Disulfiram is an Inhibitor of Human Purified Monoacylglycerol Lipase, the Enzyme Regulating 2-Arachidonoylglycerol Signaling

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Monoacylglycerol lipase (MAGL) is a key enzyme responsible for the termination of endocannabinoid signaling. Its crucial role in 2-arachidonoylglycerol (2-AG) metabolism, together with the numerous pharmacological properties mediated by this endocannabinoid, emphasize the interest in MAGL as therapeutic target, along with the need to design potent and selective inhibitors. Meanwhile, the complexity of 2-AG degradation pathways underscores the need to use a purified source of enzyme in evaluation studies of new inhibitors. We report here the first heterologous expression and purification of human MAGL. A highly pure protein was obtained and allowed us to measure the affinity of several MAGL inhibitors for the human enzyme. Importantly, disulfiram (tetraethylthiuram disulfide), a compound used to treat alcoholism, and other disulfide-containing compounds were shown to inhibit MAGL with good potency, likely through an interaction with cysteine residues.

Introduction

Fatty acid amides and esters are a class of lipid messengers that have received considerable attention since anandamide was proposed as an endogenous ligand of the cannabinoid receptors.[1] 2-Arachidonoylglycerol (2-AG), an arachidonic acid monoglyceride esterified at the sn-2 position, was subsequently identified as an endocannabinoid.[2–4] 2-AG, an agonist at both CB_1 and CB_2 receptors, acts as a retrograde messenger.[5] Measurement of the abundance of endocannabinoids in whole brain usually reveals levels of 2-AG two orders of magnitude higher than those of anandamide.[6] Note that some authors have reported equivalent extracellular levels.[7] 2-AG displays a large set of pharmacological activities, among them growth inhibition of prostate and breast cancer cells,[8–9] and immunomodulating activity[10–11] as well as neuroprotective,[12] hypotensive[13] and analgesic effects.[14] This all suggests that modulation of 2-AG levels would represent a useful pharmacological tool (for a review see ref. [15]).

As for numerous neurotransmitters, efficient enzymatic degradation pathways limit the duration of action of both endocannabinoids. To date, four enzymes have been characterized at the molecular level: fatty acid amide hydrolase (FAAH, EC 3.5.1.4)),[16] a recently discovered type-2 fatty acid amide hydrolase,[17] monoacylglycerol lipase (MAGL, EC 3.1.1.23),[18–19] and N-acylethanolamine acid amidase (NAAA).[20–21] Moreover, pharmacological and biochemical evidence suggests that endocannabinoids might be hydrolyzed by additional enzymes.[22–23] The MAGL gene, which was cloned in 1997, encodes a 33 kDa protein that is predicted to belong to the α/β-hydrolase fold family. Apart from its Ser122/His269/Asp239 catalytic triad (supported by mutagenesis studies[18]), knowledge about MAGL remains very superficial. Nevertheless, strong evidence suggests that MAGL is the main enzyme responsible for 2-AG hydrolysis in the brain. Indeed, Dinh et al. found that overexpression of MAGL in rat neurons reduced the accumulation of 2-AG,[24] and that RNA interference-mediated silencing of MAGL expression enhanced 2-AG accumulation in HeLa cells.[22] However, when MAGL was immunodepleted from cytosolic brain fractions, 50% of the 2-AG hydrolase activity was still present; this suggests the existence of additional metabolic pathways. Indeed, other enzymes are able to metabolize 2-AG—COX, LOX, and the novel microglial MAGL—this supports the need for specific and selective inhibitors to ascertain the role of MAGL.[15, 23] Few MAGL inhibitors are known and they usually exhibit poor selectivity. Currently, clarification of the precise physiological role of MAGL is hampered by the lack of pharmacological tools, inhibitors and knock-out mice models.

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This emphasizes the interest in identifying new MAGL inhibitors. However, the above-mentioned heterogeneity of mono-acylglycerol lipase activities complicates the interpretation of the enzyme assays performed on tissue or cell line homogenates. Use of a recombinant MAGL solves this problem by providing a source of pure MAGL activity. Furthermore, obtaining the human enzyme would constitute an additional advantage in drug discovery. Indeed, human and rat amino acid sequences share only 83% identity, and the 17% variation could account for differences in drug–enzyme interactions. Thus, the first aim of this study was the expression in *E. coli*, the purification, and the biochemical characterization of human MAGL.

MAGL activity in brain homogenates is sensitive to inhibition by sulfhydryl-specific agents, that is, *p*-chloromercuribenzoic acid, mercury chloride, and *N*-ethylmaleimide.[25, 26] In addition, Saario et al. elegantly suggested the presence of sulfhydryl group(s) essential for the substrate recognition.[27] Using homology modeling, they identified cysteines 208 and 242 in the vicinity of the putative 2-AG binding site and assayed a series of maleimide-type analogues of 2-AG. These compounds turned out to be irreversible MAGL inhibitors, likely involved in a Michael addition reaction on the two afore-mentioned residues.

With this in mind, our study led us to the identification of disulfide-containing inhibitors, including disulfiram, likely targeting Cys208 and/or Cys242.

Results and Discussion

Human monoacylglycerol lipase fused to two short tags, a N-terminal His$_6$-tag and a C-terminal Strep-tag was overexpressed in *Escherichia coli*. As assessed by a Western blot experiment, the entire MAGL production was recovered as a soluble protein in the supernatant (data not shown). A first separation step exploiting the affinity of the Strep-tag for a modified streptavidin (named Strep-Tactin) was performed. A considerable amount of MAGL with a very good level of purity was obtained by using this single chromatography step (Figure 1; lanes 2 to 4). Two minor bands corresponding to contaminating proteins, with molecular masses of ~65 and ~20 kDa, are still visible. Note that the latter is likely due to the endogenous biotin carboxyl carrier protein from *E. coli* (M_W of 22.5 kDa). The MAGL-containing fraction from step 1 was further purified by using N-terminal His$_6$-tag and Ni^{2+} affinity chromatography. This additional step resulted in a highly pure monoacylglycerol lipase preparation (Figure 1; lanes 5 and 6). Global yields for MAGL expression and purification typically ranged from 5 to 10 mg per liter of culture medium.

We then sought to characterize the enzymatic activity of the pure human MAGL. First, we compared its ability to hydrolyze 2-oleoylglycerol to that observed with rat brain homogenate preparations, which are known to possess high 2-OG hydrolase activity.[23] As expected, purified MAGL hydrolyzed 2-OG with higher specific activities than membrane and cytosolic fractions from rat brain homogenates did (Figure 2A). A Michaelis–Menten curve was also obtained in order to determine the enzymatic parameters of MAGL. Pure enzyme exhibited a K_m
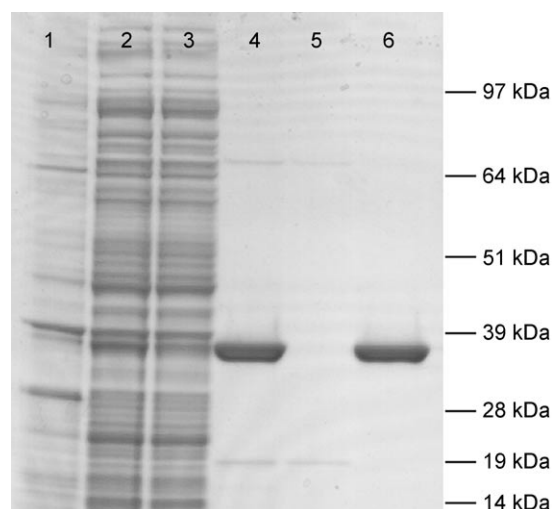

Figure 1. Expression and purification of human MAGL by SDS-PAGE electrophoresis followed by Coomassie blue staining. Lane 1: insoluble *E. coli* protein fraction after IPTG induction (4 h, 37 °C). Lane 2: soluble *E. coli* fraction. Lane 3: flow-through and lane 4: elution fractions from Strep-Tactin column chromatography. Lane 5: flow-through and lane 6: elution fractions from ion metal-affinity chromatography.

value of 16.9 ± 2.6 μM and a V_{max} of 12.2 ± 0.6 μmol min^{-1} per mg protein for 2-OG hydrolysis (Figure 2C). This constitutes the first report of the enzymatic characteristics of human MAGL. Time dependence was studied in order to find the best conditions for the inhibitor characterization assays. MAGL hydrolyzes 2-OG in a time-dependent manner with the activity reaching a plateau after 20–25 min of incubation (Figure 2B). Interestingly, the human MAGL was unable to mediate anandamide degradation (Figure 2D). Indeed, while FAAH has evolved through its unusual Ser-Ser-Lys triad to an enzyme that is able to hydrolyze amides and esters at the same rate,[28] hydrolase activity of MAGL appears to be restricted to esters. Our data with human purified MAGL are therefore consistent with the results of Dinh and co-workers, who observed no enhancement of anandamide hydrolase activity after expression of rat MAGL in HeLa cells.[24]

We then tested the ability of several known inhibitors of MAGL lipase activity—ATFMK, MAFP, and URB602—to inhibit the human purified MAGL. All three inhibitors dose-dependently inhibited 2-OG hydrolysis, whereas URB754, similarly to what was recently reported,[29–31] had no effect on the hydrolysis of 2-OG by the recombinant MAGL (Figure 3A). The pIC$_{50}$ ($\pm$ SEM) values are ATFMK = 5.20 ± 0.05, MAFP = 7.57 ± 0.07, and URB602 = 5.00 ± 0.11.

In 2005, in their MAGL molecular modeling study, Saario and colleagues located cysteines 208 and 242 in the neighborhood of the catalytic triad. They therefore proposed the maleimide scaffold as a potential candidate for covalent binding to one of the cysteine sulfhydryl residues, in a mechanism involving a Michael addition, resulting in inhibition of MAGL activity. Consistent with this hypothesis, they showed that *N*-arachidonyl-maleimide (NAM) irreversibly inhibits 2-AG esterase activity from rat cerebellar membranes.[27]
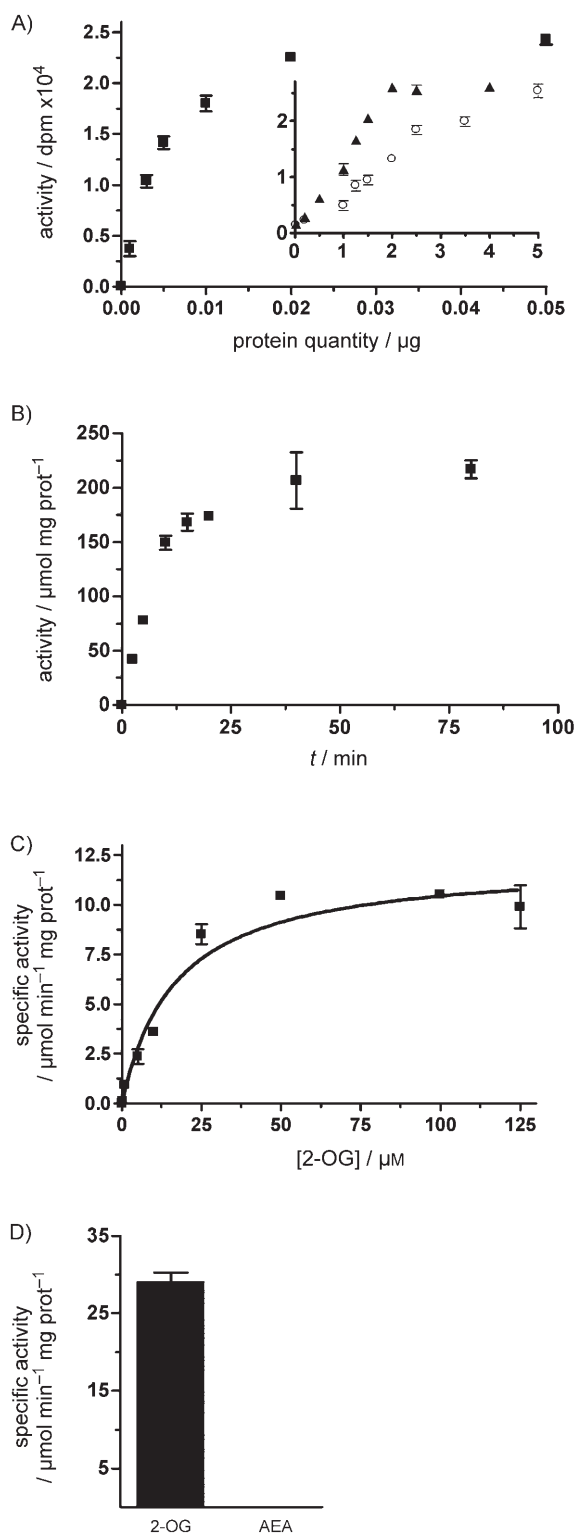
Figure 2. Characterization of purified human MAGL. A) Human MAGL hydrolyzes 2-OG in a protein-dependent manner (■). The insert represents the protein-dependency of MAGL activities from cytosolic (○) and membrane (▲) rat brain fractions. Results are expressed as dpm minus blank (i.e., without protein) values. B) Human MAGL hydrolyzes 2-OG in a time-dependent manner. C) Michaelis–Menten plot for the hydrolysis of 2-OG by human MAGL. Specific activities are expressed as μmol min^{-1} per mg of protein. D) MAGL hydrolyses the monoglyceride 2-OG but not the amide *N*-arachidonoylethanolamine (anandamide). Data represent the mean ± SEM of at least two experiments performed in duplicate.
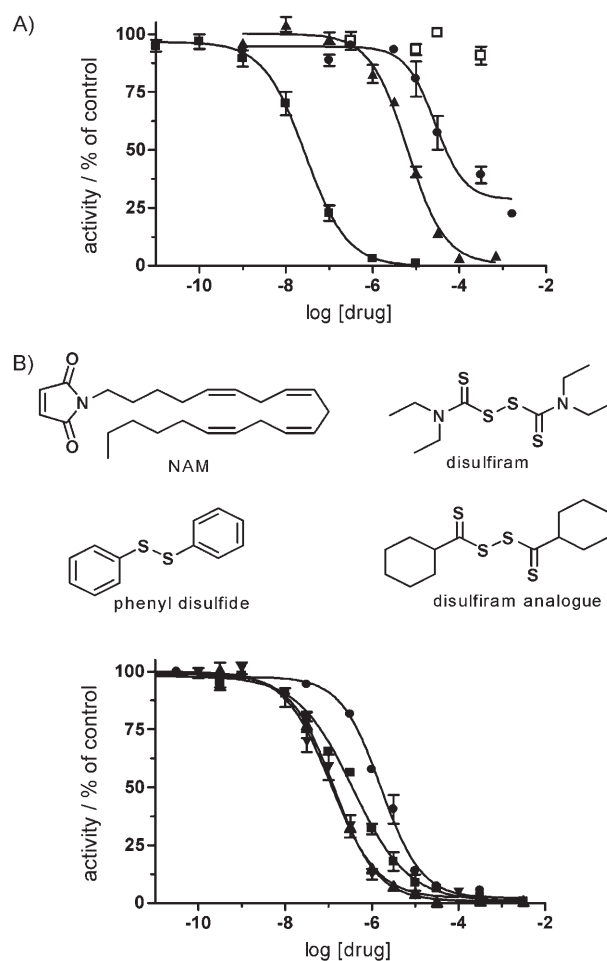

Figure 3. Determination of inhibitors' potency on human MAGL. A) Dose-dependent inhibition of MAGL by MAFP (■), URB602 (●), and ATFMK (▲). Note the absence of inhibition by URB754 (□). B) Dose-dependent inhibition of MAGL by NAM (▼), disulfiram (■), a disulfiram analogue (▲) and phenyl disulfide (●). Values are expressed as a percentage of the control and represent the mean ± SEM of at least two experiments performed in duplicate.

Using our human recombinant enzyme, we tested NAM, as well as disulfide-containing agents, with the aim of identifying new chemical entities that act as MAGL inhibitors. According to Saario's report on rat cerebellar membranes, NAM inhibited pure MAGL with a pIC$_{50}$ value of 6.94. Disulfiram, dicyclopentamethylenethiuram disulfide (a disulfiram analogue), and phenyldisulfide (Figure 3 B) were able to dose-dependently inhibit human MAGL activity with pIC$_{50}$ values of 6.44 ± 0.05, 6.87 ± 0.05, and 5.79 ± 0.05, respectively. We hypothesize than the sulfhydryl moiety of Cys208 and/or Cys242 reacts with disulfiram and its analogue, either to form a mix adduct or an intramolecular disulfide bond.

Disulfiram has been used for decades to treat alcoholism, and its therapeutic activity is thought to be mediated through irreversible inhibition of aldehyde dehydrogenase (ALDH), one of the key enzymes involved in ethanol metabolism. However, despite the numerous studies devoted to disulfiram's pharmacology, the precise mechanism of inhibition still remains ambiguous. Indeed, while disulfiram was reported to inhibit ALDH

only after in vivo metabolism,[32–33] other data suggest that disulfiram could bind to an ALDH cysteine residue, thus forming a mixed thiocarbamoyl or dithiocarbamoyl adduct, ultimately resulting in the formation of an intramolecular disulfide bond.[34–35] The latter hypothesis is likely to be the one involved in the inhibition of MAGL, because the possibility of an in vitro metabolism of disulfiram to other compounds is remote in our in vitro system consisting of pure MAGL. In order to test the hypothesis that a redox process involving sulfhydryls moieties and resulting in disulfide bond formation inactivates the enzyme, we sought to reverse the disulfiram-induced inhibition of MAGL with dithiothreitol (DTT). This reducing agent actually dose-dependently restored MAGL activity, with a full recovery observed at 10^{-2} M (Figure 4). Although additional evidence is
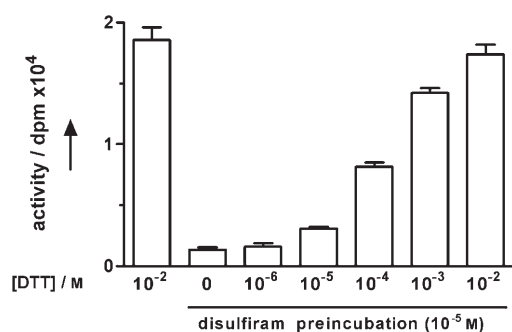

Figure 4. DTT dose-dependently reverses the disulfiram-induced inhibition of human MAGL. The enzyme was first incubated for 5 min (37 °C) in the absence (control conditions) or in the presence of 10^{-5} M disulfiram. Buffer or DTT (10^{-6} to 10^{-2} M) was then added, and MAGL was incubated for an additional 15 min to reverse the inhibition. Finally, the substrate was added, and the enzyme activity was measured according to the standard procedure. Values are expressed as dpm minus blank (without protein) values, and represent the mean ± SEM of at least two experiments performed in duplicate.

needed to assign a precise mechanism of action to disulfiram and related compounds, the fact that DTT restores the enzyme activity supports the crucial role played by cysteine residues in MAGL catalytic activity.

Conclusions

Recent advances in the field of the endocannabinoid system suggest that monoacylglycerol lipase, the enzyme likely responsible for the in vivo termination of 2-AG signaling, represents an attractive therapeutic target. However, pharmacological tools are still lacking; this makes the design of potent and selective inhibitors a key step to deepening our knowledge of MAGL.

Considering the poor understanding of the dynamic aspects that regulate endocannabinoid tone at the cellular level, and the growing number of enzymes found to be involved in this process, the use of a recombinant purified MAGL represents a precious gain for the evaluation of new inhibitors, and constitutes a required tool for conducting mechanistic and structural studies.

We have reported here the expression and purification of human monoacylglycerol lipase. This purified MAGL enabled us to measure the inhibitory potency of several known compounds on the human enzyme and to identify thiuram disulfide as a potential scaffold for the design of new inhibitors. Knowing that DTT is able to restore MAGL activity after disulfiram treatment (although the precise mechanism of this inhibition has to be further investigated), it is tempting to speculate that cysteines 208 and/or 242, previously identified as being in the vicinity of the active site,[27] are involved in the formation of an intramolecular disulfide bond that impedes MAGL activity.

In the larger context of drug discovery, obtaining milligrams quantities of pure human enzyme has allowed our group to get involved in the crystallization of MAGL. The elucidation of the 3D structure of monoacylglycerol lipase will constitute a great help for future medicinal chemistry efforts and will provide in-depth knowledge of MAGL's catalytic site and mechanism.

Experimental Section

MAGL cloning and expression: The gene encoding human MAGL was recovered by PCR from a cDNA lymphocyte preparation. Forward primer: 5′-ACCTGAAGACCCTTCCAGCAT-3′, reverse primer: 5′-CAAGCCATATCTGAGAAGCCA-3′. The MAGL gene was entirely sequenced and inserted into pASK43 vector (IBA); this allowed expression of the target protein with a N-terminal His6-tag and a C-terminal Strep-tag. Expression of human MAGL was carried out in *E. coli* (Rosetta strain). At an OD of ~0.6, cultures were induced by the addition of anhydrotetracycline (200 µg L^{-1}). After 4 h of incubation at 37 °C, cells were harvested by centrifugation, resuspended in buffer 1 (Tris 50 mM, NaCl 200 mM, DTT 10 mM, pH 9.5) and lysed by sonication. Insoluble and soluble proteins were separated by centrifugation (10 000 g, 35 min), and MAGL from supernatant fraction was submitted to further purification.

MAGL purification: The supernatant fraction from the previous step was loaded onto Strep-Tactin resin (IBA Protein Tools, Göttingen, Germany) by using the Akta Explorer Chromatography system. The column was consecutively washed with buffer 1 and buffer 2 (Tris 50 mM, NaCl 200 mM, pH 8,5). The elution was carried out by the addition of D-desthiobiotin (2.5 mM; Sigma–Aldrich). The elution fractions were pooled and loaded onto a nickel sepharose resin. After the column had been washed in buffer 2, a gradient of imidazole (0 to 500 mM) in buffer 2 allowed for the elution of MAGL from the column. Purified MAGL was then dialyzed three times against 20 volumes of buffer 3 (Tris 50 mM, NaCl 200 mM pH 9.5) at 4 °C. The protein concentration was measured by Bradford assay.

MAGL esterase activity assay: Measurement of 2-oleoyl glycerol (2-OG) hydrolysis was performed as previously described.[24,36] Briefly, 2-OG (10 µM; [^{3}H]2-OG 50 000 dpm, American Radiolabeled Chemicals) was incubated at 37 °C for 10 min in the presence of pure MAGL (5 ng in Tris buffer, pH 8.0; 200 µL of total volume assay). The incubation was stopped by adding methanol/chloroform (1:1, 400 µL), and the radioactivity in the upper aqueous phase was measured by liquid scintillation. The inhibitors were then evaluated by incubating them for 10 min with purified enzyme (5 ng). 2-AG, methyl arachidonylfluorophosphonate (MAFP), biphenyl-3-yl-carbamic acid cyclohexyl ester (URB602), and 6-

methyl-2-*p*-tolylamino-benzo[*d*][1,3]oxazin-4-one (URB754) were purchased from Cayman Chemical (Ann Arbour, MI), tetraethyl-thiuram disulfide (disulfiram) was from Fluka, dicyclopentamethyle-nethiuram disulfide was from Acros Organics, 2-OG and phenyldi-sulfide were from Sigma–Aldrich, and arachidonoyltrifluoromethyl ketone (ATFMK) was from Tocris (Ellisville, MO). *N*-arachidonylmalei-mide was kindly donated by Dr. S. Saario, from the University of Kuopio, (Finland). Where specified, cytosolic and membrane fractions from rat brain homogenates were used and therefore prepared as previously described.[36] The results are expressed either as dpm minus blank (2-OG hydrolysis in the absence of enzyme), specific activities (μmol min^{-1} per mg protein), or as percent of control activity for the inhibitors evaluation experiments. Graph Pad prism was used to treat the data and to analyze the Michaelis–Menten and dose–response curves. Inhibitors' potency is expressed as pIC_{50} values ($pIC_{50} = -\log IC_{50}$).

All experiments on animals were approved by the institutional ethics committee, and the housing conditions were as specified by the Belgian Law of November 14, 1993, on the protection of laboratory animals (agreement no. LA 1230315).

[1] W. A. Devane, L. Hanus, A. Breuer, R. G. Pertwee, L. A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, R. Mechoulam, *Science* **1992**, *258*, 1946–1949.

[2] R. Mechoulam, S. Ben Shabat, L. Hanus, M. Ligumsky, N. E. Kaminski, A. R. Schatz, A. Gopher, S. Almog, B. R. Martin, D. R. Compton, *Biochem. Pharmacol.* **1995**, *50*, 83–90.

[3] T. Sugiura, S. Kondo, A. Sukagawa, S. Nakane, A. Shinoda, K. Itoh, A. Yamashita, K. Waku, *Biochem. Biophys. Res. Commun.* **1995**, *215*, 89–97.

[4] T. Bisogno, N. Sepe, D. Melck, S. Maurelli, L. De Petrocellis, V. Di Marzo, *Biochem. J.* **1997**, *322*, 671–677.

[5] R. I. Wilson, R. A. Nicoll, *Nature* **2001**, *410*, 588–592.

[6] N. Stella, P. Schweitzer, D. Piomelli, *Nature* **1997**, *388*, 773–778.

[7] S. Caille, L. Alvarez-Jaimes, I. Polis, D. G. Stouffer, L. H. Parsons, *J. Neurosci.* **2007**, *27*, 3695–3702.

[8] K. Nithipatikom, M. P. Endsley, M. A. Isbell, C. E. Wheelock, B. D. Hammock, W. B. Campbell, *Biochem. Biophys. Res. Commun.* **2005**, *332*, 1028–1033.

[9] D. Melck, L. De Petrocellis, P. Orlando, T. Bisogno, C. Laezza, M. Bifulco, M. Di, *Endocrinology* **2000**, *141*, 118–126.

[10] S. Oka, S. Ikeda, S. Kishimoto, M. Gokoh, S. Yanagimoto, K. Waku, T. Sugiura, *J. Leukocyte Biol.* **2004**, *76*, 1002–1009.

[11] M. Gokoh, S. Kishimoto, S. Oka, M. Mori, K. Waku, Y. Ishima, T. Sugiura, *Biochem. J.* **2005**, *386*, 583–589.

[12] M. Melis, G. Pillolla, T. Bisogno, A. Minassi, S. Petrosino, S. Perra, A. L. Muntoni, B. Lutz, G. L. Gessa, G. Marsicano, M. Di, V. M. Pistis, *Neurobiol. Dis.* **2006**, *24*, 15–27.

[13] C. J. Hillard, *J. Pharmacol. Exp. Ther.* **2000**, *294*, 27–32.

[14] A. G. Hohmann, R. L. Suplita, N. M. Bolton, M. H. Neely, D. Fegley, R. Mangieri, J. F. Krey, J. M. Walker, P. V. Holmes, J. D. Crystal, A. Duranti, A. Tontini, M. Mor, G. Tarzia, D. Piomelli, *Nature* **2005**, *435*, 1108–1112.

[15] T. Sugiura, S. Kishimoto, S. Oka, M. Gokoh, *Prog. Lipid Res.* **2006**, *45*, 405–446.

[16] B. F. Cravatt, D. K. Giang, S. P. Mayfield, D. L. Boger, R. A. Lerner, N. B. Gilula, *Nature* **1996**, *384*, 83–87.

[17] B. Q. Wei, T. S. Mikkelsen, M. K. McKinney, E. S. Lander, B. F. Cravatt, *J. Biol. Chem.* **2006**, *281*, 36569–36578.

[18] M. Karlsson, J. A. Contreras, U. Hellman, H. Tornqvist, C. Holm, *J. Biol. Chem.* **1997**, *272*, 27218–27223.

[19] M. Karlsson, H. Tornqvist, C. Holm, *Protein Expression Purif.* **2000**, *18*, 286–292.

[20] K. Tsuboi, C. Hilligsmann, S. Vandevoorde, D. M. Lambert, N. Ueda, *Biochem. J.* **2004**, *379*, 99–106.

[21] K. Tsuboi, Y. X. Sun, Y. Okamoto, N. Araki, T. Tonai, N. Ueda, *J. Biol. Chem.* **2005**, *280*, 11082–11092.

[22] T. P. Dinh, S. Kathuria, D. Piomelli, *Mol. Pharmacol.* **2004**, *66*, 1260–1264.

[23] G. G. Muccioli, C. Xu, E. Cudaback, J.-A. Cisneros, D. M. Lambert, M. L. López-Rodríguez, S. Bajjalieh, N. Stella, *J. Neurosci.* **2007**, *27*, 2883–2889.

[24] T. P. Dinh, D. Carpenter, F. M. Leslie, T. F. Freund, I. Katona, S. L. Sensi, S. Kathuria, D. Piomelli, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 10819–10824.

[25] H. Tornqvist, P. Belfrage, *J. Biol. Chem.* **1976**, *251*, 813–819.

[26] T. Sakurada, A. Noma, *J. Biochem.* **1981**, *90*, 1413–1419.

[27] S. M. Saario, O. M. Salo, T. Nevalainen, A. Poso, J. T. Laitinen, T. Jarvinen, R. Niemi, *Chem. Biol.* **2005**, *12*, 649–656.

[28] M. K. McKinney, B. F. Cravatt, *Annu. Rev. Biochem.* **2005**, *74*, 411–432.

[29] J. K. Makara, M. Mor, D. Fegley, S. I. Szabó, S. Kathuria, G. Astarita, A. Duranti, A. Tontini, G. Tarzia, S. Rivara, T. F. Freund, D. Piomelli, *Nat. Neurosci.* **2007**, *10*, 134.

[30] S. M. Saario, V. Palomaki, M. Lehtonen, T. Nevalainen, T. Jarvinen, J. T. Laitinen, *Chem. Biol.* **2006**, *13*, 811–814.

[31] S. Vandevoorde, K. O. Jonsson, G. Labar, E. Persson, D. M. Lambert, C. J. Fowler, *Br. J. Pharmacol.* **2007**, *150*, 186–191.

[32] J. J. Yourick, M. D. Faiman, *Biochem. Pharmacol.* **1991**, *42*, 1361–1366.

[33] D. C. Mays, A. N. Nelson, A. H. Fauq, Z. H. Shriver, K. A. Veverka, S. Naylor, J. J. Lipsky, *Biochem. Pharmacol.* **1995**, *49*, 693–700.

[34] M. L. Shen, J. J. Lipsky, S. Naylor, *Biochem. Pharmacol.* **2000**, *60*, 947–953.

[35] T. M. Kitson, *Biochem. J.* **1983**, *213*, 551–554.

[36] N. Ghafouri, G. Tiger, R. K. Razdan, A. Mahadevan, R. G. Pertwee, B. R. Martin, C. J. Fowler, *Br. J. Pharmacol.* **2004**, *143*, 774–784.