-AG, although being almost totally devoid of FAAH activity. In this study, the hydrolyzing activity of rat cerebellar membrane preparation towards 2-AG and other endocannabinoids was examined in more detail, in order to develop a validated method for studies on novel inhibitors of 2-AG degrading enzymes. Additionally, we studied various FAAH inhibitors as inhibitors for the monoglyceride lipase-like activity found in the cerebellar preparation.

2. Materials and methods

2.1. Chemicals

2-AG, 1(3)-AG, methyl arachidonylfluorophosphonate (MAFP), arachidonoyl trifluoromethylketone (ATFMK), arachidonoyl serotonin (AA-5-HT) and URB597 were purchased from Cayman Chemical Co. Virodhamine was purchased from Tocris Cookson Ltd. OL-53 was generously provided by Professor Dale Boger (The Scripps Research Institute). AM404 was obtained from Deva Biotech Inc. and hexadecylsulphonyl fluoride (HDSF) was from Merck Biosciences Ltd. Phenylmethylsulphonyl fluoride (PMSF), DTT, ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA) (essentially fatty acid free) were purchased from Sigma-Aldrich Co. AEA and noladin ether (2-arachidonyl glyceryl ether, HU-310) were synthesized in our laboratory according to previously published procedures [1,29].

2.2. Animals

These studies used 4-week-old male Wistar rats. All animal experiments were approved by the local ethics committee. The animals lived in a 12-hr light/12-hr dark cycle (lights on at 07:00 hr) with water and food available *ad libitum*. The rats were decapitated, 8 hr after lights on (15:00 hr), whole brains were removed, dipped in isopentane on dry ice and stored at -80°.

2.3. Membrane preparation

Membranes were prepared as previously described [7,30,31]. Briefly, cerebella (minus brain stem) from eight animals were weighed and homogenized in nine volumes of ice-cold 0.32 M sucrose with a glass Teflon homogenizer. The crude homogenate was centrifuged at low speed (1000 *g* for 10 min at 4°) and the pellet was discharged. The supernatant was centrifuged at high speed (100,000 *g* for 10 min at 4°). The pellet was resuspended in ice-cold deionized water and washed twice, repeating the high-speed centrifugation. Finally, membranes were resuspended in 50 mM Tris–HCl, pH 7.4 with 1 mM EDTA and aliquoted for storage at -80°. The protein concentration of the final preparation, measured by the Bradford method [32], was 8.7 mg mL^{-1}.

2.4. Enzyme assay

The experiments were carried out with preincubations (80 μL, 30 min at 25°) containing 10 μg membrane protein, 44 mM Tris–HCl (pH 7.4), 0.9 mM EDTA, 0.5% BSA and 1.25% (v/v) DMSO as a solvent for inhibitors. The preincubated membranes were kept at 0° just prior to the experiments. The incubations (90 min at 25°), closely mimicking conditions to assess CB1 receptor-dependent G protein activity [7], were initiated by adding 40 μL of preincubated membrane cocktail, giving a final volume of 400 μL. The final volume contained 5 μg membrane protein, 54 mM Tris–HCl (pH 7.4), 1.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% BSA and 50 μM of the substrate (2-AG, 1(3)-AG, AEA, noladin ether or virodhamine). At time-points of 0 and 90 min, 100 μL samples were removed from incubation, acetonitrile

(200 μL) was added to stop the enzymatic reaction, and pH of the samples was simultaneously decreased with phosphoric acid (added to acetonitrile) to 3.0, in order to stabilize 2-AG against a possible post-incubation chemical acyl migration reaction to 1(3)-AG. Samples were centrifuged at 23,700 *g* for 4 min at RT prior to HPLC analysis of the supernatant.

The specific enzyme activity towards 2-AG, 1(3)-AG, virodhamine and AEA was determined based on the formation of arachidonic acid during the 90-min incubation period. In the case of noladin ether, the HPLC peak area of noladin ether was monitored at 0 and 90 min.

2.5. HPLC instrumentation

The analytical HPLC system consisted of a Merck Hitachi (Hitachi Ltd.) L-7100 pump, D-7000 interface module, L-7455 diode-array detector (190–800 nm, set at 211 nm) and L-7250 programmable autosampler. The separations were performed with Zorbax SB-C18 end-capped reversed-phase precolumn (4.6 mm × 12.5 mm, 5 μm) and column (4.6 mm × 150 mm, 5 μm) (Agilent). The injection volume was 50 μL. A mobile phase mixture of 28% phosphate buffer (30 mM, pH 3.0) in acetonitrile at a flow rate of 2.0 mL min^{-1} was used. Retention times were 5.8 min for 2-AG, 6.3 min for 1-AG, 7.3 min for noladin ether and 10.2 min for arachidonic acid. The relative concentrations of 2-AG, 1(3)-AG and arachidonic acid were estimated on the basis of corresponding peak areas. This was justified by the equivalence of response factors for the studied compounds, and is supported by observation that the sum of the peak areas was constant throughout the experiments. In the case of virodhamine, gradient conditions were used; solvent A was 90% acetonitrile and solvent B was phosphate buffer (30 mM, pH 3.0). Gradient conditions were as follows: 0–8 min, 60% A; 8–10 min, linear gradient to 80% A; 10–19 min, 80% A; 19–20 min, linear gradient to 60% A; 20–30 min, 60% A. The flow rate was 2 mL min^{-1}. Retention times were 7.6 min for virodhamine, 12.2 min for AEA and 18.7 min for arachidonic acid under gradient conditions.

2.6. Data analyses

For enzyme inhibition experiments, results are presented as mean ± SEM of at least three independent experiments performed in duplicate. Data analysis for dose–response curves were calculated as non-linear regressions using GraphPad Prism 3.0 for Windows.

3. Results

3.1. Degradation of endocannabinoids in rat cerebellar membrane

Degradation of 2-AG, 1(3)-AG, AEA, noladin ether and virodhamine was studied in rat cerebellar membrane preparation under assay conditions that have previously been used to monitor CB1 receptor-dependent G-protein activation [7]. The degradation of endocannabinoids was followed by reversed-phase HPLC. 2-AG was degraded to arachidonic acid by cerebellar membranes, where 1(3)-AG also formed and was degraded during the incubation (Fig. 1). Arachidonic acid formation was not observed in membrane-free buffer, indicating the enzymatic nature of 2-AG degradation. Acyl migration of 2-AG to 1(3)-AG was observed in membrane-free buffer, as expected, demonstrating the chemical nature of this reaction. Similarly, incubation of 1(3)-AG in rat cerebellar membrane preparation resulted in formation of 2-AG and arachidonic acid. The results show that the acyl migration reaction proceeds in both directions, favoring the 1(3)-configuration. During the incubation, nearly the same amount of

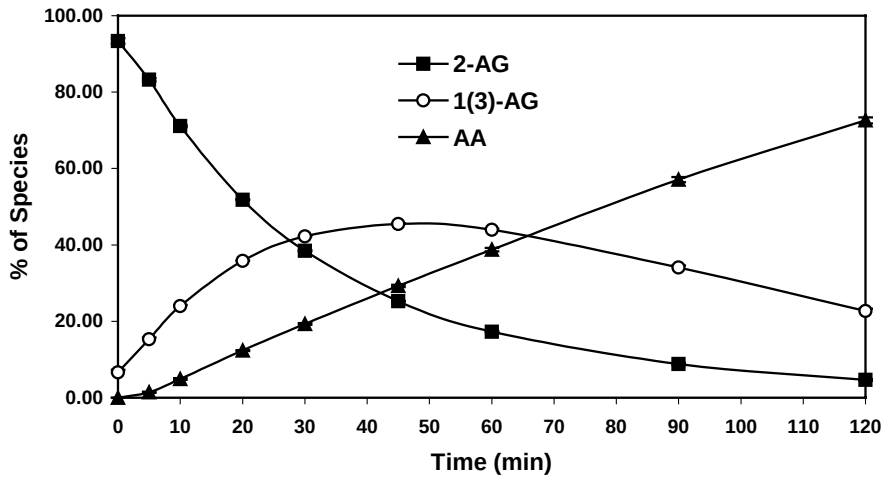


Fig. 1. Acyl migration and hydrolysis of 2-AG in rat cerebellar membranes. The data are presented as the mean ± SEM of peak area (%) from two independent experiments performed in triplicate.

Table 1
Specific enzyme activity in rat cerebellar membrane towards endocannabinoids

Compound	Specific activity (nmol min^{-1} mg^{-1} protein)
2-AG	31.8 ± 0.8[a]
1(3)-AG	29.8 ± 0.8[a]
Virodhamine	17.0 ± 0.2
Noladin ether	0
AEA	0.70 ± 0.06

The data are presented as the mean ± SEM of three independent experiments performed in duplicate or triplicate.
[a] Values represent the total enzymatic activity for 2-AG and 1(3)-AG (due to occurring chemical acyl migration during the incubation), because the determination is based on the formation of arachidonic acid.

arachidonic acid was generated when starting from either 2-AG or 1(3)-AG.

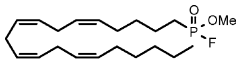
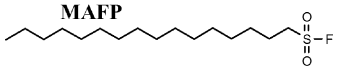
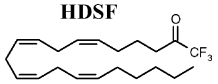
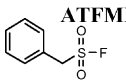
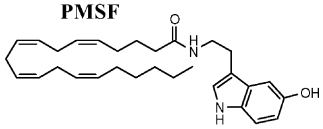
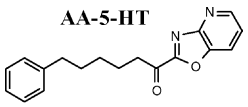
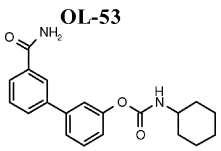
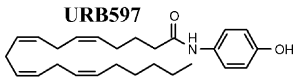
Rat cerebellar membranes showed significantly lower hydrolytic activity towards AEA when compared to 2-AG (~50-fold), and arachidonic acid formation was barely detectable in the former case (Table 1). Virodhamine hydrolysis to arachidonic acid occurred at a specific enzyme activity of 17 nmol min^{-1} mg^{-1} protein, which was about 2-fold lower than that of 2-AG. During incubation, virodhamine was partly converted to AEA, both in the absence and presence of membrane preparation. This chemical *O*- to *N*-acyl migration has been reported to be base-catalyzed and has to be taken into consideration when assessing the biological properties of virodhamine [33]. Noladin ether, an ether-linked analogue of 2-AG, was stable under the presently defined incubation conditions.

3.2. In vitro activity of inhibitors

Various potential inhibitors of 2-AG hydrolyzing activity were studied in rat cerebellar membrane preparation (Table 2). The serine hydrolase inhibitors MAFP, HDSF and PMSF, which have previously been reported to inhibit FAAH [34,35], inhibited 2-AG hydrolysis with an IC_{50} values of 2.2 nM, 241 nM and 155 μM, respectively (Fig. 2). The degradation of virodhamine was inhibited by MAFP with an IC_{50} value of 1.4 nM. ATFMK, which is a reversible electrophilic carbonyl inhibitor, inhibits FAAH with an IC_{50} value in the low micromolar range [36–38]. In our test system ATFMK inhibited 2-AG hydrolysis with an IC_{50} value of 66 μM, but the maximum inhibition was only 75% at 1 mM concentration.

A few specific FAAH inhibitors were also included to gain more information on the enzyme responsible for 2-AG hydrolysis in cerebellar membranes. AA-5-HT, a selective FAAH inhibitor with an IC_{50} value of 12 μM [39], did not inhibit 2-AG hydrolysis at 1 mM concentration. OL-53, a very potent FAAH inhibitor with a K_i value of 200 pM [40], belongs to the group of α-keto heterocycle inhibitors of FAAH that have been reported to be 100–1000 times more potent than corresponding trifluoromethyl ketones. OL-53

Table 2
IC_{50} values for the inhibition of 2-AG hydrolyzing enzyme activity in rat cerebellar membrane by various FAAH inhibitors

Compound	IC_{50} value
	2.2 ± 0.3 nM
MAFP 	241 ± 17 nM
HDSF 	66 ± 9 μM[a]
ATFMK 	155 ± 7 μM
PMSF 	No inhibition[b]
AA-5-HT 	No inhibition[b]
OL-53 	30% inhibition[b]
URB597 	No inhibition[b]
AM404	

The data are presented as the mean ± SEM of three independent experiments performed in duplicate.
[a] 75% inhibition at 1 mM ATFMK concentration.
[b] At 1 mM inhibitor concentration.

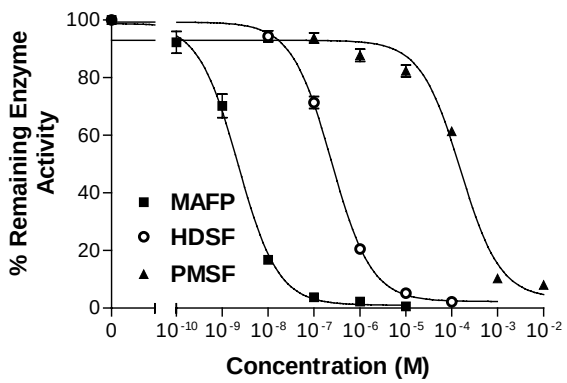


Fig. 2. Inhibition of the enzymatic hydrolysis of 2-AG by MAFP, HDSF, and PMSF. The remaining enzyme activity in the absence of inhibitors was expressed as 100%. The data represent the mean ± SEM of remaining enzyme activity (%) from three independent experiments performed in duplicate.

did not interact with the enzyme's active site, as the hydrolysis of 2-AG continued at an OL-53 concentration of 1 mM. Kathuria *et al*. [41] recently reported on the novel FAAH inhibitor URB597, with an IC_{50} value of 4.6 nM, which was shown to be highly selective towards FAAH compared to MGL. URB597 poorly inhibited 2-AG degradation in rat cerebellar membranes and the remaining enzyme activity was about 70% at 1 mM inhibitor concentration. The anandamide-transport inhibitor AM404, which has recently been reported to be a FAAH inhibitor [15], also did not prevent 2-AG degradation.

4. Discussion

2-AG is an endocannabinoid that is more abundant in the brain [3,4] and is a more potent cannabinoid receptor ligand than the previously discovered cannabinoid receptor ligand AEA [4–7]. 2-AG binds to and activates G-protein-coupled cannabinoid receptors as a full agonist, exhibiting cannabimimetic effects such as hypothermia, hypomotility and antinociception [2,42]. Nevertheless, the biologic activity of 2-AG is limited due to rapid enzymatic hydrolysis to arachidonic acid and glycerol. Earlier studies have indicated that FAAH, the enzyme responsible for AEA inactivation, may also contribute to the hydrolysis and inactivation of 2-AG [17,18]. However, more recent evidence has gathered supporting evidence for the role of monoacylglycerol lipase in the biological deactivation of 2-AG.

Goparaju *et al*. [22] purified 2-AG hydrolyzing enzymatic activity from porcine brain that was distinct from FAAH. Further, Beltramo and Piomelli [23] reported that inhibition of FAAH by BTNP does not prevent 2-AG hydrolysis in intact astrocytoma cells. Moreover, 2-AG produces similar behavioral effects in mice lacking FAAH than it does in mice having normal FAAH activity [24].

Over 20 years ago, Okazaki *et al*. [25] reported that arachidonic acid is released from 2-acyl-*sn*-glycerols by MGL activity. MGL hydrolyses arachidonic acid from the *sn*-2-monoglycerol faster than oleic acid [25,26] or palmitic acid [27] indicating an important role of polyunsaturated fatty acid chain in the binding of 2-AG at the enzymatic site of action. Recently, Karlsson *et al*. [43] cloned MGL from a mouse adipocyte cDNA, and its lipase-specific catalytic triad (Ser-122, Asp-239 and His-269) was identified by site-directed mutagenesis experiments. They also found mRNA for MGL in rat brain.

Several studies have shown that cerebellum has high levels of FAAH activity [44–46]. Contrary to those studies, there are articles where levels of FAAH activity in cerebellum have reported to be intermediate or very low [11,47]. In our recent CB1 receptor activation studies [7], PMSF-pretreatment of rat cerebellar membranes increased G-protein activation potency of 2-AG and 1(3)-AG, but left the potency of AEA unaffected. This finding suggested that the preparation contained 2-AG hydrolyzing activity distinct from FAAH. More careful investigations of the hydrolyzing activity of rat cerebellar membrane preparation in the present study confirmed that the preparation shows very low FAAH activity compared to MGL-like activity. As the specific activity of FAAH towards 2-AG has been reported to be up to 4-fold greater compared to AEA [17], the presently observed 50-fold difference in hydrolysis activities suggests that additional enzymatic activity besides FAAH was responsible for 2-AG hydrolysis. Unfortunately, no selective MGL inhibitors are currently known. Therefore, the final identity of the enzyme(s) responsible for monoglyceride lipase-like activity observed in rat cerebellar membrane remains to be established. However, the equal rates of hydrolysis for 1(3)-AG and 2-AG in this study support the role of MGL described by Tornqvist and Belfrage [20], who demonstrated that 1(3)- and 2-ester bonds of monooleoylglycerol are also hydrolyzed by MGL at equal rates.

Noladin ether, an ether-linked analogue of 2-AG was stable under experimental conditions. Hanus *et al*. [48] and Fezza *et al*. [49] reported that noladin ether is the third endocannabinoid to be found. This finding was contradicted very recently by Oka *et al*. [50], who reported that noladin ether was not detected in the brains of rat, mouse, hamster, guinea-pig or pig. Indeed, the unique structure of noladin ether and lack of a rapid enzymatic degradation pathway raises serious questions of its putative role in the endocannabinoid system. In the case of virodhamine, hydrolysis was substantial and occurred at almost the same rate as the hydrolysis of 2-AG, which indicates that both may be hydrolyzed by the same enzyme. Importantly, this suggestion is further supported by similar IC_{50} values of MAFP against 2-AG (2.2 nM) and virodhamine (1.4 nM) hydrolysis.

The possibility of FAAH for 2-AG hydrolyzing activity in rat cerebellar membranes was excluded in this study, as the very potent FAAH inhibitors OL-53 and URB597 did not inhibit the hydrolysis of 2-AG. Additionally, MAFP was over 70,000 times more effective as an inhibitor than PMSF, whereas MAFP was reported to be around 400 times more effective an inhibitor than PMSF for FAAH [34]. Very recently Dinh *et al*. [28] cloned MGL from rat brain and inhibited hydrolysis of 2-AG in MGL overexpressed HeLa cells by MAFP, ATFMK and HDSF with IC_{50} values of 800 nM, 2.5 μM and 6.2 μM, respectively. Again, in the present study 2-AG hydrolysis was inhibited by MAFP and HDSF, but in the case of ATFMK, 2-AG hydrolysis was not completely inhibited even at 1 mM inhibitor concentration. In the present study, 2-AG hydrolysis was inhibited by MAFP, with clearly higher potency than was reported by Dinh *et al*. Relative differences in IC_{50} values may be explained by different experimental conditions. However, our data regarding the potencies of MAFP and ATFMK are in close agreement with those obtained by Goparaju *et al*. [22], who reported that MAFP and ATFMK

inhibited 2-AG hydrolysis in porcine brain with IC_{50} values of 2 nM and 30 μM, respectively. Finally, the possibility of other additional enzymatic activities needs to be considered, as monoacylglycerols have also been reported to be hydrolyzed by other lipases and esterases [20,21].

In conclusion, this enzyme assay provides a useful method for further inhibition studies of 2-AG degrading enzyme(s) in brain membrane preparation having considerably higher MGL-like activity when compared to FAAH activity.

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