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Enzymes of Porcine Brain Hydrolyzing 2-Arachidonoylglycerol, an Endogenous Ligand of Cannabinoid Receptors

*Sravan Kumar Goparaju, Natsuo Ueda, Kyoko Taniguchi and Shozo Yamamoto**
DEPARTMENT OF BIOCHEMISTRY, TOKUSHIMA UNIVERSITY, SCHOOL OF MEDICINE, TOKUSHIMA 770-8503, JAPAN

ABSTRACT. Anandamide and 2-arachidonoylglycerol (2-AG) are two endogenous ligands for the cannabinoid receptors, and their cannabimimetic activities are lost when they are hydrolyzed enzymatically. Cytosol and particulate fractions of porcine brain exhibited a high 2-AG hydrolyzing activity of 100 nmol/min/mg protein. Most of the activity could be attributed to a monoacylglycerol lipase-like enzyme that did not hydrolyze anandamide. It was separated by hydroxyapatite chromatography from anandamide amidohydrolase, which is also capable of hydrolyzing 2-AG as well as anandamide. Thus, porcine brain has at least two enzymes capable of hydrolyzing 2-AG. The 2-AG hydrolase activities of both the cytosolic and particulate enzymes were irreversibly and time-dependently inhibited by methyl arachidonyl fluorophosphonate with IC_{50} values as low as 2–3 nM. BIOCHEM PHARMACOL **57**;3:417–423, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cannabinoid; anandamide; 2-arachidonoylglycerol; monoacylglycerol lipase; porcine brain; methyl arachidonyl fluorophosphonate

Anandamide (arachidonoylethanolamide) and 2-AG† are two endogenous ligands for the cannabinoid receptors CB1 and CB2 [1–3]. Both anandamide and 2-AG lose their cannabimimetic effects when they are hydrolyzed enzymatically. Anandamide is hydrolyzed to arachidonic acid and ethanolamine by an enzyme called anandamide amidase [4], or amidohydrolase [5–7], or fatty acid amide hydrolase [8]. Since the partially purified enzyme of porcine brain hydrolyzes various *N*-acylethanolamines as well as anandamide [6], the enzyme seems to be identical to *N*-acylethanolamine amidohydrolase reported earlier for rat liver [9], although the hydrolysis of anandamide was not actually shown by the latter enzyme. Very recently, we [10] and Di Marzo *et al.* [11] demonstrated that anandamide amidohydrolase could also hydrolyze 2-AG efficiently. In addition, it is well known that monoacylglycerols, including 2-AG, are hydrolyzed by other enzymes such as lipases and esterases [12–16].

The CB1 type of cannabinoid receptor is expressed abundantly in the brain [17], and 2-AG levels in the brain have been reported to be from 170 times [18] to 1000 times [3] higher than anandamide levels. Therefore, we were interested in the hydrolysis of 2-AG by brain enzyme(s), which regulates its cannabimimetic effects. Even though the presence of monoacylglycerol lipase hydrolyzing 2-AG in the brain and neuroblastoma cells was reported earlier [19–23], the enzyme has not been fully characterized with reference to the cannabimimetic compounds. The relationship between 2-AG hydrolyzing enzymes and anandamide amidohydrolase in the brain also remains unclear, and this is the subject of the present work.

MATERIALS AND METHODS

Materials

[1-^{14}C]Arachidonic acid and 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol were purchased from Amersham International; arachidonic acid and 1(3)-AG from Nu-Chek-Prep; and anandamide, MAFP, and ATFMK from the Cayman Chemical Co. 1-Stearoyl-2-arachidonoyl-*sn*-glycerol was obtained from Biomol, and 1(3)-oleoylglycerol and *Rhizopus arrhizus* lipase were from the Sigma Chemical Co. 2-AG was provided by Dr. Hiroyuki Iwamura of Japan Tobacco Inc., Central Pharmaceutical Research Institute. [*Arachidonoyl*-1-^{14}C]anandamide was chemically prepared from [1-^{14}C]arachidonic acid and ethanolamine as described previously [24]. 2-[1-^{14}C]Arachidonoylglycerol was prepared by the digestion of 1-stearoyl-2-[1-^{14}C]arachidonoylglycerol with *R. arrhizus* lipase, and was purified as described before [10]. Since *trans*-esterification of 2-AG to 1(3)-AG is accelerated by acid or base or heat [25], the purity of the 2-[1-^{14}C]arachidonoylglycerol thus prepared was checked by HPLC as described before [10], and was found to be at least 84% pure. ProbeQuant G-50 micro columns were obtained from Pharmacia Biotech, and hydroxyapatite Bio-Gel HTP Gel was obtained from Bio-Rad Laboratories.

* Corresponding author: Dr. Shozo Yamamoto, Department of Biochemistry, Tokushima University, School of Medicine, Kuramoto-cho, Tokushima 770-8503, Japan. Tel. (81) 886–33-9223; FAX (81) 886–33-6409.

† *Abbreviations*: AG, arachidonoylglycerol; MAFP, methyl arachidonyl fluorophosphonate; and ATFMK, arachidonyl trifluoromethyl ketone.

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Enzyme Preparation

Porcine brain was obtained from a local slaughterhouse. Cerebrum was homogenized in 9 times the volume (v/w) of 20 mM Tris–HCl buffer (pH 8.0) containing 0.32 M sucrose by a Potter–Elvehjem homogenizer, and centrifuged at 100 *g* for 15 min at 4° to remove tissue debris. Then the homogenate was centrifuged at 184,000 *g* for 50 min at 4°. The supernatant was used as the cytosol, and for enzyme purification, sucrose was removed from the cytosol by ultrafiltration through an Amicon Diaflo PM-10 membrane.

The pellet was resuspended in the same buffer and centrifuged again at 184,000 *g* for 50 min at 4°. The pellet was resuspended in 20 mM Tris–HCl buffer (pH 8.0), and was used as a particulate fraction. Then the particulate fraction (5 mg protein/mL) was incubated in 20 mM Tris–HCl buffer (pH 8.0) containing 0.1% Triton X-100. After incubation for 20 min on ice, the mixture was centrifuged at 184,000 *g* for 50 min at 4°. The clear supernatant obtained was used as the solubilized enzyme.

The cytosol and the Triton-X-100 solubilized protein were loaded onto a hydroxyapatite column (a bed volume of 10 mL) that was pre-equilibrated with 20 mM Tris–HCl (pH 8.0) containing 0.1% Triton X-100 and 5 mM 2-mercaptoethanol (Buffer A). The column was washed with Buffer A at a flow rate of 50 mL/hr, and 10-mL fractions were collected. The proteins adsorbed on the column were eluted with Buffer A containing 0.25 M potassium phosphate (pH 8.0). The fractions were assayed for 2-AG and anandamide hydrolase activities as described below. Protein concentration was determined by the method of Bradford [26] with BSA as standard.

Enzyme Assay

The enzyme preparations were incubated with either [^{14}C]2-AG (1000 cpm/5 nmol/5 μL DMSO) or [^{14}C]anandamide (5000 cpm/5 nmol/5 μL DMSO) in 100 μL of 50 mM sodium phosphate (pH 7.0) for 2-AG or 50 mM Tris–HCl (pH 9.0) for anandamide at 37° for 5 min. The reaction was terminated by the addition of 0.35 mL of a cold solution of diethyl ether/methanol/1 M citric acid (30:4:1, by vol.). The ethereal extract (0.1 mL) was spotted on a Merck silica gel 60 F254 glass plate (10-cm height), which was developed in a mixture of chloroform/methanol/25% ammonium hydroxide (80:20:2, by vol.) for 20 min at room temperature. Radioactivity on the plate was scanned by a Fujix BAS2000 bioimaging analyzer.

For enzyme assays with inhibitors, the enzyme preparation was preincubated with inhibitors dissolved in 2.5 μL of DMSO at 24° for 10 min. For examination of the reversible inhibition by MAFP or arachidonic acid, the porcine brain cytosol (10.5 μg protein in 8 μL) was diluted in 150 μL of 50 mM sodium phosphate (pH 7.0) containing 1 mg/mL bovine γ-globulin and 0.05% Triton X-100, and preincubated with the inhibitors for 5 min at 24°. A 50-μL aliquot of this solution was assayed in 100 μL of the reaction mixture for 2-AG hydrolysis as described above. Another 50-μL aliquot was applied onto a mini-prep gel filtration column that was pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.05% Triton X-100. The column was centrifuged at 735 *g* at 4° for 2 min, and the collected fraction in the tube was assayed in 100 μL of the reaction mixture for 2-AG hydrolysis.

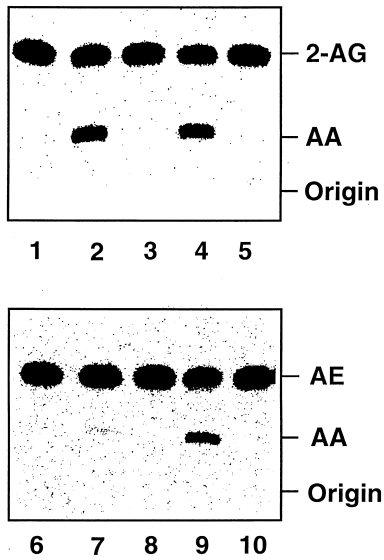

FIG. 1. Hydrolysis of 2-AG and anandamide by the cytosol and particulate fractions of porcine brain. Protein free buffer (lanes 1 and 6), the native cytosol (lanes 2 and 7), the heat-treated (kept at 95° for 5 min) cytosol (lanes 3 and 8), the native particulate fraction (lanes 4 and 9), and the heat-treated particulate fraction (lanes 5 and 10) were incubated with 50 μM [^{14}C]2-AG in 50 mM sodium phosphate buffer at pH 7.0 (lanes 1–5) or with 50 μM [^{14}C]anandamide in 50 mM Tris–HCl buffer at pH 9.0 (lanes 6–10). Protein amounts used were 3.5 μg (lanes 2–5) or 39 μg (lanes 7–10). The products were separated by TLC. Abbreviations: AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; and AE, anandamide.

RESULTS

When the cytosol and particulate fractions of porcine brain were incubated with [^{14}C]2-AG, a radioactive band corresponding to free arachidonic acid was observed on the TLC plate (lanes 2 and 4 of Fig. 1). Protein-free buffer (lane 1) and boiled proteins (lanes 3 and 5) did not catalyze this reaction, indicating the enzymatic nature of the 2-AG hydrolysis. Furthermore, the particulate fraction also hydrolyzed anandamide (lane 9), as was reported earlier [6]. The cytosol exhibited a very low hydrolyzing activity toward anandamide (lane 7).

We next compared the specific activities of 2-AG and anandamide hydrolysis of porcine brain (Table 1). Optimal pH was around 7.0 for the 2-AG hydrolysis and around 9.0 for the anandamide hydrolysis. 2-AG was hydrolyzed efficiently by both the cytosol and particulate fractions with specific activities as high as about 100 nmol/min/mg pro-

TABLE 1. Enzyme activities for 2-AG and anandamide hydrolysis in porcine brain

	Enzyme activity (nmol/min/mg protein)			
	2-AG hydrolysis		Anandamide hydrolysis	
Samples	pH 7.0	pH 9.0	pH 7.0	pH 9.0
Cytosol	120 ± 13 (3.5)	37 ± 3 (3.5)	0.11 ± 0.07 (39)	0.15 ± 0.04 (39)
Partially purified enzyme from cytosol*	3480 ± 0 (0.09)			ND (0.39)
Particulate fraction	112 ± 13 (3.5)	61 ± 2 (3.5)	2.1 ± 0.1 (39)	4.0 ± 0.1 (39)
Solubilized protein from particulate fraction†	131 ± 14 (2.0)			0.45 ± 0.02 (172)
Partially purified enzyme from the solubilized protein‡	4970 ± 190 (0.12)			ND (0.69)

Enzyme preparations were incubated with either 50 μM 2-AG or 50 μM anandamide in 50 mM sodium phosphate buffer (pH 7.0) or 50 mM Tris-HCl buffer (pH 9.0). Protein amounts in micrograms used for each assay are indicated in parentheses. Values are means ± SD (N = 3). ND: below detection limit.

* Fraction No. 3 of hydroxyapatite chromatography (Fig. 2B) was used.

† The particulate fraction was solubilized with 0.1% Triton X-100, as described in Materials and Methods.

‡ Fraction No. 2 of hydroxyapatite chromatography (Fig. 2A) was used.

tein at pH 7.0. Anandamide amidohydrolase activities of the cytosol and particulate fractions were much lower under similar assay conditions at pH 9.0 (0.15 and 4.0 nmol/min/mg protein, respectively). As we reported recently [10], a recombinant rat anandamide amidohydrolase was about fourfold more reactive with 2-AG than with anandamide. However, in the cytosol and particulate fractions of porcine brain, the 2-AG hydrolyzing activity at pH 7.0 was much higher (800- and 30-fold) than the anandamide hydrolyzing activity at pH 9.0. Therefore, most of the 2-AG hydrolase activity in porcine brain seemed to be derived from an enzyme other than anandamide amidohydrolase, and the contribution of anandamide amidohydrolase to 2-AG hydrolysis seemed to be minor.

To confirm the presence of an enzyme distinct from anandamide amidohydrolase in porcine brain, we partially purified the 2-AG hydrolyzing enzyme. Treatment of the particulate fraction with 0.1% Triton X-100 led to efficient solubilization of the 2-AG hydrolyzing activity (Table 1). However, these conditions brought about a less efficient solubilization of anandamide amidohydrolase. As shown in Fig. 2A, when the Triton X-100-solubilized proteins were applied to a hydroxyapatite column, a majority of the 2-AG hydrolyzing activity was found in the pass-through fractions, with a specific activity 38-fold higher than that of the solubilized proteins (Table 1). Anandamide amidohydrolase activity was adsorbed onto the column, followed by elution with 250 mM potassium phosphate. A part of the activity hydrolyzing 2-AG was also eluted with 250 mM potassium phosphate a little earlier than anandamide amidohydrolase.

Further, porcine brain cytosol was applied to the hydroxyapatite column (Fig. 2B). As in the case of the particulate fraction (Fig. 2A), there were two peaks of 2-AG hydrolyzing activity. The 2-AG hydrolyzing enzyme, which passed through the column, was purified 29-fold, and this partially purified enzyme (Fig. 2B, Fraction No. 3) did not hydrolyze anandamide (Table 1).

Substrate specificity of the partially purified enzymes of the cytosol and particulate fractions was examined by HPLC, as we described previously [10]. The relative activities of the cytosolic enzyme with 2-AG, 1(3)-AG, and 1(3)-oleoylglycerol at 50 μM were 100, 65, and 10%, respectively. Those of the particulate enzyme with the same substrates were 100, 41, and 21%. In contrast to monoacylglycerols, both the enzymes were almost inactive with 1-stearoyl-2-arachidonoylglycerol. The broad specificity for various monoacylglycerols and the inability to hydrolyze

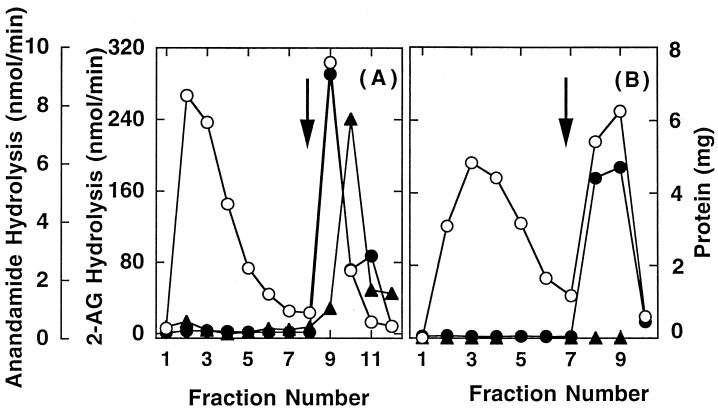

FIG. 2. Hydroxyapatite chromatography of 2-AG hydrolase in the particulate and cytosol fractions of porcine brain. The 0.1% Triton X-100-solubilized particulate fraction (A) and the cytosol fraction (B) (each 13 mg protein in 5 mL) were applied to a hydroxyapatite column as described in Materials and Methods. The fractions were assayed for the hydrolase activities of 2-AG (open circles) and anandamide (closed triangles). Protein amounts (closed circles) are also shown. Arrows indicate the change of buffer from Buffer A to Buffer A containing 250 mM potassium phosphate. The experiments were performed two times, and essentially the same results were obtained.

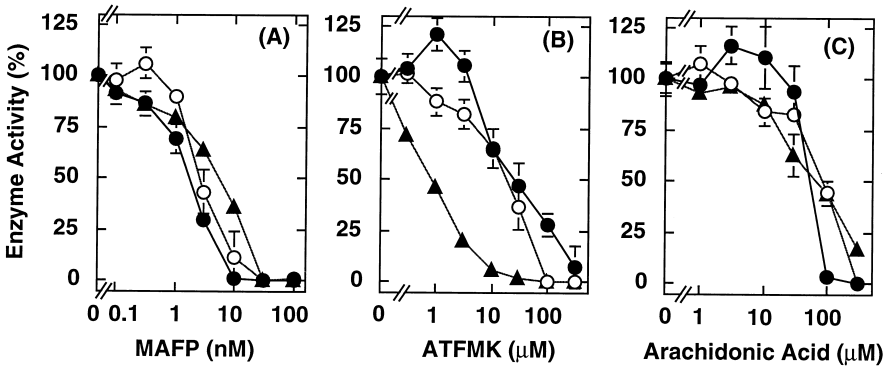

FIG. 3. Inhibition of the enzymatic hydrolysis of 2-AG and anandamide by MAFP, ATFMK, and arachidonic acid. The cytosol (3.5 μg protein, closed circles) and particulate fractions (3.5 μg protein, open circles) of porcine brain were allowed to react with 50 μM 2-AG in the presence of different concentrations of MAFP (A), ATFMK (B), or arachidonic acid (C). The particulate fraction (79 μg protein) was also allowed to react with 50 μM anandamide in the presence of each inhibitor (closed triangles). Assays were performed in triplicate, and mean values ± SD are shown. The activities in the absence of inhibitors are expressed as 100%: 2-AG hydrolysis by the cytosol, 103 ± 7 nmol/min/mg protein; 2-AG hydrolysis by the particulate fraction, 112 ± 0 nmol/min/mg protein; anandamide hydrolysis by the particulate fraction, 2.5 ± 0.1 nmol/min/mg protein.

diacylglycerol have been reported for the purified monoacylglycerol lipase from rat adipose tissue [12].

Recently, we reported that MAFP was a potent inhibitor in the hydrolysis of 2-AG catalyzed by anandamide amidohydrolase [10]. Therefore, we were interested in knowing whether MAFP could inhibit the 2-AG hydrolyzing activity of porcine brain distinct from anandamide amidohydrolase activity. MAFP inhibited 2-AG hydrolysis in the cytosol and particulate fractions in a concentration-dependent manner (Fig. 3A). The IC_{50} values were as low as 2 and 3 nM, respectively, for the cytosol and particulate fractions. MAFP could also inhibit the anandamide hydrolysis of the particulate fraction with an IC_{50} value of 6 nM under similar assay conditions (Fig. 3A), as described earlier [10, 27, 28]. In addition, ATFMK, which was reported originally as an inhibitor of cytosolic phospholipase A_2 [29], inhibited the 2-AG hydrolysis by both the cytosol and particulate fractions concentration dependently, but its IC_{50} values (around 30 and 20 μM, respectively) were much higher than those of MAFP (Fig. 3B). Under similar assay conditions, ATFMK inhibited anandamide hydrolysis by the particulate fraction with an IC_{50} value around 1 μM, as reported earlier [6, 30].

Free fatty acids were reported to cause product inhibition of monoacylglycerol lipase [20, 31, 32]. Free arachidonic acid inhibited 2-AG hydrolysis in the cytosol and particulate fractions with IC_{50} values of 60 and 75 μM, respectively (Fig. 3C). Arachidonic acid also inhibited anandamide hydrolysis in the particulate fraction with an IC_{50} value of about 65 μM.

We examined the time dependency of inhibition of the 2-AG hydrolase by MAFP. When the preincubation period of the cytosol fraction with MAFP was changed, the 2-AG hydrolysis was inhibited time-dependently (Fig. 4A). Under the same conditions, the inhibition by free arachidonic acid was time-independent (Fig. 4B). Furthermore, we tested the irreversibility of inhibition by MAFP of the 2-AG hydrolyzing enzyme. After preincubation of the cytosol fraction with MAFP for 5 min, the enzyme solution was applied onto a gel filtration column to remove the inhibitor, and the pass-through fraction was assayed for 2-AG hydrolyzing activity. It was observed that there was no recovery of enzyme activity in the MAFP-treated enzyme, whereas under the same conditions a complete recovery of the enzyme activity was observed with the enzyme preincubated with free arachidonic acid (Fig. 5). These results show that the 2-AG hydrolyzing activity is inhibited irreversibly by MAFP.

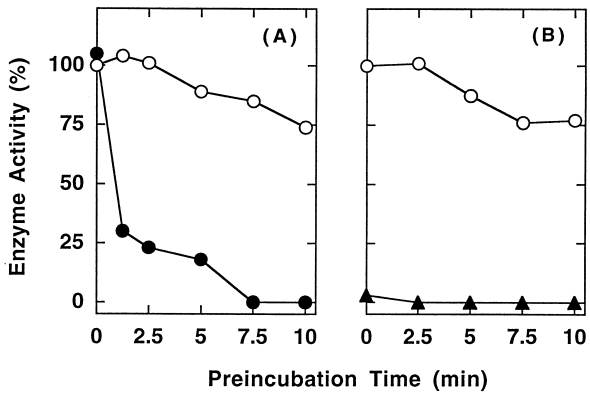

FIG. 4. Time-dependent inhibition of 2-AG hydrolysis by MAFP and arachidonic acid. The cytosol fraction of porcine brain (3.5 μg protein) was preincubated for different time periods either in the presence of (A) 3 nM MAFP (closed circles) or (B) 100 μM arachidonic acid (closed triangles) or in their absence (open circles). The remaining 2-AG hydrolyzing activity was measured as described in Materials and Methods. Mean values are shown (N = 2). The activity at time 0 in the absence of inhibitors was expressed as 100%: 149 nmol/min/mg protein (A) and 124 nmol/min/mg protein (B).

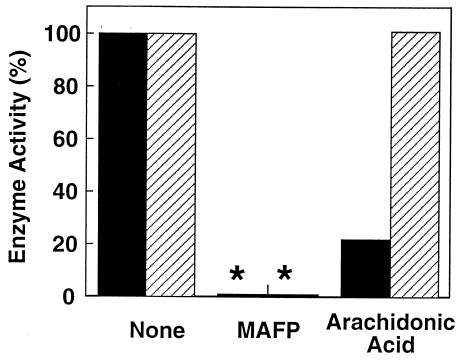

FIG. 5. Reversible inhibition of 2-AG hydrolysis by MAFP and arachidonic acid. The cytosol of porcine brain (10.5 μg protein) was preincubated with either 15 nM MAFP or 300 μM arachidonic acid for 5 min. Then a 50-μL aliquot was used for the 2-AG hydrolyzing assay after adjusting the total assay volume to 100 μL (closed columns). Another 50-μL aliquot was applied to a gel filtration column to remove the inhibitors. The collected fraction was adjusted to 100 μL, and was assayed for 2-AG hydrolyzing activity (striped columns). Mean values are shown (N = 2). Asterisks indicate the activity below detection limit. The activity in the absence of inhibitors was expressed as 100%: before and after gel filtration, 85 and 29 nmol/min/mg protein, respectively.

DISCUSSION

2-AG was identified recently as a second endogenous ligand for cannabinoid receptors [2, 3], following the earlier discovery of anandamide as a ligand. 2-AG binds to the cannabinoid receptors, and exhibits typical cannabimimetic effects such as antinociception, hypothermia, and catalepsy [2]. 2-AG also inhibits forskolin-stimulated adenylyl cyclase activity [18], modulates the intracellular Ca^{2+} level [33, 34], and suppresses hippocampal long-term potentiation [18].

2-AG has been presumed to lose its biologic activity by enzymatic hydrolysis [3]. Recently, we reported that a recombinant anandamide amidohydrolase could hydrolyze 2-AG as well as anandamide [10]. However, other enzymes of the lipase and carboxylesterase types may also be involved in the inactivation of 2-AG. In particular, monoacylglycerol lipase, which hydrolyzes monoacylglycerols including 2-AG, is distributed widely in a variety of mammalian tissues [12, 13, 16, 35]. Earlier, Tornqvist and Belfrage [12] purified a 33 kDa monoacylglycerol lipase from rat adipose tissue. Monoacylglycerol lipases of 62 and 68 kDa were also purified from rat liver [13] and human erythrocytes [16], respectively. Very recently, Karlsson *et al.* [35] cloned and characterized the cDNA for adipocyte monoacylglycerol lipase and showed a wide distribution of its mRNA in many organs including brain. In addition, rat microsomal non-specific carboxylesterase was reported to have a monoacylglycerol lipase activity [14, 15].

Although monoacylglycerol lipase activities have been reported in guinea pig, rat, and bovine brains [19–22], the enzymes responsible for 2-AG hydrolysis in the brain have not been well characterized. It is unknown what types of cells in the brain express the 2-AG hydrolyzing enzyme. In the present study, we showed that porcine brain possesses a potent 2-AG hydrolyzing activity in both the cytosol and particulate fractions. We also demonstrated a predominance of a monoacylglycerol lipase-like enzyme activity in porcine brain and a minor contribution from anandamide amidohydrolase for the hydrolysis of 2-AG. Based on the wide substrate specificity for various monoacylglycerols, our partially purified 2-AG hydrolase of porcine brain seems to be identical to monoacylglycerol lipase previously reported in the brains of other animal species [19–22].

The potent 2-AG hydrolyzing enzyme in the brain interferes with the assay for biologic activities of exogenously added 2-AG. In fact, Sugiura *et al.* [3] reported that addition of diisopropyl fluorophosphate increases the affinity of 2-AG for the receptor of rat brain. Thus, potent and selective inhibitors for enzymatic 2-AG hydrolysis are required for precise quantitative assays of biologic activities of 2-AG. However, only generally used serine hydrolase inhibitors such as diisopropyl fluorophosphate and sulfhydryl group blockers such as *p*-chloromercuribenzoic acid have been reported to inhibit monoacylglycerol lipase [12, 16, 31, 36, 37].

MAFP is an active site phosphonylating agent, and was first reported to inhibit cytosolic phospholipase A_2 [38]. Later, MAFP was also shown to inhibit calcium-independent phospholipase A_2 (IC_{50}, 0.5 μM) [39]. Binding of a cannabinoid receptor ligand (CP-55940) to CB1 was displaced by MAFP (IC_{50}, 20 nM) [27], and MAFP at 1 μM significantly attenuated the pharmacological activities of various cannabinoid receptor ligands [40]. Recently, Deutsch *et al.* [27] and De Petrocellis *et al.* [28] reported that MAFP is the most potent inhibitor of anandamide amidohydrolase thus far reported (IC_{50}, 1–3 nM). We also reported that MAFP potently inhibits recombinant anandamide amidohydrolase, using 2-AG as another substrate (IC_{50}, 6 nM) [10].

The present study indicates that MAFP at a nanomolar concentration range inhibits 2-AG hydrolysis by the cytosol and particulate fractions of porcine brain, principally attributable to an enzyme other than anandamide amidohydrolase. Thus, MAFP can inhibit hydrolysis of the two endogenous ligands, anandamide and 2-AG, at similar concentrations in the brain. The inhibition of 2-AG hydrolysis by MAFP was time dependent and irreversible, as reported previously for phospholipase A_2 [38, 39] and anandamide amidohydrolase [27, 28]. Furthermore, we showed that 2-AG hydrolyzing enzyme (monoacylglycerol lipase) was more sensitive to MAFP than phospholipase A_2, which originally was the target of MAFP. Considering that monoacylglycerol lipase is involved in arachidonic acid release from phospholipid through the phospholipase C–diacylglycerol lipase pathway [41], we should interpret cautiously experimental results obtained with MAFP as a phospholipase A_2 inhibitor.

In addition to the hydrolysis, it was reported that 2-monoacylglycerol can be converted to 1,2-diacylglycerol

by monoacylglycerol acyltransferase [42] or to lysophosphatidic acid by monoacylglycerol kinase [43]. Thus, we should note that the hydrolysis is not the sole pathway for the inactivation of 2-AG. However, these 2-AG transformations were not observed under our assay conditions.

In conclusion, an enzyme distinct from anandamide amidohydrolase is principally responsible for the 2-AG hydrolysis in porcine brain, and the enzyme was inhibited completely by extremely low concentrations of MAFP. Further attempts to develop more selective inhibitors for 2-AG hydrolysis will lead to a better understanding of the pathophysiological role of 2-AG.

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