methods

# A natural substrate-based fluorescence assay for inhibitor screening on diacylglycerol lipase α

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**Abstract The endocannabinoid 2-arachidonoylglycerol (2-AG) is predominantly biosynthesized by *sn*-1-diacylglycerol lipase α (DAGL-α) in the CNS. Selective inhibitors of DAGL-α will provide valuable insights in the role of 2-AG in endocannabinoid signaling processes and are potential therapeutics for the treatment of obesity and neurodegenerative diseases. Here, we describe the development of a natural substrate-based fluorescence assay for DAGL-α, using a coupled enzyme approach. The continuous setup of our assay allows monitoring of DAGL-α activity in real-time and in a 96-well plate format. This constitutes a major improvement to the currently available radiometric and LC/MS-based methods, which can be executed only in low-throughput formats. In addition, our assay circumvents the use of radioactive material. We demonstrate that our assay can be used to screen inhibitors of DAGL-α activity, using 1-stearoyl-2-arachidonoyl-*sn*-glycerol as the physiologically relevant natural substrate of DAGL-α. Furthermore, our method can be employed to measure DAGL activity and inhibition in the mouse brain membrane proteome. Consequently, our assay should serve as a valuable tool for rapid hit validation and lead optimization of DAGL-α inhibitors.—** van der Wel, T., F. J. Janssen, M. P. Baggelaar, H. Deng, H. den Dulk, H. S. Overkleeft, and M. van der Stelt. **A natural substrate-based fluorescence assay for inhibitor screening on diacylglycerol lipase α. *J. Lipid Res.* 2015.** 56: **927–935.**

**Supplementary key words** 2-arachidonoylglycerol • cannabinoids • endocannabinoid • enzymology • lipids • obesity

2-Arachidonoylglycerol (2-AG) is the most abundant endocannabinoid in the CNS and activates the endocannabinoid system by binding to the cannabinoid 1 receptor (1). Its local action is highly dependent on site-specific production by *sn*-1-diacylglycerol lipase α (DAGL-α), the major enzyme responsible for 2-AG biosynthesis in the CNS (2, 3). DAGL-α is an intracellular integral multi-domain membrane protein and employs a typical Ser-His-Asp catalytic triad to selectively hydrolyze the *sn*-1 acyl group of diacylglycerol (4). Selective inhibitors of DAGL-α will provide valuable insights in the role of 2-AG in endocannabinoid signaling processes and have therapeutic potential for the treatment of obesity and neurodegenerative diseases (5–7).

A limited number of DAGL-α activity assays is currently available. The first group of assays employs surrogate substrates, such as para-nitrophenol butyrate, and is generally used for the initial process of inhibitor identification. The main advantage of these assays is that product formation can be monitored in real-time, generally by absorption or fluorescence measurement. The cost-effectiveness and easy detection of surrogate substrates make these assays valuable tools for high-throughput screening applications (8, 9). However, surrogate substrates generally have an attenuated binding affinity for the enzyme active site compared with physiologically relevant natural substrates, which may lead to distorted results when determining inhibitory potency. Consequently, the use of the natural substrate of DAGL-α is preferred in the process of hit validation and lead optimization.

At present, two assay methods have been reported that utilize diacylglycerol, the natural substrate of DAGL-α. A radiometric assay has been extensively used to measure DAGL-α activity in vitro, using 1-stearoyl-2-[$^{14}$C]arachidonoyl-*sn*-glycerol as the substrate (4). This method is highly sensitive, but requires lipid extraction, fractionation by TLC, and quantification of radiolabeled 2-[$^{14}$C]arachidonoylglycerol via scintillation counting, making this assay method labor intensive. Perhaps the most important caveat is that 1-stearoyl-2-[$^{14}$C]arachidonoyl-*sn*-glycerol is not commercially available, expensive to synthesize, and requires handling of radioactive material. An alternative

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Abbreviations: ABPP, activity-based protein profiling; ABHD, α/β-hydrolase domain; ACN, acetonitrile; 2-AG, 2-arachidonoylglycerol; CV, coefficient of variation; DAGL-α, *sn*-1-diacylglycerol lipase α; GK, glycerol kinase; GPO, glycerol-3-phosphate oxidase; MAGL, monoacylglycerol lipase; SAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol; TAMRA-FP, TAMRA-fluorophosphonate; THL, tetrahydrolipstatin (Orlistat).

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method is the use of LC/MS-based methods to detect product formation (10). Although this method circumvents the use of radiolabeled substrates and is highly accurate, it also requires lipid extraction and separation of phases. Furthermore, both the radiometric and LC/MS-based assays prohibit monitoring of reaction progress in real-time due to their discontinuous setup and thus suffer from a low throughput.

Coupled enzyme glycerol assays have been used in the field of lipase research for decades and are based on an enzymatic cascade reaction to couple the production of glycerol to the generation of a fluorescent signal (11, 12). Recently, this approach was used to develop an activity assay for monoacylglycerol lipase (MAGL), α/β-hydrolase domain (ABHD)6, and ABHD12, enzymes involved in breakdown of 2-AG (13).

Here, we report on the application of this assay format in the development of a natural substrate-based DAGL-α fluorescence assay that allows monitoring of reaction progress in real-time. We demonstrate that the assay can be used to determine kinetic parameters, such as the Michaelis-Menten constant, and to screen inhibitors of DAGL-α activity.

## EXPERIMENTAL PROCEDURES

### Materials

TAMRA-fluorophosphonate (TAMRA-FP) was purchased from Thermo Fisher (Landsmeer, The Netherlands); JZL184 (irreversible selective MAGL inhibitor) and 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SAG) were purchased from Cayman Chemicals (Ann Arbor, MI). Activity-based probe MB064 (equipped with a BODIPY fluorophore), KT109 (potent irreversible DAGL inhibitor), and LEI-106 (reversible DAGL inhibitor) were synthesized in-house as previously described (10, 14, 15). All synthesized inhibitors were at least 95% pure and were analyzed by LC/MS and NMR. All other chemicals, reagents, and primers were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

### Cloning

Full-length human DAGL-α and MAGL cDNA (Source Bioscience, Nottingham, UK) was cloned into the mammalian expression vector pcDNA3.1, containing ampicillin and neomycin resistance genes. The inserts were cloned in frame of a C-terminal FLAG-tag and site-directed mutagenesis was used to generate the catalytically inactive DAGL-α S472A mutant, as previously described (14). Plasmids were isolated from transformed XL-10 Z-competent cells (Maxi Prep kit; Qiagen, Venlo, The Netherlands) and sequenced at the Leiden Genome Technology Center. Sequences were analyzed and verified (CLC Main Workbench).

### Cell culture and membrane preparation

HEK293T cells were cultured at 37°C under 7% $CO_2$ in DMEM containing stable glutamine and phenol red 10% v/v newborn calf serum (Thermo Fischer), and penicillin and streptomycin (both 200 μg/ml; Duchefa, Haarlem, The Netherlands). Medium was refreshed every 2–3 days and cells were passaged at 80–90% confluence by resuspension in fresh medium. Cell cultures were discarded after 2–3 months.

One day prior to transfection, $10^7$ cells were seeded in 15 cm plates. On the day of transfection, medium was removed by suction and a minimal amount of fresh medium (13 ml for 15 cm plates) was added. Cells were transfected by addition of 60 μg polyethylenimide and 20 μg plasmid DNA in 2 ml DMEM without serum. The empty pcDNA3.1 vector served as a mock-control. After 24 h, the medium was refreshed. Cells were harvested 72 h after transfection by resuspension and centrifuged for 10 min at 200 *g*. Supernatant was discarded and cell pellets were frozen in liquid nitrogen and stored at −80°C for at least 24 h.

Cell pellets were thawed on ice and resuspended in lysis buffer A [20 mM HEPES (pH 7.2), 2 mM DTT, 250 mM sucrose, 1 mM $MgCl_2$, and 25 U/ml benzonase]. Suspensions were homogenized by polytron (3 × 7 s, 20,000 rpm, SilentCrusher S; Heidolph, Schwabach, Germany), incubated on ice for 30 min, and subsequently centrifuged at 93,000 *g* for 30 min at 4°C (Ti70 or Ti70.1 rotor; Beckman Coulter, Woerden, The Netherlands). Pellet was resuspended in storage buffer B [20 mM HEPES (pH 7.2), 2 mM DTT]. Suspension was homogenized by polytron (1 × 10 s, 20,000 rpm). Protein concentrations were determined with Quick Start Bradford reagent (Bio-Rad, Hilversum, The Netherlands) or Qubit fluorometric quantitation (Life Technologies, Breda, The Netherlands). Membranes were diluted with storage buffer B to the desired concentration, aliquoted, frozen in liquid nitrogen, and stored at −80°C.

### Preparation of mouse brain membrane proteome

Mouse brains were isolated according to guidelines approved by the ethical committee of Leiden University (DEC#13191), frozen in liquid nitrogen, and stored at −80°C until use. Mouse brains were thawed on ice and homogenized by polytron (3 × 5 s, 20,000 rpm) in lysis buffer A [20 mM HEPES (pH 7.2), 2 mM DTT, 250 mM sucrose, 1 mM $MgCl_2$, and 25 U/ml benzonase]. The suspension was incubated on ice for 15 min, followed by low speed centrifugation (2,500 *g*, 3 min. at 4°C) to remove debris. The supernatant was subjected to ultracentrifugation (93,000 *g*, 45 min at 4°C) to yield the mouse brain membrane proteome as a pellet. The pellet was resuspended in storage buffer B [20 mM HEPES (pH 7.2), 2 mM DTT] and homogenized by polytron (1 × 7 s, 20,000 rpm). Protein concentrations were determined and membrane preparations were stored as described above.

### Activity-based protein profiling

Gels and blots were scanned with a ChemiDoc XRS system (BioRad) and images were analyzed with Image Lab software (Bio-Rad). For activity-based protein profiling (ABPP) experiments, 20 μl of protein sample (2 mg/ml) was preincubated with 0.5 μl inhibitor (10 μM final concentration) for 20 min at room temperature. Subsequently, 0.5 μl TAMRA-FP or MB064 (500 nM final concentration, in DMSO) was added and the samples were incubated for 15 min at room temperature. The reaction was quenched by addition of 10 μl 3× Laemmli sample buffer [final concentrations: 60 mM Tris-Cl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.01% (v/v) bromophenol blue]. Samples were centrifuged for 2 min at 9,300 *g* and were then loaded on a 10% acrylamide SDS-PAGE gel. Gels were run at 180 V for 60–90 min and scanned for fluorescence using Cy3 and Cy5 multichannel settings (605/50 and 695/55 filters, respectively).

Proteins on SDS-PAGE gels were transferred to 0.2 μm polyvinylidene difluoride membranes (Trans-Blot Turbo™ Transfer system; Bio-Rad). The membrane was blocked with blocking buffer [5% (w/v) milk in 10 ml TBS-Tween] and then stained with primary mouse-anti-FLAG antibody [0.1% (v/v); Sigma] and goat-anti-mouse-HRP [0.02% (v/v); Santa Cruz Biotechnology, Heidelberg, Germany], each for 1 h. Imaging solution (10 ml luminal, 100 μl ECL enhancer, 3 μl $H_2O_2$) was added and the blot was scanned using chemiluminescence settings.

### DAGL-α activity assay

The DAGL-α natural substrate assay is based on the production of 2-AG from SAG hydrolysis by DAGL-α-overexpressing membrane preparations from transiently transfected HEK293T cells. The 2-AG production is coupled to the oxidation of commercially available Amplifu™ Red via a multi-enzyme cascade, resulting in a fluorescent signal from the dye resorufin (13).

Standard assays were performed in HEMNB buffer [50 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 0.5% (w/v) BSA] in black flat clear-bottom 96-well plates (Greiner). Final protein concentration of membrane preparations from DAGL-α-overexpressing HEK293T cells was 50 μg/ml (10 μg per well). Inhibitors were added from 40× concentrated DMSO stocks. A substrate solution of SAG was prepared just prior to use. The SAG stock solution (10 mg/ml in methyl acetate) was dried under argon and subsequently dissolved in 50 mM HEPES buffer (pH 7.0) containing 0.75% (w/v) Triton X-100. The substrate solution was mixed to form an emulsion and stored on ice until use.

DAGL-α-overexpressing membranes were incubated with inhibitor for 20 min. Subsequently, assay mix containing glycerol kinase (GK), glycerol-3-phosphate oxidase (GPO), HRP, ATP, MAGL, Amplifu™ Red, and SAG was added and fluorescence was measured in 5 min intervals for 60 min on a GENios microplate reader (Tecan, Giessen, The Netherlands).

Standard assay concentrations: 0.2 U/ml GK, GPO, and HRP; 0.125 mM ATP; 5 μg/ml MAGL-overexpressing membranes; 10 μM Amplifu™ Red; 100 or 150 μM SAG; 5% DMSO; 0.0075% (w/v) Triton X-100 in a total volume of 200 μl.

### MAGL activity assay

The MAGL activity assay is based on the production of glycerol from 2-AG hydrolysis by MAGL-overexpressing membrane preparations from transiently transfected HEK293T cells, as previously reported (13). Standard assays were performed under similar conditions as for the DAGL-α activity assay, but at a final protein concentration of 1.5 μg/ml (0.3 μg MAGL-overexpressing membranes per well) and with 2-AG as the substrate. 2-AG was directly added from a stock solution in acetonitrile and no Triton X-100 was added. Fluorescence was measured in 5 min intervals for 60 min. Final assay concentrations: 0.2 U/ml GK, GPO, and HRP; 0.125 mM ATP; 10 μM Amplifu™ Red; 25 μM 2-AG; 5% DMSO; 0.5% acetonitrile (ACN) in a total volume of 200 μl.

### Mouse brain membrane proteome assay

The mouse brain membrane proteome assay was performed with mouse brain membrane preparations (75 μg/ml final concentration, 15 μg per well) as a substitute for the overexpressing membranes from transfected HEK293T cells. Endogenously expressed MAGL was used to hydrolyze 2-AG and no MAGL-overexpressing membranes were added in the assay mix. Other conditions were identical to those described above. Final assay concentrations: 0.2 U/ml GK, GPO, and HRP; 0.125 mM ATP; 10 μM Amplifu™ Red; 150 μM SAG or 25 μM 2-AG; 5% DMSO in a total volume of 200 μl. Additionally, experiments with SAG as the substrate contained 0.0075% (w/v) Triton X-100, experiments with 2-AG as the substrate contained 0.5% ACN.

### Data analysis and statistics

For the DAGL-α and MAGL activity assay, data were corrected for the average fluorescence of the negative control (mock-membranes + DMSO), unless mentioned otherwise. For the mouse brain membrane proteome assay, membranes with no added substrate served as a negative control. DAGL-α-overexpressing, MAGL-overexpressing, or mouse brain membranes + DMSO served as a positive control, respectively. Slopes of fluorescence in time were determined in the linear interval of $t = 10$ to $t = 25$ min (Michaelis-Menten experiment), $t = 0$ to $t = 15$ min (mouse brain membrane proteome assay with 2-AG), or $t = 10$ to $t = 35$ min (all other assays). If relevant, experimental data were converted into 2-AG or glycerol production rates in picomoles per minute or nanomoles per milligram protein per minute according to a glycerol standard curve. For each data set, mean, SD, and SEM were calculated. For $K_m$ and $V_{max}$ determination, the data were exported to GraphPad Prism 5.0 and subjected to Michaelis-Menten analysis. For $IC_{50}$ determination, slopes of corrected fluorescence in time were scaled to the corrected positive control as a 100% activity reference point. The data were exported to GraphPad Prism 5.0 and analyzed in a nonlinear dose-response analysis with variable slope. For the glycerol standard curve, data were exported to GraphPad Prism 5.0 and subjected to linear regression analysis. Individual points for kinetic data were presented as mean ± SEM with n = 4, and n = 8 for controls. Statistical comparison was performed using unpaired Student's *t*-test. The Z'-factor for each measurement was calculated using the formula $Z' = 1 - 3(\sigma_{pc} + \sigma_{nc})/(\mu_{pc} - \mu_{nc})$; where σ is the SD, μ is the mean, pc is the positive control, and nc is the negative control. Plates with $Z' \geqslant 0.6$ were accepted for further analysis. The inter-assay coefficient of variation (CV) was determined by calculating the mean slope of uncorrected fluorescence in time on each plate (mean of plate) for both the positive and negative controls (n = 8 and n = 4, respectively). Subsequently, the mean and SD of the means of the individual plates were calculated. The inter-assay CV was calculated as CV = (SD of means/mean of means) × 100%. $pIC_{50}$ values were calculated as $-\log(IC_{50})$.

## RESULTS AND DISCUSSION

The DAGL-α natural substrate assay is based on the production of 2-AG from SAG hydrolysis by DAGL-α-overexpressing membrane preparations from transiently transfected HEK293T cells. The produced glycerol is coupled to the oxidation of commercially available Amplifu™ Red via a coupled enzyme reaction, resulting in a fluorescent signal from the dye resorufin (**Fig. 1A**). The assay setup is derived from a previously reported MAGL assay (13). We reasoned that MAGL was the best candidate to participate in the enzymatic cascade, because previous studies indicated that this enzyme was more efficient at 2-AG hydrolysis than the other two endocannabinoid hydrolases, ABHD6 and ABHD12 (13). Fig. 1B shows an overview of the inhibitors and probes that were used in our experiments.

Activity and expression of full-length human DAGL-α and MAGL were verified by ABPP and Western blotting, respectively (**Fig. 2**). Broad-range serine hydrolase probe TAMRA-FP labeled MAGL as a doublet (~33 and ~35 kDa), likely due to alternative splicing (16). Our previously reported activity-based probe, MB064, was used to verify catalytic activity of DAGL-α (~120 kDa). The marked band at ~70 kDa likely corresponds to a degradation product of DAGL-α, whose catalytic activity is maintained. In contrast, no band is observed on Western blot, suggesting that the enzyme's C-terminal domain containing the FLAG-tag is no longer present. These observations are in line with prior experiments using a C-terminal deletion construct of DAGL-α (14). No labeling was observed when MAGL was preincubated with the selective irreversible MAGL inhibitor JZL184 (17),





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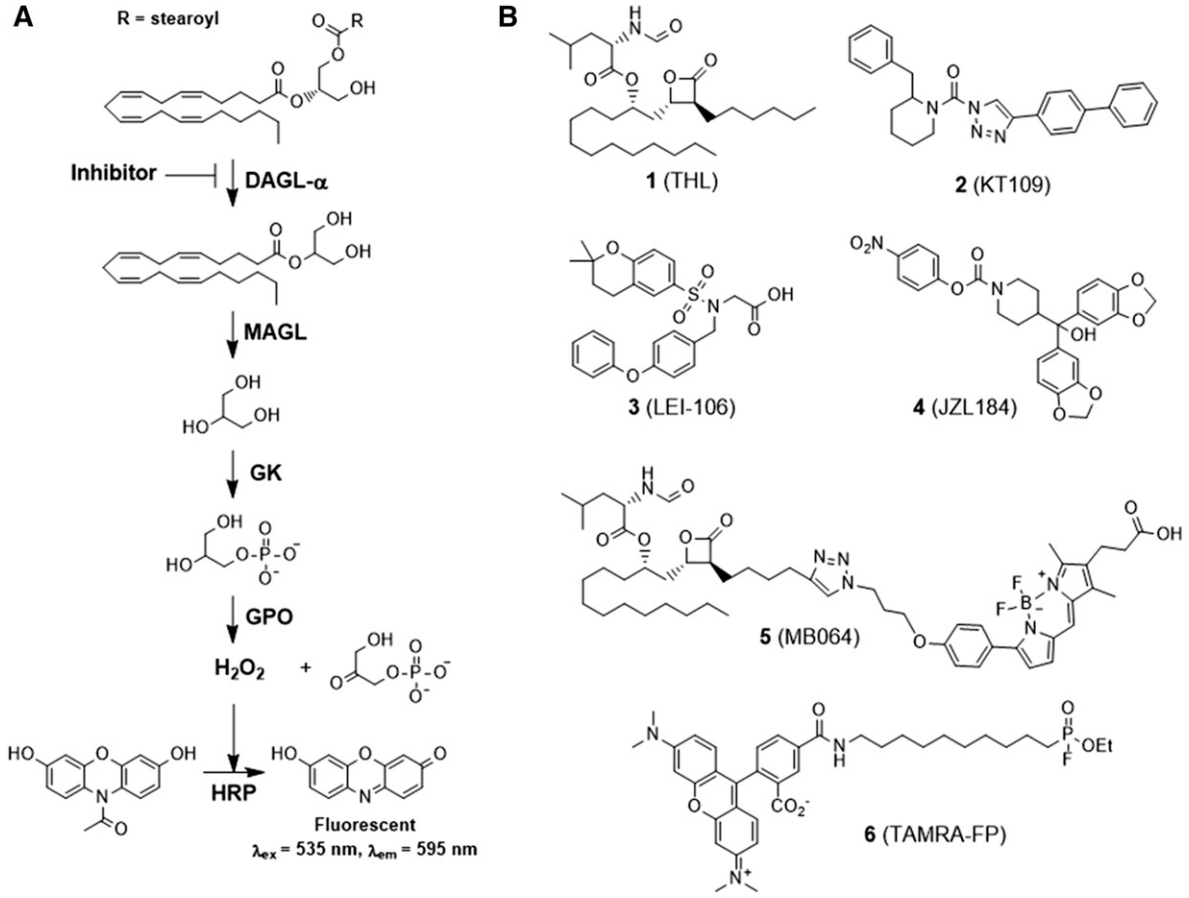


**Fig. 1.** DAGL-α natural substrate assay setup and overview of used inhibitors and probes. A: SAG is hydrolyzed by membrane preparations from DAGL-α-overexpressing HEK293T cells preincubated with DMSO or inhibitor. The produced endocannabinoid, 2-AG, is subsequently converted in a coupled enzyme reaction, initiated by hydrolysis of 2-AG into arachidonic acid and glycerol by MAGL. Glycerol is then phosphorylated by GK and oxidized to dihydroxyacetone phosphate and $H_2O_2$ by GPO. In the final step, the produced $H_2O_2$ is used by HRP to oxidize commercially available Amplifu™ Red to fluorescent resorufin, which is monitored in real-time using a plate reader ($\lambda_{ex}$ = 535 nm, $\lambda_{em}$ = 595 nm). B: Overview of used inhibitors and probes. THL [(*S*)-1-((2*S*,3*S*)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl formyl-*L*-leucinate] is a nonselective irreversible serine hydrolase inhibitor and inhibits DAGL and ABHD6, but not MAGL. KT109 [(4-([1,1′-biphenyl]4-yl)-1*H*-1,2,3-triazol-1-yl)(2-benzylpiperidin-1-yl)methanone] is a potent irreversible inhibitor of DAGL and has ABHD6 as an off-target. LEI-106 [*N*-((2,2-dimethylchroman-6-yl)sulfonyl)-*N*-(4-phenoxybenzyl)glycine] is a reversible dual inhibitor of DAGL-α and ABHD6. JZL184 [4-nitrophenyl 4-(bis(benzo[*d*][1,3]dioxol-5-yl)(hydroxy)methyl)-piperidine-1-carboxylate] is a selective irreversible inhibitor of MAGL. MB064 [3-(5,5-difluoro-6-(4-(3-(4-(4-(((2*S*,3*S*)-2-((*S*)-2-((formyl-*L*-leucyl)oxy)tridecyl)-4-oxooxetan-3-yl)butyl)-1*H*-1,2,3-triazol-1-yl)propoxy)phenyl)-1,3-dimethyl-5,5a-dihydro-4λ$^4$,5λ$^4$-cyclopenta[*c*]pyrrolo[2,1-*f*][1,2]azaborinin-2-yl)propanoic acid] is a THL-derived activity-based probe that labels, among others, DAGL-α, but not MAGL. Broad-range serine hydrolase probe TAMRA-FP [2-(6-(dimethylamino)-3-(dimethyliminio)-3*H*-xanthen-9-yl)-5-((10-(ethoxyfluorophosphoryl)decyl)carbamoyl)benzoate] labels MAGL, but not DAGL-α.

nor upon preincubation of DAGL-α with potent irreversible DAGL inhibitor KT109 (10). Mutating DAGL-α's catalytic Ser472 residue into an alanine abolished labeling by MB064, whereas protein expression was unaltered.

SAG hydrolysis by the isolated DAGL-α-overexpressing membrane preparations could be monitored by detection of fluorescent resorufin (**Fig. 3A, B**). Incubation of the DAGL-α membranes with KT109 reduced the signal to mock-levels, indicating that the signal was truly activity based. To verify that KT109 inhibited DAGL-α activity, rather than the activity of any downstream coupling enzymes, a MAGL validation assay was performed (**Fig. 4B**). Indeed, 2-AG hydrolysis by MAGL-overexpressing membrane preparations was unaffected upon treatment with KT109, which is in line with the selectivity profile of KT109 based on ABPP experiments in a previous report (10).

Under the employed assay conditions, the progression curve displayed steady state kinetics from 2 to 40 min. After 40–45 min, the fluorescence signal reached a plateau, whereas the background signal continued to increase. We used the linear interval of $t$ = 10 to $t$ = 35 min to determine the reaction rate of the enzyme. Interestingly, and in contrast to previous reports (4, 8), the addition of calcium in the assay buffer did not result in an increase of the DAGL-α-specific signal, possibly because this affected the downstream enzymatic cascade.

Limited substrate solubility and availability is a common issue encountered in lipase assay development and also proved to be a challenge in the optimization of this assay. Sonication of SAG in buffer and the use of organic solvents, such as dimethyl sulfoxide, ethanol, or ethyl acetate, resulted in a lack of DAGL-α-specific signal or an increase

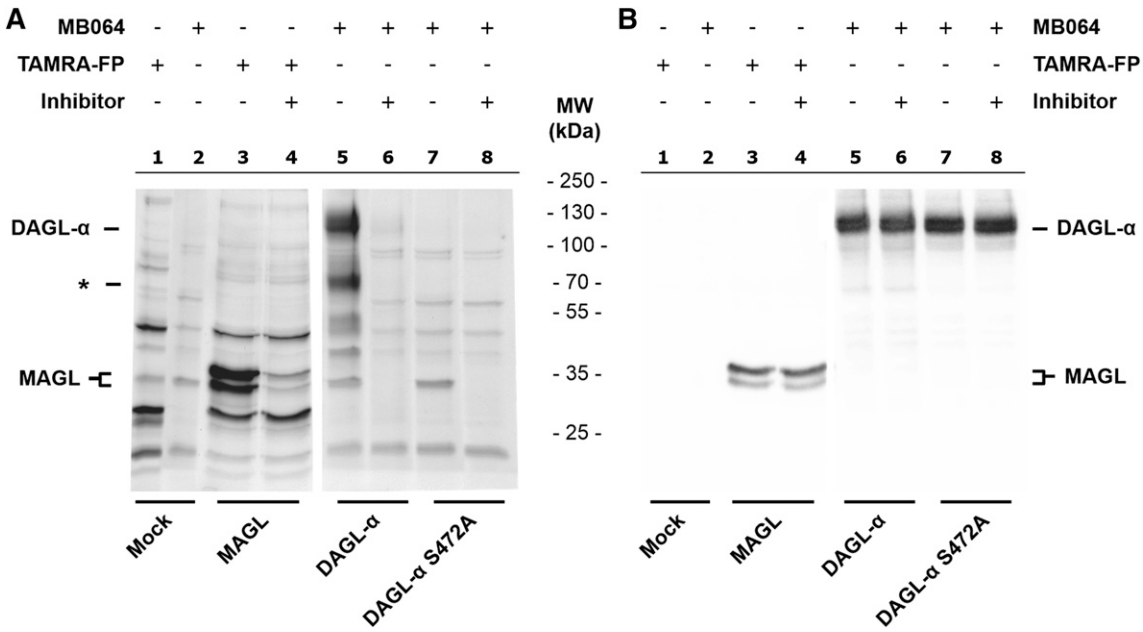


**Fig. 2.** Characterization of membrane preparations overexpressing full-length human MAGL, DAGL-α, and DAGL-α S472A. A: Membrane preparations of overexpressing HEK293T cells (2 mg/ml) were preincubated for 20 min with vehicle (DMSO) or 10 μM inhibitor (JZL184 for MAGL, KT109 for DAGL-α and DAGL-α S472A), followed by 10 min incubation with 500 nM TAMRA-FP or MB064 probe. Samples were separated by SDS-PAGE and labeling was detected by in-gel fluorescence scanning. The protein band marked with an asterisk likely corresponds to a degradation product of DAGL-α. Of note, the band at ~35 kDa labeled by MB064 corresponds to ABHD6, a known off-target of KT109. B: Expression of the enzymes was verified by Western blot analysis. Proteins were transferred to a polyvinylidene difluoride membrane and visualized with anti-FLAG antibodies. Total amount of protein loaded per lane: 12 μg for MAGL, 24 μg for other samples.

in background fluorescence. Stepwise dissolution of SAG, first in buffer with high Triton X-100 concentration, followed by dilution in the assay buffer, turned out to be crucial to establish substrate availability. DAGL-α appears to be highly susceptible to addition of detergents in the assay buffer, possibly because it is an integral membrane protein that needs an intact membrane environment to maintain its structure and catalytic activity (3). Under the employed assay conditions, we observed a DAGL-α-specific signal only in a narrow range of Triton X-100 concentrations, typically between 0.0075 and 0.075% (w/v).

To quantify the observed DAGL-α activity in the assay, we generated a glycerol standard curve with fluorescence intensity as a function of converted glycerol (Fig. 3C). This curve allowed us to convert experimentally measured slopes in relative fluorescence units per minute into 2-AG production rates in nanomoles per milligram per minute, assuming 1-to-1 conversion of 2-AG into glycerol. Subsequently, 2-AG production by DAGL-α-overexpressing membrane preparations was measured as a function of protein concentration (Fig. 3D). A linear response was observed up to approximately 50 μg/ml. Further increase of protein concentration resulted in an equal increase of background fluorescence by mock-membrane controls. For this reason, 50 μg/ml DAGL-α-overexpressing membranes (10 μg/well) was chosen as the optimal protein concentration.

In a similar way, DAGL-α activity was measured at various SAG concentrations (Fig. 3E). Nonlinear regression analysis was used to calculate an apparent $K_m$ of 150 ± 25 μM and a maximum reaction rate $V_{max}$ of 11 ± 0.7 nmol/mg/min. These values fall within the same range as those previously reported by two independent groups using a radiometric and an LC/MS-based assay (4, 8). It should be emphasized that measured kinetic parameters for lipases are apparent values, which are dependent on the employed assay conditions, such as the limited solubility of the lipophilic substrates in aqueous media and the use of membrane preparations instead of purified enzyme. Moreover, $V_{max}$ values are highly dependent on transfection efficiency and isolation procedure.

Determination of kinetic parameters using a coupled enzyme approach is only valid if the reaction of interest is rate limiting. To verify that SAG hydrolysis by DAGL-α was indeed the rate-limiting step under the optimized assay conditions, rather than downstream 2-AG hydrolysis by MAGL, we compared the reaction rates of four measurements on four independent plates (n = 4, n = 4, Z' ⩾ 0.6). The average glycerol production from 2-AG hydrolysis by 1 μg MAGL-membranes (equal to the amount used in the DAGL-α assay) was 215 ± 24 pmol/min. In contrast, 2-AG production resulting from SAG hydrolysis by 10 μg DAGL-α-membranes was approximately 69 ± 7 pmol/min. These results clearly indicate that under the employed assay conditions, the initial reaction catalyzed by DAGL-α is rate limiting.

To validate our optimized assay, we performed a number of control experiments (Fig. 3F). DAGL-α-overexpressing membranes incubated with vehicle (DMSO) served as a positive control, mock-membranes incubated with vehicle as a negative control. Incubation with broad-range serine hydrolase inhibitor tetrahydrolipstatin (Orlistat) (THL)





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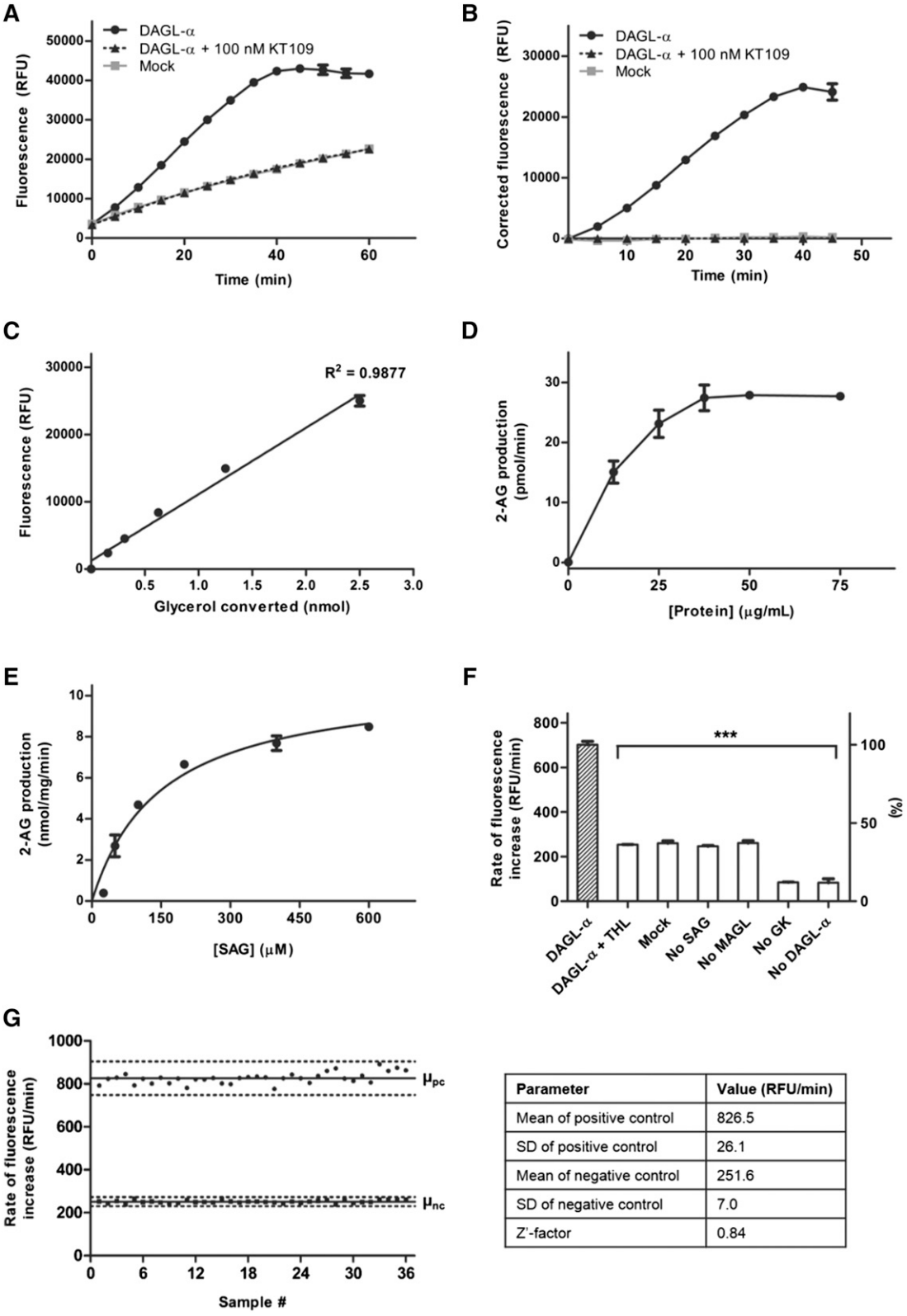


| Parameter | Value (RFU/min) |
|---|---|
| Mean of positive control | 826.5 |
| SD of positive control | 26.1 |
| Mean of negative control | 251.6 |
| SD of negative control | 7.0 |
| Z'-factor | 0.84 |

**Fig. 3.** Optimization of the DAGL-α natural substrate assay. A: Time course of SAG hydrolysis by DAGL-α (black filled circles), as measured by resorufin fluorescence. Membrane preparations from mock-transfected cells served as a negative control (gray filled boxes). A 20 min incubation with DAGL inhibitor KT109 (100 nM final concentration) resulted in reduction of the fluorescent signal to background level (black filled triangles). B: As in (A), but data was corrected for background fluorescence observed in the mock-control. C: Glycerol standard curve with fluorescence intensity as a function of converted glycerol. Instead of SAG, different concentrations of glycerol were used as substrate. No membrane preparations or inhibitors were added. After addition of the assay mix, fluorescence was measured in 2 min intervals for 30 min until full glycerol conversion. Maximum fluorescence values as a function of converted glycerol were exported to GraphPad Prism 5.0 and subjected to linear regression analysis. D: 2-AG production by DAGL-α as a function of protein concentration. Fluorescence was monitored in time for various concentrations of DAGL-α-overexpressing membrane preparations. Data was corrected for

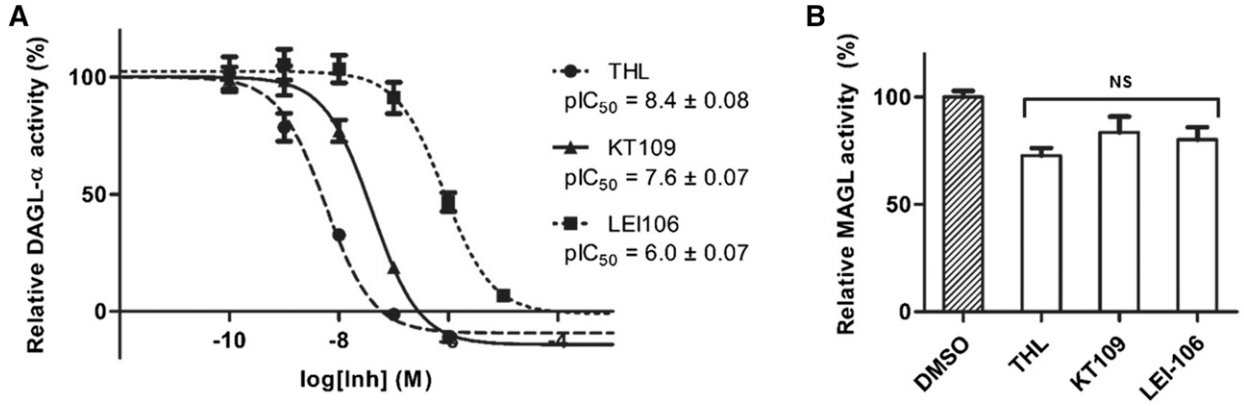


**Fig. 4.** Determination of inhibitory potency for three DAGL-α inhibitors. A: Dose-response experiment of THL (-●-), KT109 (-▲-), and LEI-106 (-■-) against DAGL-α as determined with the natural substrate-based fluorescence assay. DAGL-α-overexpressing membranes (10 μg per well, 50 μg/ml final concentration) were incubated with vehicle (DMSO) or various concentrations of inhibitor for 20 min. Data were corrected for background fluorescence observed for mock-membranes treated with vehicle. Slopes were scaled to the corrected positive control of DAGL-α membranes treated with vehicle as a 100% activity reference point, and then subjected to nonlinear dose-response analysis with variable slope using GraphPad Prism 5.0. Assay conditions: 0.2 U/ml GK, GPO, and HRP; 0.125 mM ATP; 5 μg/ml MAGL-overexpressing membranes; 10 μM Amplifu™ Red; 100 μM SAG; 5% DMSO; 0.0075% (w/v) Triton X-100 in HEMNB assay buffer in a total volume of 200 μl. B: MAGL validation assay to verify that the coupled enzyme reaction was not inhibited by the tested inhibitors. MAGL-overexpressing membranes were used instead of DAGL-α membranes, at a final protein concentration of 1.5 μg/ml (0.3 μg protein per well) and with 2-AG as the substrate (25 μM final concentration). 2-AG was directly added from a stock solution in acetonitrile (0.5% final concentration) and no Triton X-100 was added. Inhibitor concentration: 1 μM for THL and KT109, 10 μM for LEI-106. All measurements were performed in n = 4, n = 8 for controls, with Z' ⩾ 0.6. Data shown are means ± SEM. Statistical comparison was performed using unpaired Student's *t*-test. NS, $P > 0.05$ versus vehicle-treated DAGL-α.

reduced the observed signal to mock-levels. In a separate experiment, catalytically inactive mutant DAGL-α S472A also showed a signal equal to mock-levels (data not shown). In control experiments in which no substrate was added, the same background fluorescence was observed as in the mock-membranes, indicating that the observed background signal did not originate from spontaneous hydrolysis of exogenous SAG. To assess whether endogenous substrate in the mock-membranes contributed to the background signal, we incubated mock-membranes with THL, but no significant reduction in signal was observed. Removal of MAGL-membranes from the assay reaction mixture precluded the coupled enzyme reaction and reduced the fluorescent signal to mock-levels. Interestingly, removal of GK from the assay reaction mixture reduced the signal even further. This contribution can only be assigned to components that enter the coupling enzyme cascade at the stage of glycerol phosphorylation by GK, such as endogenous glycerol present in the membrane preparations or glycerol derivatives that are hydrolyzed under the employed assay conditions. A control experiment in which no DAGL-α-overexpressing membranes were added supported our hypothesis that the observed background fluorescence is directly proportional to the amount of membranes used. The residual background signal can possibly be ascribed to spontaneous oxidation of Amplifu™ Red.

To provide information about the useful dynamic range of our optimized assay, we performed an experiment with only positive and negative controls (n = 36) to generate a Z'-factor data plot (Fig. 3G). The solid lines represent the

background fluorescence observed for an equal protein concentration of mock-membranes and slopes were determined in the linear interval of $t = 10$ to $t = 35$ min. E: 2-AG production by DAGL-α as a function of SAG concentration. Fluorescence was monitored in time for various concentrations of SAG. Data were corrected for background fluorescence observed for mock-membranes in the presence of equal SAG concentrations. Slopes were determined in the linear interval of $t = 10$ to $t = 25$ min and then subjected to Michaelis-Menten analysis using GraphPad Prism 5.0. F: Control experiments for validation of assay setup. DAGL-α was incubated for 20 min with vehicle (DMSO) or broad-range serine hydrolase inhibitor THL (10 μM final concentration). Mock-membranes were included as a negative control. For the control with no SAG, an equal volume of 50 mM HEPES (pH 7.0) + 0.75% (w/v) Triton X-100 was added to the assay mix as a substitute to keep all measurement conditions equal. For the control with no MAGL, the MAGL-membranes required to establish the enzymatic cascade were left out of the assay mix. A similar experiment was performed without addition of GK. In the control with no added DAGL-α, no mock-membranes were added as a substitute. Shown data did not undergo correction. Slopes were determined in the linear interval of $t = 10$ to $t = 35$ min. G: Z'-factor data plot. Fluorescence in time was measured for positive controls (DAGL-α-membranes + DMSO, -●-) and negative controls (mock-membranes + DMSO, -■-) and slopes were determined in the linear interval of $t = 10$ to $t = 35$ min. Solid lines represent the mean slope of individual data points ($\mu_{pc}$ and $\mu_{nc}$, respectively, and dashed lines represent three SDs above and below the mean. Measurement was performed in n = 36. General assay conditions: 0.2 U/ml GK, GPO, and HRP; 0.125 mM ATP; 5 μg/ml MAGL-overexpressing membranes; 10 μM Amplifu™ Red; 100 μM (A, B, G), 150 μM (D, F), or variable concentrations (E) of SAG; 5% DMSO; 0.0075% (w/v) Triton X-100 in HEMNB assay buffer in a total volume of 200 μl, unless specifically mentioned otherwise. Amount of protein: 10 μg [50 μg/ml (A, B, E–G)] or variable amounts (D). All measurements except those in (G) were performed in n = 4, and n = 8 for controls, with Z' ⩾ 0.6. Data shown are means ± SEM, unless stated otherwise. Statistical comparison was performed using unpaired Student's *t*-test. ***$P < 0.001$ versus vehicle-treated DAGL-α. RFU, relative fluorescence unit.





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mean slope of the individual data points, and the dashed lines represent three SDs above and below the mean. The plot shows low intra-assay variability between individual data points and the Z'-factor for this experiment was 0.84. To assess the inter-assay variability of our assay, we determined the inter-assay CV from eight independently measured 96-well plates (n = 8, n = 4). The inter-assay CV was 9.4% for the positive controls and 9.6% for the negative controls.

To further validate our assay, we tested a selection of published DAGL-α inhibitors in dose-response experiments to determine their inhibitory potency using the natural substrate-based fluorescence assay (Fig. 4A). We found that of the three tested compounds, THL most potently inhibited DAGL-α activity ($pIC_{50}$ = 8.4 ± 0.08), followed by KT109 ($pIC_{50}$ = 7.6 ± 0.07). Of note, using a surrogate substrate assay with para-nitrophenol butyrate, we were unable to discriminate between THL and KT109 in terms of inhibitory potency, because low-nanomolar $IC_{50}$ values reached the detection limit of the surrogate substrate assay. This observation emphasizes the value of using natural substrate assays for lead optimization purposes. Previously identified glycine sulfonamide inhibitor LEI-106 prevented SAG hydrolysis by DAGL-α with a $pIC_{50}$ of 6.0 ± 0.07. A MAGL validation assay was performed to verify that the tested compounds inhibited DAGL-α activity and did not affect the downstream coupled enzyme reaction ($pIC_{50} < 5$, Fig. 4B).

The reversible binding mode of LEI-106 enables us to report its inhibitory potency as an inhibition constant $K_i$ via the Cheng-Prusoff equation (18). In a prior study, we determined the potency of LEI-106 in a radiometric assay employing 1-oleoyl-2-[$^{14}$C]arachidonoyl-*sn*-glycerol as the substrate, in which it inhibited DAGL-α activity with a $K_i$ of 0.7 ± 0.08 μM (15), which is within the same range as determined using our fluorescence assay ($K_i$ = 0.5 ± 0.09 μM). This demonstrates that our reported assay setup can be effectively used to evaluate DAGL-α inhibitors and that its results are consistent with those obtained using a different assay format. Evaluation of irreversible inhibitor kinetics using our real-time fluorescence assay will be a subject for future studies.

We next explored whether our assay could be used to measure DAGL activity in the mouse brain membrane proteome. As shown in **Fig. 5**, the employed assay setup allows the use of mouse brain membrane preparations to quantify endogenous DAGL activity, with only minor modifications compared with the assay employing membrane preparations from DAGL-α-overexpressing HEK293T cells. We observed that endogenously expressed MAGL was sufficiently active to establish the coupled enzyme reaction, which eliminated the need to add additional MAGL-overexpressing membranes. Under these conditions, the initial step of SAG hydrolysis remained the rate-determining step, as reflected by the reaction rates of the vehicle-treated samples. The decrease in glycerol production upon incubation of the mouse brain membrane proteome with selective MAGL inhibitor JZL184 indicated that MAGL is responsible for 2-AG hydrolysis under the employed assay conditions, whereas contribution of ABHD6 and ABHD12 could not be detected. Incubation with irreversible DAGL inhibitors THL and KT109 resulted in a significant decrease in 2-AG production (Fig. 5A). To validate that this effect was due to inhibition of DAGL activity, we performed a similar experiment with 2-AG as the substrate (Fig. 5B) to exclude potential inhibition of the downstream coupled enzyme reaction.

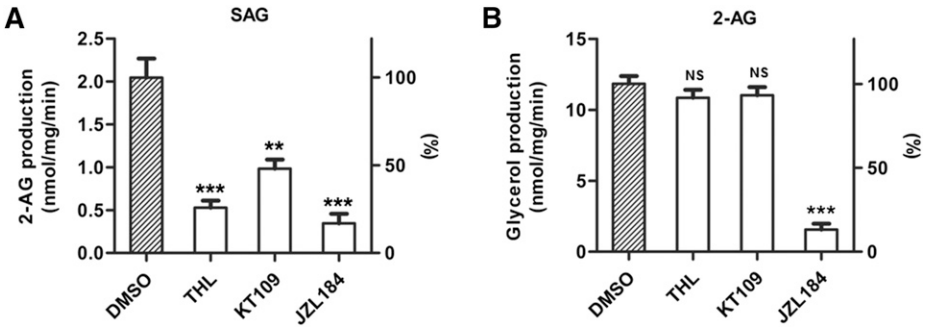


**Fig. 5.** Measurement of DAGL activity and inhibition in the mouse brain membrane proteome. A: DAGL activity and inhibition in the mouse brain membrane proteome was determined using SAG as the substrate. 2-AG production by mouse brain membranes was measured after incubation for 20 min with vehicle (DMSO) or 10 μM of the indicated inhibitor. B: Validation experiment with 2-AG as the substrate to verify that the observed decrease in signal upon incubation with THL and KT109 resulted from inhibition of DAGL activity and not from inhibition of the coupled enzyme reaction. Data were corrected for background fluorescence observed for membranes with no added SAG (A) or 2-AG (B). An equal volume of 50 mM HEPES (pH 7.0) + 0.75% (w/v) Triton X-100 (A) or ACN (B) was added as a substitute to keep all measurement conditions equal. Slopes were determined in the linear interval of $t$ = 10 to $t$ = 35 min (A) or $t$ = 0 to $t$ = 15 min (B) and converted into 2-AG or glycerol production rates, respectively. General assay conditions: 0.2 U/ml GK, GPO, and HRP; 0.125 mM ATP; 10 μM Amplifu™ Red; 150 μM SAG (A) or 25 μM 2-AG (B); 5% DMSO in a total volume of 200 μl. In addition, experiments in (A) contained 0.0075% (w/v) Triton X-100, experiments in (B) contained 0.5% ACN. Amount of protein: 15 μg (75 μg/ml). All measurements were performed in n = 4, n = 8 for controls, with Z' ⩾ 0.6. Data shown are means ± SEM. Statistical comparison was performed using unpaired Student's *t*-test. ***$P < 0.001$; **$P < 0.01$; NS, $P > 0.05$ versus vehicle-treated DAGL-α.

These results combined demonstrate that our natural substrate-based fluorescence assay is not limited to the use of DAGL-α-overexpressing membranes from transiently transfected HEK293T cells, but can also be employed to profile DAGL activity and inhibition in the mouse brain membrane proteome.

## CONCLUSIONS

In summary, we have developed a real-time natural substrate-based fluorescence assay for DAGL-α that serves as an alternative to the currently available radiometric or LC/MS-based methods. Employing a coupled enzyme approach, this assay can be used to determine DAGL-α activity in a continuous assay setup, circumventing the time-consuming isolation and fractionation of products and thus greatly improving throughput. Our method is not restricted to the use of DAGL-α-overexpressing membrane preparations, but can also be used to measure DAGL activity and inhibition in the mouse brain membrane proteome. The assay should thus be a valuable tool for rapid characterization of lead compounds and aid in the development of selective DAGL-α inhibitors that may serve as potential drug candidates for obesity and neurodegenerative diseases.

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