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Noladin ether, a putative novel endocannabinoid: inactivation mechanisms and a sensitive method for its quantification in rat tissues

Filomena Fezza[a], Tiziana Bisogno[a], Alberto Minassi[b], Giovanni Appendino[b], Raphael Mechoulam[c], Vincenzo Di Marzo[a,*]

[a] *Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Comprensorio Olivetti, Fabbricato 70, 80078 Pozzuoli (Naples), Italy*
[b] *DiSCAFF, Università del Piemonte Orientale, Viale Ferrucci 33, 28100 Novara, Italy*
[c] *Department of Medicinal Chemistry and Natural Products, Faculty of Medicine, Hebrew University, Jerusalem 91120, Israel*

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Abstract The occurrence of the novel proposed endocannabinoid, noladin ether (2-arachidonyl glyceryl ether, 2-AGE) in various rat organs and brain regions, and its inactivation by intact C6 glioma cells, were studied. 2-AGE was measured by isotope dilution liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry, with a detection limit of 100 fmol. A compound with the same mass and chromatographic/chemical properties as 2-AGE was found in whole brain, with the highest amounts in the thalamus and hippocampus. Synthetic [^{3}H]2-AGE was inactivated by intact rat C6 glioma cells by a time- and temperature-dependent process consisting of cellular uptake and partial incorporation into phospholipids. Further data suggested that 2-AGE is taken up by cells via the anandamide/2-arachidonoyl glycerol (2-AG) membrane transporter(s), and biosynthesized in a different way as compared to 2-AG. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Anandamide; 2-Arachidonoyl glycerol; 2-Arachidonyl glyceryl ether; Cannabinoid; Endocannabinoid

1. Introduction

Anandamide (arachidonoylethanolamide, AEA [1]) and 2-arachidonoyl glycerol (2-AG; [2,3]) are the best known endogenous ligands of cannabinoid receptors, and are also known as endocannabinoids. While AEA activates the CB_1 cannabinoid receptor, which is most abundant in the brain, and less efficaciously the CB_2 cannabinoid receptor, which is present almost uniquely in immune cells, 2-AG exhibits similar affinity for and efficacy at both cannabinoid receptors (see [4,5], for reviews). Recently, another putative endocannabinoid was found in porcine brain [6]. This is 2-arachidonyl glyceryl ether (noladin ether, 2-AGE) and has a chemical structure unprecedented in nature. In fact, all glyceryl ethers previously identified belong to the *sn*-1-alkyl type, and are the hydrolysis products, or the biosynthetic precursors, of the plasmalogens (Fig. 1).

*Corresponding author.
E-mail address: vdimarzo@icmib.na.cnr.it (V. Di Marzo).

Abbreviations: AEA, anandamide; AA, arachidonic acid; 2-AG, 2-arachidonoyl glycerol; 2-AGE, 2-arachidonyl glyceryl ether; TLC, thin layer chromatography

The 2-AGE binds selectively to CB_1 vs. CB_2 cannabinoid receptors, functionally activates CB_1 receptors, and induces responses typical of cannabimimetic compounds in the mouse 'tetrad' of behavioral tests in vivo [6,7]. Evidence of de novo biosynthesis and inactivation in brain cells is, however, necessary before 2-AGE can reach the status of an endogenous neuronal mediator. Both these criteria are fulfilled by AEA and 2-AG, which are produced from arachidonic acid (AA)-containing phospholipid precursors when neurons are stimulated with depolarizing agents such as ionomycin, and are inactivated by facilitated diffusion into neurons and astrocytes, followed by enzymatic hydrolysis and/or re-esterificative recycling into phospholipids [8–10]. One, or two distinct and functionally very similar, membrane transporter(s) mediate(s) the cellular uptake of AEA and 2-AG [11]. One enzyme, the 'fatty acid amide hydrolase' [12], is mostly responsible for both AEA and 2-AG hydrolysis in neuronal cells [10,13]. Nothing is known to date on the mechanisms underlying the biosynthesis and inactivation of 2-AGE. To address this issue we have investigated if 2-AGE is present in rat brain, how it is distributed among distinct brain regions, and if its biosynthesis and inactivation in neuronal and glial-like cells occur through processes similar to those previously described for 2-AG.

2. Materials and methods

2.1. Materials

d_0-, d_8- and [^{3}H]2-AGE were synthesized as described previously [6], starting from AA (Sigma), d_8-AA (Sigma) and [^{3}H]AA respectively (NEN Life Sciences, 187 Ci/mmol). In the case of [^{3}H]2-AGE, [^{3}H]AA (10 μCi) was diluted with 4 μmol of AA to obtain a final specific activity of 2.5 μCi/μmol. The compounds were purified by open bed silica chromatography [9]. AEA, 2-AG, [^{14}C]AEA and [^{3}H]2-AG were synthesized as described previously [1,2,9]. d_8-2-AG and d_8- AEA were purchased from Cayman. N18TG2, RBL-2H3 and C6 cells were cultured as described [9,11,14].

2.2. Gas chromatography and liquid chromatography-mass spectrometric analysis of 2-AGE

Analysis of synthetic 2-AGE and d_8-2-AGE by gas chromatography-electron impact mass spectrometry (GC-EIMS) was carried out as described previously [9] after derivatization with 15 μl *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide containing 1% trimethyl chlorosilane for 2 h at room temperature, thus yielding the bis-trimethylsilyl (TMS) derivatives. Analysis was carried out also by high performance liquid chromatography-atmospheric pressure chemical ionizaion-mass spectrometry (HPLC-APCI-MS) by using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole

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MS via a Shimadzu APCI interface. MS analyses were carried out either in the full scan or the select ion monitoring (SIM) mode. The temperature of the APCI source was 400°C; the HPLC column was a Phenomenex (5 μm, 150×4.5 mm) reverse phase (RP) column, eluted with an isocratic step of methanol:water:acetic acid (85:15:0.1, by volume) at a flow rate of 1 ml/min. Tissues from adult male Sprague–Dawley rats were dissected within 5 min from decapitation and immediately frozen at −80°C, and lipid extracts were obtained and pre-purified as described previously [9].

2.3. Biosynthesis experiments

Almost confluent N18TG2 cells in 10 cm Petri dishes were incubated overnight with [^{3}H]AA (0.2 μCi/ml in serum-supplemented culture medium), and then washed twice with serum-free medium prior to stimulation with 3 μM ionomycin (Sigma, in 3 ml/dish of serum-free medium) for 10 min at 37°C. Ten dishes (about 50×10^6 cells) for each data point were used. After the incubation 2 ml of ice-cold methanol were added to each dish, cells were transferred to 50 ml Falcon tubes, and 2 ml chloroform were added. The mixture was sonicated for 3 min, and the organic phase separated from debris by centrifugation at 1000 rpm. The organic phase was lyophilized and pre-purified by open bed chromatography as described elsewhere [9]. RP-HPLC of the extracts was carried out as by using a semi-preparative Spherisorb column [9] eluted with methanol:water (92:8 by volume), at a flow rate of 2 ml/min. Under these conditions synthetic standards of AEA, 2-AG and 2-AGE are eluted after 21, 22 and 29 min. Each HPLC fraction was transferred into scintillation vials, 5 ml of scintillation liquid added, and radioactivity counted by a β counter.

2.4. Inactivation experiments

Confluent C6 or RBL-2H3 cells in 6× multi-well dishes were incubated with [^{3}H]2-AGE (10 000 cpm in 0.5 ml, 7 μM) for increasing intervals of time (0, 5, 15 and 30 min) at 37°C or 0°C. In a second set of experiments C6 cells were incubated for 10 min at 37°C with [^{3}H]2-AGE at increasing concentrations (0, 0.5, 1, 5, 10, 50 μM). In another series of experiments, cells were incubated for 10 min at 37°C with [^{3}H]2-AGE (2 μM) with or without 30 μM AEA or 2-AG. In a fourth set of experiments, cells were incubated for 10 min at 37°C with either [^{3}H]2-AG (4 μM) or [^{14}C]AEA (4 μM) with or without increasing concentrations (1, 10, 25 and 50 μM) of 2-AGE. In each experiment the incubation was terminated by lowering the temperature at 4°C. In the first set of experiments, [^{3}H]2-AGE amounts in the incubation medium were determined after extraction with chloroform:methanol (2:1 by volume) followed by thin layer chromatography (TLC) on silica gel polyethylene plates (Merck) eluted with the organic phase of ethyl acetate:water:2,2,4-trimethyl-pentane:acetic acid (39.3:35.7:17.8:7.2 by volume). The amounts of [^{3}H]2-AGE, [^{3}H]phospholipids and [^{3}H]AA+[^{3}H]diacylglycerols+[^{3}H]triacylglycerols in the cells was determined after extraction of the cells with chloroform/methanol/DMEM (2:1:1 by volume), followed by TLC on polyethylene plates eluted with the solvent system described above. In the second and third series of experiments, the amount of residual extracellular [^{3}H]2-AGE was determined after extraction and purification of the medium as described above, and used to calculate the amount taken up by cells. The uptake observed at 4°C was kept into account for the calculation of net uptake at 37°C. In the fourth set of experiments, the amount of residual [^{3}H]2-AG or [^{14}C]AEA in the incubation medium was determined as described previously [11]. In all cases, the radioactivity contained in each lipid component was determined by cutting the corresponding TLC bands, transferring them to scintillation vials, adding 1 ml methanol followed by 5 ml scintillation liquid, and counting by a β counter.

3. Results and discussion

In order to undertake this study we needed a sensitive technique to measure 2-AGE levels in little amounts of tissue. We synthesized d_8-2-AGE from d_8-AA by a procedure previously described [6]. The identity and purity of synthetic d_8-2-AGE were checked by means of GC-EIMS of its TMS derivative, which yielded a single peak with a retention time of 15.91 min identical to that of 2-AGE-TMS. This peak exhibited an

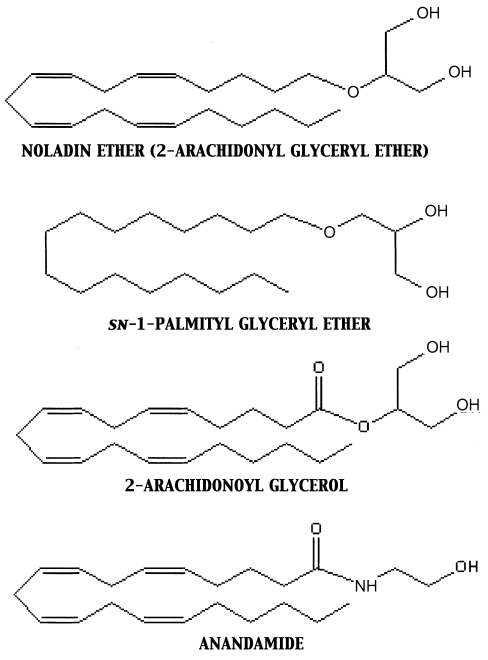

Fig. 1. Chemical structures of noladin ether (2-AGE) together with a more frequently found *sn*-1-alkyl-glyceryl ether, and the two previously discovered endocannabinoids, 2-AG and AEA.

EI-MS spectrum identical to that reported by Hanus and co-workers for synthetic 2-AGE-TMS [6] except for the expected 8 mass unit shift of the molecular ion at $m/z = 516$ and of the acyl fragments ($m/z = 280$, 297, 323, 370 and 413). No mass shift was observed in the fragment corresponding to the glyceryl moiety at $m/z = 219$. The amount of synthetic d_8-2-AGE was determined by GC-EIMS of the TMS derivative after dilution with a known amount of synthetic 2-AGE-TMS. d_8-2-AGE was then analyzed by LC-MS by using the full scan mode. While 2-AGE exhibited a retention time of 14.9 min and a most abundant fragment at $m/z = 365.3$, corresponding to the M+1 ion, d_8-2-AGE displayed an almost identical retention time (14.7 min) and a cluster of fragment ions from $m/z = 366.3$ up to 374.3, the most abundant of which was at $m/z = 369.3$, corresponding to the M+1 ion of d_4-2-AGE (data not shown). This was very likely due to proton–deuterium exchange with the solvent at the high temperature of the APCI source. The two regio-isomers of 2-AGE, 1-AGE and 3-AGE were both eluted 3.8 min after 2-AGE under the same chromatographic conditions. For LC-MS analysis of 2-AGE, increasing amounts (100, 500, 1000, 2000 and 4000 fmol) of the synthetic non-deuterated compound were diluted with 500 fmol of d_8-2-AGE and MS analysis carried out in the SIM mode by monitoring $m/z = 365.3$, 369.3 and 373.3 (d_8-2-AGE). Over this range of 2-AGE amounts the ratios between the areas of the LC-MS peaks at $m/z = 365.3$ and 369.3, and at $m/z = 365.3$ and 373.3 varied in a linear way with varying concentrations of 2-AGE (data not shown). The detection limit of this method was ∼100 fmol of d_8-2-AGE.

We next analyzed with this LC-MS method the amounts of the native compound in lipid extracts from different rat organs. To prevent the over-load of the HPLC column only a part (5–50%) of the purified extracts was analyzed. Therefore, the actual detection limit of our method was ∼2 pmol/g for

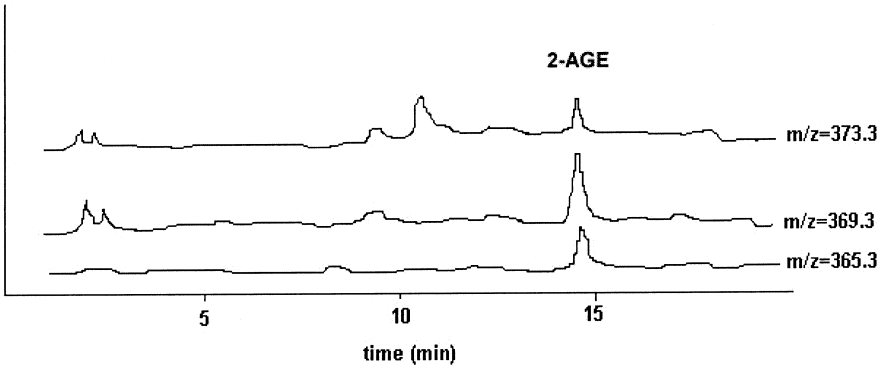

Fig. 2. LC-MS chromatogram of a purified lipid extract from rat brain diluted with 250 fmol of synthetic d_8-2-AGE. APCI-MS was run in the selected ion monitoring mode at the *m/z* values corresponding to the molecular weight+1 of 2-AGE (365.3), d_8-2-AGE (373.3) and d_4-2-AGE (369.3), formed from the deuterium–proton exchange of d_8-2-AGE. The retention time of synthetic 2-AGE, as determined after the analysis of the lipid extract, is shown. Synthetic standards of the two regio-isomers of 2-AGE, 1-AGE and 3-AGE were both eluted 3.8 min after 2-AGE under the same chromatographic conditions The chromatogram is representative of two distinct analyses.

the organs and 200 fmol/g wet tissue weight for the brain areas. The brain was the only organ where we found measurable amounts (25.4 ± 3.8 pmol/g of whole brain wet tissue) of a HPLC component with the same molecular weight as 2-AGE and the same retention time as d_8-2-AGE (Fig. 2). Derivatization of purified brain lipid extract with formation of TMS derivatives led to the disappearance of this component from LC-MS chromatograms, and to the appearance of a component in GC-EIMS analyses with the same retention time and fragment ions (*m/z* = 272, 289, 315, 362, see above) as synthetic 2-AGE (data not shown). By using the same LC-MS method, and by monitoring the ions at *m/z* = 356.2 and 387.3 (M+1 for d_8-AEA and d_8-2-AG) and 348.2 and 379.3 (M+1 for AEA and 2-AG), we quantified also endogenous AEA and 2-AG in whole brain (26.3 ± 5.2 pmol/g and 4.5 ± 1.1 nmol/g, respectively). These compounds, but not 2-AGE, were also found in the spleen, heart and liver (data not shown). Taken together, the tissue-specific occurrence of 2-AGE in the brain, its selectivity for CB_1 vs. CB_2 receptors [6], and the high concentration of CB_1 receptors in the brain as compared to other tissues, support the hypothesis that this compound is an endogenous CB_1 receptor ligand. The regional distribution of 2-AGE in rat brain overlapped only in part with that of cannabinoid CB_1 receptors, or with that of AEA and 2-AG [15], with the thalamus and the hippocampus exhibiting the highest concentrations, and the cerebellum, spinal cord and brainstem the lowest (Table 1).

Having demonstrated that a 2-AGE-like lipid is present in rat brain, we next wanted to assess whether this compound, like 2-AG [9], is produced on stimulation of N18TG2 cells with ionomycin. We found that treatment of [^{3}H]AA-prelabelled cells with 3 μM ionomycin leads to the formation of de novo synthesized [^{3}H]2-AG, but of very little, if any, [^{3}H]2-AGE (from 209 ± 90 to 450 ± 125 cpm for 2-AG, $P < 0.05$ by ANOVA, and from 35 ± 15 to 63 ± 14 cpm for 2-AGE, not significant by ANOVA, means ± S.D., $n = 3$). These data suggest that the biochemical mechanism for the synthesis of 2-AGE might be different from that of 2-AG [16], as indicated also by the different brain regional distribution of the two compounds (Table 1). It is possible that Ca^{2+} entry and neuronal membrane depolarization do not trigger 2-AGE biosynthesis, or that this process is not dependent on AA-containing precursors. The latter possibility is rather unlikely since, in the case of *sn*-1-alkyl-glycerols, it was shown that these compounds derive ultimately from the corresponding fatty acids via the fatty acid alcohols ([17] for review). On the other hand, the possibility that 2-AGE is not produced on Ca^{2+} influx into neurons, under conditions sufficient to elicit endocannabinoid biosynthesis, might suggest that this novel compound is not produced during neuronal depolarization. More in-depth experiments are needed in order to understand the pathway for 2-AGE biosynthesis and whether it is regulated by $[Ca^{2+}]_i$-modulating stimuli different from Ca^{2+} ionophores.

Table 1
Concentration of 2-AGE and 2-AG in several rat brain regions as determined by isotope dilution LC-MS

Brain region	2-AGE (pmol/g)	2-AG (nmol/g)
Thalamus	64.5 ± 4.0	6.5 ± 3.1
Hippocampus	57.9 ± 28.5	4.4 ± 0.4
Striatum	29.5 ± 6.9	3.0 ± 1.1
Cerebral cortex	10.1 ± 1.5	5.6 ± 1.0
Brainstem	4.3 ± 2.1	3.3 ± 0.9
Spinal cord	1.4 ± 0.8	7.8 ± 0.2
Cerebellum	N.D.	14.6 ± 0.2

Data are means ± S.D. ($n = 2$). N.D., not detectable. The whole cerebral cortex was used.

Finally, we wanted to determine if at least the mechanisms for 2-AGE disposal by living cells are similar to those previously described for AEA and 2-AG. Clearly, 2-AGE, unlike the other two endocannabinoids, is not likely to be hydrolyzed enzymatically. If other means for the degradation of this compound were lacking, this property of 2-AGE would strongly argue against a role for this compound as an endogenous mediator. However, we found here that [^{3}H]2-AGE could be efficaciously taken up in a time-, concentration- and temperature-dependent manner by intact C6 glioma and RBL-2H3 cells ($t_{1/2} \sim 5$ min, Fig. 3A and data not shown), where an AEA membrane transporter was previously partially characterized [9,11]. The apparent K_m and V_{max} for [^{3}H]2-AGE uptake were 12.8 ± 2.1 μM and 0.21 ± 0.08 nmol min^{-1} mg protein^{-1} (means ± S.D., $n = 3$), which were comparable to those previously described for 2-AG in the same cells [11]. A minor part (~10%) of [^{3}H]2-AGE taken up by C6 cells was transformed into phospholipids as well as into a lipid mixture with the same mobility on TLC as AA, diacylglycerols and triacylglycerols (Fig. 3B). The small amounts available

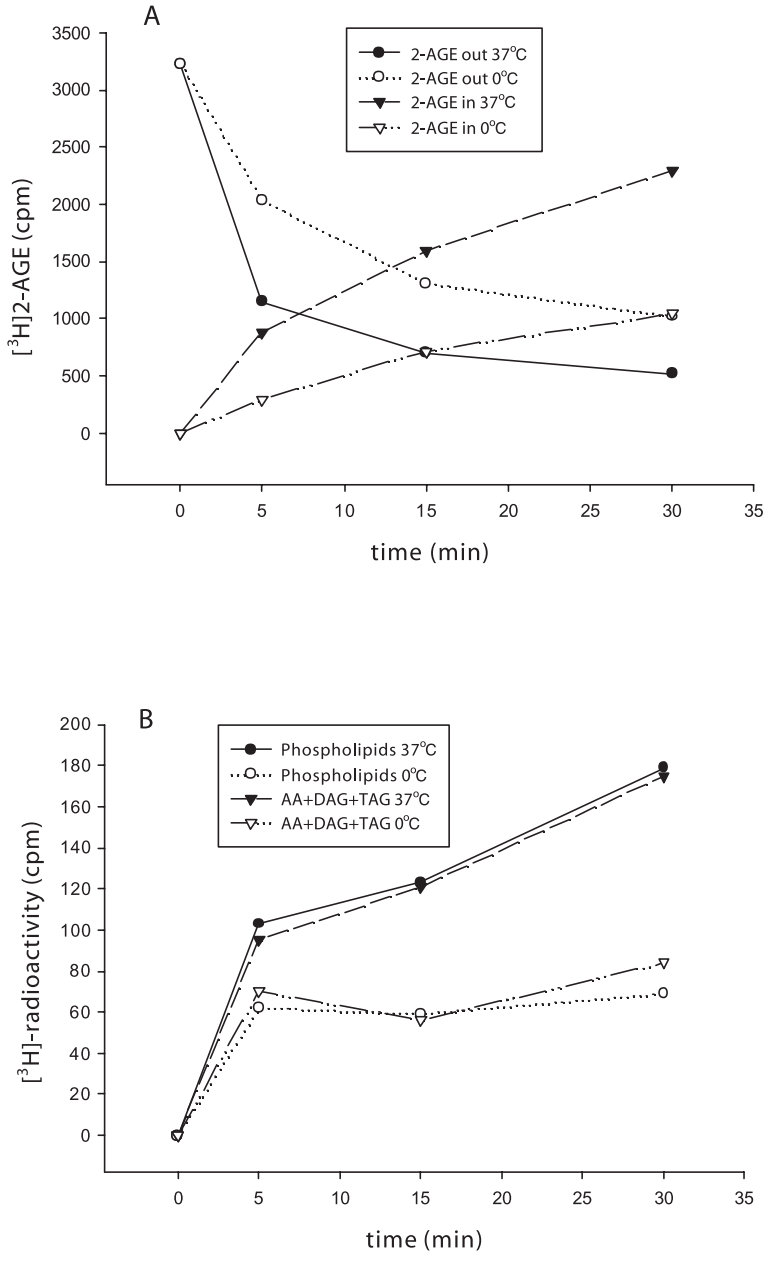

Fig. 3. Inactivation of [^{3}H]2-AGE by intact C6 glioma cells. Cells were incubated for increasing intervals of time, and either at 37°C or 0°C, with synthetic [^{3}H]2-AGE (10 000 Cpm, 7 μM). The amounts of (A) residual [^{3}H]2-AGE in the incubation medium and of unmetabolized [^{3}H]2-AGE found in cells, and (B) [^{3}H]radioactivity found in TLC bands corresponding to phospholipids (R_f = 0–0.1) or AA+diacylglycerols+triacylglycerols (R_f = 0.9–1.0), are shown for each time point. Data are means of n = 3 experiments carried out in duplicate. Error bars are not shown for the sake of clarity and were never higher than 5% of the means.

of this mixture prevented its further characterization. However, if the analogy between 2-AGE and *sn*-1-alkyl-glycerol ethers is to be pushed further, it is more likely that the major metabolic pathway of this novel endocannabinoid consists of the acylation of the *sn*-1 and/or *sn*-3 hydroxy groups [17]. Furthermore, 2-AG is also directly re-esterified into glycerolipids [10]. Interestingly, no radioactive band other than those described above could be detected in TLC analyses of C6 cell extracts after incubation with [^{3}H]2-AGE.

C6 cells were previously shown to contain one or two distinct, albeit functionally very similar, membrane transporters for the uptake of AEA and 2-AG [11]. Therefore, we examined whether the uptake of 2-AGE by these cells was mediated by this(ese) transporter(s). 2-AGE dose-dependently inhibited the uptake of both [^{14}C]AEA and [^{3}H]2-AG by C6 cells, with estimated K_i values of 15.6 ± 2.3 and 22.2 ± 2.7 μM, respectively (means ± S.D., n = 3). Under the same conditions, AEA and 2-AG inhibited [^{14}C]AEA and [^{3}H]2-AG uptake by the same cells with K_i values ranging between 13.4 and 30.1 μM, respectively [11]. These observations suggest that 2-AGE may diffuse through the cell membrane by means of the previously identified AEA/2-AG transporter(s), and this hypothesis was supported by the finding that AEA and 2-AG (30 μM) produced a 47.9 ± 3.5 and 41.3 ± 3.9% inhibition of [^{3}H]2-AGE uptake by C6 cells, respectively ($P < 0.01$ by ANOVA, means ± S.D., n = 3).

In conclusion, we have developed a method for the quantification of 2-AGE in tissues, and have presented evidence for the occurrence in rat brain of a lipid substance with the same chromatographic mobility, molecular weight and chemical behavior as 2-AGE. The brain concentration of 2-AGE is similar to that of AEA and lower than that of 2-AG, and its brain regional distribution reflects that of CB_1 receptors only in part. In glial cells 2-AGE was: (1) rapidly taken up from the extracellular medium, very likely through the same proteins that facilitate AEA and 2-AG re-uptake; and (2) slowly metabolized into phospholipids, thus supporting the role of this novel metabolite as an endogenous mediator. The way 2-AGE is biosynthesized in the CNS remains, however, still unexplored, and represents an interesting area for further investigations.

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