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N-Arachidonyl Maleimide Potentiates the Pharmacological and Biochemical Effects of the Endocannabinoid 2-Arachidonylglycerol through Inhibition of Monoacylglycerol Lipase

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ABSTRACT

Inhibition of the metabolism of the endocannabinoids, anandamide (AEA) and 2-arachidonyl glycerol (2-AG), by their primary metabolic enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively, has the potential to increase understanding of the physiological functions of the endocannabinoid system. To date, selective inhibitors of FAAH, but not MAGL, have been developed. The purpose of this study was to determine the selectivity and efficacy of *N*-arachidonyl maleimide (NAM), a putative MAGL inhibitor, for modulation of the effects of 2-AG. Our results showed that NAM unmasked 2-AG activity in a tetrad of in vivo tests sensitive to the effects of cannabinoids in mice. The efficacy of 2-AG (and AEA) to produce hypothermia was reduced compared with Δ^9-tetrahydrocannabinol; however, 2-AG differed from AEA by its lower efficacy for catalepsy. All tetrad effects were partially CB_1 receptor-mediated because they were attenuated (but not eliminated) by SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*H*-pyrazole-3-carboxamide HCl] and in $CB_1^{-/-}$ mice. In vitro, NAM increased endogenous levels of 2-AG in the brain. Furthermore, NAM raised the potency of 2-AG, but not AEA, in agonist-stimulated guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding assay, a measure of G-protein activation. These results suggest that NAM is an MAGL inhibitor with in vivo and in vitro efficacy. NAM and other MAGL inhibitors are valuable tools to elucidate the biological functions of 2-AG and to examine the consequences of dysregulation of this endocannabinoid. In addition, NAM's unmasking of 2-AG effects that are only partially reversed by SR141716A offers support for the existence of non-CB_1, non-CB_2 cannabinoid receptors.

The endocannabinoid system is comprised of two main receptors and various endogenous ligands. The CB_1 cannabinoid receptor is found in both the CNS and periphery and is believed to interact with and modulate various neurotransmitter systems (Howlett, 2002; Szabo and Schlicker, 2005). The CB_2 cannabinoid receptor is found principally in the immune system (Pertwee, 1997), although recent reports suggest that it may also be present in the brain stem (Van Sickle et al., 2005). To date, the two main cannabinoid ligands that have been isolated from the brain are anandamide (AEA) and 2-arachidonylglycerol (2-AG) (Hillard, 2000). Discovery and isolation of these ligands have led to significant advances in the cannabinoid field, ranging from the possible therapeutic application of endocannabinoids to the physiological role of the endocannabinoid system.

Despite these advances, understanding the full role of these ligands has proven difficult because of their extremely short biological half-life, which is mediated by degradation

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ABBREVIATIONS: CNS, central nervous system; AEA, anandamide; 2-AG, 2-arachidonylglycerol; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; URB597, (3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate; URB602, [1,1′-biphenyl]-3-yl-carbamic acid, cyclohexyl ester; MAFP, methyl arachidonyl fluorophosphonate; NAM, *N*-arachidonyl maleimide; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*H*-pyrazole-3-carboxamide HCl; SR144528, *N*-((1*S*)-endo-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide); WIN 55,212-2, *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; BSA, bovine serum albumin; ANOVA, analysis of variance; THC, Δ^9-tetrahydrocannabinol; GPR, G protein-coupled receptor.

enzymes (Laine et al., 2002). The enzymes primarily responsible for inactivation of AEA and 2-AG are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (Basavarajappa, 2007). To study these endocannabinoid inactivation pathways, significant work has been undertaken to develop selective enzyme inhibitors. To date, there has been some success with developing potent and selective inhibitors of FAAH. For example, the use of the FAAH inhibitor URB597 has revealed a potential role for AEA degradation inhibitors in the treatment of chronic pain (Jayamanne et al., 2006).

In contrast, the development of inhibitors of MAGL has been slower, in part because of the fact that most previous research focused on AEA, the first endocannabinoid to be discovered (Devane et al., 1992). However, recent research has indicated the importance of 2-AG in various physiological processes, including appetite regulation, energy balance, and stress-induced opioid-independent analgesia (Hohmann et al., 2005; Cota, 2007). Other studies have shown that 2-AG levels may be altered in pathological conditions such as celiac disease (D'Argenio et al., 2007). These converging lines of research have prompted renewed interest in developing inhibitors of 2-AG synthesis and inactivation. This increased focus on 2-AG may aid in the understanding of its physiological properties and in the discovery of potential therapeutic indications for 2-AG modulation.

Currently, two main compounds have been shown to inhibit 2-AG degradation: URB602 and methyl arachidonyl fluorophosphonate (MAFP) (Savinainen et al., 2003; Makara et al., 2005). Both of these compounds have significant limitations. In addition to inhibiting MAGL, MAFP inhibits FAAH, directly activates CB_1 receptors, and has noncannabinoid targets (Lio et al., 1996). Although URB602 is far more selective for MAGL than MAFP, the main limitations to use of this compound in vivo are low potency and solubility. The IC_{50} of URB602 for MAGL in mice is 28 μM, and its maximal solubility is approximately 1 mg/ml (Makara et al., 2005). These two factors prevent effective systemic administration of this compound.

Recent research with *N*-arachidonyl maleimide (NAM) is more promising. NAM prevented cerebellar membrane-mediated degradation of 2-AG at a relatively low concentration (IC_{50}, 140 nM) (Saario et al., 2005). Despite these initial results, there have been no reports of the effect of NAM on 2-AG action within in vivo systems. However, very recently, Blankman et al. (2007) showed that NAM inhibited up to 80% of 2-AG degradation, thus confirming the results of Saario et al. (2005). Based on this research and the fact that there is little information on the in vivo effects of NAM, the aims of this study were to examine NAM modulation of the tetrad effects of 2-AG (a four-factor test that includes suppression of spontaneous activity, antinociception, hypothermia, and catalepsy) (Martin et al., 1991), to determine the effects of NAM on CB_1 receptor binding and activation, to assess the selectivity of NAM for 2-AG versus AEA, and to examine the effect of NAM on endogenous 2-AG levels.

Materials and Methods

Subjects. Female ICR mice (outbred albino mouse strain developed by Dr. T.S. Hauschka, Fox Chase Cancer Center, Philadelphia, PA), purchased from Harlan (Indianapolis, IN), were housed five per cage. All animals were kept in a temperature-controlled (23°C) environment with a 12-h light/dark cycle (lights on at 7:00 AM). Separate mice (n = 6/group) were used for testing each drug dose in the in vivo procedures. The mice were free fed and had free access to water. The studies reported in this manuscript were carried out in accordance with guidelines published in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Chemicals. *N*-Arachidonyl maleimide was provided by Cayman Chemical (Ann Arbor, MI). SR141716A and SR144528 were provided by the Drug Supply Program of the National Institute of Drug Abuse (Rockville, MD). 2-AG (Organix identification no. O-1361) and AEA were synthesized in our labs (Organix, Inc., Woburn, MA). All compounds were dissolved in a vehicle of ethanol, Emulphor-620 (Sanofi-aventis, Bridgewater, NJ), and physiological saline in a ratio of 1:1:18. WIN 55,212-2, GDP, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Guanosine 5′-3-*O*-(thio)triphosphate was purchased from Roche Diagnostics (Indianapolis, IN). [^{35}S]GTPγS (1150–1300 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). [^{3}H]-SR141716A (44.0 Ci/mmol) was purchased from GE Healthcare (Chalfont St. Giles, UK). Scintillation fluid (ScinitSafe Econo 1) was purchased from Thermo Fisher Scientific (Waltham, MA). Adenosine deaminase was purchased from Sigma-Aldrich.

Apparatus. Measurement of spontaneous activity in mice occurred in square mouse chambers (20 × 20 × 20 cm) surrounded by panels of photocell beams (Open Field Activity System; Med Associate Inc., St. Albans, VT). A tail-flick apparatus and digital thermometer (Thermo Fisher Scientific) were used to measure antinociception and rectal temperature, respectively. The ring immobility device was constructed in the investigator's laboratory and consisted of a metal ring (diameter = 5.5 cm) centered at right angles to an elevated (height = 16 cm) board that was painted black. A Micromass Quattro II (Triple Quad) equipped with EI/CI Source, CPI (atmospheric pressure chemical ionization) with Megaflow and Nanoflow options was used to measure 2-AG levels.

In Vivo Procedures. Mice were acclimated to the experimental setting for 1 h before the first injection. Baseline values for rectal temperature (in degrees Celsius) and tail-flick latency (in seconds) were obtained immediately before any injection. After injection(s), each mouse was tested in two procedures (spontaneous activity and tail-flick or rectal temperature and ring immobility). Tail-flick latency or rectal temperature was measured at 6 min after the last injection. Antinociception was calculated as percentage of maximum possible effect: {((test − control time)/(10 − control time)) × 100}. To avoid damage to the tail, the ambient heat source was turned off after a 10-s maximal latency. Rectal temperature values were expressed as the difference between control temperature and temperature after drug administration. Five minutes after measurement of antinociception or rectal temperature, mice were placed in individual activity chambers, and spontaneous activity was measured for 10 min, or they were placed on the ring immobility apparatus for 5 min, respectively. Spontaneous activity was measured as total number of interruptions of 16 photocell beams/chamber during the 10-min test. During placement on the ring immobility apparatus, the total amount of time (in seconds) that the mouse remained motionless was measured. This value was divided by 300 s and multiplied by 100 to obtain a percent immobility rating. NAM, SR144528, and SR141716A were administered to the mice via intraperitoneal injection, 5 min before 2-AG was administered. 2-AG was injected intravenously via the tail vein 6 min before testing. Compounds were injected intraperitoneally or intravenously at a volume of 0.01 ml/g b.wt. (e.g., a 30-g mouse would receive an injection volume of 0.3 ml).

In Vitro Procedures. For all in vitro procedures, mice were sacrificed by decapitation, and the cerebellum was dissected out. Tissue was stored at −80°C until use.

Agonist-Stimulated [^{35}S]GTPγS Binding. Tissue was placed in 5 ml of cold membrane buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4) and homogenized. Endocannabinoid degradation inhibitors (0.1–50 μM) were then incubated with the homogenate for 30 min at 30°C to ensure that there was significant inhibition of FAAH/MAGL before 2-AG/AEA was added to the protein. The samples were then centrifuged at 50,000*g* at 5°C for 10 min. The supernatant was removed, and samples were resuspended in 5 ml of assay buffer A (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4). Protein concentration was determined by the Bradford method (Bradford, 1976). Before assay, membranes (4–8 μg of protein) were preincubated for 25 min at 30°C with adenosine deaminase (3 mU/ml) in assay buffer. Concentration-effect curves were generated by incubating the appropriate amount of membrane protein (4–8 μg) in assay buffer B (assay buffer A plus 1.25 g/l BSA) with 0.1 to 60 μM cannabinoid WIN/AEA/2-AG plus inhibitors (20–300 nM) in the presence of 30 μM GDP and 0.1 nM [^{35}S]GTPγS in 0.5-ml total volume for 2 h at 30°C. Basal binding was measured in the absence of agonist, and nonspecific binding was measured in the presence of 20 μM unlabeled guanosine 5′-3-O-(thio)triphosphate. The reaction was terminated by vacuum filtration though Whatman GF/B glass fiber filters, followed by three washes with 4°C Tris buffer (50 mM Tris-HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency after 10-h extraction in ScintiSafe Econo 1 scintillation fluid.

[^{3}H]SR141716A Binding. Membranes were prepared as described above. Membrane proteins (8 μg) were incubated with 0.2 to 3 nM [^{3}H]SR141716A in assay buffer B in the presence or absence of 5 μM unlabeled SR141716A (to determine nonspecific binding) for 90 min at 30°C. A second set of samples was prepared using the same protocol but with varying concentrations of NAM (0.01–10 μM). The reaction was terminated by vacuum filtration though a Whatman GF/B glass fiber filter (Whatman, Clifton, NJ) that was presoaked in Tris buffer containing 5 g/l BSA (Tris-BSA), followed by three washes with 4°C Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency after extraction in ScinitSafe Econo 1 scintillation fluid.

Quantification of 2-AG and AEA Levels. Adult female mice received injections intraperitoneally with either vehicle (1:1:18) or 5 mg/kg NAM. One hour later, mice were decapitated and the cerebellum was harvested and rapidly cooled by immersion in liquid nitrogen. 2-AG and AEA were then extracted using a methanol/chloroform extraction (Hardison et al., 2006). After extraction, quantification of 2-AG and AEA was conducted by liquid chromatography/mass spectrometry analysis (Kingsley and Marnett, 2003).

Data Analysis. Data for [^{35}S]GTPγS binding experiments are reported as mean and S.E. of at least four experiments, which were each performed in triplicate. Nonspecific binding was subtracted from each sample. Net stimulated [^{35}S]GTPγS binding is defined as agonist-stimulated minus basal [^{35}S]GTPγS binding, and percentage stimulation is defined as (net − stimulated/basal [^{35}S]GTPγS binding) × 100%. Nonlinear iterative regression analyses of agonist concentration-effect curves were performed with Prism 4.0 (GraphPad Software Inc., San Diego, CA). For SR141716A displacement study, data are expressed as mean and S.E. for percentage SR141716A bound for each concentration point of NAM, which was calculated as follows: (specific radiolabeled SR141716 at each concentration of NAM/specific radiolabeled SR141716 in the absence of NAM) multiplied by 100. Statistical significance was determined by ANOVA followed by Dunnett's post hoc test. For mass spectrometry data, mean and S.E. were determined for 2-AG concentration (nanomolar) per gram of cerebellum for each condition. ANOVA was used to determine significant differences between control and test groups followed by Dunnett's post hoc test. Statistical analysis was performed using SigmaStat, version 3.1 (Systat Software, Inc., San Jose, CA). Significance was defined as $p < 0.05$.

For behavioral data, means and S.E. were derived for percentage antinociception, percentage inhibition of locomotor activity, percentage catalepsy/ring immobility, and change in degrees Celsius. ANOVA was used to determine significant differences between control and test groups ($n = 6$ for all groups) followed by Dunnett's post hoc test. Statistical analysis was performed using SigmaStat, version 3.1.

Results

To determine whether the putative MAGL inhibitor NAM enhanced the in vivo activity of 2-AG, this endocannabinoid was exogenously administered (intravenously) to mice that had been pretreated (intraperitoneally) with 1 mg/kg NAM or vehicle, and a tetrad of in vivo measures that are characteristic of cannabinoid agonists was assessed. As shown in Fig. 1, 2-AG alone did not affect any of the tetrad measures at doses up to 10 mg/kg. However, when combined with a 1 mg/kg dose of NAM, 2-AG produced significant and dose-dependent hypothermia, inhibition of locomotor activity, antinociception, and catalepsy. These results are similar to those previously observed with other cannabinoid agonists (Martin et al., 1991), although the magnitude of the catalepsy effect was comparatively modest. Moreover, NAM alone did not produce any of these in vivo effects. These results suggest that NAM acted in a permissive manner to reveal cannabimimetic pharmacological effects of 2-AG.

Experiments were then conducted to determine whether the in vivo effects of 2-AG in the presence of NAM were mediated by cannabinoid receptors. Results showed that all of these effects were significantly, but not completely, reversed by the CB_1 receptor antagonist SR141716A (Fig. 2). In contrast, administration of the CB_2 antagonist SR144528 did not reduce the effects of the 2-AG + NAM combination (data not shown). Likewise, the hypothermic, antinociceptive, and cataleptic effects of 2-AG + NAM were significantly reduced, but were not completely absent, in CB_1 receptor knockout mice compared with C57BL/6 wild-type littermates (Fig. 3). It is interesting to note that mice of both genotypes showed significant inhibition of locomotor activity that was similar in magnitude. These results indicate that the majority of the in vivo activity of 2-AG in the presence of NAM was CB_1 receptor-mediated. However, the residual activity of 2-AG + NAM in CB_1 knockout mice (especially the high level of locomotor inhibition) suggests the possibility of additional mechanisms of action.

The findings that NAM had a permissive effect on 2-AG in vivo and that the effects of 2-AG + NAM were predominantly CB_1 receptor-mediated suggested that NAM was acting to inhibit metabolic inactivation of 2-AG. However, it is possible that NAM could be positively acting on CB_1 receptors along with 2-AG. Therefore, the effects of NAM and 2-AG on CB_1 receptor binding and signaling were assessed directly in membranes prepared from mouse cerebellum, using [^{3}H]SR141716 competition and ligand-mediated [^{35}S]GTPγS binding assays. As shown in Fig. 4, NAM alone inhibited [^{3}H]SR141716A binding in a concentration-dependent manner and decreased basal [^{35}S]GTPγS binding (Fig. 5). In contrast, the CB_1 receptor agonist WIN 55,212-2 produced an increase in [^{35}S]GTPγS binding. Thus, NAM alone did not activate CB_1 receptor-mediated G proteins. However, more importantly, when NAM was combined with 2-AG (Fig. 4), a significant leftward shift in the 2-AG concentration-effect curve was observed, with no difference in maximal stimulation. Curve-fitting analysis confirmed that NAM decreased the 2-AG EC_{50} value from 4.45 ± 0.34 to 0.68 ± 0.21 μM,

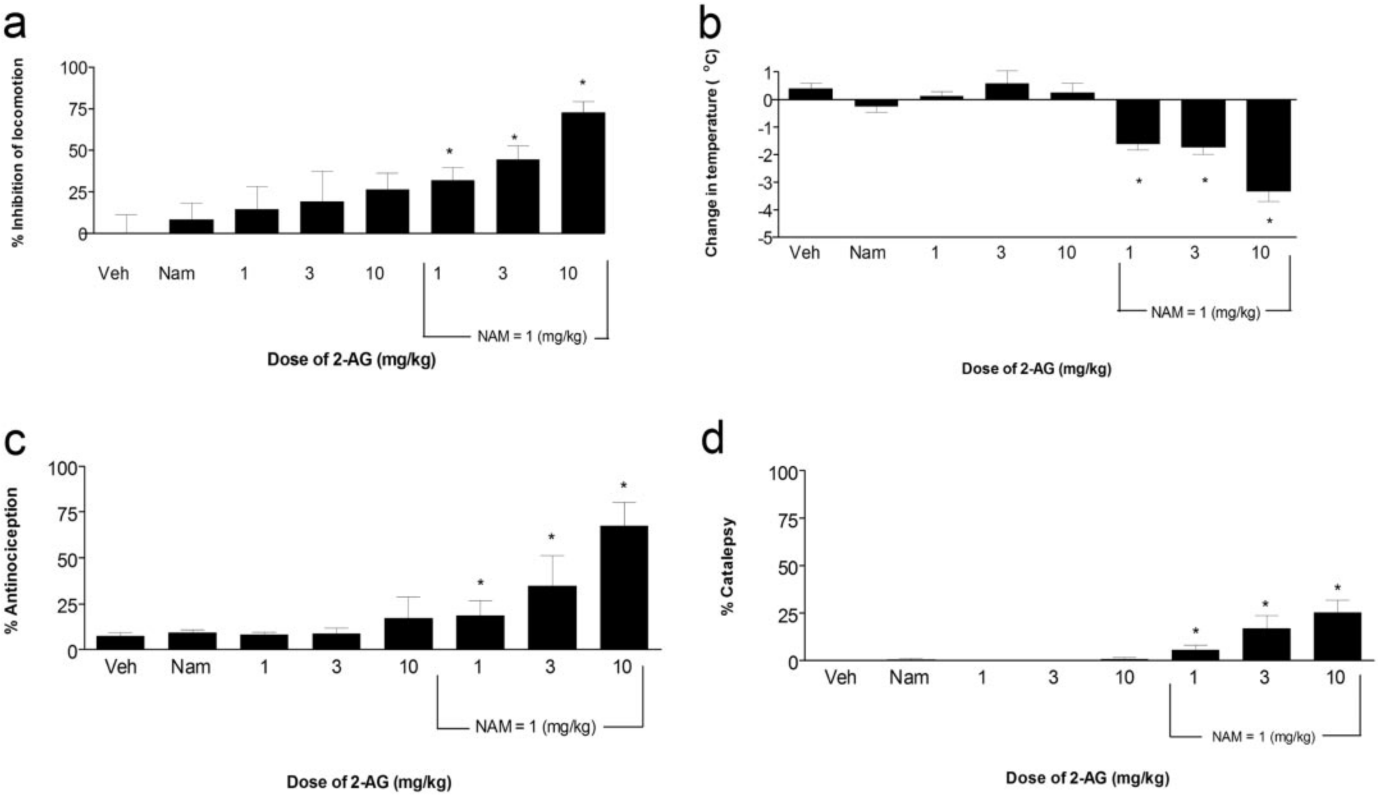

Fig. 1. Effects of NAM and 2-AG alone and in combination on locomotor activity (a), rectal temperature (b), antinociception (c), and catalepsy (d). n = 6 mice/group. *, significance from vehicle ($p < 0.05$).

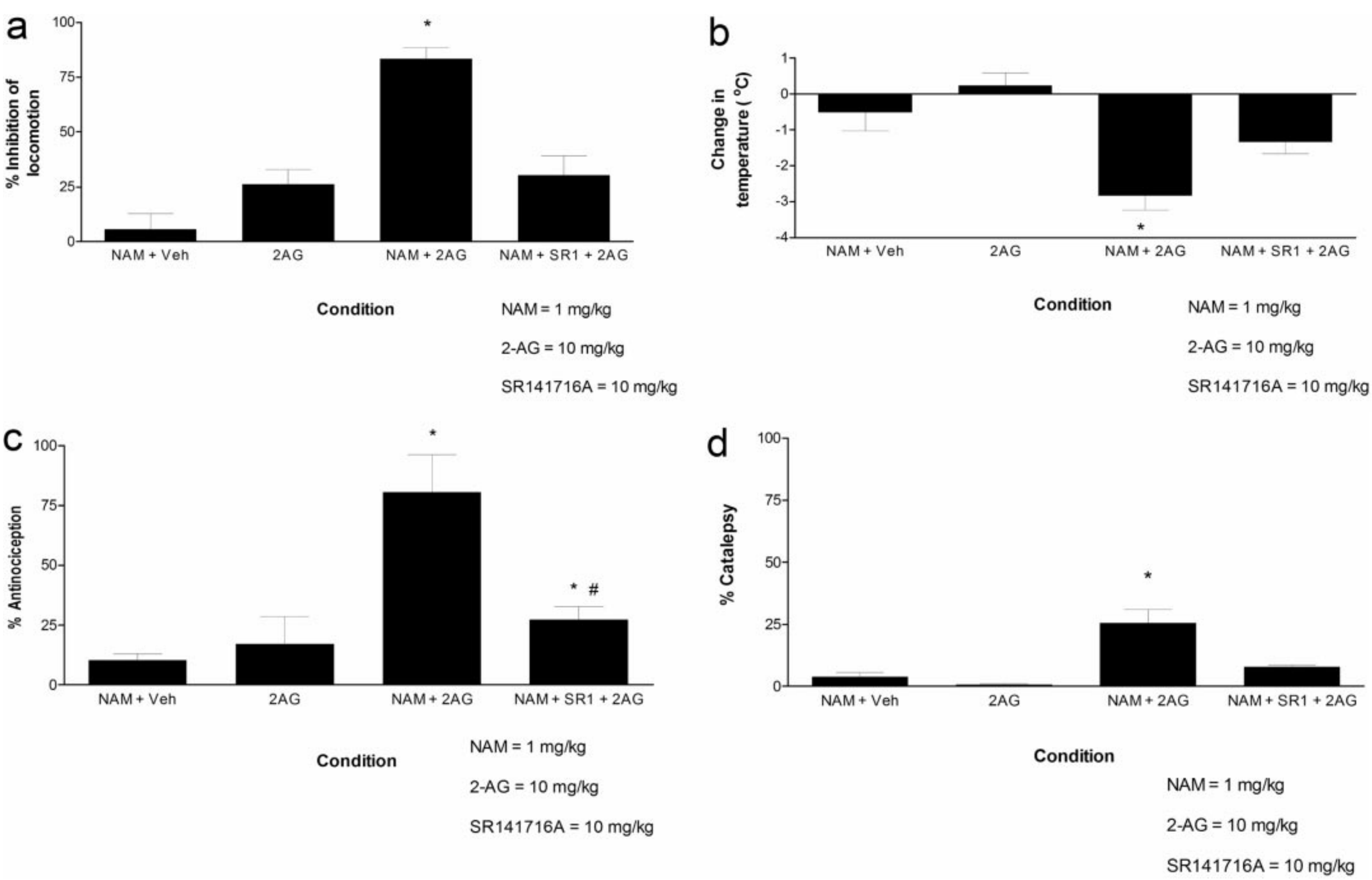

Fig. 2. Effect of SR141716A on locomotor activity (a), rectal temperature (b), antinociception (c), and catalepsy (d), induced by NAM and 2-AG. n = 6 mice/group. *, significantly different effect ($p < 0.05$) from vehicle baseline; #, significantly ($p < 0.05$) reduced effect compared with 2-AG + NAM.

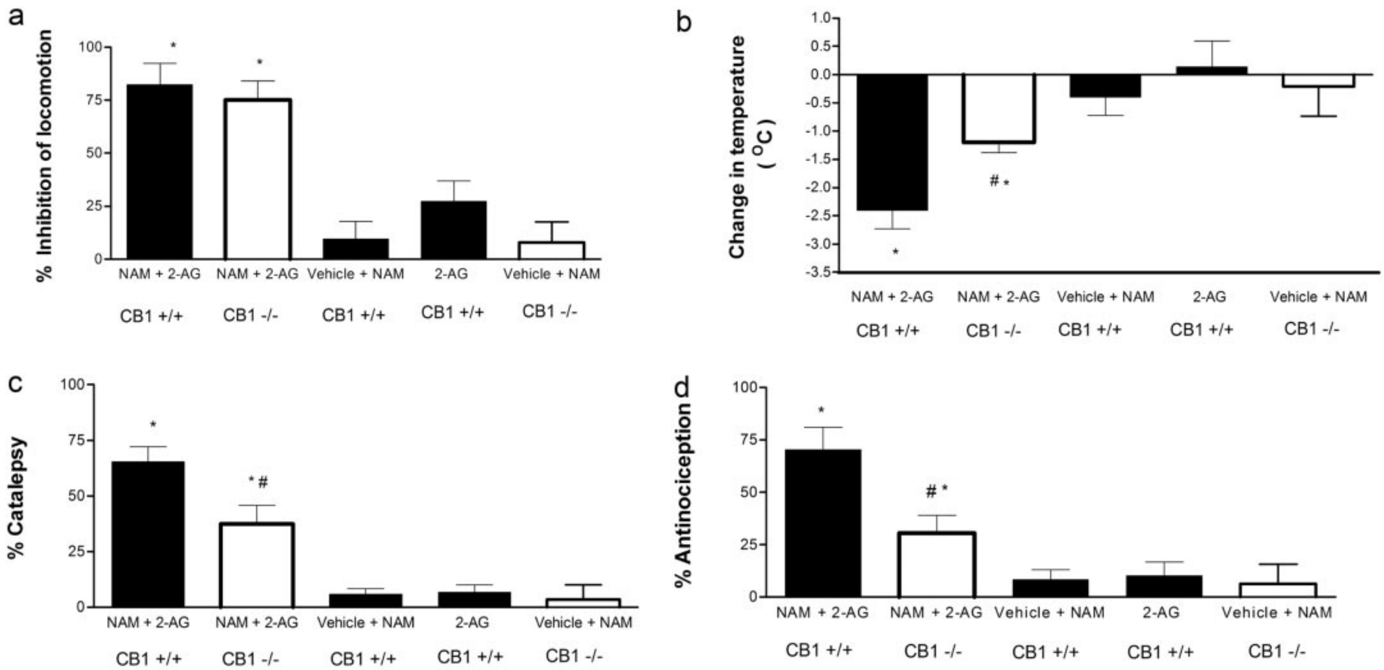

Fig. 3. Effects of NAM (1 mg/kg) + 2-AG (10 mg/kg) on locomotor activity (a), rectal temperature (b), catalepsy (c), and antinociception (d) in $CB_1^{-/-}$ knockout and $CB_1^{+/+}$ wild-type mice. $n = 6$ mice/group. CB1 KO mice. *, significant difference from VEH + NAM; #, significant difference between wild-type and knockout mice ($p < 0.05$).

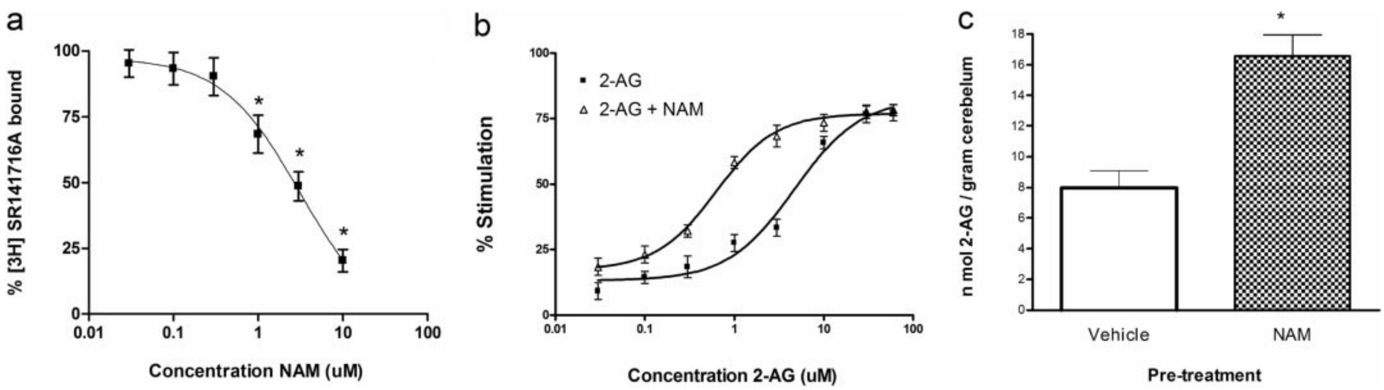

Fig. 4. a, effect of NAM on the percentage of radiolabeled SR141716A bound to cerebellum protein. b, stimulation of CB_1-mediated G proteins by 2-AG alone and with NAM. c, effect of vehicle versus NAM on endogenous 2-AG levels in the cerebellum. a to c, *, significance (compared with baseline) ($p < 0.05$).

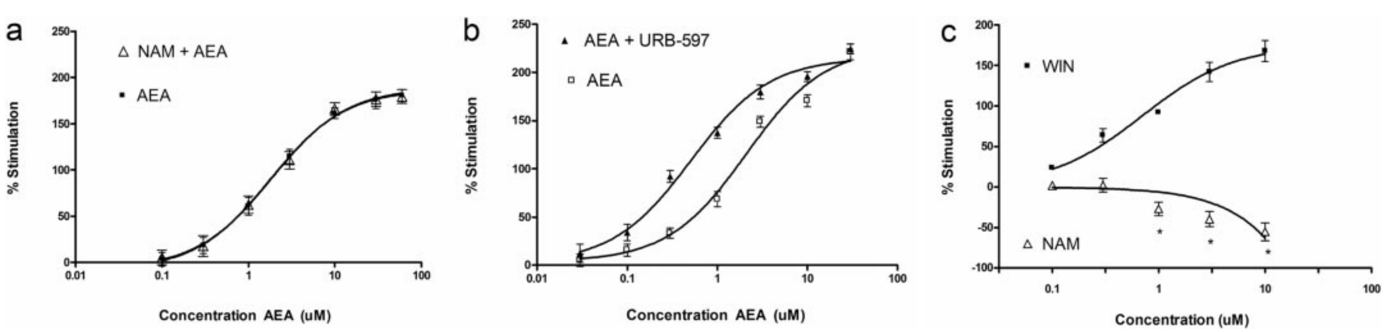

Fig. 5. a, effect of NAM (300 nM preincubation followed by a 150 nM incubation) on AEA-stimulated CB_1-mediated G-protein activation. b, effect of URB597 (50 nM preincubation followed by a 10 nM incubation) on AEA-stimulated CB_1-mediated G-protein activation. c, effects of NAM (open triangles) to prevent $[^{35}S]GTP\gamma S$ binding. *, significant difference ($p < 0.05$).

whereas the E_{max} value was unaffected (80.40 ± 5.67% versus 77.45 ± 4.03% in the absence or presence of NAM, respectively). These results indicate that at a concentration of 150 nM, NAM enhanced the potency of 2-AG without affecting CB_1 receptor binding sites or altering basal G-protein activation. Furthermore, this concentration of NAM did not affect either the EC_{50} or E_{max} of the CB_1 full agonist WIN 55,212-2 (data not shown). NAM + 2-AG did not cause

[^{35}S]GTPγS binding in CB_1 knockout tissue; here, 2-AG produced an E_{max} in $CB_1{}^{+/+}$ cerebellar tissue homogenates of 93.25 ± 8.76%, whereas the E_{max} in $CB_1{}^{-/-}$ cerebellar tissue homogenates (data not shown) was 5.54 ± 3.31% (not significantly different from basal), which is surprising because NAM + 2-AG produced tetrad effects in CB_1 knockout mice (however, it should be noted that the highest concentration of 2-AG tested in this tissue was 10 μM). However, the [^{35}S]GTPγS assay is primarily designed to detect activation of $G_{i/o}$ proteins; hence, it is entirely feasible that 2-AG may be activating a receptor(s) distinct from CB_1 that is coupled to a non-$G_{i/o}$ protein such as G_S or G_q.

To determine whether NAM selectively enhances the potency of 2-AG, G-protein activation by the endocannabinoid AEA was examined in mouse cerebellar membranes in the presence and absence of NAM or the established FAAH inhibitor URB597 (Kathuria et al., 2003). Figure 5 showed that although URB597 decreased the EC_{50} value of AEA from 2.577 ± 0.31 M to 0.31 ± 0.27 μM, NAM (at a concentration of 150 nM) had no significant effect on the EC_{50} value of AEA. Neither enzyme inhibitor significantly affected the E_{max} value of AEA (210.89 ± 11.23% with NAM, 207.56 ± 9.45% with URB597, and 212 ± 10.44% in the absence of inhibitor). These results indicate that NAM selectively increases the potency of 2-AG but not AEA. Note that these results also suggest that NAM does not act as an allosteric modulator of CB_1 receptors at the concentration examined because neither the EC_{50} nor E_{max} value of AEA or WIN (data not shown) was altered by NAM at a concentration of 150 nM.

The finding that NAM selectively enhanced the in vitro potency rather than maximal effect of 2-AG is consistent with the concept that NAM inhibits degradation of 2-AG. However, these findings do not demonstrate a protective effect of NAM on 2-AG levels in vivo. To determine whether NAM protects against 2-AG degradation in vivo, mass spectrometry analysis of 2-AG and AEA levels was performed in mouse cerebellar tissue that was collected 1 h after administration of 5 mg/kg NAM. Figure 4 shows that approximately twice the level of endogenous 2-AG was detected in the presence of NAM compared with mice that received vehicle injections, whereas the level of AEA was not significantly altered by NAM 30.7 ± 3.21 pM/g in vehicle-treated tissue and 33.1 ± 2.69 pM/g in NAM-treated tissue. Furthermore, NAM (5 mg/kg) was shown to elevate the level of exogenously administered 2-AG (1 mg/kg) from 60 to 135 nM/g cerebellum. These findings strongly suggest that NAM augments the in vivo action of 2-AG by selectively protecting 2-AG from metabolic degradation without affecting the degradation of AEA.

Discussion

The role of 2-AG in the CNS has not been well defined, probably because of its lability in the presence of endogenous MAGL. Previous studies showed that 2-AG was rapidly degraded (20 min) (Laine et al., 2002), suggesting that prevention of enzymatic degradation is necessary to reveal its pharmacological properties and biological functions. Consistent with this premise, the present results show that 2-AG produced significant dose-dependent effects in all tests in the cannabinoid tetrad when mice were pretreated with NAM, but not when 2-AG was administered alone. Furthermore, tetrad effects observed with the combination of NAM and 2-AG were significantly attenuated by the CB_1-selective antagonist SR141716A, but unaffected by the CB_2-selective antagonist SR144528. These results are consistent with a large body of research demonstrating that cannabinoids of various classes, including AEAs (when metabolism is inhibited), THC-like cannabinoids, bicyclic cannabinoids, and aminoalkylindoles, produce dose-dependent and CB_1-mediated effects in these tests (Compton et al., 1992a,b; Compton and Martin, 1997; Bourne et al., 2007; Wise et al., 2007).

The finding that 2-AG produced significant tetrad effects only when mice were pretreated with 1 mg/kg NAM suggests that NAM may be preventing enzymatic degradation of 2-AG by inhibiting MAGL. In contrast with the 1 mg/kg dose of NAM, a 10-fold lower dose (0.1 mg/kg) only moderately enhanced the tetrad effects of 2-AG (data not shown), suggesting insufficient inhibition of MAGL at this lower dose. The fact that NAM's enhancement of 2-AG was dose-dependent lends further support to the hypothesis of a saturable substrate (e.g., enzyme inhibition) is its mechanism of action.

However, it was important to rule out other explanations for the enhancement observed. First, we showed that 2-AG levels in the cerebellum increased substantially after administration of NAM but that AEA levels were unaltered. These data suggest that NAM inhibits MAGL, which is in agreement with previous research (Saario et al., 2005). Second, NAM increased the potency, but not the efficacy, of 2-AG, thus ruling out the idea that NAM might alter the number of CB_1 receptors activated or the magnitude of activation by 2-AG. In contrast, NAM did not alter either potency or efficacy of AEA, suggesting that it is selective for metabolic inhibition of 2-AG and not AEA at the concentration tested. These results further suggest that MAGL does not degrade AEA and are consistent with previous research reports showing that FAAH preferentially degrades AEA (Boger et al., 2000; McKinney and Cravatt, 2005). However, it is worth noting that FAAH also degrades 2-AG to some extent (Basavarajappa, 2007). In addition, NAM is not exclusively selective for MAGL but at a high concentration (50 μM) also interacts with FAAH (Blankman et al., 2007). Because this concentration is approximately 300 times greater than that used in the present study and because we saw no elevation of AEA levels in NAM-treated animals, NAM-mediated inhibition of FAAH is unlikely to account for the findings here.

Another possibility that we considered was that NAM might enhance the tetrad effects of 2-AG by directly activating the CB_1 receptor, although it did not produce tetrad effects when administered alone. To exclude this possibility more conclusively, the effects of NAM on CB_1 receptor binding and activation were examined. Results showed that, although NAM binds to the CB_1 receptor (at micromolar concentrations), it did not activate G proteins. In fact, NAM inhibited basal G-protein activity, suggesting that it may be an antagonist/inverse agonist for the CB_1 receptor rather than an agonist. However, it is worth noting that NAM inhibits MAGL at a lower concentration than is required to block the CB_1 receptor, as shown by the fact that the 150 nM concentration of NAM enhanced 2-AG-mediated [^{35}S]GTPγS binding but did not cause significant SR141716A displacement or alter basal [^{35}S]GTPγS binding when presented alone. Based on these results, we concluded that NAM did not potentiate the tetrad effects of 2-AG through coactivation of the CB_1 receptor.

Although the tetrad effects observed with the combination of NAM and 2-AG were similar to those obtained with traditional THC-like cannabinoids (Martin et al., 1991), differences were also apparent. First, the maximal magnitude of hypothermia produced by 2-AG + NAM was approximately −3°C, as it is for AEA in phenylmethylsulfonyl fluoride-pretreated mice (Compton and Martin, 1997). In contrast, THC typically produces maximal temperature decreases up to −6°C. A second difference is that 2-AG (+NAM) produced a maximum of only 40 to 60% catalepsy at doses that produced approximately 80% antinociception and suppression of locomotion. Although this degree of catalepsy is often seen with THC-like cannabinoids, it is far lower than that observed with AEA (approximately 80–90%) at doses that produced a similar magnitude of locomotor inhibition and antinociception (Compton and Martin, 1997). Hence, the pharmacological effects of 2-AG do not entirely resemble either THC or AEA.

One possible explanation for these apparent differences is that the dose of NAM used in these experiments may not fully inhibit MAGL. The presence of residual MAGL activity would result in effectively lower doses of 2-AG. For example, AEA causes a greater degree of hypothermia in mice that lack FAAH than in mice that were treated with phenylmethylsulfonyl fluoride and AEA (Compton and Martin, 1997; Wise et al., 2007). To investigate whether residual MAGL may have contributed to the results here, we tested a higher dose of NAM (3 mg/kg) in combination with 2-AG, but obtained similar results (data not shown). A more plausible explanation is that 2-AG is not only degraded by MAGL, but also by a compensatory/backup 2-AG hydrolyzing enzyme that is not affected by NAM. This alternative enzyme may be a member of the cyclooxygenase family (Hu et al., 2008).

In addition to testing 2-AG in wild-type C57BL/6 mice, we also assessed its effects in CB_1 knockout mice in the tetrad. These data were interesting for a couple of reasons. First, although the hypothermic, cataleptic, and antinociceptive effects of 2-AG (+NAM) were reduced in CB_1 knockout mice (versus wild-type littermates), these effects were not absent because the magnitude of each effect was still significantly different from vehicle in these mice. We surprisingly found that, unlike the other three measures, locomotor inhibition was not reduced significantly in CB_1 knockout mice treated with 2-AG (+NAM) compared with wild-type mice. Although somewhat surprising, these results were consistent with the observation that AEA produced the same level of locomotor inhibition, regardless of CB_1 genotype (Wise et al., 2007). Although these results suggest that endocannabinoid action at non-CB_1, non-CB_2 cannabinoid receptor(s) might play a role in these findings, the possibility of a developmental compensatory process in the knockout mice cannot be entirely eliminated (Mackie, 2007). It is interesting to note that previous research has suggested that there may be other receptor targets for cannabinoids that, to date, have not been identified (Breivogel et al., 2001; Wiley and Martin, 2002). One possible non-CB_1 receptor candidate is G protein-coupled receptor (GPR) 55. Studies have shown that 2-AG and AEA stimulate [^{35}S]GTPγS binding in GPR 55-transfected cells (Pertwee, 2007; Ryberg et al., 2007). However, our behavioral and biochemical data suggest that 2-AG may be activating a non-$G_{i/o}$-coupled receptor, ruling out the GPR 55 receptor because it seems to be coupled to a $G_{i/o}$ protein.

In conclusion, NAM treatment revealed the in vivo activity of 2-AG. Although there were similarities in the profile of effects of 2-AG (+NAM) and other cannabinoids in the tetrad, there were also differences. It is noteworthy that 2-AG (+NAM) was less efficacious in producing hypothermia and catalepsy. Furthermore, our in vitro data suggest that NAM enhanced the effect of 2-AG through inhibition of MAGL. However, it is interesting to note that some of the findings from this study also point to the possibility that 2-AG may have a target in the CNS that is distinct from the CB_1/CB_2 receptor. These results suggest that NAM (and other MAGL inhibitors) will be valuable tools to elucidate the biological functions of 2-AG. Moreover, dysregulation of 2-AG homeostasis might contribute to certain disorders, and NAM could provide a new therapeutic lead for development of MAGL inhibitors for treatment of conditions such as chronic neuropathic pain, depression, and traumatic brain injury (Panikashvili et al., 2001; Petrosino et al., 2007; Hill et al., 2008).

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