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An optimized approach to study endocannabinoid signaling: evidence against constitutive activity of rat brain adenosine A_1 and cannabinoid CB_1 receptors

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1 At nanomolar concentrations, SR141716 and AM251 act as specific and selective antagonists of the cannabinoid CB_1 receptor. In the micromolar range, these compounds were shown to inhibit basal G-protein activity, and this is often interpreted to implicate constitutive activity of the CB_1 receptors in native tissue. We show here, using [^{35}S]GTPγS binding techniques, that micromolar concentrations of SR141716 and AM251 inhibit basal G-protein activity in rat cerebellar membranes, but only in conditions where tonic adenosine A_1 receptor signaling is not eliminated.

2 Unlike lipophilic A_1 receptor antagonists (potency order DPCPX $\gg$ N-0840 $\approx$ cirsimarin $>$ caffeine), adenosine deaminase (ADA) was not fully capable in eliminating basal A_1 receptor-dependent G-protein activity. Importantly, all antagonists reduced basal signal to the same extent (20%), and the response evoked by the inverse agonist DPCPX was not reversed by the neutral antagonist N-0840. These data indicate that rat brain A_1 receptors are not constitutively active, but that an ADA-resistant adenosine pool is responsible for tonic A_1 receptor activity in brain membranes.

3 SR141716 and AM251, at concentrations fully effective in reversing CB_1-mediated responses (10^{-6} M), did not reduce basal G-protein activity, indicating that CB_1 receptors are not constitutively active in these preparations.

4 At higher concentrations ($1-2.5\times10^{-5}$ M), both antagonists reduced basal G-protein activity in control and ADA-treated membranes, but had no effect when A_1 receptor signaling was blocked with DPCPX. Moreover, the CB_1 antagonists right-shifted A_1 agonist dose–response curves without affecting maximal responses, suggesting competitive mode of antagonist action. The CB_1 antagonists did not affect muscarinic acetylcholine or $GABA_B$ receptor signaling.

5 When further optimizing G-protein activation assay for the labile endocannabinoid 2-arachidonoylglycerol (2-AG), we show, by using HPLC, that pretreatment of cerebellar membranes with methyl arachidonoyl fluorophosphonate (MAFP) fully prevented enzymatic degradation of 2-AG and concomitantly enhanced the potency of 2-AG. In contrast to previous claims, MAFP exhibited no antagonist activity at the CB_1 receptor.

6 The findings establish an optimized method with improved signal-to-noise ratio to assess endocannabinoid-dependent G-protein activity in brain membranes, under assay conditions where basal adenosinergic tone and enzymatic degradation of 2-AG are fully eliminated.
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Keywords: Adenosine A1 receptor; AM251; constitutive activity; cryptic adenosine; endocannabinoid; inverse agonist; monoacylglycerol lipase; SR141716

Abbreviations: AA-5HT, arachidonoyl serotonin; ADA, adenosine deaminase; AEA, arachidonoyl ethanolamide; 2-AG, 2-arachidonoylglycerol; 2-AGE, 2-arachidonoylglyceryl ether, noladin ether; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; ANOVA, analysis of variance; ATFMK, arachidonoyl trifluoromethylketone; BSA, bovine serum albumin; CB_1, central cannabinoid receptor; CCh, carbachol; ClAdo, 2-chloroadenosine; CP55,940, (−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol; DMSO, dimethyl sulfoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxantine; DTT, dithiothreitol; FAAH, fatty acid amide hydrolase; GTPγS, guanosine-5′-*O*-(3-thio)-triphosphate; HU-210, (6a*R*)-*trans*-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[b,d]pyran-9-methanol; MAFP, methyl arachidonoyl fluorophosphonate; MGL, monoacylglycerol lipase; PMSF, phenylmethylsulfonyl fluoride; [^{35}S]GTPγS, guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate; SR141716, *N*-piperidin-*O*-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; 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; WIN-55212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone

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Introduction

The compounds SR141716 and AM251 are highly potent and selective CB_1 receptor antagonists that bind to this receptor with K_i values around 10^{-8} M (Rinaldi-Carmona *et al*., 1994; 1995; Lan *et al*., 1999). In line with this, the two compounds inhibit CB_1-mediated responses with IC_{50} values in the low nanomolar concentration range (Rinaldi-Carmona *et al*., 1994; 1995; Landsman *et al*., 1997; Griffin *et al*., 1998; Savinainen *et al*., 2001). At micromolar concentrations, however, SR141716 in particular has been reported to inhibit basal G-protein signalling in native tissues (Sim-Selley *et al*., 2001; Bass *et al*., 2002; Mato *et al*., 2002; Ooms *et al*., 2002) and this has been variously interpreted to implicate inverse agonism, and therefore constitutive activity, of the CB_1 receptors in native environment. As our previous studies indicated little, if any, inverse agonism for micromolar concentrations of SR141716 and AM251 towards the CB_1 receptors in rat cerebellar membranes (Savinainen *et al*., 2001), one major goal for the present studies was to resolve inconsistencies regarding the occurrence of constitutively active CB_1 receptors, as detected using [^{35}S]GTPγS membrane-binding assay, in their native cellular environment. We have paid special attention to tonic adenosine A_1 receptor activity, as these receptors are abundant and widely distributed throughout the central nervous system and since previous studies have revealed tonic adenosine A_1 receptor-dependent G-protein activity in basal conditions of rat brain [^{35}S]GTPγS autoradiography (Laitinen & Jokinen, 1998; Laitinen, 1999; Moore *et al*., 2000).

Another goal for the present studies was to improve the existing methodology to assess the initial steps of endocannabinoid-dependent and CB_1 receptor-mediated signaling in brain membranes. Endocannabinoids, 2-arachidonoylglycerol (2-AG), arachidonoyl ethanolamide (AEA) and 2-arachidonoylglyceryl ether (2-AGE, also named as noladin ether or HU-310) are thought to be the principal endogenous ligands that bind and activate brain cannabinoid CB_1 receptors (Devane *et al*., 1992; Mechoulam *et al*., 1995; Sugiura *et al*., 1995; Hanus *et al*., 2001). In particular, 2-AG and AEA are labile compounds that are rapidly degraded by enzymatic activity in brain tissue preparations, most notably by fatty acid amide hydrolase (FAAH) or in the case of 2-AG, also by monoacylglycerol lipase (MGL) (for reviews, see Dinh *et al*., 2002a; Ueda, 2002). Previously, we have demonstrated that rat cerebellar membranes, in conditions of [^{35}S]GTPγS-binding assay suitable for the labile endocannabinoids, showed no enzymatic activity towards AEA or 2-AGE but that 2-AG was efficiently degraded to arachidonic acid (Savinainen *et al*., 2001). The degradation of 2-AG was substantially inhibited by pretreatment of membranes with the nonspecific serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Savinainen *et al*., 2001). Other studies have revealed that methyl arachidonoyl fluorophosphonate (MAFP) is clearly a more potent inhibitor of MGL and FAAH, than PMSF (Deutsch *et al*., 1997; Goparaju *et al*., 1999). In spite of this, MAFP is not being commonly used as an inhibitor of endocannabinoid degradation in G-protein-activation studies. One reason might be an observation that describes MAFP as an irreversible CB_1 antagonist (Fernando & Pertwee, 1997).

We demonstrate here that a basal tone of A_1 receptor-dependent G-protein activity is present in brain membrane preparations. This tone can be eliminated by the use of lipophilic A_1 receptor antagonists, but is only partially removed by treatment with the adenosine-depleting enzyme ADA, indicating that an ADA-resistant pool, rather than constitutive A_1 receptor activity, is responsible for the basal adenosinergic tone in membrane preparations. Further, we show that CB_1 receptors are not constitutively active in brain membrane preparations, and that in the micromolar range (10^{-5}–2.5×10^{-5} M), the CB_1 receptor antagonists SR141716 and AM251 act as competitive antagonists of A_1 receptors, thereby explaining the previously reported basal G-protein activity-decreasing property of these compounds in membrane [^{35}S]GTPγS-binding assays. Finally, we establish an optimized methodology to assess endocannabinoid-dependent and CB_1 receptor-mediated G-protein activity in brain membranes under conditions where signal-to-noise ratio is significantly improved due to elimination of tonic A_1 receptor activity, and where enzymatic degradation of endocannabinoids is fully prevented by treatment with MAFP. These studies also indicate that, under the assay conditions employed, MAFP has neither agonist nor antagonist activity at the CB_1 receptors.

Methods

Animals and preparation of rat cerebellar membranes

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 07:00 h), with water and food available *ad libitum*. The rats were decapitated, 8 h after lights on (15:00 h), whole brains were removed, cerebellum was cut off, dipped in isopentane on dry ice and stored thereafter at −80°C. Cerebellar membranes were prepared as previously described (Savinainen *et al*., 2001).

Chemicals

2-AG, MAFP, arachidonoyl serotonin (AA-5HT) and ATFMK were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). AEA and 2-AGE were synthesized at the Department of Pharmaceutical Chemistry, University of Kuopio. SR141716 and SR144528 were obtained from Sanofi Recherche (Montpellier, France). Cirsimarin was a generous gift from Professor Arnold Vlietinck and Dr John A. Hasrat (Department of Pharmaceutical Sciences, University of Antwerp, Belgium). CP55,940, AM251 and HU-210 were from Tocris Cookson Ltd (Bristol, U.K.). 2ClAdo, CCh, atropine, BSA (essentially fatty acid free), DTT, PMSF, GDP and GTPγS were purchased from Sigma (St Louis, MO, U.S.A.). ADA was purchased from Roche Diagnostics GmbH (Mannheim, Germany). DPCPX, WIN-55212-2 and R(+)-Baclofen HCl were from RBI/Sigma (Natick, MA, U.S.A.) and [^{35}S]GTPγS (initial specific activity 1250 Ci mmol^{-1}) from NEN Life Science Products, Inc. (Boston, MA, U.S.A.). All cannabinoids were dissolved in ethanol as 10^{-2} M stock solutions and stored at −80°C. SR141716, AM251 and SR144528 were dissolved in DMSO as 10^{-2} or 2×10^{-2} M stocks. The stock solution of 2-AG (initially in acetonitrile) was prepared just prior to experiments by evaporating the

organic solvent and reconstitution with ethanol. All other chemicals were of the highest purity available.

[^{35}S]GTPγS membrane-binding assay

Incubations were carried out as previously described (Savinainen *et al*., 2001). For experiments with the enzyme inhibitors, membranes were preincubated for 30 min at 25°C with PMSF, MAFP, ATFMK or AA-5HT (dissolved in DMSO, of which the final concentration in preincubation was 1.25% (vol vol^{-1}), or the vehicle as control in the presence of 0.5% (wt vol^{-1}) BSA. Preincubated membranes were kept at 0°C prior to experiments. ADA, DPCPX, N-0840, cirsimarin or caffeine (all antagonists were dissolved in DMSO, final concentration 0.5% vol vol^{-1}) in appropriate concentrations were included in the incubations to block the signaling of endogenous adenosine, as indicated in the results. Nonspecific binding was determined in the presence of 10^{-5} M GTPγS, and was subtracted from all other values. In a typical assay with fresh radioligand, basal and nonspecific binding was ~8500 and ~500 c.p.m., respectively. These values represented ~4 and ~0.2% of total radioactivity, respectively.

HPLC

In order to monitor 2-AG degradation, incubations mimicking [^{35}S]GTPγS membrane-binding assays were carried out, as previously described (Savinainen *et al*., 2001) with the following modifications. The concentration of 2-AG was 5×10^{-5} M. At time points of 0 and 90 min, 100 μl samples were removed from incubations, acetonitrile (200 μl) was added to stop the enzymatic reaction, and simultaneously pH of the samples was decreased with phosphoric acid to 3.0, in order to stabilize 2-AG against chemical acyl migration reaction yielding 1(3)-AG. Samples were centrifuged at 23,700 × *g* for 4 min at 20°C prior to HPLC analysis of the supernatant. The analytical HPLC system consisted of a Merck Hitachi (Hitachi Ltd, Tokyo, Japan) L-7100 pump, Merck Hitachi D-7000 interface module, Merck Hitachi L-7455 diode-array detector (190–800 nm, set at 211 nm) and a Merck Hitachi L-7250 programmable autosampler. The separations were performed with Zorbax SB-C18 endcapped reversed-phase precolumn (4.6 × 12.5 mm^2, 5 μm) and column (4.6 × 150 mm^2, 5 μm). The injection volume was 50 μl. A mobile phase mixture of a 28% phosphate buffer (30 mM, pH 3.0) in acetonitrile at a flow rate of 2.0 ml min^{-1} was used. The retention times were 5.8 min for 2-AG, 6.3 min for 1-AG and 10.2 min for arachidonic acid. The relative concentrations of 2-AG, 1(3)-AG and arachidonic acid were estimated on the basis of corresponding peak areas. This was justified by the equivalence of response factors of the compounds, which is supported by the observation that the sum of the peak areas was constant throughout the experiments.

Data analysis

For agonist dose-response, antagonist and HPLC experiments, results are presented as mean ± s.e.m. of at least three independent experiments performed in duplicate. Data analysis for dose–response curves were calculated as nonlinear 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.

Results

At micromolar concentrations, CB_1 receptor antagonists block adenosine A_1 receptors

As illustrated in Figure 1, AM251 and SR141716, at the concentration (10^{-6} M) capable of fully reversing CB_1 receptor-mediated responses (Rinaldi-Carmona *et al*., 1995; Landsman *et al*., 1997; Savinainen *et al*., 2001), had no effect on basal

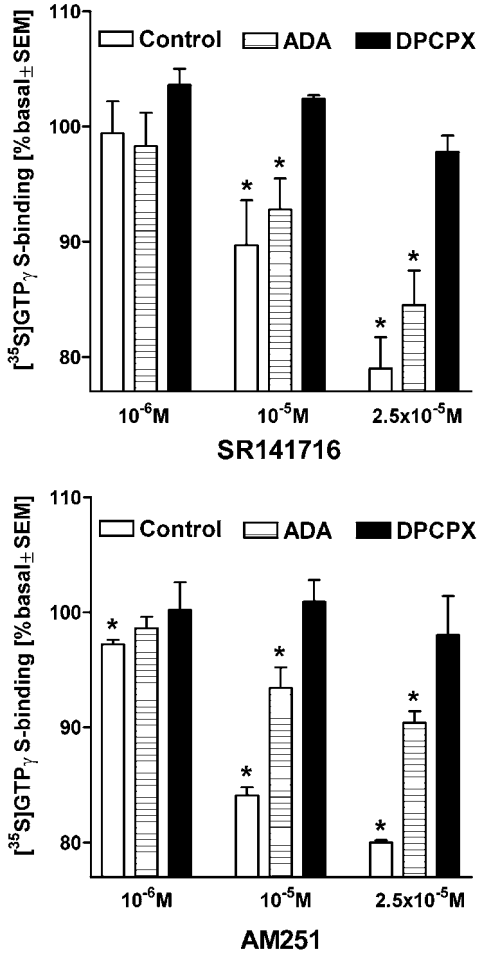

Figure 1 Low micromolar concentrations of the CB_1 receptor antagonists SR141716 and AM251 inhibit basal G-protein activity in untreated and ADA-treated rat cerebellar membranes, but have no effect in the presence of the adenosine A_1 receptor selective antagonist DPCPX. Following a 30-min preincubation in the absence (control and DPCPX conditions) or presence of ADA (0.5 U ml^{-1}), [^{35}S]GTPγS-binding assay was conducted, as described in methods, in control conditions or in the presence of ADA (0.5 U ml^{-1}) or DPCPX (10^{-6} M). Control incubations contained the vehicle for DPCPX (0.5% DMSO vol vol^{-1}). The vehicle for ADA (0.06% glycerol) did not affect basal binding (data not shown). The data represent the mean of [^{35}S]GTPγS binding over basal ± s.e.m. from three independent experiments performed in duplicate. An asterisk (*) denotes a statistically significant decrease ($P<0.05$).

G-protein activity in rat cerebellar membranes under the three incubation conditions tested (control, 0.5 U ml^{-1} ADA or 10^{-6} M DPCPX). In contrast, higher concentrations (10^{-5} and 2.5×10^{-5} M) of the CB_1 antagonists inhibited basal G-protein activity in a dose-dependent manner. The effect was most dramatic (~20%) in control conditions where no attempts were made to deplete endogenous adenosine by ADA treatment, or to block adenosine A_1 receptors by inclusion of the highly selective antagonist DPCPX.

In the presence of ADA, the antagonist effect was clearly reduced, and, in the presence of DPCPX, the antagonists no more affected basal [^{35}S]GTPγS binding at the used concentrations. These data indicate that, at the low micromolar concentration range (10^{-5}–2.5×10^{-5} M), the CB_1 receptor antagonists interact with A_1 receptors.

As shown in Figure 2, the dose–response curve for the adenosine receptor agonist 2-chloroadenosine was right-shifted in the presence of the two antagonists (−log $EC_{50} \pm$ s.e.m., $n = 3$), control 6.7±0.1; 10^{-5} M SR141716 6.4±0.1*; 10^{-5} M AM251 6.4±0.0* (the asterisk denotes significant difference ($P < 0.05$) as compared to control) with no change in maximal response (E_{max}, % basal ± s.e.m. ($n = 3$), control 347±9; 10^{-5} M SR141716 362±6; 10^{-5} M AM251 364±2), suggesting a competitive mode of antagonist action.

To explore the specificity of this action, we assessed the effects of AM251 and SR141716 (both at 10^{-5} M) on receptor-dependent G-protein activity following stimulation of $GABA_B$ and muscarinic acetylcholine receptors. We used the agonists baclofen and carbachol (CCh) at concentrations producing near half-maximal and maximal stimulation of $GABA_B$ and muscarinic receptors, respectively. As shown in Figure 3, the CB_1 antagonists had no effect on $GABA_B$- or muscarinic receptor-dependent responses. Additional control experiments indicated that similar concentrations of the CB_2 receptor selective antagonist SR144528 (Rinaldi-Carmona *et al*., 1998) did not affect A_1-, $GABA_B$ or muscarinic responses (data not shown), suggesting that the observed effects were specific for the A_1 receptors and CB_1 antagonists.

Collectively, these experiments demonstrate that the CB_1 receptors in rat brain membrane preparations are not constitutively active in the [^{35}S]GTPγS binding assay, and that the CB_1 receptor antagonist AM251 and SR141716, at micromolar concentrations, selectively antagonize adenosine A_1 receptor signaling.

Rat brain adenosine A_1 receptors are not constitutively active

We were surprised to see the somewhat differential behavior of the CB_1 receptor antagonists in ADA- *versus* DPCPX-treated

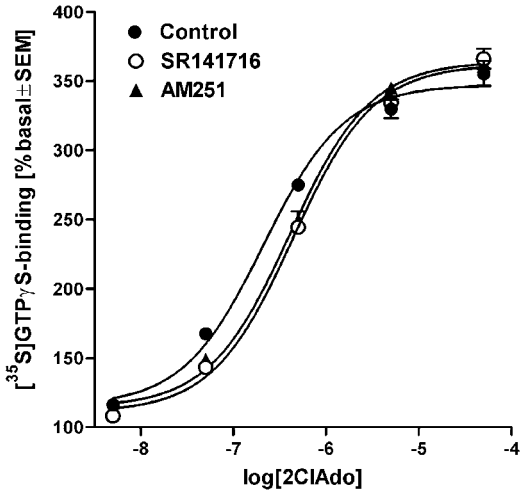

Figure 2 At micromolar concentrations, the CB_1 receptor antagonists inhibit adenosine A_1 receptor signaling. Rat cerebellar membranes were preincubated for 30 min in the presence of ADA (0.5 U ml^{-1}), and then incubated with increasing concentrations of the adenosine receptor agonist 2-chloroadenosine (2ClAdo) in the absence (control) or presence of the CB_1 antagonists SR141716 or AM251 (both at 10^{-5} M), as indicated. Vehicle for all conditions was 0.5% DMSO (vol vol^{-1}). The data represent the mean ± s.e.m. from three independent experiments performed in duplicate. When not visible, error bars fell within the size of the symbol.

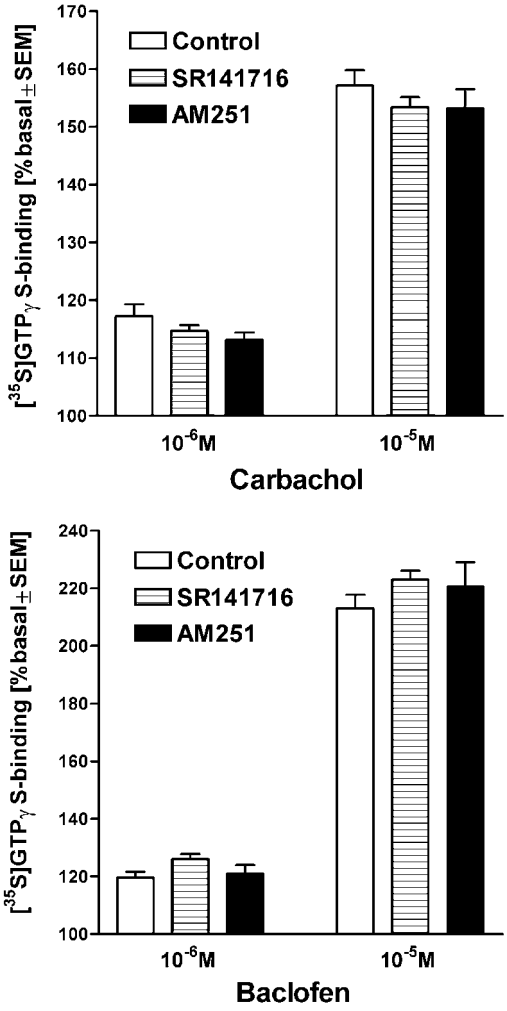

Figure 3 Micromolar concentrations of the CB_1 receptor antagonists do not affect mACh- or $GABA_B$ receptor-dependent G-protein activity. Rat cerebellar membranes were incubated with the muscarinic agonist CCh or the $GABA_B$ agonist baclofen at concentrations, producing near half-maximal and maximal stimulation (10^{-6} M and 10^{-5} M, respectively). The CB_1 antagonists were present at 10^{-5} M concentration and agonist responses were determined in the presence of 1 μM DPCPX, to block basal A_1 receptor-mediated G-protein activity. The data represent the mean ± s.e.m. from three independent experiments performed in duplicate.

membranes, as both treatments were previously found equally effective in eliminating the tonic and widespread A_1 receptor dependent G-protein activity in rat brain [^{35}S]GTPγS autoradiography studies (Laitinen, 1999). The outcome was essentially the same, regardless of the ADA concentration used (0.01–2 U ml^{-1}) (data not shown). Two possibilities were considered likely to explain this. Either A_1 receptors will gain constitutive activity in membrane preparation (in contrast to brain tissue sections) or an ADA-resistant pool of adenosine is formed in membrane preparation, as previously suggested (Prater *et al*., 1992). To resolve between these possibilities, we assessed the basal G-protein activity-lowering capacity of a panel of lipophilic adenosine receptor antagonists, including compounds with inverse agonist (DPCPX) and neutral antagonist (N-0840) properties at the A_1 receptors under heterologous expression (Shryock *et al*., 1998). As illustrated in Figure 4a, all adenosine receptor antagonists inhibited basal G-protein activity to the same extent (approximately 15%) in ADA-treated cerebellar membranes and with the expected pharmacology at the A_1 receptors (potency order: DPCPX $\gg$ N-0840 = cirsimarin > caffeine) (Shryock *et al*., 1998; Laitinen, 1999). When ADA was omitted from the incubations, the effect was even more pronounced (data not shown). Additional studies revealed that the combined effect of DPCPX (10^{-6} M) plus each of the three antagonists were non-additive and, more importantly, the neutral antagonist N-0840 (5×10^{-5} M) failed to even partially reverse the 'inverse agonism' of DPCPX (Figure 4b). These data are fully consistent with the artificial formation of ADA-resistant adenosine pool in membrane preparation (Prater *et al*., 1992), and strongly suggest that the rat brain A_1 receptors exhibit no constitutive activity in the absence of adenosine.

MAFP prevents 2-AG degradation and has no antagonist or agonistic activity at the CB_1 receptors

In order to find an inhibitor that would fully prevent enzymatic degradation of 2-AG in rat cerebellar membranes, various known inhibitors, including MAFP, arachidonoyl trifluoromethylketone (ATFMK), AA-5HT and PMSF, were tested in conditions of [^{35}S]GTPγS-binding assay. Initial studies revealed that MAFP, ATFMK and AA-5HT stimulated G-protein activity less than 106% basal at 10^{-5} and 10^{-6} M, except ATFMK which evoked a 112% basal response at 10^{-5} M. Moreover, in contrast to the results obtained with MAFP or PMSF (see Figure 5a), responses to 2-AG were not potentiated by pretreatment of membranes with ATFMK and AA-5HT (data not shown). Therefore, ATFMK and AA-5HT were not tested further in these experiments. As depicted in Figure 5a, membrane pretreatment with MAFP (10^{-5} M) or PMSF (10^{-3} M) significantly potentiated responses to 2-AG at 10^{-6} M, a concentration previously shown to produce near half-maximal G-protein activation (Savinainen *et al*., 2001). As further evident from Figure 5a, MAFP was significantly more effective than PMSF, although it was used at a 100-fold smaller concentration. This is consistent with previous findings demonstrating that MAFP potently (IC_{50} ~3 nM) inhibits brain 2-AG hydrolzing enzymatic activity (Goparaju *et al*., 1999). Indeed, our recent work indicates that MAFP is ~70,000-fold more potent than PMSF in inhibiting 2-AG hydrolyzing activity in this preparate (S.M. Saario *et al*. manuscript submitted).

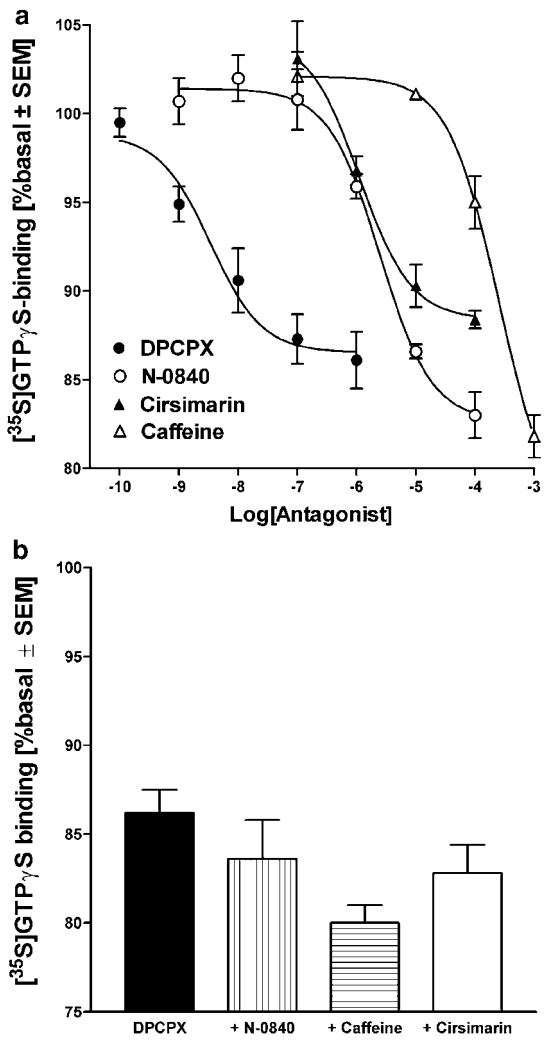

Figure 4 Lipophilic adenosine receptor antagonists decrease basal G-protein activity in rat cerebellar membranes to the same extent and with the potency expected at A_1 receptors (a). Membranes were incubated in the presence of ADA (0.5 U ml^{-1}) and the indicated concentrations of the antagonists, as described in Methods (b). Inhibitory responses to the inverse A_1 receptor agonist DPCPX (10^{-6} M) cannot be reversed by the neutral A_1 antagonist N-0840 (5×10^{-5} M) or other A_1 antagonists, cirsimarin (5×10^{-5} M) and caffeine (5×10^{-4} M). The vehicle for all conditions was 0.5% DMSO (vol vol^{-1}). The data represent the mean ± s.e.m. from at least three independent experiments performed in duplicate.

We wished to monitor, by using HPLC, the enzymatic degradation of 2-AG in parallel incubations closely mimicking conditions of the G-protein activation assay (Figure 5b). In the buffer system without tissue, 2-AG is spontaneously isomerized to 1(3)-AG. We have previously shown that 2-AG is more potent and more efficacious than 1(3)-AG in stimulating CB_1-dependent G-protein activity in this preparate (Savinainen *et al*., 2001). Upon addition of membranes, enzymatic activity hydrolyzes both 2-AG and 1(3)-AG, generating a single end product that co-eluted at the position of arachidonic acid (AA) (Savinainen *et al*., 2001). Our present work also confirmed this, as the material eluting with the retention times of 2-AG, 1(3)-AG and AA represented practically 100% of intial material (data not shown). Consistent with the data on G-protein activation assay, HPLC analysis revealed that

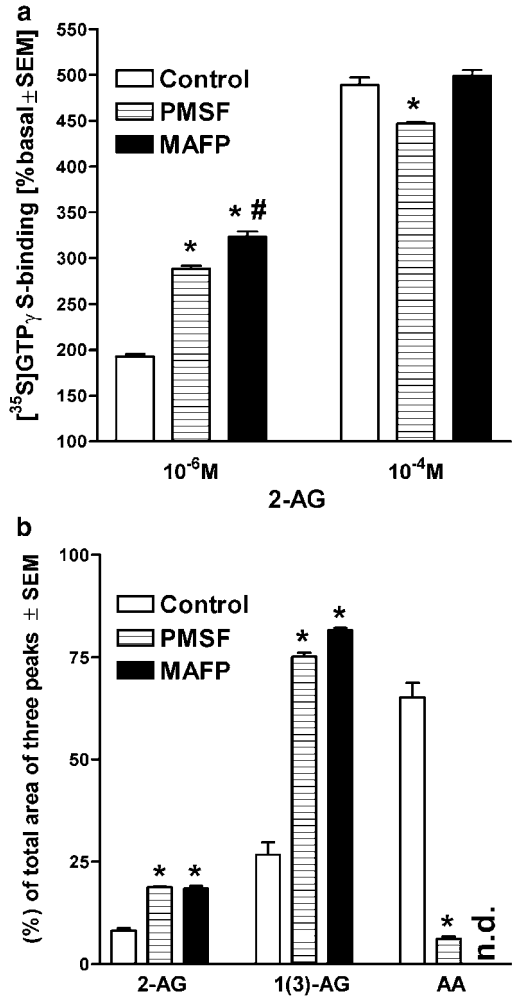

Figure 5 Pretreatment of rat cerebellar membranes with MAFP concomitantly potentiates 2-AG-stimulated G-protein activity (a) and prevents enzymatic degradation of 2-AG to AA more efficiently than PMSF (b). Membranes were pretreated with MAFP (10^{-5} M), PMSF (10^{-3} M) or the vehicle (DMSO) as a control for 30 min at $+25^{\circ}$C in the presence of 0.5% BSA. In (a), membranes were used for [^{35}S]GTPγS-binding assay to determine G-protein activation in response to 2-AG concentrations, producing near half-maximal (10^{-6} M) or maximal stimulation (10^{-4} M). In (b), pretreated membranes were used for HPLC to assess enzymatic hydrolysis of 2-AG (5×10^{-5} M) under incubation conditions closely mimicking G-protein activation assay. By HPLC analysis, initial purity of 2-AG was 98% with the rest of the material (2%) representing 1(3)-AG. For (a), the data represent the mean$\pm$s.e.m. of [^{35}S]GTPγS binding from basal and, for (b), the mean$\pm$s.e.m. of relative (%) peak areas, each from at least three independent experiments performed in duplicate. n.d.: not detectable. An asterisk (*) denotes the statistically significant ($P<0.05$) difference from the respective control; # indicates statistically the significant ($P<0.05$) difference between MAFP and PMSF treatment.

enzymatic degradation of 2-AG was totally prevented with 10^{-5} M MAFP and largely so also with 10^{-3} M PMSF (Figure 5b). In contrast to 2-AG, AEA and 2-AGE were not degraded, not even in control conditions (Savinainen *et al*., 2001; data not shown).

Of note, [^{35}S]GTPγS-binding studies revealed that membrane preincubation with PMSF had a small, but statistically significant inhibitory effect on 2-AG- and HU-210-evoked maximal responses (Figure 5a; data not shown). This can be

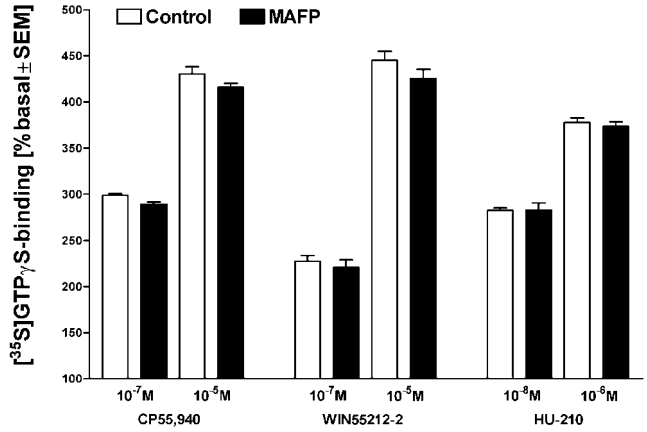

Figure 6 MAFP has no antagonist activity towards cannabinoid CB_1 receptor-dependent G-protein activity in rat cerebellar membranes. Membranes were pretreated with MAFP (10^{-5} M) or solvent (DMSO) as control for 30 min at $+25^{\circ}$C in the presence of 0.5% BSA. The cannabinoid agonists were tested near to their EC_{50} and E_{max} concentrations to reveal possible antagonism. The data represent the mean$\pm$s.e.m. from at least three independent experiments performed in duplicate.

partly explained by the small increase (16 ± 1%; s.e.m., $n=3$) of basal [^{35}S]GTPγS binding in the presence of PMSF. On the other hand, MAFP pretreatment (10^{-5} M) also slightly increased basal G-protein activity (8 ± 1% s.e.m., $n=7$), but concomitantly did not blunt maximal agonist responses (Figures 5a and 6).

Previously, MAFP was reported to behave as an irreversible antagonist of the CB_1 receptors (Fernando & Pertwee, 1997). To clarify whether MAFP exhibits antagonistic effects in rat cerebellar membranes, we assessed responses to the well-established cannabinoid agonists HU-210, CP55,940 and WIN55212-2 representing diverse chemical structures near to their EC_{50} and E_{max} values following treatment of membranes with MAFP. As illustrated in Figure 6, MAFP had no inhibitory effect on cannabinoid agonist-stimulated G-protein activity.

An optimized method to assess endocannabinoid-dependent G-protein activity

Based on the above results, we finally determined dose–response curves for to the three endocannabinoids (2-AG, AEA and 2-AGE) and the stable cannabinoid CP55,940 in the presence of MAFP (10^{-5} M in preincubation) and DPCPX (10^{-6} M). As shown in Figure 7 and Table 1, 2-AG was the most efficacious agonist among the tested compounds, producing a maximal response of 6.2-fold basal. CP55,940, AEA and 2-AGE all behaved as partial agonists by generating responses of 5.1-, 4.8-, and 4.2-fold basal, respectively. CP55,940 was the most potent CB_1 agonist (EC_{50} $\sim 7.5 \times 10^{-8}$ M), whereas the EC_{50} values for 2-AG was $\sim 10^{-6}$ M. AEA and 2-AGE were approximately equipotent in these experiments (EC_{50} $\sim 6 \times 10^{-6}$ M). Since 2-AG is more potent than 1(3)-AG in [^{35}S]GTPγS-binding assay and since it is continuously isomerized to 1(3)-AG during the 90-min incubation (Savinainen *et al*., 2001), we tested whether a shorter incubation time (10 min) would further increase the potency for 2-AG. These experiments (not shown) revealed,

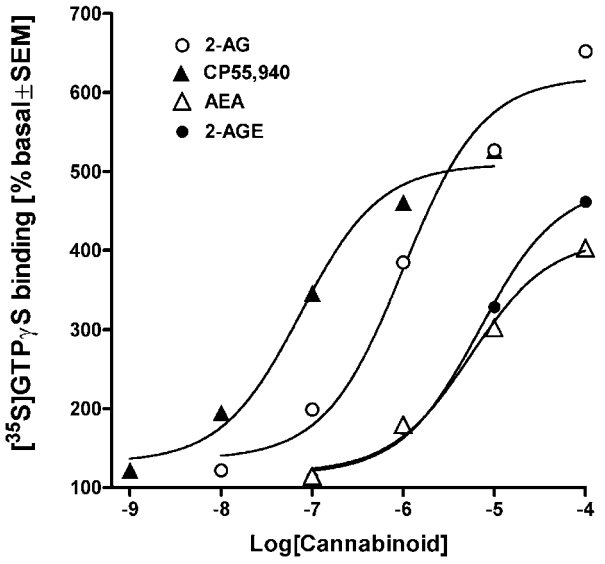

Figure 7 Dose–response curves to various cannabinoid agonists in optimized assay conditions where tonic adenosine A_1 receptor activity and enzymatic degradation of 2-AG is fully eliminated. Rat cerebellar membranes were pretreated with 10^{-5} M MAFP in the presence of 0.5% BSA, as described in Methods. [^{35}S]GTPγS-binding assay was conducted for 90 min at 25°C in the presence of 10^{-6} M DPCPX. The data represent the mean±s.e.m. from three independent experiments performed in duplicate. When not visible, error bars fell within the size of the symbol. AEA, arachidonoyl ethanolamide; 2-AG, 2-arachidonoylglycerol; 2-AGE, 2-arachidonoylglyceryl ether; CP55,940, (−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol.

Table 1 Comparison of the efficacy (E_{max}) and potency (pEC_{50}) of the four cannabinoid agonists tested in MAFP- (10^{-5} M) pretreated rat cerebellar membranes in the presence of 10^{-6} M DPCPX

Compound	E_{max} *(%Basal±s.e.m.)*	pEC_{50}*±s.e.m.*
2-AG	620±5	6.0±0.0
CP55,940	510±4	7.1±0.0
2-AGE	484±7	5.2±0.0
AEA	415±3	5.3±0.1

EC_{50} and E_{max} values were calculated from the [^{35}S]GTPγS-binding experiments depicted in Figure 7. Values are means±s.e.m. from three independent experiments performed in duplicate.

however, that the potency of 2-AG was not further increased using shorter incubation times. Similarly, the potency of the stable cannabinoid CP55,940 remained unchanged.

Discussion

Tonic signaling by an endogenous compound bears direct relevance to the issue of constitutive receptor activity which, by definition, means receptor activity in the absence of activating ligand (for review see Seifert & Wenzel-Seifert, 2002). Recent mutation studies have revealed crucial amino-acid residues responsible for constitutive activity and inverse agonism at cannabinoid CB_1 receptors under heterologous expression (Nie & Lewis, 2001; Hurst *et al*., 2002). Nevertheless, it is still unresolved whether constitutive activity is present in native tissues. Some previous studies have concluded that the CB_1 receptors are constitutively active also in brain tissue (Bass *et al*., 2002; Mato *et al*., 2002; Ooms *et al*., 2002). These observations were based on the effects of micromolar concentrations of the CB_1 receptor antagonist and inverse agonist, SR141716, on basal G-protein activity. Importantly, similar inhibitory effects of SR141716 at these concentrations were also reported in brain membranes of CB_1 knockout mice, indicating non-CB_1 receptor-dependent actions (Breivogel *et al*., 2001).

We demonstrated here that micromolar concentrations of SR141716 and its structural derivative AM251 (Lan *et al*., 1999) inhibited adenosine A_1 receptor-, but not muscarinic or $GABA_B$ receptor-mediated G-protein activity in brain membranes. The inhibition of basal [^{35}S]GTPγS binding by the CB_1 antagonists was most evident in untreated membranes, still present in ADA-treated membranes but not detected in incubations with the selective A_1 receptor antagonist DPCPX. These data directly indicate that, at the low micromolar range, the CB_1 antagonists can antagonize A_1 receptor activity.

Some laboratories routinely include ADA in membrane [^{35}S]GTPγS-binding assays (Breivogel *et al*., 1998; Savinainen *et al*., 2001; Rouleau *et al*., 2002), but mainly such studies are conducted without any attempts to eliminate endogenous adenosine activity. Since ADA and DPCPX were found to be equally effective in decreasing the basal adenosinergic tone in rat brain [^{35}S]GTPγS autoradiography studies (Laitinen, 1999), we were rather surprised to learn that ADA was not fully competent in the membrane preparations.

An obvious explanation for this differential outcome emerges from the findings of Prater *et al*. (1992), who have demonstrated that, in membrane preparations, a cryptic adenosine pool is trapped in compartments that are not accessible to ADA. In agreement, we found that all the lipophilic adenosine receptor antagonists of this study, including the inverse agonist DPCPX and the neutral antagonist N-0840, inhibited basal [^{35}S]GTPγS binding to the same extent, even in the presence of ADA.

It was previously demonstrated that the inverse agonism at constitutively active A_1 receptors could be reversed by neutral antagonists (Shryock *et al*., 1998). Based on these findings, we tested further whether N-0840 could reverse the inhibitory response evoked by DPCPX. As was clearly shown, this was not the case, indicating therefore that tonic A_1 receptor activity is not constitutive, but is mediated by an ADA-resistant pool of adenosine.

So far, various enzymes participating in the degradation of 2-AG have been established, with MGL and FAAH being the most prominent candidates (for reviews see, Dinh *et al*., 2002a; Ueda, 2002). Very recently, Dinh *et al*. (2002b) provided strong evidence that MGL is the primary enzyme degrading 2-AG in brain tissue and, concomitantly, is not capable of degrading AEA. In contrast, FAAH also degrades AEA (Ueda, 2002). Previously, we reported that 2-AG, but not AEA or 2-AGE, was degraded by rat cerebellar membranes, and that this degradation was substantially (~80%) inhibited by PMSF (Savinainen *et al*., 2001). Therefore, we concluded that, under the assay conditions employed, FAAH activity was not apparent and additional enzymatic activity, possibly MGL, was responsible for 2-AG degradation. This led us to test more selective and efficacious inhibitors, such as MAFP, which has been shown to be a potent inhibitor of 2-AG degradation

(Goparaju *et al*., 1999). Contrary to this status, MAFP has also been described as an irreversible CB_1 receptor antagonist (Fernando & Pertwee, 1997). As clearly shown here, MAPF had absolutely no antagonist activity towards the CB_1 receptor when tested against compounds representing four major classes of CB_1 receptor agonists. It was also clearly demonstrated that MAFP totally prevented enzymatic degradation of 2-AG. These results show that MAFP, when used under the presently defined conditions, is the inhibitor of choice to prevent endocannabinoid degradation without any disturbing side effects. In contrast, ATFMK and AA-5HT, both of which were previously described as novel FAAH inhibitors (Koutek *et al*., 1994; Bisogno *et al*., 1998), potentiated 2-AG responses only marginally and, moreover, had small stimulatory effects on G-protein activity of their own at higher concentrations.

We anticipated that pretreatment of cerebellar membranes with hydrophobic arachidonic acid derivatives, such as MAFP, must be performed in the presence of BSA. Interestingly, these experiments also revealed that basal G-protein activity was slightly increased both in MAFP- and PMSF-pretreated membranes (8 and 16%, respectively), as compared with control. This may be taken as an indication of reduced degradation of endocannabinoids or other endogenous agonists that can stimulate G-protein activity. Whether endocannabinoids and/or other endogenous ligands are indeed generated under the presently defined assay conditions in sufficient quantities to stimulate [^{35}S]GTPγS binding remains an interesting question for future studies.

Since MAFP and DPCPX were identified as the compounds of choice to fully prevent enzymatic degradation of 2-AG and to fully eliminate tonic A_1 receptor activity, respectively, these compounds are now routinely included in our assay protocols assessing CB_1 receptor-mediated G-protein activity in rat cerebellar membranes. When comparing the potencies of the cannabinoids from this study with those reported previously (Savinainen *et al*., 2001), all cannabinoids now exhibit slightly increased potency, but, as expected, the effect is most dramatic in the case of 2-AG. Furthermore, maximal responses to all cannabinoids under the optimized conditions are throughout higher than in any previously published study assessing CB_1 receptor-dependent G-protein activity. These effects on agonist dose responses can be explained by the following reasons. First, inclusion of DPCPX decreases basal G-protein activity by blocking the basal adenosinergic tone. Since agonist responses are expressed as % basal, the inclusion of DPCPX results in improved signal-to-noise ratio for the [^{35}S]GTPγS-binding assay, thus allowing detection of higher maximal responses for all other studied GPCRs, including the CB_1. Secondly, the presence of 0.5% BSA with the hydrophobic inhibitor (MAFP) in membrane preincubations, besides fully preventing enzymatic degradation of 2-AG, may additionally produce 'an entourage-like-effect', where binding of the lipophilic agonists to non-CB_1 receptor sites is minimized.

To conclude, we have demonstrated here that two abundant and widely distributed GPCRs in the brain tissue, cannabinoid CB_1 and adenosine A_1 receptors, are not constitutively active in membrane [^{35}S]GTPγS-binding assays. Instead, a cryptic ADA-resistant adenosine pool is responsible for a tonic adenosinergic G-protein activity in basal conditions of this technique. We further demonstrated that micromolar concentrations of the commonly used CB_1 antagonists act as competitive antagonists of the A_1 receptor. Finally, an optimized method to detect endocannabinoid-evoked and CB_1 receptor-mediated G-protein activity was described.

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References

BASS, C.E., GRIFFIN, G., GRIER, M., MAHADEVAN, A., RAZDAN, R.K. & MARTIN, B.R. (2002). SR-141716A-induced stimulation of locomotor activity A structure–activity relationship study. *Pharmacol. Biochem. Behav.,* **74,** 31–40.

BISOGNO, T., MELCK, D., DE PETROCELLIS, L., BOBROV, M.Y., GRETSKAYA, N.M., BEZUGLOV, V.V., SITACHITTA, N., GERWICK, W.H. & DI MARZO, V. (1998). Arachidonoylserotonin and other novel inhibitors of fatty acid amide hydrolase. *Biochem. Biophys. Res. Commun.,* **248,** 515–522.

BREIVOGEL, C.S., GRIFFIN, G., DI MARZO, V. & MARTIN, B.R. (2001). Evidence for a new G-protein-coupled cannabinoid receptor in mouse brain. *Mol. Pharmacol.,* **60,** 155–163.

BREIVOGEL, C.S., SELLEY, D.S. & CHILDERS, S.R. (1998). Cannabinoid receptor agonist efficacy for stimulating [^{35}S]GTPγS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. *J. Biol. Chem.,* **273,** 16865–16873.

DEUTSCH, D.G., OMEIR, R., ARREAZA, G., SALEHANI, D., PRESTWICH, G.D., HUANG, Z. & HOWLETT, A. (1997). Methyl arachidonyl fluorophosphonate: a potent irreversible inhibitor of anandamide amidase. *Biochem. Pharmacol.,* **53,** 255–260.

DEVANE, W.A., HANUS, L., BREUER, A., PERTWEE, R.G., STEVENSON, L.A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science,* **258,** 1946–1949.

DINH, T.P., CARPENTER, D., LESLIE, F.M., FREUND, T.F., KATONA, I., SENSI, S.L., KATHURIA, S. & PIOMELLI, D. (2002a). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc. Natl. Acad. Sci. USA.,* **99,** 10819–10824.

DINH, T.P., FREUND, T.F. & PIOMELLI, D. (2002b). A role for monoglyceride lipase in 2-arachidonoylglycerol inactivation. *Chem. Phys. Lipids,* **121,** 149–158.

FERNANDO, S.R. & PERTWEE, R.G. (1997). Evidence that methyl arachidonyl fluorophosphonate is an irreversible cannabinoid receptor antagonist. *Br. J. Pharmacol.,* **121,** 1716–1720.

GOPARAJU, S.K., UEDA, N., TANIGUCHI, K. & YAMAMOTO, S. (1999). Enzymes of porcine brain hydrolysing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. *Biochem. Pharmacol.,* **57,** 417–423.

GRIFFIN, G., ATKINSON, P.J., SHOWALTER, V.M., MARTIN, B.R. & ABOOD, M.E. (1998). Evaluation of cannabinoid receptor agonists and antagonists using the guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate binding assay in rat cerebellar membranes. *J. Pharmacol. Exp. Ther.,* **285,** 553–560.

HANUS, L., ABU-LAFI, S., FRIDE, E., BREUER, A., VOGEL, Z., SHALEV, D.E., KUSTANOVICH, I. & MECHOULAM, R. (2001). 2-Arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB_1 receptor. *Proc. Natl. Acad. Sci. USA.,* **98,** 3662–3665.

HURST, D.P., LYNCH, D.L., BARNETT-NORRIS, J., HYATT, S.M., SELTZMAN, H.H., ZHONG, M., SONG, Z.-H., NIE, J., LEWIS, D. & REGGIO, P.H. (2002). *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A) interaction with LYS 3.28(192) is crucial for its inverse agonism at the cannabinoid CB1 receptor. *Mol. Pharmacol.,* **62,** 1274–1287.

KOUTEK, B., PRESTWICH, G.D., HOWLETT, A.C., CHIN, S.A., SALEHANI, D., AKHAVAN, N. & DEUTSCH, D.G. (1994). Inhibitors of arachidonoyl ethanolamide hydrolysis. *J. Biol. Chem.,* **269,** 22937–22940.

LAITINEN, J.T. (1999). Selective detection of adenosine A_1 receptor-dependent G-protein activity in basal and stimulated conditions of rat brain [^{35}S]guanosine 5′(γ-thio)triphosphate autoradiography. *Neuroscience,* **90,** 1265–1279.

LAITINEN, J.T. & JOKINEN, M. (1998). Guanosine 5′-(gamma-[^{35}S]thio)triphosphate autoradiography allows selective detection of histamine H_3 receptor-dependent G-protein activation in rat brain tissue sections. *J. Neurochem.,* **71,** 808–816.

LAN, R., LIU, Q., FAN, P., LIN, S., FERNANDO, S.R., MCCALLION, D., PERTWEE, R. & MAKRIYANNIS, A. (1999). Structure–activity relationships of pyrazole derivatives as cannabinoid receptor antagonists. *J. Med. Chem.,* **42,** 769–776.

LANDSMAN, R.S., BURKEY, T.H., CONSROE, P., ROESKE, W.R. & YAMAMURA, H.I. (1997). SR141716A is an inverse agonist at the human cannabinoid CB_1 receptor. *Eur. J. Pharmacol.,* **334,** R1–R2.

MATO, S., PAZOS, A. & VALDIZÁN, E.M. (2002). Cannabinoid receptor antagonism and inverse agonism in response to SR141716A on cAMP production in human and rat brain. *Eur. J. Pharmacol.,* **443,** 43–46.

MECHOULAM, R., BEN-SHABAT, S., HANUS, L., LIGUMSKY, M., KAMINSKI, N.E., SCHATZ, A.R., GOPHER, A., ALMOG, S., MARTIN, B.R., COMPTON, D.R., PERTWEE, R.G., GRIFFIN, G., BAYEWHICH, M., BARG, J. & VOGEL, Z. (1995). Identification of an endogenous 2-monoglyceride, present in canine cut, that binds to cannabinoid receptors. *Biochem. Pharmacol.,* **50,** 83–90.

MOORE, R.J., XIAO, R., SIM-SELLEY, L.J. & CHILDERS, S.R. (2000). Agonist-stimulated [^{35}S]GTPgammaS binding in brain modulation by endogenous adenosine. *Neuropharmacology,* **39,** 282–289.

NIE, J. & LEWIS, D.L. (2001). Structural domains of the CB1 cannabinoid receptor that contribute to constitutive activity and G-protein sequestration. *J. Neurosci.,* **21,** 8758–8764.

OOMS, F., WOUTERS, J., OSCARI, O., HAPPAERTS, T., BOUCHARD, G., CARRUPT, P.A., TESTA, B. & LAMBERT, D.M. (2002). Exploration of the pharmacophore of 3-alkyl-5-arylimidazolidinediones as new CB_1 cannabinoid receptor ligands and potential antagonists: synthesis, lipophilicity, affinity, and molecular modeling. *J. Med. Chem.,* **45,** 1748–1756.

PRATER, M.R., TAYLOR, H., MUNSHI, R. & LINDEN, J. (1992). Indirect effect of guanine nucleotides on antagonist binding to A_1 adenosine receptors: occupation of cryptic binding sites by endogenous vesicular adenosine. *Mol. Pharmacol.,* **42,** 765–772.

RINALDI-CARMONA, M., BARTH, F., HEAULME, M., ALONSO, R., SHIRE, D., CONGY, C., SOUBRIE, P., BRELIERE, J.C. & LE FUR, G. (1995). Biochemical and pharmacological characterization of SR141716A, the first potent and selective brain cannabinoid receptor antagonist. *Life Sci.,* **56,** 1941–1947.

RINALDI-CARMONA, M., BARTH, F., HEAULME, M., SHIRE, D., CALANDRA, B., CONGY, C., MARTINEZ, S., MARUANI, J., NELIAT, G., CAPUT, D., FERRARA, P., SOUBRIE, P., BRELIERE, J.C. & LE FUR, G. (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.,* **350,** 240–244.

RINALDI-CARMONA, M., BARTH, F., MILLAN, J., DEROCQ, J.-M., CASELLAS, P., CONGY, C., OUSTRIC, D., SARRAN, M., BOUABOULA, M., CALANDRA, B., PORTIER, M., SHIRE, D., BRELIERE, J.-C. & LE FUR, G. (1998). SR144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J. Pharmacol. Exp. Ther.,* **284,** 644–650.

ROULEAU, A., LIGNEAU, X., TARDIVEL-LACOMBE, J., MORISSET, S., GBAHOU, F., SCHWARTZ, J.-C. & ARRANG, J.-M. (2002). Histamine H_3-receptor-mediated [^{35}S]GTPγS binding: evidence for constitutive activity of the recombinant and native rat and human H_3 receptors. *Br. J. Pharmacol.,* **135,** 383–392.

SAVINAINEN, J.R., JÄRVINEN, T., LAINE, K. & LAITINEN, J.T. (2001). Despite substantial degradation, 2-arachidonoylglycerol is a potent full efficacy agonist mediating CB1 receptor-dependent G-protein activation in rat cerebellar membranes. *Br. J. Pharmacol.,* **134,** 664–672.

SEIFERT, R. & WENZEL-SEIFERT, K. (2002). Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **366,** 381–416.

SHRYOCK, J.C., OZECK, M.J. & BELARDINELLI, L. (1998). Inverse agonists and neutral antagonists of recombinant human A1 adenosine receptors stably expressed in chinese hamster ovary cells. *Mol. Pharmacol.,* **53,** 886–893.

SIM-SELLEY, L.J., BRUNK, L.K. & SELLEY, D.E. (2001). Inhibitory effects of SR141716A on G-protein activation in rat brain. *Eur. J. Pharmacol.,* **414,** 135–143.

SUGIURA, T., KONDO, S., SUKAGAWA, A., NAKANE, S., SHINODA, A., ITOH, K., YAMASHITA, A. & WAKU, K. (1995). 2-arachidonoylglycerol: A possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.,* **215,** 89–97.

UEDA, N. (2002). Endocannabinoid hydrolases. *Prostag. Oth. Lipid M.,* **68-69,** 521–534.

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