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Despite substantial degradation, 2-arachidonoylglycerol is a potent full efficacy agonist mediating CB_1 receptor-dependent G-protein activation in rat cerebellar membranes

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1 Two endocannabinoids, arachidonoyl ethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) bind and activate G-protein-coupled cannabinoid receptors, but limited data exist on their relative ability to activate G-proteins.

2 Here we assess agonist potency and efficacy of various cannabinoids, including 2-AG, HU-310 (2-arachidonoyl glyceryl ether, a third putative endocannabinoid), HU-313 (another ether analogue of 2-AG), AEA, *R*-methanandamide (an enzymatically stable analogue of AEA), and CP-55,940 at rat brain CB_1 receptors using agonist-stimulated [^{35}S]-GTPγS binding to cerebellar membranes and whole brain sections. Degradation of endocannabinoids under experimental conditions was monitored by HPLC.

3 To enhance efficacy differences, agonist dose-response curves were generated using increasing GDP concentrations. At 10^{-6} M GDP, all compounds, except HU-313, produced full agonists responses $\sim$2.5 fold over basal. The superior efficacy of 2-AG over all other compounds became evident by increasing GDP (10^{-5} and 10^{-4} M).

4 In membrane incubations, 2-AG was degraded by 85% whereas AEA and HU-310 were stable. Pretreatment of membranes with phenylmethylsulphonyl fluoride inhibited 2-AG degradation, resulting in 2 fold increase in agonist potency. Such pretreatment had no effect on AEA potency.

5 Responses in brain sections were otherwise consistent with membrane binding data, but 2-AG evoked only a weak signal in brain sections, apparently due to more extensive degradation.

6 These data establish that even under conditions of substantial degradation, 2-AG is a full efficacy agonist, clearly more potent than AEA, in mediating CB_1 receptor-dependent G-protein activity in native membranes.

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Abbreviations: ADA, adenosine deaminase; AEA, arachidonoylethanolamide; 1-AG, 1-arachidonoylglycerol; 2-AG, 2-arachidonoylglycerol; ANOVA, analysis of variance; BSA, bovine serum albumin; CB_1, type 1 cannabinoid receptor; CB_2, type 2 cannabinoid receptor; CHO, Chinese hamster ovary; CP-55,940, (−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol; Δ^9-THC, Δ^9-tetrahydrocannabinol; DMSO, dimethyl sulphoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxantine; DTT, dithiothreitol; FAAH, fatty acid amide hydrolase; [^{35}S]-GTPγS, guanosine-5′-*O*-(3-[^{35}S]-thio)-triphosphate; GTPγS, guanosine-5′-*O*-(3-thio)-triphosphate; HU-310, 2-*O*-arachidonoylglyceryl ether (noladin ether); HU-313, 2-isopropoxyethyl ether; PMSF, phenylmethylsulphonyl fluoride; *R*-α-mAEA (*R*-methanandamide), arachidonoylamide of *R*-(−)-2-amino-1-propanol; SR141716, *N*-piperidin-*O*-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carbonxamide; SR144528, *N*-[1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carbonxamide

Introduction

Cannabinoids are a group of chemically diverse compounds, derived from Δ^9-tetrahydrocannabinol (Δ^9-THC), which is the major psychotropic constituent of *Cannabis sativa* (Pertwee, 2000). To date, two G-protein-coupled cannabinoid receptors, termed CB_1 and CB_2, have been molecularly identified from the brain and peripheral tissues, respectively (Matsuda *et al*., 1990; Munro *et al*., 1993). Arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) are postulated to act as the endogenous ligands for these receptors (Devane *et al*., 1992; Mechoulam *et al*., 1995; Sugiura *et al*., 1995). Both the potency and efficacy of these endocannabinoids at the CB receptors have been determined by measuring second messenger responses, such as inhibitory effect on cyclic AMP-accumulation (Stella *et al*., 1997; Gonsiorek *et al*., 2000) and increases in intracellular Ca^{2+} concentrations in intact cells (Sugiura *et al*., 1999). Both responses are downstream steps in the signalling cascade initiated by

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agonist-liganded receptor. In the above-cited whole cell studies, 2-AG turned out to be more potent and efficacious than AEA, suggesting that the CB receptors might be preferentially activated by 2-AG. Regarding CB_1, this reasoning is also supported by the fact that levels of 2-AG in the CNS are considerably higher than those of AEA (Di Marzo *et al*., 2000).

Although several studies have evaluated the potency and efficacy of AEA and its stable analogue *R*-methanandamide (*R*-α-mAEA) at the more proximal step in cannabinoid signalling, namely receptor-dependent G-protein activation (Selley *et al*., 1996; Breivogel & Childers, 2000), only two articles have reported on the potency and efficacy of 2-AG in G-protein activation (Gonsiorek *et al*., 2000; Hillard, 2000). In membranes prepared from insect Sf9 cells engineered to overexpress the human CB_2 receptor together with mammalian $G_{\alpha i3}$, 2-AG and AEA were equipotent, both showing full agonism in stimulating [^{35}S]-GTPγS binding (Gonsiorek *et al*., 2000). In the same study, using CHO cells as a host for the CB_2 receptor, 2-AG behaved as a full agonist, whereas AEA was clearly less potent, producing partial agonist responses, and capable of even antagonizing the 2-AG-evoked responses. In a [^{35}S]-GTPγS binding assay with rat cerebellar membranes endogenously expressing the CB_1 receptor, 2-AG acted as a full efficacy agonist whereas AEA, although being considerably more potent than 2-AG, gave only a partial agonist response (Hillard, 2000). Clearly more detailed studies, assessing also the metabolic fate of 2-AG, are needed to provide a more comprehensive picture on the G protein activation capacity of these endocannabinoids.

Recently, 2-arachidonyl glyceryl ether (noladin ether, HU-310) was identified as a third putative endocannabinoid (Hanus *et al*., 2001). Although HU-310 binds to CB_1 receptors (Hanus *et al*., 2001) and stimulates cannabinoid receptor-dependent second messenger responses (Sugiura *et al*., 1999; 2000), no studies have assessed the capacity of this compound in G-protein activation.

In the present study, the superior agonist behaviour of 2-AG is demonstrated over AEA and HU-310, as well as over several other cannabinoids. We tested various cannabinoids, including the three endocannabinoids, and used increasing GDP concentrations to systematically distinguish between partial and full agonists in CB_1-dependent G-protein activation assay using rat cerebellar membranes. CB_1-dependent G-protein activity was detected also in brain sections using [^{35}S]-GTPγS autoradiography. Degradation of 2-AG was monitored by HPLC to examine the different metabolic behaviours of 2-AG and HU-310 in membrane binding and autoradiographic assays.

Methods

Animals

These studies were conducted using 4-week-old male Wistar rats. All animal experiments were approved by the local ethics committee. The animals lived in a 12-h light/12-h dark cycle (lights on at 0700 h) with water and food available *ad libitum*. The rats were decapitated, 8 h after lights on (1500 h), whole brains were removed, dipped in isopentane on dry ice and stored at −80°C.

Preparation of membranes and autoradiographic sections

Membranes were prepared as previously described (Lorenzen *et al*., 1993; Kurkinen *et al*., 1997). Cerebella (minus brain stem) from eight animals were weighed and homogenized in nine volumes of ice-cold 0.32 M sucrose with a glass Teflon homogenizer. The crude homogenate was centrifuged at low speed ($1000 \times g$ for 10 min at 4°C) and the pellet was discharged. The supernatant was centrifuged at high speed ($100{,}000 \times g$ for 10 min at 4°C). The pellet was resuspended in ice-cold deionized water and washed twice, repeating the high speed centrifugation. Finally, membranes were resuspended in 50 mM Tris-HCl, pH 7.4 with 1 mM EDTA and aliquoted for storage at −80°C. The protein concentration, measured by the Bradford method (Bradford, 1976), was 5.8 mg ml^{-1}. For autoradiography, coronal or sagittal sections (20 μm thick) were cut at −14°C using a Leica cryo stat. Tissue was thaw-mounted onto SuperFrost*/Plus slides (Menzel-Gläser, Germany) at 20°C and air-dried under a fan. Typically, two coronal and two sagittal sections were collected per slide. The sections were stored at −80°C for up to 6 months without any apparent loss in binding response.

Chemicals

2-AG was purchased from Calbiochem (San Diego, CA, U.S.A.) or from Cayman Chemical (Ann Arbor, MI, U.S.A.), 1-AG was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.), *R*-α-mAEA, AEA and arachidonic acid from Deva Biotech (Hatboro, PA, U.S.A.). HU-310 and HU-313 were generous gifts from Prof R. Mechoulam (Hebrew University, Israel). SR141716 and SR144528 were obtained from Sanofi Recherche (Montpellier, France), AM251 was purchased from Tocris Cookson Ltd. (Bristol, U.K.), and CP-55,940 was from Pfizer (Groton, CT, U.S.A.). BSA (essentially fatty acid free), DTT, PMSF, GDP and GTPγS were purchased from Sigma (St. Louis, MO, U.S.A.). Adenosine deaminase (ADA) was purchased from Roche Diagnostics GmbH (Mannheim, Germany), 8-cyclopentyl-1,3-dipropylxantine (DPCPX) from RBI/Sigma (Natick, MA, U.S.A.) and [^{35}S]-GTPγS (initial specific activity 1250 Ci/mmol) from NEN Life Science Products, Inc. (Boston, MA, U.S.A.). All cannabinoids were dissolved in ethanol as 10 mM stock solutions and stored at −80°C AM251, SR141716 and SR144528 were dissolved in DMSO as 10 or 20 mM stocks. The stock solution of 2-AG (initially in hexane or in acetonitrile) was prepared just prior to experiments by evaporating the organic solvent and reconstitution with ethanol. All other chemicals were of highest purity available.

[^{35}S]-GTPγS-membrane binding assay

Incubations were carried out as previously described (Kurkinen *et al*., 1997) with the following modifications. The final incubation volume was 400 μl, containing 5 μg membrane protein, (mM) Tris-HCl 55, pH 7.4, EDTA 1.1, NaCl 100, $MgCl_2$ 5, DTT 1, 0.5% (wt/vol) BSA, 1–100 μM GDP as indicated in the results, 0.5 U ml^{-1} ADA to deplete endogenous adenosine, ~150 pM [^{35}S]-GTPγS and cannabinoids in ethanol (final concentration 1%, v v^{-1}) in combination with the CB-antagonists in DMSO (0.5%,

v v^{-1}), as detailed in the results. For experiments with PMSF, membranes were preincubated for 30 min at 25°C with 10^{-3} M PMSF (dissolved in DMSO) or the vehicle as control, and kept at 0°C prior to experiments. The final PMSF concentration in [^{35}S]-GTPγS binding assay was 10^{-4} M. Incubations were initiated by adding 40 μl of the membrane dilution, and continued routinely for 90 min at 25°C under diminished illumination. Non-specific binding was determined in the presence of 10 μM GTPγS and was subtracted from all other values. The reaction was quenched by the addition of 4 ml ice-cold washing buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4), followed by rapid filtration through glass fibre filters (Whatman GF/B) and two additional 4-ml washes with the buffer. The filters were transferred into scintillation vials and 2 ml of liquid scintillation cocktail (HiSafe3, Wallac, Turku, Finland) was added. After horizontal shaking for 15 min, the vials were centrifuged at $1000 \times g$ for 10 min at 20°C to force filters to the bottom. Vials were left overnight at 20°C protected from light, before counting with a Wallac LKB 1214 Rackbeta, having 95% counting efficiency for [^{14}C].

[^{35}S]-GTPγS autoradiography

The assay was conducted under optimized conditions, where noise due to tonic adenosine A_1 receptor activity has been eliminated (Laitinen, 1999). Briefly, the assay consisted of preincubation (step 1) for 20 min at 20°C in buffer A ((mM) Tris-HCl 50, pH 7.4, EDTA 1, NaCl 100, $MgCl_2$ 5), followed by GDP loading (step 2) for 1 h at 20°C in buffer A, routinely containing 2 mM GDP and 1 μM DPCPX. For [^{35}S]-GTPγS binding (step 3), sections were incubated for 90 min (or 45 min) at 20°C in buffer A, which also contained ~60 pM [^{35}S]-GTPγS, 2 mM GDP, 1 μM DPCPX, 0.1% (wt/vol) BSA, 1 mM DTT and cannabinoids at concentrations as indicated in the results. In some experiments, 10^{-4} M PMSF was present throughout steps 2 and 3. Final concentrations of ethanol or DMSO was 0.5% (v v^{-1}). Non-specific binding was determined in the presence of 10 uM GTPγS. The sections were washed twice at 0°C for 5 min each time in washing buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4), rinsed in ice-cold deionized water for 30 s, air dried and apposed to Hyperfilm™-βmax (Amersham) for 2–4 weeks in conjunction with autoradiographic [^{14}C]-microscale standards (Amersham). Films were developed in Kodak D-19 developer for 4 min at 4°C.

HPLC

In order to study the degradation of endocannabinoids, incubations mimicking [^{35}S]-GTPγS binding assays were carried out in triplicates. [^{35}S]-GTPγS was replaced with GTPγS. The concentrations of endocannabinoids were 5×10^{-5} M or 10^{-4} M, depending on the experiment. The analytical HPLC system consisted of a Merck Hitachi (Hitachi Ltd., Tokyo, Japan) L-6200A intelligent pump, Merck Hitachi L-6000A interface module, Merck Hitachi L-4500 diode array detector (200–400 nm, set at 215 nm) and a Merck Hitachi AS-2000 autosampler. The separations were performed with a Purospher RP-18 endcapped reverse-phase column (125×4 mm, 5 μM). A mobile phase mixture of an 18% phosphate buffer (20 mM, pH 5.0 for 2-AG and AEA, pH 3.0 for HU-310) in acetonitrile at a flow rate of 1.2 ml min^{-1} was used. Retention times varied somewhat between different HPLC runs and were 5.1–7.7 min for 2-AG, 8.4 min for 1-AG, 6.9 min for HU-310, 8.9 min for AEA and 7.6–12.5 min for arachidonic acid. In each HPLC run, synthetic reference compounds were included to facilitate identification of eluted material.

Data-analysis

For agonist dose-response and antagonist experiments, results are presented as mean ± s.e.mean of at least three (2–4 for data of Figure 5B) independent experiments performed in duplicate. Data-analysis for dose-response curves were calculated as non-linear regressions. Statistical differences between groups were tested using one-way ANOVA, followed by Tukey's Multiple comparison test with $P < 0.05$ considered as statistically significant. Data-analysis was performed by using GraphPad Prism 3.0 for Windows. Autoradiographic images were scanned as black and white negatives and magnified to twice their original size using an HP PhotoSmart S20 Scanner at 1200 DPI resolution. Figure 3 was generated by Corel Photo Paint 7 and Corel Draw 7 softwares.

Results

GDP concentration in membrane [^{35}S]-GTPγS binding experiments dramatically affects cannabinoid agonist efficacy

The differences in efficacy between partial agonists at various G-protein-coupled receptors can be increased by altering GDP concentrations in the [^{35}S]-GTPγS binding assay. To systematically clarify how GDP affected cannabinoid-evoked [^{35}S]-GTPγS binding responses in rat cerebellar membranes, agonist dose-response studies were conducted using 10^{-6}, 10^{-5} and 10^{-4} M GDP. Agonist dose-response curves are depicted in Figure 1 and the efficacies (E_{max}) and potencies (inverse $LogEC_{50}$-values) are shown in Figure 1 (bottom right) and Table 1, respectively. The GDP concentrations significantly affected both agonist potency and efficacy. The potencies of all cannabinoids, except HU-313, significantly decreased when GDP concentration increased from 10^{-6} M to 10^{-4} M (Table 1). The effect of GDP was more dramatic in efficacy, especially in the case of 2-AG, where increasing GDP from 10^{-6} M to 10^{-4} M resulted in ~4 fold increase in E_{max}. For HU-310 and CP-55,940, this increase was ~2.5 fold. At 10^{-6} M GDP, all compounds, except HU-313, behaved as full agonists, that produced maximal responses approximately 2.5 fold over basal. HU-313 evoked about 50% of this response (Figure 1). At 10^{-5} and 10^{-4} M GDP, the differences between agonist efficacies became more evident. At 10^{-4} M GDP, where the maximal response of 2-AG was approximately 10 fold over basal, all other compounds produced only partial agonist responses with the following relative efficacy order: 2-AG > > CP-55,940 ≈ HU-310 > *R*-α-mAEA > HU-313 (Figure 1, bottom right). It is important to point out that the maximal responses to HU-313 and *R*-α-mAEA were reached at 10^{-5} M GDP, in sharp contrast to the behaviour of all the other compounds at higher GDP concentrations. We used the metabolically stable *R*-α-mAEA instead of AEA in these initial studies to avoid

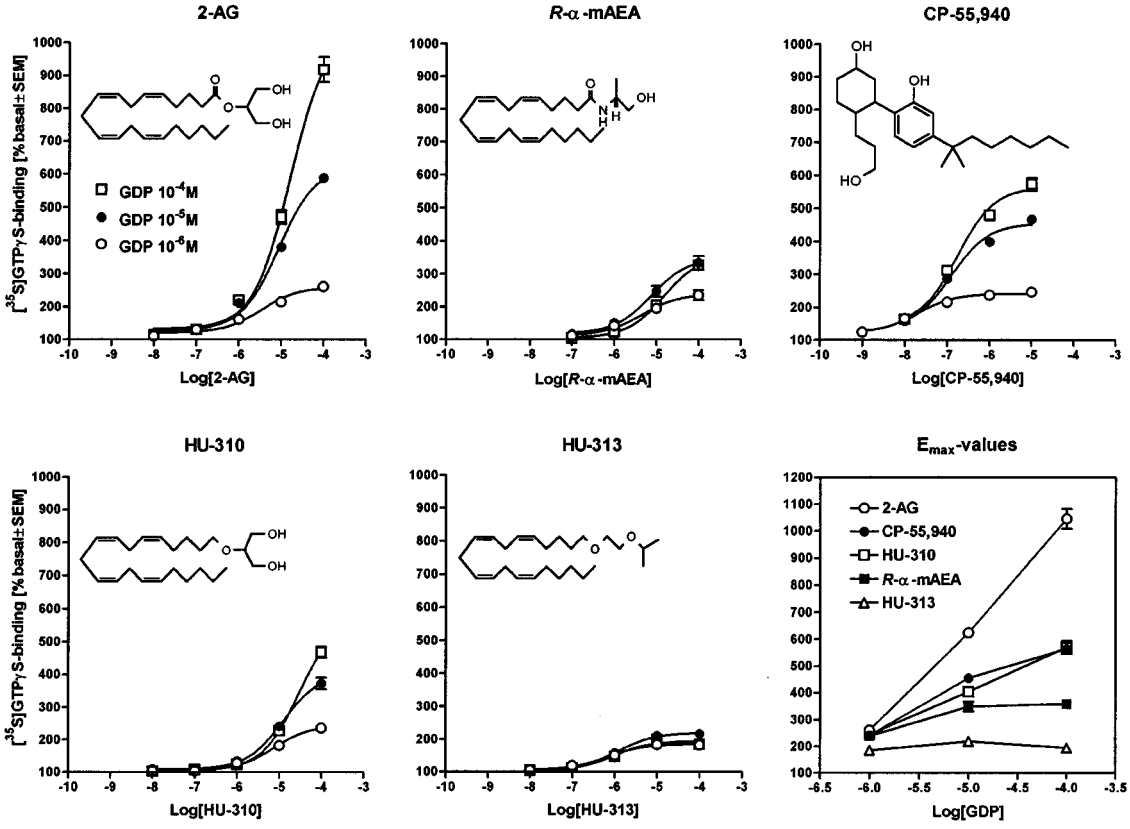

Figure 1 Dose-response curves and maximal efficacy (E_{max}) of cannabinoid ligands in activating G-proteins in rat cerebellar membranes at various GDP concentrations. The data represent the mean ± s.e.mean of [^{35}S]-GTPγS binding over basal from three to four independent experiments performed in duplicate. When not visible, the error bars fell within the size of the symbol.

Table 1 Comparison of potency of the five cannabinoid agonists at various GDP concentrations

Compound	*−LogEC50 ± SE*		
	GDP 10^{-6} M	*GDP 10^{-5} M*	*GDP 10^{-4} M*
CP-55,940	7.7 ± 0.2*	6.9 ± 0.1	6.8 ± 0.1
R-α-mAEA	5.3 ± 0.2*	5.1 ± 0.2	4.8 ± 0.1
2-AG	5.5 ± 0.1*	5.1 ± 0.1*	4.8 ± 0.1
HU-310	5.1 ± 0.1*	4.9 ± 0.1*	4.6 ± 0.1
HU-313	6.2 ± 0.2	5.9 ± 0.1	6.0 ± 0.2

EC_{50}-values were calculated from the [^{35}S]GTPγS binding experiments depicted in Figure 1. Values are presented as means ± s.e.mean from three to four independent experiments performed in duplicate. *Significantly different from values at 10^{-4} M GDP.

potential problems due to lability of AEA. Subsequent studies revealed, however, that AEA was not degraded to a detectable degree in membrane incubations and that *R*-α-mAEA and AEA behaved very similarly in this assay (illustrated in Figure 5). These data demonstrate that 2-AG was clearly more efficacious and at least as potent as R-α-mAEA in activating G-proteins in rat cerebellar membranes.

2-AG activates G-proteins in rat cerebellar membranes via *CB_1 receptors; AEA does not antagonize this response*

The CB_1 receptor antagonist SR141716 reversed the response evoked by 10^{-6} M 2-AG in cerebellar membranes in a dose dependent manner, abolishing the signal totally at 10^{-6} M (Figure 2). The CB_2 receptor antagonist SR144528 was approximately 1000 fold less potent (Figure 2), consistent with the involvement of CB_1 receptors in this response. Further, responses to 10^{-4} M 2-AG (554 ± 6% basal, mean ± s.e.mean, $n=3$ at 10^{-5} M GDP) were largely reversed by coincubation with 10^{-6} and 10^{-5} M SR141716 (152 ± 4 and 129 ± 1% basal, mean ± s.e.mean, $n=4$, respectively). Similarly, coincubation with AM251, another potent CB_1-selective antagonist, brought these responses to 140 ± 2 and 125 ± 3% basal (mean ± s.e.mean, $n=4$), respectively. When tested alone, SR141716 did not affect basal binding (102 ± 1% and 98 ± 1% at 10^{-6} and 10^{-5} M, mean ± s.e.mean, $n=2$, respectively). For AM251, these Figures (mean ± s.e.mean, $n=2$) were 105 ± 2 and 93 ± 3% at 10^{-6} and 10^{-5} M, respectively. Additional studies revealed further that responses to all cannabinoids were abolished in the presence of 10^{-6} M SR141716, when agonists were tested at concentrations close to their EC_{50}-values (data not shown). Since a previous G-protein activation study implicated AEA as an antagonist at the human CB_2 receptor (Gonsiorek *et al*., 2000), its ability to reverse 2-AG-dependent G-protein activity in rat cerebellar membranes was tested. However, AEA had no antagonistic activity at any of the concentrations used (10^{-4}–10^{-6} M), but produced closely additive responses with 2-AG, consistent with its partial agonist action (data not shown). These results indicate that 2-AG activated G-proteins solely *via* CB_1-receptors in rat cerebellar

membranes and that AEA did not antagonize this response.

Regional distribution of CB_1 receptor-dependent G-protein activity in rat brain using [^{35}S]-GTPγS autoradiography

Tonic adenosine A_1 receptor-dependent G-protein activity is widespread in rat brain sections under basal conditions in [^{35}S]-GTPγS autoradiography (Laitinen, 1999). Such activity is prominent also in many of the CB_1 receptor-enriched regions. To increase the signal-to-noise ratio in this assay, we routinely block the adenosine signal by including 10^{-6} M DPCPX, a A_1-selective antagonist (Laitinen, 1999). Under such conditions, results from cannabinoid-stimulated [^{35}S]-GTPγS binding to brain sections were consistent with those obtained from membrane-binding assays (particularly using 10^{-5} M GDP), with one notable exception: 2-AG produced only a weak, yet detectable signal (Figure 3). This was most evident in the substantia nigra. All tested cannabinoids activated G-proteins in identical brain regions, most notably the molecular layer of the cerebellum, hippocampus, globus pallidus (not illustrated) and the substantia nigra pars reticulata (Figure 3), fully consistent with known distribution of CB_1 receptors in the CNS, as revealed using [^{3}H]-CP-55,940 (Herkenham *et al*., 1991) or [^{35}S]-GTPγS autoradiography (Sim *et al*., 1996). Regardless of brain region, the relative rank order of agonist efficacy was CP-55,940 > > HU-310 > *R*-α-mAEA > HU-313 ≈ 2-AG. Throughout the brain, the cannabinoid-evoked [^{35}S]-GTPγS signal was abolished in the presence of 10^{-6} M SR141716 (data not shown).

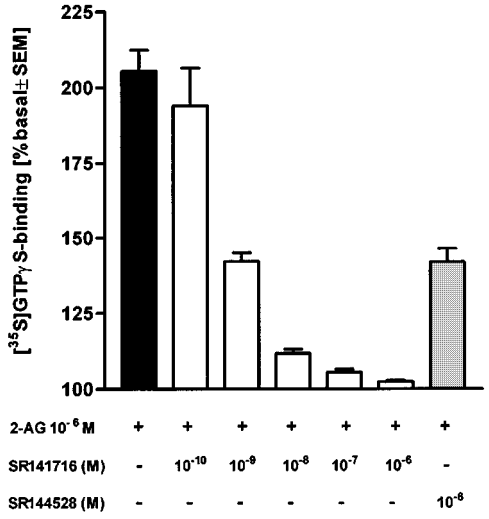

Figure 2 2-AG activates G-proteins in rat cerebellar membranes *via* CB_1 receptors. Membranes were incubated in the presence of 10^{-6} M 2-AG together with increasing concentrations of the CB_1 receptor antagonist SR141716 or with 10^{-6} M SR144528 (a CB_2 receptor antagonist) as indicated. The data represent the mean of [^{35}S]-GTPγS binding over basal ± s.e.mean from at least three independent experiments performed in duplicate.

Degradation of 2-AG under conditions mimicking G-protein activation assays in membranes and in brain sections

2-AG was highly efficacious in [^{35}S]-GTPγS membrane binding experiments, although it may have degraded substantially during the experiment. Thus, the degradation of 2-AG in cerebellar membranes was investigated. From the original material, approximately 95% eluted in HPLC as a single peak, representing 2-AG. The remaining 5% eluted as a single peak corresponding to 1-AG (Figure 4). During a 45 min incubation with cerebellar membranes, approximately 50% of the 2-AG was degraded to 1-AG and to a compound eluting at the position of synthetic arachidonic acid. After a 90 min incubation, some 15% of the 2-AG remained intact. In contrast, both AEA and HU-310, the ether analogue of 2-AG, were stable under identical conditions (data not shown). In the absence of

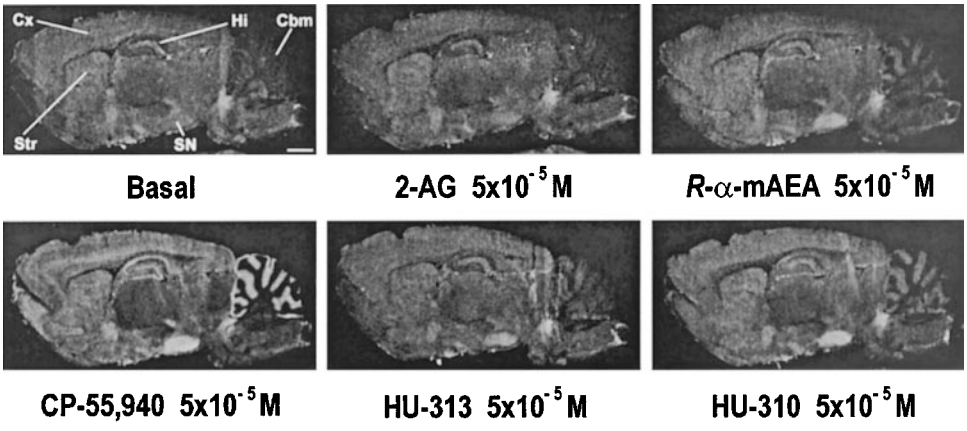

Figure 3 [^{35}S]-GTPγS autoradiography reveals CB_1 receptor-dependent G-protein activity in rat brain sections. Adjacent sagittal sections, obtained from 4-week-old male Wistar rats, were processed for [^{35}S]-GTPγS autoradiography, as described in Methods using a 3-step incubation protocol. This consisted of preincubation (step 1), loading with 2 mM GDP (step 2) and [^{35}S]-GTPγS binding for 45 min at 20°C (step 3). Cannabinoids (5×10^{-5} M) were present during step 3, whereas the A_1 receptor antagonist DPCPX (10^{-6} M) was present throughout steps 2 and 3. Note weak responses to 2-AG (most evident in the substantia nigra) and the completely overlapping distribution of activated G-proteins in response to all five cannabinoids, especially in the cerebellar molecular cell layer (Cbm), cerebral cortex (Cx), hippocampus (Hi), substantia nigra (SN) and striatum (Str). Scale bar = 2 mm.

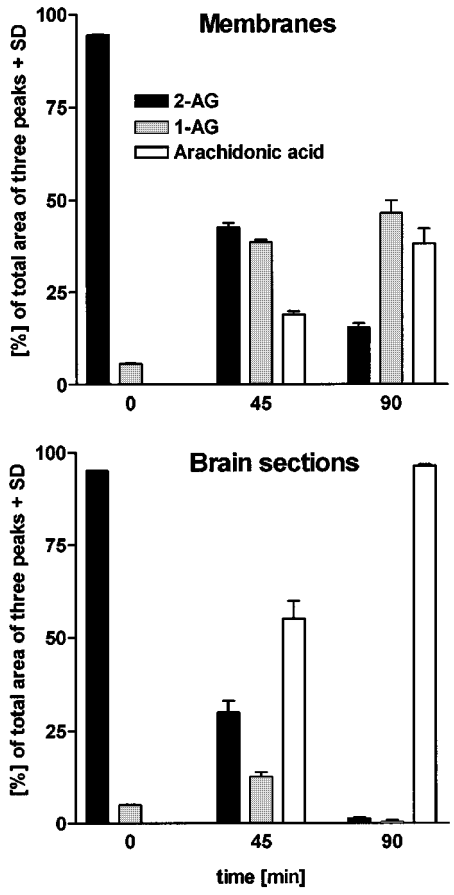

Figure 4 Degradation of 2-AG during incubations mimicking [^{35}S]-GTPγS binding assays in cerebellar membranes and brain sections. The data represent the mean ($\pm$s.d. $n=3$) of relative (%) peak areas for each compound.

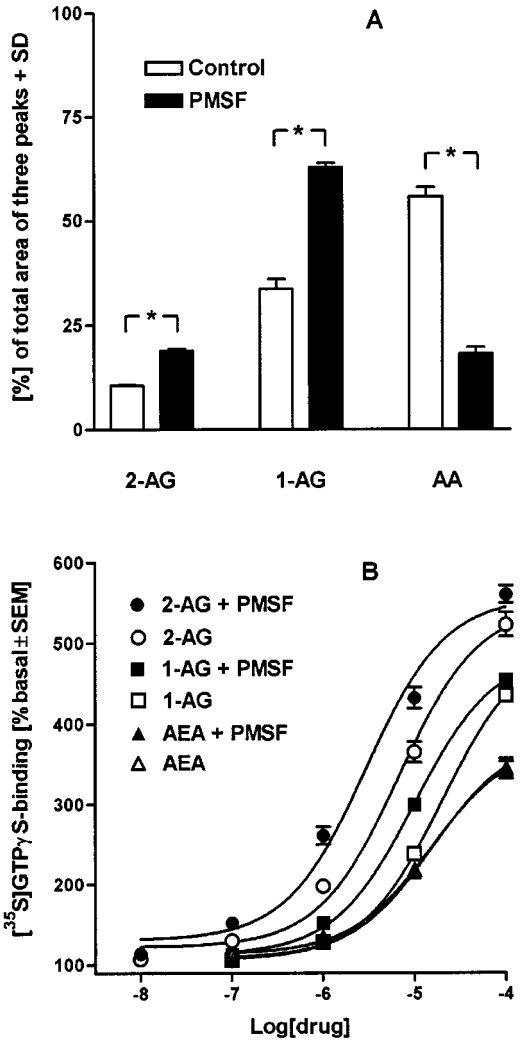

Figure 5 Pretreatment of cerebellar membranes with PMSF inhibits 2-AG degradation with concomitant increase in G-protein activation potency. Membranes were incubated for 30 min at 25°C in the presence or absence of 10^{-3} M PMSF, followed by 90 min incubation with 2-AG (5×10^{-5} M) to study degradation (A) or with the indicated concentrations of 2-AG, 1-AG or AEA to assess CB_1 receptor-dependent G-protein activity (B). Note significant inhibition of arachidonic acid (AA) formation and concomitant accumulation of 2-AG and 1-AG in PMSF-treated membranes. Note also significant leftward shifts in dose-response curves for 2-AG and 1-AG and total lack of PMSF in the case of AEA. For (A) the data represent the mean ($\pm$s.d. $n=3$) of relative (%) peak areas for each compound. For (B) the data represent the mean$\pm$s.e.mean of [^{35}S]-GTPγS binding over basal from two (AEA and 1-AG) to four independent experiments performed in duplicate. When not visible, the error bars fell within the size of the symbol. An asterisk denotes significant difference between pairs ($P<0.05$).

tissue, 2-AG was spontaneously converted to 1-AG in an aqueous environment with BSA but no arachidonic acid was formed (data not shown).

Degradation of 2-AG and HU-310 was also monitored under conditions mimicking autoradiography. During the routine 90 min incubation with brain sections, 2-AG was practically totally degraded to a product eluting as a single peak with a retention time corresponding to arachidonic acid. After a 45 min incubation, some 30% of 2-AG remained intact. Based on this observation, we used 45 min incubation time for [^{35}S]-GTPγS autoradiography in order to be able to visualize 2-AG-stimulated G-proteins (see Figure 3). In brain sections, HU-310 was also degraded ($\sim$50%), producing two, clearly separate peaks with relative amounts of $\sim$25% each (data not shown). One of the peaks eluted at the retention time of synthetic arachidonic acid, while the second peak remains to be identified.

PMSF-sensitive enzymatic activity, distinct from FAAH, degrades 2-AG in cerebellar membranes

As an initial step to reveal enzymatic activity responsible for 2-AG degradation, cerebellar membranes were pretreated with phenylmethylsulphonyl fluoride (PMSF), a widely used nonspecific enzyme inhibitor. HPLC analysis (Figure 5A) revealed that PMSF significantly prevented 2-AG degradation. Most notably, a significant drop in the accumulation of arachidonic acid was evident with concomitant increase in the amounts of 1-AG and 2-AG. Since both compounds possess cannabinoid agonist activity, we compared the potencies of the two isomers in G-protein activation assay both in control- and PMSF-treated cerebellar membranes. As shown in Figure 5B, 2-AG was clearly more efficacious and $\sim$3 fold more potent as 1-AG, and that PMSF treatment resulted in $\sim$2 fold increase in the potency of both agonists. Arachidonic acid (10^{-4} M)

was inactive in this assay (data not shown). AEA was tested in parallel incubations, but as evident from Figure 5B, PMSF did not affect the potency of this agonist at all (dose-response curves for AEA in PMSF-treated and control membranes were practically indistinguishable). Furthermore, HPLC analysis revealed that AEA was not degraded to a detectable degree in control or PMSF-treated membranes (data not shown). It is also evident from these experiments, that when degradation of 2-AG could be even partially inhibited, this endocannabinoid was at least 5 fold more potent than AEA in activating G-proteins *via* CB_1 receptors.

Pretreatment of brain sections with PMSF (at 10^{-4} M present throughout steps 2 and 3) did not enhance the 2-AG-evoked signal suggesting that other factors than PMSF-sensitive enzymatic activity must explain the weak signal of 2-AG in [^{35}S]-GTPγS autoradiography.

Discussion

In agreement with results obtained from several G-protein-coupled receptors (Lorenzen *et al*., 1993; 1996; Selley *et al*., 1997; Pauwels *et al*., 1998), including the cannabinoid receptors (Breivogel *et al*., 1998; Griffin *et al*., 1998), increasing GDP concentration in [^{35}S]-GTPγS binding assays dramatically increased efficacy differences between cannabinoid agonists in our study. Increasing GDP also shifted dose-response curves of most agonists to the right. It was clearly shown that from the tested compounds only 2-AG acted as a full agonist at all GDP concentrations, even though its dose-response curves did not fully saturate at the used concentrations. This can be attributed to the significant enzymatic degradation of 2-AG to arachidonic acid, as clearly demonstrated here. At the two highest GDP-concentrations, all other cannabinoids behaved as partial agonist, producing variable maximal responses. Among the tested drugs, CP-55,940 which was previously classified as a high efficacy partial agonist in G-protein activation assays (Breivogel *et al*., 1998), was the most potent and second most efficacious cannabinoid in this study. The potency of HU-310 resembled that of *R*-α-mAEA, but HU-310 was clearly more efficacious than *R*-α-mAEA, as revealed with increasing GDP concentration. HU-313 had, in spite of its low efficacy, the second highest potency. It also had relatively similar maximal responses regardless of GDP concentrations, comparable to the behaviour of the other low efficacy partial agonist, *R*-α-mAEA. This GDP-insensitive behaviour clearly distinguishes these two compounds from all other compounds of this study.

An emerging concept to explain agonist efficacy among G-protein-coupled receptors is that partial agonists may favour distinct conformational changes in the receptor molecule, perhaps allowing specific activation of a subset of G-protein-signalling pathways, whereas a full agonist would be capable of turning on all available pathways. In coexpression and reconstitution studies, the types of G-proteins available for interaction with a given receptor clearly affects agonist efficacy (Lorenzen *et al*., 1998; Yang & Lanier, 1999). This applies to cannabinoid receptors as well, as AEA acted as a full agonist at the CB_1 receptors when reconstituted with $G_{\alpha i}$, whereas partial agonism was evident after reconstitution of CB_1 with $G_{\alpha o}$ (Glass & Northup, 1999). Interestingly, recent studies have shown that the rat cerebellar CB_1 receptors can interact with at least five distinct α-subunits of the $G_{i/o}$ family (Mukhopadhyay *et al*., 2000; Prather *et al*., 2000). Moreover, distinct intracellular domains of the CB_1 receptor are being implicated as responsible for the interaction with the specific α-subunits (Mukhopadhyay & Howlett, 2001). In light of these recent findings, we interpret our data to indicate that the endogenous agonist 2-AG likely favoured multiple receptor conformations capable of activating all naturally interacting $G_{i/o}$ proteins, while the partial agonists HU-313, AEA and *R*-α-mAEA favoured receptor conformations resulting in more restricted activation of only a subset of these G-proteins. We anticipate that further testing of these predictions is now feasible and such studies will add to our understanding regarding general function of the cannabinoid receptors, as well as the diversity of chemical structures capable of selective activation of these receptors.

Our results establish that even under conditions of substantial degradation, 2-AG was clearly more potent than AEA in stimulating [^{35}S]-GTPγS binding to rat cerebellar membranes (Figure 5B), in contrast to previous work, where AEA was reported to be substantially more potent than 2-AG in mediating G-protein activation (Hillard, 2000). PMSF, the non-specific enzyme inhibitor, enhanced the potency of 2-AG (and 1-AG) over 2 fold without affecting the potency of AEA. The inhibition of 2-AG degradation was also demonstrated clearly by HPLC.

The third putative endocannabinoid, 2-arachidonyl glyceryl ether (HU-310), binds to CB_1 receptors (Hanus *et al*., 2001) and stimulates both CB_1 and CB_2 receptor-dependent second messenger responses (Sugiura *et al*., 1999; 2000). In the present study, HU-310 was less potent and less efficacious than 2-AG, despite its metabolic stability, suggesting that the ether-modification resulted in decreased agonist efficacy and potency at the CB_1 receptor. This is fully consistent with previously described agonist behaviour of this compound, independently synthesized as an 'ether-linked analogue of 2-arachidonoylglycerol', in cannabinoid receptor-dependent Ca^{2+} mobilization using intact cells (Sugiura *et al*., 1999; 2000).

In rat cortical neurons, 1-AG was found to be more potent than 2-AG and AEA in CB_1 receptor-mediated inhibition of forskolin-stimulated cyclic AMP accumulation (Stella *et al*., 1997). On the other hand, 2-AG was clearly more potent and at least as efficacious than 1-AG in stimulating cannabinoid receptor-dependent Ca^{2+} responses (Sugiura *et al*., 1999; 2000). In the present G-protein activation assay, 2-AG was clearly more potent and more efficient than 1-AG both in the absence and presence of PMSF, in line with the work of Sugiura *et al*. Based on these observations, we conclude that the overall activity of 2-AG in cerebellar membranes likely consist of activity of its all isomeric forms.

[^{35}S]-GTPγS autoradiography is emerging as a powerful tool to reveal receptor-dependent G-protein activity in anatomically defined brain regions. To our knowledge, this is the first report to visualize 2-AG-stimulated G-proteins using this approach. Although 2-AG generated only a weak response in brain sections, this signal was consistently detectable in CB_1 receptor enriched regions, most notably the substantia nigra. By monitoring 2-AG degradation with

HPLC, the incubation time was shortened to 45 min, as some intact 2-AG was still present at this time point. Degradation analysis suggests that the weak signal of 2-AG in brain sections resulted from complete enzymatic breakdown of both 2-AG and 1-AG to arachidonic acid. Both, 2-AG and 1-AG were previously shown to be good substrates for FAAH (Goparaju *et al*., 1998). However, in brain sections, which despite extensive preincubation steps, likely retain considerable enzymatic activity in contrast to washed membranes, HU-310 was also degraded, indicating that membranes and brain sections exhibit distinct enzymatic activities. This was further supported by the findings that although PMSF-pretreatment significantly increased the potency of 2-AG in cerebellar membranes, pretreatment of brain sections with PMSF did not result in enhanced 2-AG-evoked signal.

Naturally, structural differences between AEA and 2-AG affect their substrate specificity towards degradable enzymes. Both endocannabinoids can be hydrolysed by FAAH to produce arachidonic acid in a pH-sensitive manner (Goparaju *et al*., 1998). However, the metabolism of 2-AG seems to be rather complex, and degradation may also be attributed to other types of enzymes such as monoacylglycerol lipases, esterases and cyclooxygenase-2 (Bisogno *et al*., 1997; Goparaju *et al*., 1999; Kozak *et al*., 2000; Dinh *et al*., 2000). We found, that *R*-α-mAEA and AEA had similar potency in G-protein activation in cerebellar membranes suggesting that AEA was not degraded. While PMSF-treatment clearly inhibited 2-AG degradation and increased its potency in G-protein activation, PMSF did not affect the potency or the efficacy of AEA at all. Moreover, HPLC analysis confirmed that AEA was not degraded. This indicates that the currently used membrane preparation was essentially devoid of AEA degrading activity, particularly that of cytosolic FAAH, probably due to the relatively intensive purification steps. However, FAAH may preferentially degrade 2-AG over AEA (Goparaju *et al*., 1998), and this may be one of the reasons for the weak signal of 2-AG in autoradiography. It is possible that monoacylglycerol lipase activity accounted for 2-AG degradation in cerebellar membranes, as a preliminary report (Dinh *et al*., 2000) is implicating this enzyme in the physiological role in terminating 2-AG signalling in the brain.

To conclude, our data document for the first time in a systematic manner that 2-AG rather than 2-arachidonoyl glyceryl ether (HU-310) or AEA is the endocannabinoid capable of potently and fully activating rat brain G-proteins *via* CB_1 receptors, adding strong support to previous conclusions drawn from studies assessing cannabinoid receptor-mediated second messenger responses (Stella *et al*., 1997; Sugiura *et al*., 1999; 2000). The development of potent and metabolically stable cannabinoids, having selectivity for desired effects, and the search for potent specific inhibitors of endocannabinoid degradation remain as interesting challenges for future research. The results herein demonstrate, that the metabolic fate of endocannabinoids can be readily monitored by HPLC, both in brain membranes and tissue sections, and that the potency and efficacy of cannabinoid agonists on G-protein activation can be determined under carefully controlled experimental conditions using brain membranes or autoradiography sections.

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