

# Hydrolysis of Prostaglandin Glycerol Esters by the Endocannabinoid-Hydrolyzing Enzymes, Monoacylglycerol Lipase and Fatty Acid Amide Hydrolase<sup>†</sup>

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**ABSTRACT:** Cyclooxygenase-2 (COX-2) can oxygenate the endocannabinoids, arachidonyl ethanolamide (AEA) and 2-arachidonylglycerol (2-AG), to prostaglandin-H<sub>2</sub>-ethanolamide (PGH<sub>2</sub>-EA) and -glycerol ester (PGH<sub>2</sub>-G), respectively. Further metabolism of PGH<sub>2</sub>-EA and PGH<sub>2</sub>-G by prostaglandin synthases produces a variety of prostaglandin-EA's and prostaglandin-G's nearly as diverse as those derived from arachidonic acid. Thus, COX-2 may regulate endocannabinoid levels in neurons during retrograde signaling or produce novel endocannabinoid metabolites for receptor activation. Endocannabinoid-metabolizing enzymes are important regulators of their action, so we tested whether PG-G levels may be regulated by monoacylglycerol lipase (MGL) and fatty acid amide hydrolase (FAAH). We found that PG-Gs are poor substrates for purified MGL and FAAH compared to 2-AG and/or AEA. Determination of substrate specificity demonstrates a 30–100- and 150–200-fold preference of MGL and FAAH for 2-AG over PG-Gs, respectively. The substrate specificity of AEA compared to those of PG-Gs was ~200–300 fold higher for FAAH. Thus, PG-Gs are poor substrates for the major endocannabinoid-degrading enzymes, MGL and FAAH.

COX-2 can oxygenate arachidonyl ethanolamide (AEA)<sup>1</sup> and 2-arachidonylglycerol (2-AG) to prostaglandin-H<sub>2</sub>-ethanolamide (PGH<sub>2</sub>-EA) and -glycerol ester (PGH<sub>2</sub>-G), respectively (1, 2). Prostaglandin isomerases/synthases metabolize the PGH<sub>2</sub>-derived species to the corresponding PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2α</sub>, and PGI<sub>2</sub>, (but not TxA<sub>2</sub>) ethanolamides or glyceryl esters in vitro (3). Peritoneal macrophages synthesize PGE<sub>2</sub>-G and PGI<sub>2</sub>-G in response to zymosan treatment, and RAW 264.7 cells synthesize PGD<sub>2</sub>-G when exposed to ionomycin following lipopolysaccharide and interferon-γ pretreatment (4, 5). An important consideration when assessing the impact of PG-G synthesis in cells or tissues is the rate of degradation of this bioactive lipid. For example, primary macrophages do not significantly degrade PG-Gs over several hours, but PGE<sub>2</sub>-G is rapidly metabolized to its free acid species in rat

plasma [*t*<sub>1/2</sub> ~ 14 s (6)]. In contrast to PG-Gs, PG-EAs are relatively stable to hydrolysis in rat whole blood (6). Instead, PGE<sub>2</sub>-EA undergoes slow dehydration and/or isomerization to produce PGB<sub>2</sub>-EA (approximately two-thirds of the starting material converted over 40 h at 37 °C) (6).

Although previous cellular studies demonstrate PG-G formation (4, 5), evidence for the production of these species in intact tissue is lacking. However, it has been shown that COX-2 participates in regulating 2-AG levels in neurons during retrograde signaling (7), and COX-2 metabolites of 2-AG metabolism (PGE<sub>2</sub>-G, D<sub>2</sub>-G, and F<sub>2α</sub>-G) increase the frequency of miniature inhibitory postsynaptic currents in primary hippocampal neurons (8). This latter effect is independent of endocannabinoid and prostanoid signaling pathways. However, it appears that COX-2 inhibitors specifically block inhibitory retrograde signaling in the hippocampus. Others show that COX-2 does not contribute to the regulation of 2-AG levels during inhibitory or excitatory retrograde signaling in mouse cerebellum or hippocampus, respectively (9, 10). Characterization of the enzymatic activity responsible for PG-G degradation could be important in explaining the potential biological function of PG-Gs. Also, identification of an enzyme or enzymes that degrade PG-Gs would allow specific targeting of esterases that recognize these species. Therefore, an accurate model of PG-G synthesis and biological function could be assessed.

Two enzymes that may participate in the degradation of PG-Gs include monoacylglycerol lipase (MGL) and fatty acid

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<sup>1</sup> Abbreviations: COX-2, cyclooxygenase-2; AEA, arachidonyl ethanolamide (anandamide); AG, arachidonylglycerol; EA, ethanolamide; PG, prostaglandin; MGL, monoacylglycerol lipase; FAAH, fatty acid amide hydrolase; 2-OG, 2-oleoylglycerol; ELSD, evaporative light scattering detection; MS, mass spectrometry.

amide hydrolase (FAAH). MGL is responsible for the degradation of monoacylglycerols, such as 2-AG, in the brain (11). FAAH degrades *N*-acyl ethanolamides such as AEA but also degrades 2-AG in vitro (12). Both of these hydrolase enzymes regulate the levels of endocannabinoids (2-AG and AEA) during retrograde synaptic signaling in the hippocampus (7, 13). Recently, it was reported that the COX products of 2-AG and AEA only slightly inhibit degradation of 2-oleoylglycerol (2-OG) and AEA by cytosolic (MGL-containing) and membrane (FAAH-containing) fractions isolated from rat brain cerebellum, respectively (14). Similarly, another study showed that the ethanolamide derivatives of PGE<sub>2</sub>, PGD<sub>2</sub>, and PGF<sub>2α</sub> were unable to inhibit AEA hydrolysis by FAAH activity (15). Therefore, PG-Gs and PG-EAs may weakly interact with MGL and FAAH. This study evaluates the ability of MGL and FAAH to degrade PG-Gs in vitro. The hydrolysis of PG-Gs was tested with purified MGL and FAAH, and the efficiency of catalysis for PG-Gs was shown to be much lower compared to that of 2-AG and/or AEA.

## MATERIALS AND METHODS

**Materials.** PGE<sub>2</sub>, PGE<sub>2</sub>-d<sub>4</sub>, PGD<sub>2</sub>, PGF<sub>2α</sub>, PGE<sub>2</sub>-G, PGD<sub>2</sub>-G, PGF<sub>2α</sub>-G, PGE<sub>2</sub>-EA, PGD<sub>2</sub>-EA, PGF<sub>2α</sub>-EA, 2-AG, AEA, AA, and dihomolinolenic acid (LLA) were obtained from Cayman Chemical Co. (Ann Arbor, MI). PGE<sub>2</sub>-G-d<sub>5</sub> was synthesized as described previously (2). HPLC solvents were purchased from Fisher Scientific (Chicago, IL). PEEK tubing and fittings were purchased from Upchurch Scientific (Oak Harbor, WA). Rat brain MGL was cloned and expressed in COS-7 cells (16) and purified in the Piomelli laboratory. Rat liver FAAH was expressed in *Escherichia coli* and purified as described previously in the Cravatt laboratory (17). A Luna C18 column (10 cm × 2.0 mm, 3 μm particle size) was obtained from Phenomenex (Torrance, CA). HLB oasis columns were from Waters Chromatography (Milford, MA).

**PGE<sub>2</sub>-G, 2-AG, and AEA Hydrolysis Assay.** Hydrolysis of PGE<sub>2</sub>-G, PGD<sub>2</sub>-G, PGF<sub>2α</sub>-G, and 2-AG by purified rat brain MGL was assayed in 50 mM potassium phosphate (pH 8.0) as described previously (18). MGL was incubated at 37 °C for 2 min prior to the addition of substrates. All substrates were prepared as 100× stock solutions in MeOH and diluted to 1% (v/v) in 500 μL of reaction mixture. MGL concentration-dependent hydrolysis of 50 μM 2-AG or PGE<sub>2</sub>-G was assessed with 0.35–3.5 μg/mL MGL in 500 μL at time points of 0, 5, 10, and 30 min at 37 °C. Substrate concentration-dependent hydrolysis rates were determined with 2–100 μM 2-AG and 25–400 μM PGE<sub>2</sub>-G using 0.35 and 0.7 μg/mL MGL, respectively. Time points for the substrate concentration dependence with MGL were 30 min for 2-AG and 60 min for PGE<sub>2</sub>-G (both hydrolysis rates were linear up to these time points). Samples containing 2-AG were quenched with 1 volume of an ice-cold CHCl<sub>3</sub>/MeOH solution (2:1, v:v) containing 10 μM butylated hydroxyanisole (BHA, Sigma, St. Louis, MO.). BHA was added to minimize autooxidation of polyunsaturated fatty acid chains of 2-AG, AEA, AA, and LLA. For 0 min time points, substrate was added to reaction buffer containing 1 volume of CHCl<sub>3</sub>/MeOH (2:1, v:v) on ice. LLA (25 nmol per reaction; 50 μM final concentration) was added as an internal standard to quantify formation of AA from 2-AG hydrolysis. Distilled H<sub>2</sub>O (250 μL) was added followed by vortexing

and centrifugation (2500 rpm for 2 min). The lower phase was removed, and the upper phase was reextracted with 500 μL of chloroform. The extract was dried under argon and then redissolved in 100 μL of a distilled H<sub>2</sub>O/MeOH mixture (3:7, v:v) for HPLC–UV analysis.

Reactions that included PG-Gs were quenched with addition of 1.6 volumes of ice-cold 20 mM ammonium acetate (pH 4.0). The samples were then loaded onto HLB Oasis columns (30 mg capacity; Waters) and washed successively with 1 mL each of 0.2% aqueous acetic acid and 0.2% aqueous acetic acid with 10% MeOH, and then PG-Gs and PGs were eluted with 1 mL of 100% MeOH. The extracts were dried under argon, redissolved in 100 μL of a distilled H<sub>2</sub>O/MeOH mixture (7:3, v:v), and analyzed by HPLC with evaporative light scattering detection (ELSD) as described below. Quantification of PG-Gs was carried out relative to the appropriate internal standard (e.g., PGE<sub>2</sub>-EA was used for PGE<sub>2</sub>-G). PGD<sub>2</sub> was used as an internal standard for PGE<sub>2</sub> and PGF<sub>2α</sub> quantification, and PGE<sub>2</sub> was used for an internal standard for PGD<sub>2</sub> quantification. Internal standards in MeOH were added in 10 μL aliquots to quenched reaction mixtures. PG-EA and PG free acid internal standards were added to each reaction mixture (e.g., 25 and 1.25 nmol per reaction and 50 and 2.5 μM for PG-EA and PG free acid, respectively). PG-EAs were used as internal standards because they are stable in brain homogenates that contain FAAH and MGL over several hours. All reactions were performed in triplicate.

Hydrolysis of PG-G, 2-AG, and AEA with FAAH was performed in 125 mM Tris (pH 9.0) and 1 mM EDTA with 0.1% Triton X-100 (v:v) at 37 °C as previously described (17). Time points of 0, 5, 10, and 30 min were used to determine FAAH concentration dependence (0.5–25 μg/mL). To determine substrate concentration dependence, 25–1000 μM AEA and 2-AG and 25–2000 μM for PG-Gs were used with 1 μg/mL FAAH. Also, substrate concentration dependence hydrolysis rates for AEA and 2-AG were determined after incubation for 5 min, whereas rates for PG-G hydrolysis were determined after 30 min (both hydrolysis rates were linear up to these time points). 2-AG or AEA was extracted with CHCl<sub>3</sub> and MeOH as described above for the MGL assay and analyzed by HPLC–UV, and samples containing PG-Gs were extracted as described above and analyzed by HPLC–ELSD.

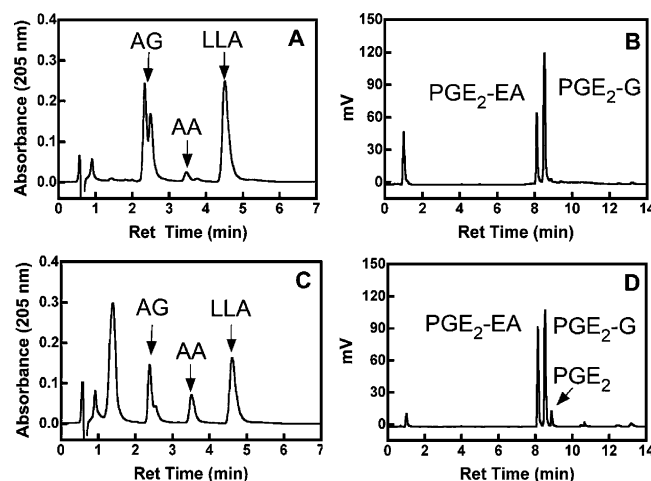
**Hydrolysis of PG-Gs in RAW 264.7 Cells.** RAW 264.7 cells were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with GlutaMax, high glucose, sodium pyruvate, and pyridoxine HCl (Invitrogen, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS) (Atlas, Fort Collins, CO) (DMEM/FCS). Cells at 90% confluency were passaged into 35 mm plates at a density of 5 × 10<sup>5</sup> cells/mL in 1 mL of DMEM/FCS as previously described (19). After incubation for 2 h, 1 mL of DMEM/FCS containing 40 ng/mL GM-CSF was added to each plate for an additional 22 h. Subsequently, the medium was aspirated and 2 mL of fresh DMEM/FCS containing 20 ng/mL GM-CSF, 100 ng/mL lipopolysaccharide, and 10 ng/mL interferon-γ was added. RAW cells were incubated for an additional 5 h followed by aspiration of the medium and two washes with calcium- and magnesium-free phosphate-buffered saline (PBS) (pH 7.4). Fresh DMEM

(1 mL) was added followed by 1 mL of DMEM with 1% DMSO (v:v) containing either 100 nM PGE<sub>2</sub>-G or 2  $\mu$ M 2-AG to start the reaction. The amount of PGE<sub>2</sub>-G-*d*<sub>5</sub> remaining was assayed at 0, 0.5, 1, 2, and 4 h intervals. At each time point, the medium was removed and spiked with deuterated internal standards, PGF<sub>2 $\alpha$</sub> -G-*d*<sub>5</sub> (50 pmol/mL) and PGE<sub>2</sub>-*d*<sub>4</sub> (50 pmol/mL), and 10  $\mu$ L of glacial acetic acid. The samples were loaded onto HLB Oasis columns followed by washing and elution steps as described above. Dried samples were redissolved in a distilled H<sub>2</sub>O/MeOH mixture (65:35, v:v) and analyzed by HPLC with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).

For 2-AG-containing samples, the medium was removed at 0, 5, 10, 20, 30, and 60 min intervals and added to 1 mL of ice-cold acetonitrile (ACN) containing deuterated standards, 2-AG-*d*<sub>8</sub> (100 ng/mL) and AA-*d*<sub>8</sub> (10 ng/mL), and 10  $\mu$ M BHA. Subsequently, RAW cells were scraped into 500  $\mu$ L of ice-cold MeOH (two times) and added to the same collected medium. The lipids were extracted twice with 5 mL of an ethyl acetate/hexane/acetic acid mixture (9:1:0.01, v:v:v). The sample was dried under argon, redissolved in 1 mL of an ethyl acetate/methanol/acetic acid mixture (95:5:0.1, v:v:v), and loaded onto unconditioned silica Sep-Pak columns from Waters. The same solvent was used to wash the Sep-Pak columns (three times). Both breakthrough and wash fractions then were combined, dried under argon, and redissolved in 100  $\mu$ L of a distilled H<sub>2</sub>O/MeOH mixture (25:75, v:v) for LC-ESI-MS/MS analysis.

URB-602 [a specific MGL inhibitor (13)] was used to test whether PGE<sub>2</sub>-G-*d*<sub>5</sub> was a substrate for MGL in intact RAW cells. URB-602 (100  $\mu$ M) was added to pretreated RAW cells in 1 mL of DMEM with 1% DMSO (v:v) for 15 min. Subsequently, 1 mL of either PGE<sub>2</sub>-G-*d*<sub>5</sub> (100 nM) or 2-AG (2  $\mu$ M) was added to RAW cells in DMEM for a final concentration of 50 nM or 1  $\mu$ M, respectively. The amount of 2-AG or PGE<sub>2</sub>-G-*d*<sub>5</sub> remaining was determined in the presence or absence of URB-602 after 1 or 4 h, respectively. All reactions were performed in triplicate.

**Metabolism of PGE<sub>2</sub>-G in Brain Particulate Fractions.** A 10% homogenate of dog brain frontal cortex in lysis buffer [20 mM Tris-HCl buffer (pH 7.5) containing 0.32 M sucrose, 1 mM EDTA, and 2.5 mM DTT] was produced with a polytron homogenizer (model PCU11, VWR, West Chester, PA). Unbroken cells and nuclei were removed by centrifugation (500g for 10 min at 4 °C), and the supernatant was recovered as the crude homogenate. The crude homogenate was centrifuged (180000g for 50 min at 4 °C), and the pellet was recovered as the particulate fraction. The particulate fraction was then adjusted to 0.1 mg/mL protein in 50 mM potassium phosphate buffer (pH 7.4) and incubated for 15 min at 37 °C with URB-597 (a specific FAAH inhibitor) (0.0001, 0.001, 0.005, 0.500, and 10  $\mu$ M) or vehicle control (MeOH). Both AEA and PGE<sub>2</sub>-G were prepared as 100 $\times$  stock solutions in MeOH and diluted to 1% (v:v) for final concentrations of 50 and 10  $\mu$ M, respectively. The amount of PGE<sub>2</sub>-G or AEA remaining was determined at 30 min by using HPLC-ELSD or HPLC-UV analysis, respectively (described below). Percent remaining activity was determined at each URB-597 concentration relative to the vehicle control (designated 100% activity). All reactions were performed in triplicate.



**FIGURE 1:** MGL degradation of 2-AG and PGE<sub>2</sub>-G. Panels A and C represent 2-AG hydrolysis in the absence and presence, respectively, of 0.7  $\mu$ M MGL after incubation for 10 min. The HPLC chromatograms for panels A and C show 2-AG and 1(3)-AG (2.3 min), AA (3.6 min), and dihomolinolenic acid (LLA) (4.8 min). The peak at 1.5 min in panel C is Triton X-100 from the stock solution of MGL. 2-AG and LLA were both added at 50  $\mu$ M (5 nmol on column). The amount of AA formed 30 min after the injection was 14.7  $\mu$ M (1.47 nmol on column). Panels B and D represent PGE<sub>2</sub>-G hydrolysis in the presence of 0.7 and 3.5  $\mu$ M MGL, respectively, after incubation for 30 min. The HPLC chromatograms in panels B and D show PGE<sub>2</sub>-EA (8.1 min), PGE<sub>2</sub>-G (8.5 min), and PGE<sub>2</sub> (9.0 min). The ELSD drift tube temperature was set to 50 °C; the nitrogen source was set to 2.4 psi, and the gain was set to 6 mV. PGE<sub>2</sub>-EA and PGE<sub>2</sub>-G were both at 50  $\mu$ M (5 nmol on column). The levels of PGE<sub>2</sub> formed after 30 min in panels B and D were 3.0 and 8.6  $\mu$ M, respectively (0.30 and 0.86 nmol on column, respectively).

**HPLC Analysis.** Analysis of prostanoids was accomplished by reverse-phase (RP) HPLC-ELSD, and endocannabinoid analysis was achieved via RP HPLC-UV. The HPLC system used was a Waters 2695 separation module with an autosampler and pump. Resolution of PG-EAs, PG-Gs, and PGs was afforded by isocratic elution on a Phenomenex Luna C18 column (100 mm  $\times$  2.0 mm, 3  $\mu$ m particle size) at a column temperature of 40 °C. For the assay of PG-G hydrolysis, two different conditions were used. The first condition quantified both the degradation of PGE<sub>2</sub>-G and the formation of PGE<sub>2</sub>. Solvent A was distilled H<sub>2</sub>O with 0.1% acetic acid, and solvent B was ACN with 0.1% acetic acid. The samples were injected onto the column at a solvent composition of 80% A and 20% B with a flow rate of 0.3 mL/min. After a 1 min hold under the same solvent conditions, the level of solvent B was linearly increased from 20 to 50% over 3 min and then held at 50% for an additional 5 min, at which time PGE<sub>2</sub>-EA, PGE<sub>2</sub>-G, and PGE<sub>2</sub> eluted off the column [8.1, 8.5, and 9.0 min, respectively (Figure 1B,D)]. The ELSD detector drift tube was set to 50 °C and the nitrogen source to 2.4 psi, and the gain was 6 mV. The second condition was used to assess the amount of PG free acid produced as a function of the concentration of the respective PG-G species. The solvent composition was 65:35:0.1 (v:v:v) distilled H<sub>2</sub>O:ACN:acetic acid, and the flow rate was 0.3 mL/min. The ELSD detector drift tube and nitrogen source were the same, but the gain was set to 10 mV. PG-EAs eluted at ~2.3–2.4 min, PG-Gs at ~3.1–3.5 min, and PGs at 4–5 min (Figure 3A,B). The signal from the ELSD detector is a logarithmic response, so a plot of log(nanograms of com-



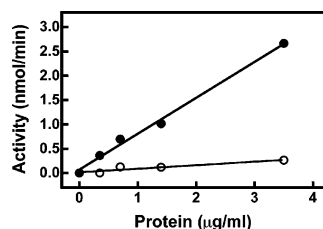


FIGURE 2: Enzyme concentration dependence for hydrolysis of 2-AG and PGE<sub>2</sub>-G by MGL. 2-AG (●) or PGE<sub>2</sub>-G (○) was incubated with MGL for 0, 5, 10, and 30 min. The samples were extracted and assessed by HPLC–UV analysis for 2-AG hydrolysis or HPLC–ELSD for PGE<sub>2</sub>-G hydrolysis as described above. Experiments were performed in triplicate.

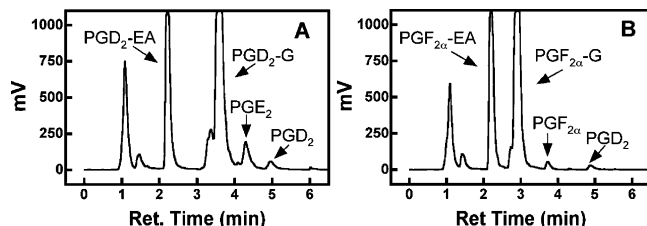


FIGURE 3: HPLC–ELSD chromatograms for MGL-mediated degradation of PGD<sub>2</sub>-G and PGF<sub>2α</sub>-G. Panels A and B depict data for 200 μM PGD<sub>2</sub>-G and PGF<sub>2α</sub>-G incubated with 0.7 μg/mL MGL for 60 min, respectively. Panel A shows data for PGD<sub>2</sub>-G (3.7 min) and its internal standard, PGD<sub>2</sub>-EA (2.3 min), and PGD<sub>2</sub> (4.9 min) and its internal standard, PGE<sub>2</sub> (4.3 min). Panel B shows data for PGF<sub>2α</sub>-G (3.0 min) and its internal standard, PGF<sub>2α</sub>-EA (2.2 min), and PGF<sub>2α</sub> (3.7 min) and its internal standard, PGD<sub>2</sub> (4.9 min). The PG-EA peaks are representative of 50 μM analyte (5 nmol on column) for the corresponding PG-G (100 μM, 10 nmol on column) being studied. The ELSD detector was set to a gain of 10 mV to accurately assess PG free acid formation, which results in the PG-EA and PG-G peaks being off-scale. Panel A shows PGD<sub>2</sub> formation (5.34 μM, 0.53 nmol on column) and panel B PGF<sub>2α</sub> formation (4.72 μM, 0.47 nmol on column), relative to their respective internal standards of PGE<sub>2</sub> (5 μM, 0.5 nmol on column) and PGD<sub>2</sub>, respectively (2.5 μM, 0.25 nmol on column).

pound) versus log(peak area) produces a straight line. The response of the instrument has a dynamic range of approximately 1 order of magnitude. Therefore, the first and second condition had to be done separately with different gain settings. Also, the volumes of injection were changed with the expected sample load of PGs to fall within the dynamic range. For a gain of 10 mV, the dynamic concentration range was 0.04–2 nmol/injection, and for a gain of 6, the dynamic concentration range was 0.2–10 nmol/injection.

Separation of AEA, 2-AG, and AA was afforded by RP-HPLC with the same column described above using an isocratic elution of an ACN/distilled H<sub>2</sub>O/acetic acid mixture (75:25:0.1, v:v:v) at 0.7 mL/min. Peaks were detected with a Waters 2487 dual-wavelength absorbance detector set at 205 nm. Arachidonylglycerol eluted as a mixture of the 2- and 1(3)-isomer (Figure 1A) after incubation in aqueous buffer for 30 min. Retention times for AEA, 2- and 1(3)-AG, AA, and LLA were 1.8, 2.3, 3.4, and 4.4 min, respectively (panels A and C of Figure 1 for 2-AG and 1(3)-AG, AA, and LLA, respectively).

**HPLC with Tandem Mass Spectrometry.** Analysis of endocannabinoids, AA, and prostanoids was accomplished through use of reverse-phase HPLC coupled to electrospray tandem mass spectrometry (LC–ESI-MS/MS) as previously described (19, 20). HPLC separation of endocannabinoids was accomplished with a solvent composition of 70 μM

silver acetate in a distilled H<sub>2</sub>O/MeOH mixture (15:85, v:v) with a flow rate of 0.3 mL/min on a Phenomenex Luna C18 column (50 mm × 2.0 mm, 3 μm particle size) at a column temperature of 40 °C. Silver ion coordination was used for electrospray ionization of endocannabinoids, resulting in [M + Ag<sup>+</sup>] species. The selected reaction monitoring (SRM) *m/z* transitions were *m/z* 454 → 436 for [AEA + Ag<sup>107</sup>], *m/z* 485 → 411 for [2-AG + Ag<sup>107</sup>], and *m/z* 519 → 412 for [AA<sup>−</sup> + 2Ag<sup>+</sup>]. Mass spectrometric conditions for 2-AG, AEA, and AA fragmentation were as follows; capillary temperature, 350 °C; capillary offset, 35 V; spray voltage, 5.0 kV; sheath gas, 49 psi; auxiliary gas, 25 psi; CID pressure, 1.5 mTorr; collision energy, 30 V; and tube lens offset, 130 V.

PG-G and PG analytes were separated by HPLC using 2 mM ammonium acetate in distilled H<sub>2</sub>O and acetic acid (pH 3.3) and ACN (65:35, v:v) at a flow rate of 0.3 mL/min using the same column conditions described above. The prostanoids coordinate with the NH<sub>4</sub><sup>+</sup> cation, resulting in formation of the [M + NH<sub>4</sub><sup>+</sup>] adduct, resulting in addition of 18 mass units. The SRM *m/z* transitions for [PGE<sub>2</sub>-G-*d*<sub>5</sub> + NH<sub>4</sub><sup>+</sup>], [PGF<sub>2α</sub>-G-*d*<sub>5</sub> + NH<sub>4</sub><sup>+</sup>], [PGE<sub>2</sub>-*d*<sub>4</sub> + NH<sub>4</sub><sup>+</sup>], and [PGE<sub>2</sub> + NH<sub>4</sub><sup>+</sup>] were *m/z* 449 → 391, *m/z* 451 → 393, *m/z* 374 → 321, and *m/z* 370 → 317, respectively. Mass spectrometric conditions for PG-G and PG fragmentation were as follows; capillary temperature, 300 °C; capillary offset, 35 V; spray voltage, 4.3 kV; sheath gas, 35 psi; auxiliary gas, 21 psi; and CID pressure, 1.0 mTorr. The collision energy was varied from 16 to 22 V and the tube lens offset from ~80 to 130 V.

## RESULTS

**Hydrolysis of 2-AG, AEA, PGE<sub>2</sub>-G, PGD<sub>2</sub>-G, and PGF<sub>2α</sub>-G by MGL.** The relative ability of MGL to hydrolyze PG-Gs to 2-AG was assessed by HPLC–ELSD. Panels A and C of Figure 1 demonstrate that approximately 30% of 50 μM 2-AG was converted to AA after incubation for 10 min with 0.7 μg/mL MGL. However, the same concentration of MGL produced a barely detectable level of PGE<sub>2</sub> after incubation for 30 min with 50 μM PGE<sub>2</sub>-G (Figure 1B). At the highest concentration of MGL that was tested (3.5 μg/mL), approximately 18% of 50 μM PGE<sub>2</sub>-G was hydrolyzed to PGE<sub>2</sub> (Figure 1D). The initial rates of 2-AG hydrolysis compared to that of PGE<sub>2</sub>-G as a function of MGL concentration show an ~10-fold higher value for the former at a constant substrate concentration of 50 μM [740 ± 67.5 nmol of 2-AG min<sup>−1</sup> (mg of protein)<sup>−1</sup> vs 74.6 ± 14.7 nmol of PGE<sub>2</sub>-G min<sup>−1</sup> (mg of protein)<sup>−1</sup> (Figure 2)]. The relative efficiency of hydrolysis of PG-G by MGL compared to that of 2-AG was tested by varying the substrate concentration. For these experiments, 0.7 μg/mL MGL was used to determine PG-G hydrolysis rates, and the time course was extended to 60 min. At 0.7 μg/mL MGL, the hydrolysis rates of PG-Gs were linear through incubation for 60 min. The substrate concentration dependence for 2-AG hydrolysis was determined with 0.35 μg/mL using a reaction time of 30 min. As shown in Figure 3, the percent conversion of PGD<sub>2</sub>-G and PGF<sub>2α</sub>-G to their corresponding free acids with MGL was also low. The amount of PG free acid formed at initial substrate concentrations of 25–400 μM ranged from 5 to 1% conversion after incubation for 60 min (data not shown). Nonlinear regression analysis of a plot of the rate of 2-AG hydrolysis

Table 1: Kinetic Parameters for MGL Degradation of 2-AG and PG-Gs<sup>a</sup>

species	$k_{\text{cat}}$ (min <sup>-1</sup> )	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1} \text{min}^{-1}$ )
2-AG	21 $\pm$ 2	10 $\pm$ 3	2.1
PGE <sub>2</sub> -G	9.6 $\pm$ 1.5	150 $\pm$ 40	6.4 $\times 10^{-2}$
PGD <sub>2</sub> -G	4.1 $\pm$ 0.3	133 $\pm$ 30	3.1 $\times 10^{-2}$
PGF <sub>2\alpha</sub> -G	8.6 $\pm$ 1.7	311 $\pm$ 104	2.7 $\times 10^{-2}$

<sup>a</sup> 2-AG (2–100  $\mu\text{M}$ ) was incubated with 0.35  $\mu\text{g/mL}$  MGL. PGE<sub>2</sub>-G, PGD<sub>2</sub>-G, or PGF<sub>2 $\alpha$</sub> -G (25–400  $\mu\text{M}$ ) was incubated with 0.7  $\mu\text{g/mL}$  MGL. Initial rates were determined for 2-AG and the PG-Gs after incubation for 30 and 60 min, respectively. Initial rates for 2-AG and PG-G degradation were linear throughout the reaction. Samples were extracted as described above and analyzed by HPLC with either UV (2-AG hydrolysis) or ELSD detection (PG-G hydrolysis). The hyperbolic plot of initial rate vs initial concentration was determined with nonlinear regression in Prism. Experiments were performed in triplicate.

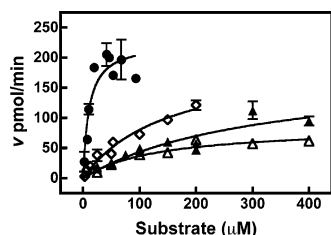


FIGURE 4: Substrate concentration dependence for hydrolysis of PG-G and 2-AG by MGL. 2-AG (●) hydrolysis was evaluated with 0.35  $\mu\text{g/mL}$  MGL, and hydrolysis of PG-Gs [(▲) PGF<sub>2 $\alpha$</sub> -G, (Δ) PGD<sub>2</sub>-G, and (◇) PGE<sub>2</sub>-G] was evaluated with 0.7  $\mu\text{g/mL}$  MGL. 2-AG was incubated with MGL for 30 min, and PG-Gs were incubated with MGL for 60 min. Samples were analyzed by HPLC with either UV (2-AG hydrolysis) or ELSD (PG-G hydrolysis) detection, and experiments were performed in triplicate.

with 0.35  $\mu\text{g/mL}$  MGL versus initial 2-AG concentration yielded values of  $K_m$  and  $k_{\text{cat}}$  for the reaction (Table 1). These values are approximate because the critical micellar concentration of 2-AG is 25  $\mu\text{M}$ , making free substrate limiting at higher concentrations (data not shown). PG-Gs (E<sub>2</sub>, D<sub>2</sub>, and F<sub>2 $\alpha$</sub> ) were incubated at concentrations ranging from 25 to 400  $\mu\text{M}$  with 0.7  $\mu\text{g/mL}$  MGL. As shown in Figure 4 and Table 1,  $k_{\text{cat}}$  values for PG-Gs were much lower (50–80%) than that of 2-AG and the  $K_m$  values were much higher (13–30-fold). The high error associated with the  $K_m$  values for the PG-G species may be from the lack of complete saturation of MGL. The relative substrate efficiency for MGL was 32–100 times higher for 2-AG (Table 1). There were only small differences in the  $k_{\text{cat}}$  and  $K_m$  values among PG-G species. The  $k_{\text{cat}}$  for PGE<sub>2</sub>-G hydrolysis was ~2-fold higher than those of PGD<sub>2</sub>-G and PGF<sub>2 $\alpha$</sub> -G, and the  $K_m$  for PGE<sub>2</sub>-G was similar to that of PGD<sub>2</sub>-G and 2-fold lower than that of PGF<sub>2 $\alpha$</sub> -G (Table 1).

**Metabolism of 2-AG, AEA, PGE<sub>2</sub>-G, PGD<sub>2</sub>-G, and PGF<sub>2 $\alpha$</sub> -G by FAAH.** The relative ability of FAAH to degrade PG-Gs relative to those of AEA and 2-AG also was assessed. Comparison of the initial rates of hydrolysis for AEA compared to that of PGE<sub>2</sub>-G as a function of FAAH concentration showed a 22-fold higher rate for the former [692  $\pm$  53.9 nmol of AEA min<sup>-1</sup> (mg of protein)<sup>-1</sup> vs 31.8  $\pm$  2.6 nmol of PGE<sub>2</sub>-G min<sup>-1</sup> (mg of protein)<sup>-1</sup> (Figure 5)]. The effect of substrate concentration on PG-G, AEA, and 2-AG hydrolysis with 1  $\mu\text{g/mL}$  FAAH was also tested (Figure 6). The  $k_{\text{cat}}$  for 2-AG hydrolysis was 2-fold lower than that of AEA, and the  $K_m$  for 2-AG was 3-fold higher than that for AEA (Table 2 and Figure 6). The substrate

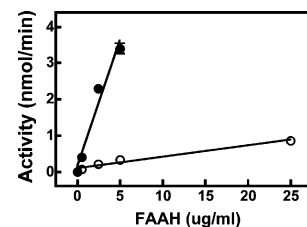


FIGURE 5: Enzyme concentration dependence for hydrolysis of AEA and PGE<sub>2</sub>-G by FAAH. AEA at 50  $\mu\text{M}$  (●) or PGE<sub>2</sub>-G at 50  $\mu\text{M}$  (○) was incubated with FAAH for 0, 5, 10, and 30 min. Samples were extracted and analyzed by HPLC–UV (AEA hydrolysis) and HPLC–ELSD (PGE<sub>2</sub>-G hydrolysis) as described above. Experiments were performed in triplicate.

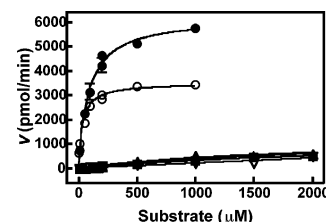


FIGURE 6: Substrate concentration dependence for hydrolysis of PG-G, AEA, and 2-AG by FAAH. FAAH was at 1  $\mu\text{g/mL}$ ; concentrations of 2-AG (●) and AEA (○) varied from 25 to 1000  $\mu\text{M}$ , and concentrations of PG-Gs [(▲) PGF<sub>2 $\alpha$</sub> -G, (Δ) PGD<sub>2</sub>-G, and (◇) PGE<sub>2</sub>-G] varied from 25 to 2000  $\mu\text{M}$ . 2-AG and AEA were incubated with FAAH for 5 min, and PG-Gs were incubated for 30 min. Experiments were performed in triplicate and analyzed by HPLC with either UV detection or ELSD.

Table 2: Kinetic Parameters for FAAH Degradation of AEA, 2-AG, and PG-Gs<sup>a</sup>

species	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1} \text{s}^{-1}$ )
2-AG	28 $\pm$ 1	89 $\pm$ 10	0.31
AEA	16 $\pm$ 1	34 $\pm$ 4	0.46
PGE <sub>2</sub> -G	6.5 $\pm$ 1.2	3600 $\pm$ 1000	1.8 $\times 10^{-3}$
PGD <sub>2</sub> -G	1.7 $\pm$ 0.2	1100 $\pm$ 400	1.6 $\times 10^{-3}$
PGF <sub>2\alpha</sub> -G	4.8 $\pm$ 1.1	2400 $\pm$ 800	2.0 $\times 10^{-3}$

<sup>a</sup> The concentration of FAAH was 1  $\mu\text{g/mL}$  with the concentrations of 2-AG and AEA varied from 25 to 1000  $\mu\text{M}$ , and concentrations of PGF<sub>2 $\alpha$</sub> -G, PGD<sub>2</sub>-G, and PGE<sub>2</sub>-G were varied from 25 to 2000  $\mu\text{M}$ . 2-AG and AEA were incubated with FAAH for 5 min, and PG-Gs were incubated for 30 min. Initial rates for AEA, 2-AG, and PG-G degradation were linear throughout the reaction. Samples were extracted as described above and analyzed by HPLC with either UV (2-AG and AEA hydrolysis) or ELSD (PG-G hydrolysis) detection. The hyperbolic plot of initial rate vs initial concentration was determined with nonlinear regression in Prism. Experiments were performed in triplicate.

specificity was similar for AEA and 2-AG with  $k_{\text{cat}}/K_m$  values of 0.462 and 0.313  $\mu\text{M}^{-1} \text{s}^{-1}$ , respectively. Estimated  $k_{\text{cat}}$  values for PGE<sub>2</sub>-G, PGD<sub>2</sub>-G, and PGF<sub>2 $\alpha$</sub> -G were significantly lower (Table 2 and Figure 6). The high error associated with PG-G  $k_{\text{cat}}$  values is expected because FAAH was not completely saturated at the highest concentration of PG-Gs that was used. The  $K_m$  values for PGE<sub>2</sub>-G, PGD<sub>2</sub>-G, and PGF<sub>2 $\alpha$</sub> -G were much higher than those for AEA or 2-AG (Table 2). As shown with MGL, it is clear that PG-Gs are poor substrates for FAAH compared to AEA and 2-AG. The estimated  $k_{\text{cat}}/K_m$  values were 200–300- and 150–200-fold higher for AEA and 2-AG hydrolysis than for PG-Gs (Table 2).

**Metabolism of PGE<sub>2</sub>-G-d<sub>5</sub> in Intact RAW 264.7 Macrophages by MGL.** A specific MGL inhibitor, URB-602 (13), was used as a tool to discern whether MGL could metabolize

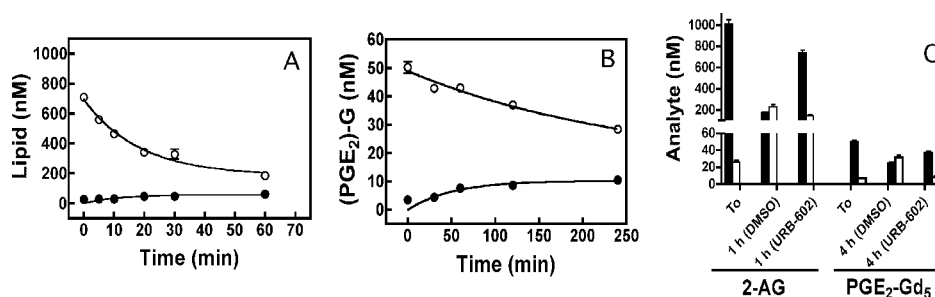


FIGURE 7: Metabolism of PG-Gs in RAW 264.7 cells by MGL in the absence and presence of URB-602. Panel A depicts the degradation of 1000 nM 2-AG (○) with subsequent formation of AA (●). Panel B depicts the degradation of 50 nM PGE<sub>2</sub>-G-d<sub>5</sub> (○) with subsequent formation of PGE<sub>2</sub> (●). Panel C shows metabolism of 2-AG and PGE<sub>2</sub>-G-d<sub>5</sub> in the presence of URB-602. The left part of the panel, labeled 2-AG, shows 2-AG levels (black bars) and AA levels (white bars) at time zero (T<sub>0</sub>), 1 h, and 1 h with URB-602 treatment. The right part of the panel, labeled PGE<sub>2</sub>-G-d<sub>5</sub>, shows PGE<sub>2</sub>-G-d<sub>5</sub> levels (black bars) and PGE<sub>2</sub> levels (white bars) at time zero (T<sub>0</sub>), 4 h, and 4 h with URB-602.

PG-Gs in RAW 264.7. These cells were chosen because they make PG-Gs (4) and also possess PG-G hydrolytic activity (data not shown). 2-AG (1  $\mu$ M) and PGE<sub>2</sub>-G-d<sub>5</sub> (50 nM) were applied exogenously to RAW 264.7 cells in the presence or absence of 100  $\mu$ M URB-602. Only 50 nM PGE<sub>2</sub>-G-d<sub>5</sub> was added to the RAW cells because it has previously been shown that only nanomole amounts of PG-Gs are produced in macrophages (4). The concentrations of 2-AG and PGE<sub>2</sub>-G-d<sub>5</sub> given above were chosen because these levels approximate what is produced from endogenous sources following pharmacological challenge. In the absence of URB-602, the RAW cells reduced the concentration of 2-AG to  $18.5 \pm 1.1\%$  of its starting value (Figure 7A), and arachidonic acid levels reached  $\sim 6\%$  of initial 2-AG levels ( $61.1 \pm 13.3$  nM) over a period of 1 h. Arachidonate recovery may not be 100% because of its ability to enter other metabolic pathways (i.e., phospholipid, triacylglycerol synthesis). Degradation of PGE<sub>2</sub>-G-d<sub>5</sub> was much slower with  $\sim 50\%$  ( $24.7 \pm 1.8$  nM) of this species remaining after incubation for 4 h (Figure 7B). The increase in PGE<sub>2</sub> levels accounted for  $\sim 42\%$  of the initial PGE<sub>2</sub>-G-d<sub>5</sub> concentration ( $10.4 \pm 1.3$  nM). As with arachidonic acid, PGE<sub>2</sub> may be subject to further metabolism (i.e., by 15-hydroxyprostaglandin dehydrogenase), thus limiting its recovery. In the presence of 100  $\mu$ M URB-602, RAW cells reduced the level of exogenously applied 2-AG to  $74.0 \pm 4.2\%$  after incubation for 1 h [control levels were  $17.5 \pm 0.9\%$  (Figure 7C)]. PGE<sub>2</sub>-G-d<sub>5</sub> levels were  $37.1 \pm 2.4$  nM after exposure to URB-602-treated RAW cells for 4 h, whereas those of the control samples were  $24.7 \pm 1.8$  nM (Figure 7C). These results show that there is some inhibition of PGE<sub>2</sub>-G hydrolysis in the presence of URB-602 when 2-AG hydrolysis is  $\sim 70\%$  inhibited. The total level of hydrolysis of PGE<sub>2</sub>-G is much lower than the level of 2-AG hydrolysis. Therefore, 50% inhibition of PGE<sub>2</sub>-G hydrolysis with URB-602 accounts for a rather small inhibition of PGE<sub>2</sub>-G turnover. The *in vitro* enzymatic assays show PG-Gs are poor substrates for MGL ( $\sim 33$ – $100$ -fold), so it is possible that URB-602 inhibits another PG-G hydrolase to some extent. RAW cells do not possess FAAH protein as judged by Western blot analysis (data not shown), suggesting that these cells do not metabolize PGE<sub>2</sub>-G through FAAH enzymatic activity.

*Metabolism of PGE<sub>2</sub>-G by FAAH Activity in Brain Particulate Fractions.* We tested whether FAAH activity from particulate fractions of dog brain frontal cortices

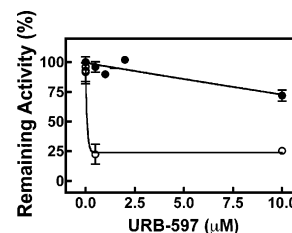


FIGURE 8: Inhibition of PG-G hydrolysis with URB-597 in dog brain particulate fractions. Brain particulate fractions (0.1 mg/mL) were incubated with URB-597 (0.0001–10  $\mu$ M) for 15 min followed by addition of PGE<sub>2</sub>-G (10  $\mu$ M) or AEA (50  $\mu$ M). Percent inhibition is the amount of PGE<sub>2</sub>-G (●) or AEA (○) hydrolyzed after 30 min compared to vehicle control values (AEA hydrolysis,  $21.4 \text{ nmol min}^{-1} \text{ mg}^{-1}$ ; and PGE<sub>2</sub>-G hydrolysis,  $2.92 \text{ nmol min}^{-1} \text{ mg}^{-1}$ ).

degrades PGE<sub>2</sub>-G in the presence or absence of a specific FAAH inhibitor, URB-597 (21). In the presence of 0.5  $\mu$ M URB-597, AEA hydrolysis activity was inhibited 75%, whereas PGE<sub>2</sub>-G hydrolysis was not significantly inhibited at URB-597 concentrations up to 2.5  $\mu$ M and was  $\sim 30\%$  inhibited at 10  $\mu$ M (Figure 8). Thus, PGE<sub>2</sub>-G appears to be metabolized by an enzymatic activity independent of FAAH in crude brain particulate fractions, and URB-597 appears to modestly inhibit its activity.

## DISCUSSION

Endocannabinoid hydrolases, MGL and FAAH, were tested for their ability to degrade PG-Gs. Both enzymes hydrolyze 2-AG, the precursor for PG-G from COX-2 oxygenation, very efficiently and are suggested to participate in the rapid inactivation of 2-AG during retrograde signaling. Rat brain MGL hydrolyzes monooleoylglycerol at a rate 5-fold higher than that of 2-AG (18). It is estimated that the concentration of 2-AG released during hippocampal retrograde signaling is 10  $\mu$ M (18), which is the  $K_m$  for MGL hydrolysis of 2-AG. Recently, a specific MGL inhibitor (URB-602) has been developed as a tool for discerning the role of the enzyme in 2-AG metabolism (13). Inclusion of this inhibitor with hippocampal slice preparations significantly extends DSI duration, suggesting that MGL plays an important role in regulating 2-AG levels during retrograde signaling (13). Specific inhibitors of COX-2 (e.g., nimesulide and meloxicam) also extend the duration of DSI, suggesting that COX-2 may regulate 2-AG levels (7). Another study using primary hippocampal neuron cultures showed that PG-Gs (PGE<sub>2</sub>-G, PGD<sub>2</sub>-G, and PGF<sub>2 $\alpha$</sub> -G) and PGD<sub>2</sub>-EA have



potent effects on retrograde signaling distinct from that of 2-AG (8). Although the studies mentioned above suggest that COX-2 is involved in regulating 2-AG levels in the hippocampus, COX-2 inhibitors do not affect inhibitory retrograde signaling in mouse cerebellar slices (10) or excitatory signaling in hippocampal slices (9). Similar to 2-AG levels, PG-G levels may be tightly regulated by hydrolases during inhibitory retrograde signaling in the hippocampus. The rates of PG-G hydrolysis by MGL were found to be low compared to the rate of 2-AG hydrolysis (the  $k_{\text{cat}}/K_m$  value is  $\sim 30$ –100-fold lower for the former).

FAAH also degrades 2-AG in addition to AEA, at least in vitro. It has been shown that 2-AG is hydrolyzed at a 4–5-fold higher rate than AEA in recombinant rat, porcine, and cellular FAAH preparations. However, in those studies, the enzyme was not or only partially purified (22–24). It has since been demonstrated that there is 2-AG hydrolysis activity in particulate fractions of rat brain cerebellum that is independent of FAAH activity (25). Microglia cells also contain a novel MGL activity in the mitochondria that is distinct from FAAH (26). No comparison of hydrolysis for 2-AG and AEA with purified FAAH has been reported. However, the esterase and amidase activity have been compared. The hydrolysis kinetics of oleoyl methyl ester and oleoyl methyl amine with purified FAAH showed  $k_{\text{cat}}$  values of  $2.8 \text{ s}^{-1}$  for the former and  $1.9 \text{ s}^{-1}$  for the latter. The  $K_m$  value for oleoyl methyl ester was  $21 \mu\text{M}$  and for oleoyl methyl amine  $9 \mu\text{M}$ , giving  $k_{\text{cat}}/K_m$  values of 0.13 and  $0.21 \mu\text{M}^{-1} \text{ s}^{-1}$ , respectively (27). Also, the  $k_{\text{cat}}/K_m$  values for oleoylethanolamide and monooleoylglycerol were 0.23 and  $0.11 \mu\text{M}^{-1} \text{ s}^{-1}$ , respectively (28). The results of this study show that  $k_{\text{cat}}/K_m$  values for FAAH hydrolysis of AEA and 2-AG are similar, 0.47 and  $0.31 \mu\text{M}^{-1} \text{ s}^{-1}$ , respectively. Also, as observed with PG-G degradation with MGL, FAAH activity with PG-Gs is very low compared to that with 2-AG and AEA. Although complete substrate saturation of FAAH by PG-Gs was not reached (up to 2 mM PG-G), the estimated  $k_{\text{cat}}/K_m$  values are at least 150–200-fold higher for 2-AG hydrolysis and 200–300-fold higher for AEA hydrolysis.

RAW 264.7 cells possess both 2-AG and PG-G hydrolytic activity. Therefore, this cell line was used as an in vivo model to establish that 2-AG and PG-G hydrolytic activities are separate. These studies suggest that MGL contributes little to PGE<sub>2</sub>-G hydrolysis in vivo. However, there is some inhibition of PGE<sub>2</sub>-G<sub>d5</sub> hydrolysis in RAW cells treated with URB-602. Although in vitro experiments show that PG-Gs are poor substrates for purified MGL, longer time points (e.g., 4 h) may allow for MGL-mediated PGE<sub>2</sub>-G hydrolysis. Alternatively, URB-602 may partially inhibit an unknown lipase/esterase that is responsible for PGE<sub>2</sub>-G hydrolysis. Others showed that URB-602 was not a specific MGL inhibitor over FAAH in C6 glioma and RBL2H3 basophilic leukemia cells (29). We observed very low FAAH activity in RAW cells under our experimental conditions (data not shown). Therefore, FAAH activity contributed very little to 2-AG degradation. Because FAAH activity in RAW cells was very low, we assumed that these cells do not metabolize PGE<sub>2</sub>-G through FAAH enzymatic activity. We used particulate fractions from dog brain cortex to establish that PGE<sub>2</sub>-G hydrolysis activity is distinct from AEA hydrolysis activity. When using URB-597 with brain particulate fractions, PGE<sub>2</sub>-G hydrolysis is unaffected. Therefore, degrada-

tion of PG-Gs occurs in a manner independent of FAAH enzymatic activity from isolated brain particulate fractions.

These results suggest that MGL and FAAH contribute very little to the degradation of PG-Gs in cells. However, it is noteworthy that long chain fatty acyl taurine derivatives are poor substrates for FAAH in vitro, but in vivo experiments using FAAH knockout animals indicate that FAAH plays a major role in their hydrolysis (28). Identification of additional enzyme(s) responsible for PG-G degradation could provide important information for inhibitor development or the generation of knockout mice that would represent critical tools for evaluating the biological significance of PG-Gs in vivo.

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