Mutation of Cys242 of Human Monoacylglycerol Lipase Disrupts Balanced Hydrolysis of 1- and 2-Monoacylglycerols and Selectively Impairs Inhibitor Potency

Tuomo Laitinen, Dina Navia-Paldanius, Roosa Rytilahti, Joona J. T. Marjamaa, Julie Kařízková, Teija Parkkari, Tatu Pantsar, Antti Poso, Jarmo T. Laitinen, and Juha R. Savinainen

Institute of Biomedicine/Physiology, School of Medicine (D.N.-P., R.R., J.J.T.M., J.K., Te.P., J.T.L., J.R.S.), and School of Pharmacy (T.L., Ta.P., A.P.), Faculty of Health Sciences, University of Eastern Finland, Kuopio, Finland

Received November 15, 2013; accepted December 24, 2013

ABSTRACT

Considerable progress has been made in recent years in developing selective, potent monoacylglycerol lipase (MAGL) inhibitors. In the investigations of measures to inhibit this enzyme, less attention has been paid to improving our understanding of its catalytic mechanisms or substrate preferences. In our study, we used site-directed mutagenesis, and we show via versatile activity assays combined with molecular modeling that Cys242 and Tyr194, the two opposing amino acid residues in the catalytic cavity of MAGL, play important roles in determining the rate and the isomer preferences of monoacylglycerol hydrolysis. In contrast to wild-type enzymes that hydrolyzed 1- and 2-monoacylglycerols at similar rates, mutation of Cys242 to alanine caused a significant reduction in overall activity (maximal velocity, \( V_{\text{max}} \)) particularly skewing the balanced hydrolysis of isomers to favor the 2-isomer. Molecular modeling studies indicate that this was caused by structural features unfavorable toward 1-isomers as well as impaired recognition of OH-groups in the glycerol moiety. Direct functional involvement of Cys242 in the catalysis was found unlikely due to the remote distance from the catalytic serine. Unlike C242A, mutation of Tyr194 did not bias the hydrolysis of 1- and 2-monoacylglycerols but significantly compromised overall activity. Finally, mutation of Cys242 was also found to impair inhibition of MAGL, especially that by fluorophosphonate derivatives (13- to 63-fold reduction in potency). Taken together, this study provides new experimental and modeling insights into the molecular mechanisms of MAGL-catalyzed hydrolysis of the primary endocannabinoid 2-arachidonoylglycerol and related monoacylglycerols.

Introduction

In the central nervous system, three serine-hydrolases—monoacylglycerol lipase (MAGL), \( \alpha/\beta \)-hydrolase-domain (ABHD) 6, and ABHD12—are responsible for inactivation of the primary endocannabinoid 2-arachidonoylglycerol (2-AG) (Dinh et al., 2002; Blankman et al., 2007; Savinainen et al., 2012). The lion’s share of 2-AG hydrolysis (~85%) is accounted for by MAGL (Saario et al., 2005; Blankman et al., 2007). Traditional role of MAGL in catalyzing the last step in the hydrolysis of stored triglycerides in fat cells has recently expanded to encompass modulation of 2-AG-dependent cannabinoid CB1 receptor signaling in the brain (for review, see Savinainen et al., 2012). Moreover, as MAGL liberates arachidonic acid as a result of 2-AG hydrolysis, it may have a pivotal task in regulating the formation of inflammatory prostaglandins in the central nervous system. Ultimately, heightened MAGL activity may lead to development of inflammatory brain diseases, such as Parkinson’s and Alzheimer’s diseases (Nomura et al., 2011; Piro et al., 2012). Another potential pathophysiological role for MAGL relates to development of cancer; in cancer cells, MAGL activity has been demonstrated to redirect lipid stores toward protumorigenic signaling lipids (Nomura et al., 2010). It is obvious that these observations together make MAGL a very attractive therapeutic target.

MAGL represents an enzyme capable of catalyzing hydrolysis of monoacylglycerols with different acyl chain lengths (Tornqvist and Belfrage, 1976; Dinh et al., 2002; Navia-
Paldanius et al., 2012). It has structural characteristics typical for serine hydrolases, including the catalytic triad (Ser122-His269-Asp239) and the lipase motif GXXGXG (Karlsson et al., 1997). Recent crystallographic studies have revealed structural features of the enzyme (Bertrand et al., 2010; Labar et al., 2010; Schalk-Hihi et al., 2011) and have offered important complementary support for the first homology-comparison model, which was initially constructed to get a view on the conserved cysteine residues (Cys201, Cys208, Cys242) potentially residing near the active site (Saario et al., 2005). More recently, these cysteines have been suggested to act as potential targets for selective sulphydryl-reactive MAGL inhibitors, N-arachidonoylmalonamide (NAM) in particular (Saario et al., 2005; Zvonok et al., 2008; King et al., 2009b; Labar et al., 2010). However, their role in this context has remained partly unclear due to inconsistent results. In investigations of measures to inhibit the enzyme, less attention has been paid to improving our understanding of the catalytic mechanisms important for substrate and isomer preferences in MAGL-driven hydrolysis. Besides the catalytic triad, recent structural and computational studies have provided evidence for the importance of additional amino acids in the catalytic center but mainly without strong experimental support.

It is well known that MAGL is capable of hydrolyzing 1-monoacylglycerol and 2-monoacylglycerol isomers at similar rates. This was first demonstrated by measuring 1-oleoylglycerol (1-OG) and 2-OG hydrolysis with purified rat adipose tissue membranes or recombinant MAGL (Saario et al., 2004; Navia-Paldanius et al., 2012). Here, by combining kinetic enzymatic measurements with molecular modeling and simulations, we show that Cys242 and Tyr194, the two opposed amino acid residues in the MAGL catalytic cavity, play important roles in determining the rate of monoaoylglycerol hydrolysis. Functional roles of Cys201, Cys208, Cys242, and Tyr194 in the activation and inhibition of MAGL were evaluated by determining substrate preferences, relative hydrolysis rates of 1- and 2-isomers, and inhibition profiles by a sensitive and validated fluorescence-based glycerol assay (Navia-Paldanius et al., 2012). Activity assays were complemented by molecular dynamic simulations and docking of substrates and inhibitors into crystal structures of human MAGL.

### Materials and Methods

All reagents for the glycerol assay and the following substrates were obtained from Sigma-Aldrich (St. Louis, MO): 1-capryloyl-rac-glycerol (1-CG) [C8:0], 1-decanoyl-rac-glycerol (1-DG) [C10:0], 1-lauroyl-rac-glycerol (1-LaurG) [C12:0], 1-myristoyl-rac-glycerol (1-MG) [C14:0], 2-palmitoyl-rac-glycerol (2-PG) [C16:0], and the 1- and 2-oleoylglycerol (1- and 2-OG) [C18:1]. The 1- and 2-linoleoylglycerol (1- and 2-LG) [C18:2], 1- and 2-AG [C20:4], prostaglandin D2, prostaglandin E2 [PGD2, PGE2], and 15-deoxy-D12,14-prostaglandin J2 [PGJ2] were obtained from Cayman Chemicals (Ann Arbor, MI). The hydrolysis inhibitors were purchased from the following sources: MAPP (methylarachidonoylfluorophosphate), IPDP (isopropylideneacyclohexyl- phosphonate), JZL184 [4-nitrophenyl 4-dibenzo[1,13]dioxol-5-y1 (hydroxy)methyl]piperidine-1-carboxylate], and NAM (N-arachidonoylmalonamide) were obtained from Cayman Chemicals. Pristimerin was obtained from Sigma-Aldrich. JYKK-048 [4-[bis-[benzol][1,3]dioxol-5-y1methyl]-piperidin-1-yl][1H-1,2,4-triazol-1-y1]methanone] (MAGL) was synthesized in our laboratory, as described previously elsewhere (Aaltolen et al., 2013). TAMRA-FP [2-[3,6-bis(dimethylamino)anilinio]-9-yl-5-(10-ethoxyfluorophosphoryl)deciylcarbamoyl]benzoate] was obtained from Thermofisher Scientific (Rockford, IL). All the other reagents were of highest purity available.

### Site-Directed Mutagenesis. Plasmid material for the study was generated by subcloning hrMAGL-DNA (transcript variant 2; Origen Technologies, Rockville, MD) into a pcDNA3.1-vector containing a N-terminal 5x-hemagglutinin (3xHA) tag. Mutagenesis of cysteines to alanine (C201A, C208A, and C242A) and Tyr194 to phenylalanine as well as the double mutant C242A+T194F were generated by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the following synthetic primers (Infinzymes, Vantaa, Finland): hMAGL-Y194F-Fwd: 5' - GAGGTCGACATTAAAACTCAGACCCCC-3'; hMAGL-Y194F-Rev: 5'-GGGGGCTCTGTAGTTAAAA GTTCTGACCTC-3'; hMAGL-C201F-A: 5' - GACCCCCCGTATGCCGG CGGGCAGGCG-3'; hMAGL-C201F: 5'-GCCGCTGCCTGGGCC CGTACGGTCC-3'; hMAGL-C208F: 5'-GGCGCTGCGATGTCG GCCACCTCACC-3'; hMAGL-C208F-Rev: 5'-GGCATGCGGAGGCCAC CTCTACCCG-3'; hMAGL-C242F-A: 5'-GGCTGCGGCTGAGTG GCCACCTCACC-3'. Finally, after transformation of mutated material into competent bacterial cells, the DNA was isolated, purified and sequenced for confirmation of correct mutant constructs.

### Expression of Human MAGL and Its Mutants in Human Embryonic Kidney Cells. Human embryonic kidney 293 (HEK293) cells were cultured as monolayers in Dulbecco’s modified Eagle’s medium (EuroClone, Milan, Italy) containing 10% fetal bovine serum (EuroClone) under antibiotics (penicillin/streptomycin; EuroClone) at 37°C in a humidified atmosphere of 5% CO2/95% air. Plasmids containing wild-type (WT) and mutant MAGL were introduced to cells by a standard (transient) transfection procedure using X-tremeGENE Hp DNA transfection reagent (Roche, Mannheim, Germany) following the manufacturer’s instructions and as previously described elsewhere (Savinainen et al., 2005; Navia-Paldanius et al., 2012). Mock cells were transfected with pcDNA3.1-vector but otherwise were treated as MAGL cells. Cell lysates were prepared, and protein concentrations were measured as previously described elsewhere (Navia-Paldanius et al., 2012).

### Analysis of MAGL Expression by Western Blot. Expression of 3HA-tagged WT and mutant MAGL was analyzed by Western blot, mainly as previously described elsewhere (Savinainen et al., 2005; Navia-Paldanius et al., 2012). Briefly, cell lysate samples from each HEK293 clone carrying WT or mutant MAGL (5–10 µg protein) together with a molecular weight marker (PugeRuler Prestained Protein Ladder; ThermoFisher Scientific) were fractionated by SDS-PAGE (10%) and transferred to a nitrocellulose membrane (Protran; Schleicher and Schuell, Dassel, Germany). To block nonspecific binding, membranes were incubated with 5% fat-free milk solution for 1 hour at room temperature. The membranes were treated overnight at +4°C with an anti-HA antibody (anti-HA high affinity [3F10], 1:10,000; Roche) and washed for 4 times for 10 minutes each in Tris-buffered saline containing 0.1% Tween. Membranes were then incubated with a secondary antibody (Alexa Fluor 680 goat anti-rat IgG, 1:1,000; Invitrogen/Life Technologies, Carlsbad, CA) for 1 hour at 20°C, followed by washing 4 times for 10 minutes each with Tris-buffered saline containing 0.1% Tween. Membranes were treated overnight with an anti-HA antibody (anti-HA high affinity [3F10], 1:10,000; Roche) and washed for 4 times for 10 minutes each in Tris-buffered saline containing 0.1% Tween. Membranes were then incubated with a secondary antibody (Alexa Fluor 680 goat anti-rat IgG, 1:1,000; Invitrogen/Life Technologies, Carlsbad, CA) for 1 hour at 20°C, followed by washing 4 times for 10 minutes each with Tris-buffered saline containing 0.1% Tween.

The results were normalized against endogenous expression of β-actin by using anti-β-actin primary antibody (monoclonal mouse, Sigma A5441, 1:2000) and secondary antibody (goat anti-mouse IgG [H+L], DyLight 800 conjugated, 1:10,000; ThermoFisher Scientific). Immunoblots were visualized by Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE) and quantified by using an image analysis software ImageJ.

### Activity-Based Protein Profiling of MAGL and Its Cysteine Mutants. Activity-based protein profiling was conducted to visualize active-site binding of a fluorescent fluorophosphate probe TAMRA-FP, as described elsewhere (Navia-Paldanius et al., 2012). Briefly, cell lysates (50 µg) were treated with 20 nM to 2 µM TAMRA-FP for
1 hour at RT, labeled proteins were separated in electrophoresis gels together with standards and visualized (ex.552 nm/em.575 nm) by a fluorescent reader (FLA-3000 laser fluorescence scanner; FujiFilm, Tokyo, Japan) and compared with the pattern of labeled serine hydrolases in mock-transfected cells. The intensity of bands were quantified by an image analysis software ImageJ.

**Fluorescence- and High-Performance Liquid Chromatography-Based MAGL Activity Assays.** A sensitive fluorescence-based hydrolysis assay detecting formation of glycerol was used to determine the rate of monooacylglycerol hydrolysis, $K_m$ and $V_{\text{max}}$ values as well as inhibitor dose-responses in a 96-well format, as previously described elsewhere (Navia-Paldanius et al., 2012). The amount of lysate protein per well was 0.3 μg. Comparative analysis to determine the hydrolysis rates of 2- and 1-AG by high-performance liquid chromatography (HPLC) was performed as previously described elsewhere (Saario et al., 2005). S-Nitrosylation experiments were performed as previously reported elsewhere (Kokkola et al., 2005; Saario et al., 2005).

**Molecular Modeling.** Molecular modeling was mainly performed using Schrödinger’s Maestro software package (Schrödinger Suite 2012: Maestro version 9.3; LigPrep version 2.5; Protein Preparation Wizard: Epik version 2.3, Impact version 5.8, Prime version 3.1; QM-Polarized Ligand Docking protocol: Glide version 5.8, Jaguar version 7.9, QSite version 5.8; Schrödinger, New York, NY). Structures of small molecules were prepared using the LigPrep module. X-ray crystal structure of the human MAGL was used (PDB ID 3PE6) (Schalk-Hihi et al., 2011), and the biomolecule was preprocessed using the protein preparation wizard (Schrödinger) to optimize the hydrogen bonding network and to remove any possible crystallographic artifacts. Graphical illustrations and molecular interaction potentials were generated using Molecular Operating Environment software, version 2012.10 (Chemical Computing Group, 2012).

Unconstrained QM-polarized ligand docking protocol was used for studying binding properties of endogenous ligands to the MAGL active site (PDB ID 3PE6) (Cho et al., 2005). In docking studies, the grid box was centered using the corresponding X-ray ligand as a template. In QM-polarized ligand docking, the initial docking poses were generated using the default SP settings of the Glide module (Schrödinger), after which quantum mechanical treatment was performed with the QSite module (Schrödinger). Fast settings of the Jaguar module were used for charge treatment, and redocking was performed using the default SP settings of Glide. The final selection was made using Coulomb–Van Der Waals energy. Covalent docking of NAM to cysteine residue C201 was performed using Michael Addition reaction embedded to improved covalent docking protocol of the Schrödinger Small-Molecule Drug Discovery Suite 2013-1 (Glide version 5.9 and Prime version 3.2; Schrödinger).

**Molecular Dynamics Simulations.** Periodic water box simulations of 10 nanoseconds were calculated for selected docking poses by using the AMBER 12 program (Case et al., 2012). Hydrogen atoms were added to protein atoms with the leap module of the AMBER 12 program. For the protein, force field parameters and partial charges from the ff99sb force field were used (Hornak et al., 2006). For the small molecules, the GAFF parameter assignments (Wang et al., 2004) were made by using the antechamber module, and the atom-centered partial charges were generated by using the AM1-BCC method (Jakalian et al., 2000). Structures were solvated with a box of TIP3P water molecules, and the structural crystal water molecules were included in the simulations.

Water molecules and the hydrogens of the protein were first energy minimized for 1000 steps followed by a heating step of the solvent box to 300 K in 7.5 ps and then equilibrated for 50 ps at a constant temperature of 300 K and at a pressure of 1 atm. Subsequently, the entire simulation system was minimized for 1000 steps, and the temperature of the system was increased to 300 K in 7.5 ps and equilibrated for 300 ps by using a time step of 1.5 fs and the sander module. The SHAKE algorithm was used to constrain the bonds involving hydrogen atoms to their equilibrium values (Ryckaert et al., 1977). Consequently, production NPT constant number of atoms, pressure, and temperature) simulations of 10.0 nanoseconds were computed using the pmd module. The cutoff for Lennard–Jones interactions was 8 Å, and the particle mesh Ewald (PME) method was used for the treatment of electrostatic interactions (Darden et al., 1993).

The stability of the structures was checked from the root-mean-square (rms)-deviation (Supplemental Fig. 1) curves of the backbone Ca, and the atomic positional fluctuations of backbone atoms, which were calculated with the ptraj program of Amber Tools 1.3 (Case et al., 2005). The relative occupation (0–100%) of main hydrogen bond contacts between ligands and MAGL was analyzed using the ptraj module. Ligand specific rms-deviations were calculated for nine ligand atoms at the glycerol substructure of monooacylglycerols. Selected snapshot and average structures from the molecular dynamics runs were visually examined with the assistance of the Molecular Operating Environment software, and the supplemental videos were generated by using UCSF Chimera Software (Pettersen et al., 2004).

**Statistical Analyses.** The $K_m$ and $V_{\text{max}}$ values, inhibitor dose-response curves, and IC$_{50}$ values derived thereof were calculated from nonlinear regressions using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA), and results are presented as mean ± S.E.M. of at least three independent experiments performed in duplicate. Statistical differences between groups were tested using one-way analysis of variance, followed by Tukey’s multiple comparison test, with $P < 0.05$ considered statistically significant, or by using unpaired Student’s t-test ($^*P < 0.05; ^{*}{^*}P < 0.01; ^{*}{^*}{^*}P < 0.001$).

**Results**

**Substrate Preferences of WT MAGL and Its Cysteine Mutants.** To produce a sufficient amount of biologic material for the study, transient transfections in HEK293 cells were repeated at least 3 times, and all the measurements were performed together with mock-transfected cell material. Expression levels of 3HA-MAGL WT and mutant enzymes were determined from cellular lysates by Western blot analysis, as described in Materials and Methods. These studies indicated that the cell lysates expressed mutant and WT MAGL at comparable levels (Supplemental Fig. 2). Next, we assessed activities of enzyme preparations to hydrolyze a panel of monoaoylglycerol substrates using a sensitive fluorescence-based glycerol assay (Navia-Paldanius et al., 2012). All the 14 substrates tested represent 1- or 2-isomers of monoaoylglycerols with growing acyl chain length and degree of saturation (C8:0 to C20:4) and three prostaglandin glycerol esters.

We first determined the general substrate profiles using a single substrate concentration (25 μM). Figure 1 illustrates that, in spite of some difference between total enzyme activities, the shapes of substrate profiles were similar among WT, C201A, and C208A enzymes when compared with 2-AG hydrolysis. In contrast, the profile shape of the C242A mutant was strikingly deviating; the relative capacity of this mutant to hydrolyze 1-monoacylglycerols was decelerated as compared with that of 2-monoacylglycerols (Fig. 1). To study this in further detail, we subjected three 1- and 2-monoaoylglycerol pairs with different acyl chain lengths (1-OG/2-OG, 1-LG/2-LG, and 1-AG/2-AG) to hydrolysis by the MAGL mutants. In line with the initial profiling data (Fig. 1), WT MAGL as well as C201A and C208A mutants hydrolyzed each 1-2-monoacylglycerol pair with equal rates, whereas a statistically significant drop in the relative hydrolysis rates of 1-monoaoylglycerols versus 2-monoaoylglycerols was evident in the case of the C242A mutant: 1- and 2-AG (29% ± 1%, S.E.M.,...
n = 3), 1- and 2-LG (36% ± 4%, S.E.M., n = 3), and 1- and 2-OG (24% ± 2%, S.E.M., n = 3) (Fig. 2). To obtain independent methodological confirmation, we measured the hydrolysis rates of 1- and 2-AG also by a validated high-performance liquid chromatography–based MAGL-assay (Saario et al., 2005). Similarly, this analysis confirmed that the mutation of Cys242 significantly dampened the relative rate of 1-AG hydrolysis (Supplemental Fig. 3).

Because Cys242 was found to be a critical residue required for unbiased hydrolysis of monoacylglycerol isomers, we tested whether S-nitrosylation might affect balanced hydrolysis of monoacylglycerol isomers and in particular whether Cys242 serves a possible target for this modification, which has emerged as a potential regulator of both enzyme and G protein–coupled receptor activity by targeting free sulfhydryl groups of proteins (Stamler et al., 2001; Kokkola et al., 2005). To this end, we measured the hydrolysis rates of 1- and 2-AG after treating WT MAGL and the C242A mutant with two S-nitrosothiols, S-nitrosoglutathione and S-nitrosocysteine, as previously reported elsewhere (Kokkola et al., 2005). However, the hydrolysis rates of 1- and 2-AG were not affected by S-nitrosothiol treatment (Supplemental Fig. 3), which confirmed and extended our previous results on the effect of S-nitrosothiols on 2-AG hydrolysis by rat cerebellar membrane MAGL preparation (Saario et al., 2005).

In addition to monoacylglycerols, we assessed the capacity of the MAGL mutants to hydrolyze prostaglandin glycerol esters, the products of cyclooxygenase-2 catalyzed 2-AG oxygenation (Rouzer and Marnett, 2011). The three tested...
prostaglandin glycerol esters (15d-PGJ\(_2\)-G, PGD\(_2\)-G, and PGE\(_2\)-G) were feasible substrates for MAGL when compared with the primary endocannabinoid 2-AG (Fig. 1). Furthermore, Michaelis-Menten analysis revealed that when compared with 2-AG, 15d-PGJ\(_2\)-G had 10- to 14-fold higher affinity (\(K_m\)) toward WT MAGL and C201A and C208A mutants and approximately 6-fold higher toward the C242A-mutant (Fig. 3; Supplemental Table 1). However, none of the mutations caused statistically significant changes in \(V_{\text{max}}\) or \(K_m\) values compared with WT MAGL (Fig. 3).

**Mutation of Cys242 Induces Unfavorable Structural Changes Impacting More 1-Monoacylglycerols than 2-Monoacylglycerols.** To ensure that our preliminary observations of diminished hydrolysis rate of 1-monoacylglycerols versus 2-monoacylglycerols by the C242A mutant were not restricted to the single concentration of substrates used, we performed a Michaelis–Menten analysis for the WT and mutant enzymes with 1- and 2-AG as the substrates (Fig. 4; Supplemental Table 1). Even though the relative expression of the C242A mutant was somewhat higher compared with that of WT MAGL and other cysteine mutants (see the Western blot data in Supplemental Fig. 2), it was the only cysteine mutant that significantly affected the rate (\(V_{\text{max}}\)) and affinity (\(K_m\)) of 1- or 2-AG when compared with the WT enzyme (Fig. 4; Supplemental Table 1). Importantly and in line with the previous results, the \(V_{\text{max}}\) and \(K_m\) values for WT MAGL and mutants C201A and C208A were comparable (Supplemental Table 1). However, the ability of C242A-mutant to hydrolyze 1-AG was significantly diminished, indicating that Cys242 is more critical for the hydrolysis of 1- than 2-AG.

However, whether this feature was the result of plain structural changes in the binding cavity or a direct consequence of severing the catalytic mechanism was not clear. To resolve this interesting question, we used molecular modeling studies to point to the locations of the C201A, C208A, and C242A mutations relative to substrate binding cavity, followed by extensive docking studies. Because docking of flexible monoacylglycerols was found challenging for the current docking algorithms, we restricted the docking studies to 1- and 2-AG, which both produced equally placed docking poses and similar interactions with the human MAGL.

The orientation of the polar head of monoacylglycerols is well established based on earlier modeling studies as well as recent experimental structures of bacterial MAGL in various conformations (Bertrand et al., 2010; Rengachari et al., 2013). To gain further understanding on the binding properties in the ground state, a series of unconstrained molecular dynamic simulations were calculated for WT and mutant complexes with selected docking poses of 2- and 1-AG. In contrast with recent speculations (Karageorgos et al., 2010), our modeling suggested that although Cys242 is involved in the hydrogen bonding network, its side chain does not form direct hydrogen bond contacts with residues of the catalytic triad (Ser122-His269-Asp239). Indeed, during the simulations, the distance of Cys242 from the catalytic serine remained in most part longer than 4.5 Å (see Supplemental Fig. 4). However, the key interactions of substrates to the oxyanion hole (N-H, Ala51 and N-H, Met123) were well retained during the simulations, although the average distance to Met123 was shorter (Supplemental Table 2).

According to the hydrogen bond analysis, other key interactions with His121, Tyr194, and Ala51 were occupied most of the time, especially in the case of 2-AG (Supplemental Table 2). For comparison, the coordination of the polar head of 1-AG was more...
labile according to rms-deviations, calculated for heavy atoms forming the polar head and hydrogen bond analysis (Supplemental Fig. 5; Supplemental Table 2). Interestingly, modeling of C242A into human MAGL did not cause any considerable effect on ligand rms-deviation profile of 2-AG simulations. In contrast, it caused clear fluctuations to the position of the 1-AG (Supplemental Fig. 5). Based on structures and molecular interaction field energies (Supplemental Fig. 6), the main chain carbonyl of Ala51 offers an important site for interactions with Tyr194 in terms of binding a crystal water, or alternatively in terms of recognition of single OH group of monoacylglycerols. When the coordinations of 1- and 2-AG were compared

Fig. 4. Michaelis–Menten kinetics for (A) WT MAGL, (B) cysteine mutants C201A, (C) C208A, (D) C242A, and (E) tyrosine mutant Y194F, as well as (F) the double mutant C242A/Y194F using 1- and 2-AG as substrates. The data (see also Supplemental Table 1) reveal a statistically significant decrease in maximal velocity ($V_{\text{max}}$) and affinity ($K_m$) for C242A (but not the other mutants) to hydrolyze 1-AG as compared with 2-AG. In addition, $V_{\text{max}}$ values for C242A, Y194F, and double mutant to hydrolyze 1- and 2-AG are significantly decreased compared with the WT enzyme. Lineweaver Burk plots are shown as insets. Incubations were performed at room temperature for 60 minutes with increasing substrate concentrations (cellular protein 0.3 µg/well). The data are mean ± S.E.M. from three independent experiments performed in duplicate.
against this location during molecular dynamics, relative occurrences were found to be different (Supplemental Table 2). This prompted us to test whether mutation of Tyr194, a residue located at the opposite side of the active site cavity as compared with Cys242 (Fig. 5), would potentially cause similar effects as those observed with the C242A mutant. To address this, we generated cell lines transiently expressing the Y194F-mutant as well as a double mutant Y194F+C242A. The expression levels of these mutants versus WT MAGL were found to be sufficiently similar to allow further comparison of enzymatic parameters (Supplemental Fig. 2).

As shown in Fig. 4 and Supplemental Table 1, the overall activity, as well as kinetic values ($V_{\text{max}}$ and $K_m$), of both mutants were significantly compromised when compared with WT MAGL. However, in contrast with the C242A mutation that caused an unbalanced rate of hydrolysis between 1- and 2-AG, the Y194F mutant hydrolyzed these isomers with similar rates (Supplemental Table 1). The activity of the double mutant to hydrolyze 1- and 2-AG was severely attenuated, but no statistically significant difference was found between the rates of 1- and 2-AG hydrolysis due to the low signal-to-noise ratio resulting in relatively high error ($\text{P} = 0.124$ ($K_m$); $\text{P} = 0.053$ ($V_{\text{max}}$), unpaired t test).

Collectively, these findings suggest that C242A (with or without Y194F) selectively disturbs the balanced hydrolysis of monoacylglycerol isomers by causing unfavorable structural effects on substrate binding, but this deficit can be partly restored with more optimal hydrogen bonding interactions in the case of 2-isomers.

**Potency of Established MAGL Inhibitors in WT MAGL and Its Cysteine Mutants.** To test the effect of cysteine mutations on inhibitor potencies, we determined the full inhibition dose responses for several MAGL selective and nonselective inhibitors (Supplemental Figs. 7, structures, and 8; Table 1). 2-AG was used as the substrate in these experiments, as 1-monoacylglycerols were shown to lose their natural hydrolysis rate due to C242A mutation. Here, Cys201 was confirmed to be the principal NAM target, as C201A mutation clearly blunted the inhibitory potency and efficacy of NAM (Supplemental Fig. 8). In contrast, the potencies of the other tested inhibitors were not altered in the C201A mutant (Supplemental Fig. 8; Table 1).

To gain further insight into the interaction of NAM with MAGL, we covalently docked this inhibitor into Cys201. The modeling data indicated that NAM binding likely disturbs the organization of the glycerol exit hole (Supplemental Fig. 9), a finding implicating that the inhibition may be due to blocking the enzyme from the outside (masking the glycerol exit hole) rather than due to steric hindrance of substrate entry and/or inhibition of substrate hydrolysis by targeting Cys208 or Cys242.

None of the cysteine mutations had statistically significant effects on the potency of pristimerin (Table 1), another postulated sulfhydryl-reactive MAGL inhibitor. Interestingly, all the other tested inhibitors, including the highly potent and selective MAGL inhibitor JKKK-048 (Aaltonen et al., 2013), the MAGL-inhibitor JZL184, and the potent and nonselective serine hydrolase inhibitors MAFP and IDFP, lost their potency in the C242A mutant (Supplemental Fig. 8; Table 1). The most dramatic potency drop was witnessed for the fluorophosphonates IDFP (63-fold drop) and MAFP (13-fold drop), indicating that targeting of the MAGL catalytic serine by these inhibitors was especially sensitive to C242A mutation. For comparison, the potency decrease in the case of JZL184 and JKKK-048 was.

---

**Fig. 5.** Selected docking poses of 2-AG (orange, A) and 1-AG (green, B) to MAGL (PDB ID 3PE6) showing the key hydrogen bonding interaction found among highest ranking poses. The location of the carbonyl oxygen interaction potential of 8.5 kcal/mol is presented using magenta cage. Note that, in contrast to 1-AG, 2-AG does not form direct hydrogen bonding interactions (light blue bars) with Tyr194 in docking.
We used a kinetic enzymatic assay that has proven useful in assessing comparisons of natural monoacylglycerol hydrolysis in a simple 96-well format. This approach has many advantages over the traditional approaches for assessing monoacylglycerol hydrolysis, as they rely on degradation of labeled/nonlabeled 2-AG or 2-OG by HPLC, liquid scintillation, or liquid chromatography/mass spectrometry technology (Saario et al., 2005; King et al., 2009a,b; Labar et al., 2010). In contrast with the previously discussed methods that typically use only a single substrate, the kinetic glycerol assay enables determination of hydrolysis of all-natural 1- or 2-monoacylglycerols by the endocannabinoid hydrolases MAGL, ABHD6, and ABHD12 (Navia-Paldanious et al., 2012).

In this study, we tested the catalytic activity of WT MAGL and C201A, C208A, and C242A mutants using a repertoire of substrates to find any possible alterations on substrate preference resulting from cysteine substitutions. We demonstrated that Cys242 was important for the hydrolysis of monoacylglycerols, which was especially relevant for 1-monoacylglycerols. This issue was addressed in detail by testing the hydrolysis rates of the endocannabinoids 1- and 2-AG.

Previously, it had been suggested based on NMR analysis that Cys242 possibly interacts directly with the catalytic triad and, thus, potentially participates in the catalysis (Karageorgos et al., 2010). In contrast, our modeling studies indicate that the long distance between the catalytic triad and Cys242 is unlikely to offer direct interactions with the catalytic triad. Instead, both the mutational and molecular modeling studies suggested that the C242A mutation leads to structural disturbance of the oxyanion hole. This conclusion was supported by the experimental observations that the fluorophosphonate inhibitors MAFFP and IDFP targeting the catalytic Ser122 dramatically lost their potency as a result of the C242A mutation. We propose, in this case, that the catalytic triad is locked in a nonreactive state and thereby prevents catalysis. Unlike these particular fluorophosphonates, the reactivity of natural substrates is retained by the formation of a sufficient network of favorable interactions between the active site residues and the OH groups of the polar head of monoacylglycerols. Our experimental data supported by the atomic level molecular modeling analysis suggest that in the C242A mutant the hydroxyl groups of 2-monoacylglycerols are able to form more stable interactions with active site residues as opposed to 1-monoacylglycerols (Supplemental Videos 1 and 2).

Intriguingly, the three tested prostaglandin glycerol esters (15d-PGJ2-G, PGD2-G, and PGE2-G) appeared to be excellent substrates for human MAGL. This, together with recent findings from another laboratory demonstrating efficient hydrolysis of PGE2-G and PGF2α-G by human MAGL (Xie et al., 2010), challenges previous claims stating that prostaglandin glycerol esters act as poor MAGL substrates (Vila et al., 2007). Our preliminary data, as shown here, suggest that under the assay conditions employed, 15d-PGJ2-G has even higher affinity for MAGL than the primary endocannabinoid 2-AG. We have more thoroughly examined prostaglandin glycerol esters as MAGL substrates, and those results will be reported in a separate publication.

As mentioned in the Introduction, Cys201, Cys208, and Cys242 have been suggested to act as potential targets for selective sulfhydryl-reactive inhibitors, NAM in particular (Saario et al., 2005; Zvonok et al., 2008; King et al., 2009a,b; Labar et al., 2010), but their role in this respect has remained unclear, we used site-directed mutagenesis to comprehensively analyze the impact of these cysteine residues as well as additional amino acid substitutions in the binding and function of a panel of MAGL substrates and inhibitors. Tyr194 was included for its suggested role in the catalysis of substrate hydrolysis, as speculated earlier by Bertrand et al. (2010).

### Discussion

MAGL has emerged as a promising and druggable target, and inhibitors targeting this hydrolase may show great promise in treatments of cancer, neurodegenerative diseases, and metabolic disorders. Although progress in inhibitor development has been rapid, our knowledge regarding determinants that regulate the catalytic activity and substrate preferences of human MAGL is limited. As the role of conserved cysteine residues Cys201, Cys208, and Cys242 in the activity of MAGL has remained unclear, we used site-directed mutagenesis to comprehensively analyze the impact of these cysteine residues as well as additional amino acid substitutions in the binding and function of a panel of MAGL substrates and inhibitors. Tyr194 was included for its suggested role in the catalysis of substrate hydrolysis, as speculated earlier by Bertrand et al. (2010).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>C201A</th>
<th>C208A</th>
<th>C242A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-logIC50 ± S.E.M.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAM</td>
<td>6.06 ± 0.12</td>
<td>Ambiguous</td>
<td>6.51 ± 0.10</td>
<td>6.34 ± 0.06</td>
</tr>
<tr>
<td>MAFFP</td>
<td>7.69 ± 0.11</td>
<td>7.79 ± 0.05</td>
<td>7.59 ± 0.20</td>
<td>6.08 ± 0.80</td>
</tr>
<tr>
<td>JZL184</td>
<td>6.19 ± 0.12</td>
<td>5.81 ± 0.04</td>
<td>6.70 ± 0.11**</td>
<td>5.74 ± 0.06***</td>
</tr>
<tr>
<td>JJKK-048</td>
<td>9.39 ± 0.04</td>
<td>9.38 ± 0.02</td>
<td>9.59 ± 0.07</td>
<td>9.01 ± 0.06***</td>
</tr>
<tr>
<td>IDFP</td>
<td>9.36 ± 0.22</td>
<td>9.42 ± 0.20</td>
<td>9.58 ± 0.34</td>
<td>7.55 ± 0.06***</td>
</tr>
<tr>
<td>Pritisterin</td>
<td>5.36 ± 0.20</td>
<td>5.37 ± 0.05</td>
<td>5.33 ± 0.41</td>
<td>5.60 ± 0.08</td>
</tr>
</tbody>
</table>

*P < 0.001; **P < 0.05; ***P < 0.01.
unclear due to inconsistent results. However, Cys201 was recently identified as the target of NAM (King et al., 2009b; Labar et al., 2010), and we can verify this based on our previous study. However, unlike previous studies, we provide evidence to suggest that the inhibition of MAGL by NAM may be due to masking the glycerol exit hole, thereby resulting in de- celerated hydrolysis of substrates. As stated earlier, we chose 2-AG as the substrate for the inhibitor studies because 1-monoacylglycerols were shown to be more sensitive toward the C242A mutation. This important factor has not been addressed in any of the previous studies assessing inhibitor potencies in MAGL cysteine mutants, further highlighting the value of the observations made in our current study.

Another interesting detail was that the potencies of the catalytic Ser122-targeting fluorophosphonates (MAFP, IDFP, and TAMRA-FP) were found to be sensitive to the C242A mutation. In particular, contrast with a previous report (King et al., 2009a), the potency of pristimerin was not significantly affected by the C208A mutation (or any other cysteine mutation), suggesting that the trимере may not target these cysteine residues after all.

To conclude, we have pinpointed here two amino acid residues in the human MAGL structure—namely, Cys242 and Tyr194—that are crucial in determining the rate and/or isomer selectivity of monoacylglycerol hydrolysis. Mutation of Cys242 to alanine was shown to skew the balanced hydrolysis of 1- and 2-monoacylglycerols to favor 2-monoacylglycerols, and Cys201 to alanine was shown to skew the balanced hydrolysis of 1-monoacylglycerols to favor 2-monoacylglycerols. To conclude, we have pinpointed here two amino acid residues in the human MAGL structure—namely, Cys242 and Tyr194—that are crucial in determining the rate and/or isomer selectivity of monoacylglycerol hydrolysis. Mutation of Cys242 to alanine was shown to skew the balanced hydrolysis of 1- and 2-monoacylglycerols to favor 2-monoacylglycerols, and Cys201 to alanine was shown to skew the balanced hydrolysis of 1-monoacylglycerols to favor 2-monoacylglycerols.


Address correspondence to: Dr. Juha R. Savinainen, School of Medicine, Institute of Biomedicine/Physiology, University of Eastern Finland, Kuopio, Finland. E-mail: juha.savinainen@uef.fi