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# An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity

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## Abstract

2-Arachidonoyl-glycerol (2-Ara-Gl) has been isolated from various tissues and identified as an endogenous ligand for both cannabinoid receptors, $CB_1$ and $CB_2$. Here we report that in spleen, as in brain and gut, 2-Ara-Gl is accompanied by several 2-acyl-glycerol esters, two major ones being 2-linoleoyl-glycerol (2-Lino-Gl) and 2-palmitoyl-glycerol (2-Palm-Gl). These two esters do not bind to the cannabinoid receptors, nor do they inhibit adenylyl cyclase via either $CB_1$ or $CB_2$; however, they significantly potentiate the apparent binding of 2-Ara-Gl and its apparent capacity to inhibit adenylyl cyclase. Together these esters also significantly potentiate 2-Ara-Gl inhibition of motor behavior, immobility on a ring, analgesia on a hot plate and hypothermia caused by 2-Ara-Gl in mice. 2-Lino-Gl, but not 2-Palm-Gl, significantly inhibits the inactivation of 2-Ara-Gl by neuronal and basophilic cells. These data indicate that the biological activity of 2-Ara-Gl can be increased by related, endogenous 2-acyl-glycerols, which alone show no significant activity in any of the tests employed. This effect ('entourage effect') may represent a novel route for molecular regulation of endogenous cannabinoid activity. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Anandamide; Endocannabinoid; Cannabinoid receptor; 2-Arachidonoyl-glycerol inactivation

## 1. Introduction

We have identified anandamide (arachidonoylethanolamide) in porcine brain and 2-arachidonoyl-glycerol (2-Ara-Gl) in canine gut (Devane et al., 1992b; Mechoulam et al., 1995). Both ligands bind to the $CB_1$ and $CB_2$ cannabinoid receptors and exhibit cannabinoid-type activities. Later Sugiura et al. (1995) and Stella et al. (1997) reported the presence of 2-Ara-Gl in brain, while Bisogno et al. (1997b) found that 2-Ara-Gl is biosynthesized and released in a $Ca^{2+}$-dependent fashion by mouse neuroblastoma cells. 2-Ara-Gl inhibits forskolin-stimulated adenylyl cyclase in mouse spleen cells (Mechoulam et al., 1995) and rat neurons (Stella et al., 1997). In mice, 2-Ara-Gl is active in a tetrad of assays, which together have been shown to be highly predictive of cannabinoid-induced activity (Mechoulam et al., 1995; Fride and Mechoulam, 1993; Martin et al., 1991).

In view of the identification of $CB_2$ cannabinoid receptor in immune cells (Munro et al., 1993) and of the inhibition by 2-Ara-Gl of T- and B-cell proliferation (Lee et al., 1995), we decided to look for the presence of active endogenous ligands in the spleen, an organ with well established immune functions, using a fractionation guided by a binding assay.

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## 2. Materials and methods

### 2.1. Isolation of fatty acid esters of glycerol

Mouse spleen tissue (280 mg from three mice) was homogenized in chloroform/methanol (2:1 v/v) with a Kontex glass tissue grinder. The homogenate was filtered via a sintered glass and the residue reextracted. The chloroform layer, which contained the extracted lipids, was partitioned against 0.8% aqueous NaCl, dried under a stream of nitrogen and redissolved in 1 ml of chloroform. Ten volumes of acetone were added to the solution and after 20 min (at −20°C) the mixture was centrifuged at $3500 \times g$ for 10 min. The supernatant was evaporated to dryness and the residue was dissolved in 1 ml chloroform, 100 ml was spotted on a thin layer chromatography (TLC) plate (silica gel 60, Merck) and developed in hexane/diethylether/acetone/acetic acid (40:20:30:1 v/v/v/v). The TLC plate was divided into five bands, which were eluted from the TLC plate with a solvent mixture of chloroform/methanol (9:1 v/v). Activity was assayed by inhibition of binding of the high affinity cannabinoid ligand [$^3$H]HU-243 to rat brain synaptosomal membranes ($CB_1$ cannabinoid receptors) (Devane et al., 1992a), and to membranes of Chinese hamster ovary (CHO) cells transiently transfected with $CB_2$ cannabinoid receptor (see below). The only TLC band that showed cannabinoid binding activity had an $R_f$ of 0.5. This sample was analysed by gas chromatography–mass spectrometry (GC–MS) for the presence of anandamide and acylglycerols (Devane et al., 1992b; Mechoulam et al., 1995).

### 2.2. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses were carried out with a Finnigan TSQ 700 mass spectrometer coupled to a Varian 3400 gas chromatograph. Chromatographic separation were performed on a cross-linked methyl silicone (DB-5MS) capillary column (length, 15 m; i.d. 0.25 mm; film thickness, 0.25 μm); column temperature was programmed to increase from 150 to 280°C at a rate of 25°C/min following a 5 min holding time at 280°C. Helium was used as the carrier gas at a head pressure of 6 psi. Injection temperature was 220°C in the splitless mode. Mass spectra were obtained in electron impact (EI) mode with electron energy of 70 eV. Ion source and transfer-line temperatures were 150°C and 280°C, respectively. The quadrupole was scanned in the $m/z$ range 50–550 at 1 scan/s. Silylation of the endogenous compounds was made by adding bis-trimethylsilyl (TMS) trifluoroacetamide to the dry sample. After 30 min of incubation at room temperature, the silylated material was injected into the GC–MS.

For quantitative analysis, an internal standard, 1(3)-eicosanoyl-glycerol (25 nmol) (Nu-Check Prep, Elysian, MN), was added during homogenization and the same procedure described above was followed. The isolated fraction was reacted with 50 μl of bis-TMS trifluoroacetamide (30 min at room temperature), dried under nitrogen, and resuspended in 50 μl chloroform. The sample was analysed by GC–MS in a Hewlett-Packard G 1800 A GCD system. The capillary column (HP5MS, 30 m × 0.25 mm i.d.) was temperature programmed from 150–280°C at 50°C/min, using selective ion monitoring. The calibration curve (area ratio vs. weight ratio of 2-Ara-Gl to 1(3)eicosanoyl-glycerol) was linear.

### 2.3. Binding of [$^3$H]HU-2432 to $CB_1$ or $CB_2$ cannabinoid receptors

The monoacylglycerols 2-Ara-Gl, 2-linoleoyl-glycerol (2-Lino-Gl) and 2-palmitoyl-glycerol (2-Palm-Gl) were assayed for competition in binding [$^3$H]HU-243 to the $CB_2$-receptor in CHO cells in a centrifugation based ligand binding assay which has been described in detail in previous publications (Devane et al., 1992a,b; Rhee et al., 1997). To measure the binding to $CB_1$ and $CB_2$ receptors in African green monkey kidney cells (COS-7 cells), the latter were transiently transfected (using DEAE-dextran method) with plasmids (5 μg/100 mm dish) encoding $CB_1$ or $CB_2$. Two days later the cells were washed with phosphate-buffered saline, scraped, pelleted, and stored at −80°C. Cell pellets were homogenized in 50 mM Tris–HCl, 5 mM $MgCl_2$, and 2.5 mM EDTA, pH 7.4, and 50 μg protein aliquots were assayed for binding of [$^3$H]HU-243 in this buffer supplemented with 10 mM $CaCl_2$. The final concentration of [$^3$H]HU-243 in the binding mixture was 300 pM. For more detailed information on the assay and the calculation of the $K_i$ values see Devane et al. (1992a) and Rhee et al. (1997).

### 2.4. Adenylyl cyclase assay

In brief, COS-7 cells in 100 mm dishes were cotransfected with adenylyl cyclase type V and either $CB_1$ or $CB_2$ cDNAs. The cells were replated in 24-well plates, labelled with [$^3$H]adenine for 2 h and the adenylyl cyclase was stimulated with 1 μM forskolin (in the presence of the tested compounds) for 10 min at 37°C. Incubation was stopped by the addition of perchloric acid followed by neutralization and the amount of [$^3$H]cAMP formed was assayed by a two column separation procedure. For more details see Rhee et al. (1997) and Bayewitch et al. (1996).

### 2.5. Pharmacological tests in mice

Female mice (C57BL/6, 2–3 months old) were injected i.p. with 2-Ara-Gl, 2-Lino-Gl and 2-Palm-Gl (1, 10 and 5 mg/kg, respectively), in a vehicle consisting of ethanol:emulphor:saline (1:1:18), with each drug alone, or together. We employed a tetrad of tests commonly used to demonstrate cannabinoid activity (Martin et al., 1991; Fride and Mechoulam, 1993). These tests include ambulation in

an open field for 8 min; immobility on a ring of 5.5 cm diameter; change in rectal temperature measured with a telethermometer (Yellow Springs Instrument Yellow Springs, OH) and analgesia on a hot plate (Columbus Instruments, Columbus, OH). The effects in mice were observed 15 min after injections. Data from the tetrad of observations were analysed by one-way analyses of variance with Newman–Keuls multiple comparison tests.

### 2.6. Effect of monoacylglycerols on the hydrolysis/inactivation of 2-Ara-Gl

Experiments on 2-Ara-Gl and 1-Ara-Gl hydrolysis were performed with both particulate fractions and intact cells. Particulate fractions ($10\,000 \times g$) from N18TG2 and RBL-2H3 cells were prepared as previously described (Bisogno et al., 1997a,b). These fractions (0.05–0.1 mg total proteins) were incubated for 30 min at 37°C in 0.25 ml of a 50 mM Tris–HCl buffer, pH 7.4 with 10 000 cpm [$^3$H]2-Ara-Gl or [$^3$H]1-Ara-Gl (8.0 μM), and with increasing concentrations (0, 10, 50, 100 and/or 250 μM) of various monoacylglycerols or with unlabelled 1- or 2-Ara-Gl (100 μM). In a separate set of experiments, the effect of a mixture of 2-Palm-Gl and 2-Lino-Gl on the hydrolysis of [$^3$H]2-Ara-Gl or [$^3$H]1-Ara-Gl was also studied. [$^3$H]-Arachidonic acid produced from the hydrolysis of [$^3$H]2-Ara-Gl or [$^3$H]1-Ara-Gl was quantified by TLC carried out as described previously (Bisogno et al., 1997b). For experiments with intact cells (Di Marzo et al., 1994), confluent RBL-2H3 or N18TG2 cells in 6-well dishes were washed three times with serum-free minimal essential medium and then incubated for increasing periods of time at 37°C with 0.5 ml serum-free minimal essential medium containing 10 000 cpm of [$^3$H]2- or [$^3$H]1-Ara-Gl (4.0 μM) with or without 1- or 2-Lino-Gl (100 μM) or 1- or 2-Palm-Gl (100 μM). In a separate set of experiments, RBL-243 cells were incubated for 30 min in the presence of a mixture of 2-Palm-Gl and 2-Lino-Gl (in a 5:12:1 molar ratio with [$^3$H]2-Ara-Gl). After the incubation, the media were extracted with chloroform/methanol 2:1 (v/v) and the organic phase was analysed as described previously (Bisogno et al., 1997b). After three washings with 2 ml of serum minimal essential medium containing 1% bovine serum albumin, cells from each well were extracted by sonication with chloroform/methanol/50 mM Tris–HCl buffer pH 7.4, 2:1:1 (v/v/v). The organic phase was then analysed for the presence of [$^3$H]2-Ara-Gl and [$^3$H]-arachidonic acid as described above.

## 3. Results

### 3.1. Isolation and identification

Mouse spleen was extracted with methanol/chloroform (1:2) and the extract was chromatographed to yield a fraction that was found to bind to both $CB_1$ and $CB_2$ cannabinoid receptors. The active fraction was silylated with bis-TMS trifluoroacetamide and the resulting mixture was analysed by GC–MS. Several of the single peaks observed before silylation were transformed into pairs of compounds which, on the basis of our previous work, are the silylated derivatives of 1- and 2- monoacylglycerols (Mechoulam et al., 1995) (Fig. 1). The 1-acyl-glycerols

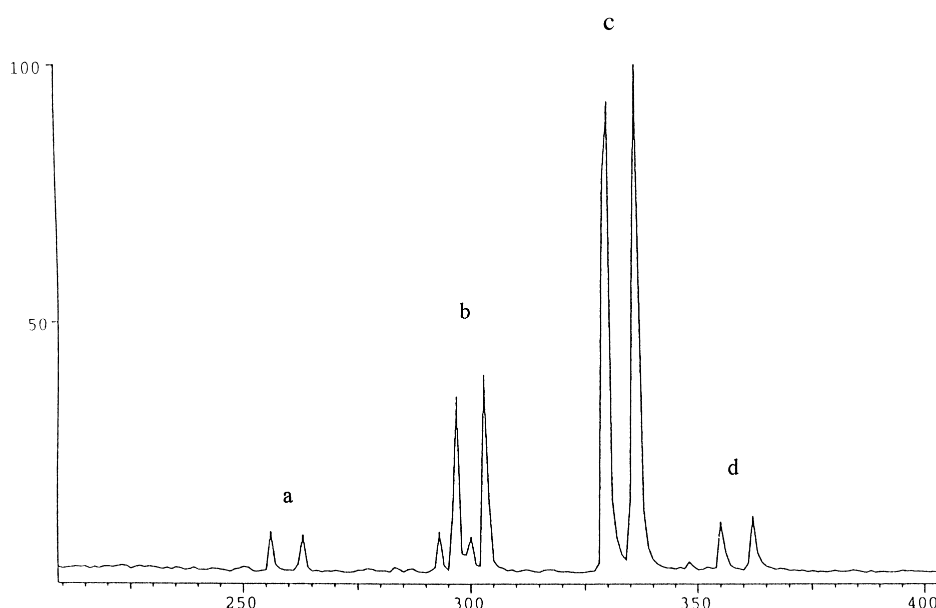


Fig. 1. Capillary gas chromatography analysis of the endogenous compounds after silylation with bis-TMS trifluoroacetamide performed on DB-5MS capillary column (15 m × 0.25 mm). Peak identities: tetramethylsilyl ethers of the glycerol esters of (a) myristic acid; (b) palmitic acid; (c) linoleic acid; (d) arachidonic acid.

derivatives are formed by rearrangement from the 2-acylglycerol derivatives (Mechoulam et al., 1995). The molecular weights of the compounds in each pair, as determined by MS, were identical, being 450, 474, 498 and 522. As monoacylglycerols form bis-TMS ethers, the molecular weights recorded represent the molecular weight of the original monoacylglycerols plus 144 units (as each bis-TMS moiety has a molecular weight of 72). On silylation we thus obtained the bis-TMS ethers of the glycerol esters of myristic (14:0), palmitic (16:0), linoleic (18:2, $n-6$), and arachidonic (20:4, $n-6$) acids, respectively (approximate ratio of peaks 1:5–6:10–12:1) (Fig. 1). The EI spectra of the endogenous bis-silylated compounds were compared with those of the corresponding synthetic bis-TMS-monoacylglycerols and were found to be identical. For a mass spectrometric figure comparing endogenous 2-Ara-Gl with synthetic 2-Ara-Gl (as their bis-TMS derivatives) see Mechoulam et al. (1995). The amounts of 2-Ara-Gl, 2-Lino-Gl and 2-Palm-Gl (see Section 2) were $5.0 \pm 1.3$, $60.0 \pm 6.5$ and $23.0 \pm 6.5$ nmol/g wet weight of spleen tissue, respectively.

### 3.2. Binding to $CB_1$ and $CB_2$

In view of the high levels of 2-Lino-Gl and of 2-Palm-Gl present together with 2-Ara-Gl, as recorded here in spleen and previously in gut (Mechoulam et al., 1995) and brain (Sugiura et al., 1995), we carried out a series of experiments aimed at assessing the possible biological role of these two esters. In binding assays, based on competition with the binding of [$^3$H]HU-243 using membranes of CHO cells transfected with $CB_2$, 2-Palm-Gl and 2-Lino-Gl were found to be inactive up to 20 μM, while 2-Ara-Gl, in the same system, had a $K_i$ of $1640 \pm 260$ nM. These three esters were then mixed (in molar ratio of 5:12:1, for 2-Palm-Gl, 2-Lino-Gl and 2-Ara-Gl, respectively, as present in the spleen) and the mixture assayed for its effects on cannabinoid binding. The mixture competes with [$^3$H]HU-243 for $CB_2$ with an apparent $K_i$ value of $273 \pm 22$ nM (see Fig. 2a) calculated for the concentration of 2-Ara-Gl in the mixture. The apparent $K_i$ values for 2-Ara-Gl in mixtures with each of the two esters were $476 \pm 23$ nM for 2-Ara-Gl with 2-Lino-Gl (ratio 1:12), and $339 \pm 5$ nM for 2-Ara-Gl with 2-Palm-Gl (ratio 1:5). The potentiation by 2-Palm-Gl was thus significantly higher than that induced by 2-Lino-Gl ($P < 0.01$) despite its lower concentration in the reaction mixture. Further binding experiments with various ratios of the 'entourage compounds' to 2-Ara-Gl, indicated that there is a range of concentrations in which 2-Palm-Gl and 2-Lino-Gl can potentiate 2-Ara-Gl binding. Thus, we found apparent $K_i$ values close to those reported above, with ratios of 2-Ara-Gl to 2-Lino-Gl varying from 1:5 to 1:12 and of 2-Ara-Gl to 2-Palm-Gl varying from 1:2 to 1:5 (data not shown). The $K_i$ values, or apparent $K_i$ values, resulting from all combinations of compounds (2-Ara-Gl alone; 2-Ara-Gl + 2-Lino-Gl; 2-Ara-Gl + 2-Palm-Gl; all three) were first compared with analysis of variance. Individual post hoc comparisons were subsequently made using Newman–Keuls multiple comparison test.

Using COS-7 cells transfected with $CB_2$, 2-Ara-Gl had a $K_i$ of $145 \pm 39$ nM. A mixture of 2-Palm, 2-Lino-Gl and 2-Ara-Gl (applied in the same molar ratio as described above) was shown to compete with [$^3$H]HU-243 with an apparent $K_i$ value of $58 \pm 14$ nM (calculated for the concentration of 2-Ara-Gl). The differences in $K_i$ values observed for the two cell lines are probably due to the different methodologies used in the assays.

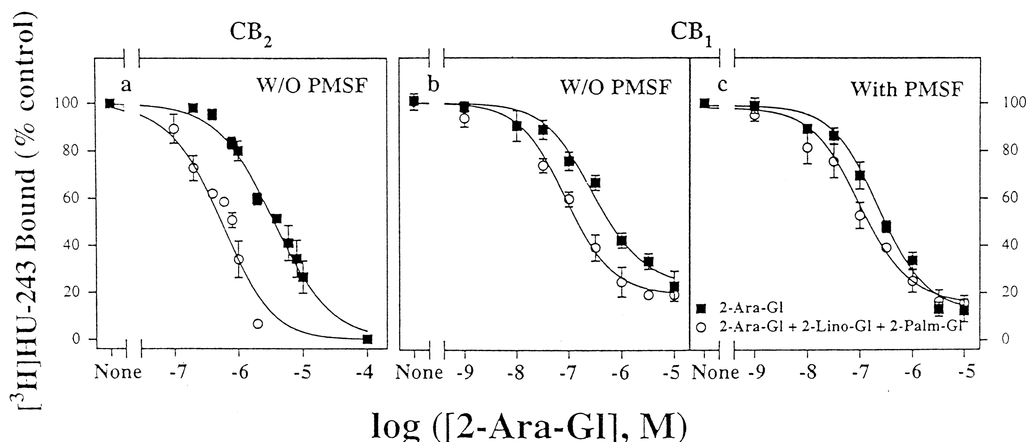


Fig. 2. Binding of 2-Ara-Gl to $CB_1$ and $CB_2$ cannabinoid receptors. (a) Binding of [$^3$H]HU-243 to membranes of $CB_2$ cannabinoid receptor transfected CHO cells was assayed in the presence of the indicated concentrations of 2-Ara-Gl and in the absence or the presence of the inactive acyl-glycerols 2-Lino-Gl and 2-Palm-Gl (in a ratio of 1:12:5). Data are the means ± S.E. of three experiments performed in triplicate. (b–c) Binding of [$^3$H]HU-243 to membranes of $CB_1$ cannabinoid receptor transfected COS-7 cells was determined as in (a) except that the concentration of [$^3$H]HU-243 was raised to 300 pM and 10 mM $CaCl_2$ were added to the assay mixture. When indicated, the membranes were preincubated for 10 min with 2 mM PMSF (final concentration of PMSF in the assay 0.2 mM). Data are represented as the means ± S.E. of three experiments performed in triplicate. The PMSF treatment as well as those of 2-Lino-Gl and 2-Palm-Gl did not affect the binding of [$^3$H]HU-243.

The above mixture of 2-Lino-Gl and 2-Palm-Gl also potentiated the binding of 2-Ara-Gl to the $CB_1$ receptor in COS cells (Fig. 2b). The apparent $K_i$ value observed for 2-Ara-Gl was shifted from 58.3 ± 10.7 nM (for 2-Ara-Gl alone) to 13.9 ± 2.1 nM when assayed in the presence of the two other glycerol esters. Pretreatment of the membranes with phenyl-methylsulphonyl fluoride (PMSF), a non-selective inhibitor of a variety of esterases, as well as of 2-Ara-Gl enzymatic hydrolysis (Bisogno et al., 1997b), potentiated the binding of 2-Ara-Gl to the $CB_1$ receptor, but to a lesser extent (reaching an apparent $K_i$ of 34.6 ± 5.6 nM), compared with the mixture of the two esters (Fig. 2c). The final concentration of PMSF in the assay was 0.2 mM. The combination of PMSF with the two esters yielded essentially the same apparent $K_i$ value (13.5 ± 5.1 nM) as observed with the two esters alone, indicating the high efficacy of the esters in enhancing the activity of 2-Ara-Gl.

### 3.3. Inhibition of adenylyl cyclase

COS-7 cells were transfected with plasmids encoding either $CB_1$ or $CB_2$ and adenylyl cyclase type V (Fig. 3a–d). The addition of 2-Ara-Gl to the cells transfected with $CB_1$ led to inhibition of adenylyl cyclase activity with $IC_{50}$ of 1463 ± 170 nM (Fig. 3a). Pretreatment of the membranes with PMSF (with a final concentration of 0.2 mM during the assay) reduced the $IC_{50}$ to 428 ± 45 nM (Fig. 3b), while the addition of the two fatty acid esters (in the ratio described above) reduced the $IC_{50}$ to 307 ± 51 nM. The addition of the two esters to PMSF treated cells yielded a similar $IC_{50}$ value (385 ± 26 mM). A similar, albeit lower, potentiation of 2-Ara-Gl inhibition of adenylyl cyclase by 2-Lino-Gl and 2-Palm-Gl was found in cells transfected with $CB_2$ (Fig. 3c,d). The addition of these two esters reduced the apparent $IC_{50}$ of 2-Ara-Gl from 2724 ± 371 to 794 ± 111 mM and in cells treated with PMSF from 1884 ± 297 to 784 ± 197 mM demonstrating again the entourage effect of the two esters on the activity of 2-Ara-Gl (Fig. 3c,d).

### 3.4. In vivo assays

The 2-acyl-glycerols were tested in mice in the tetrad of tests which together are generally considered to reflect

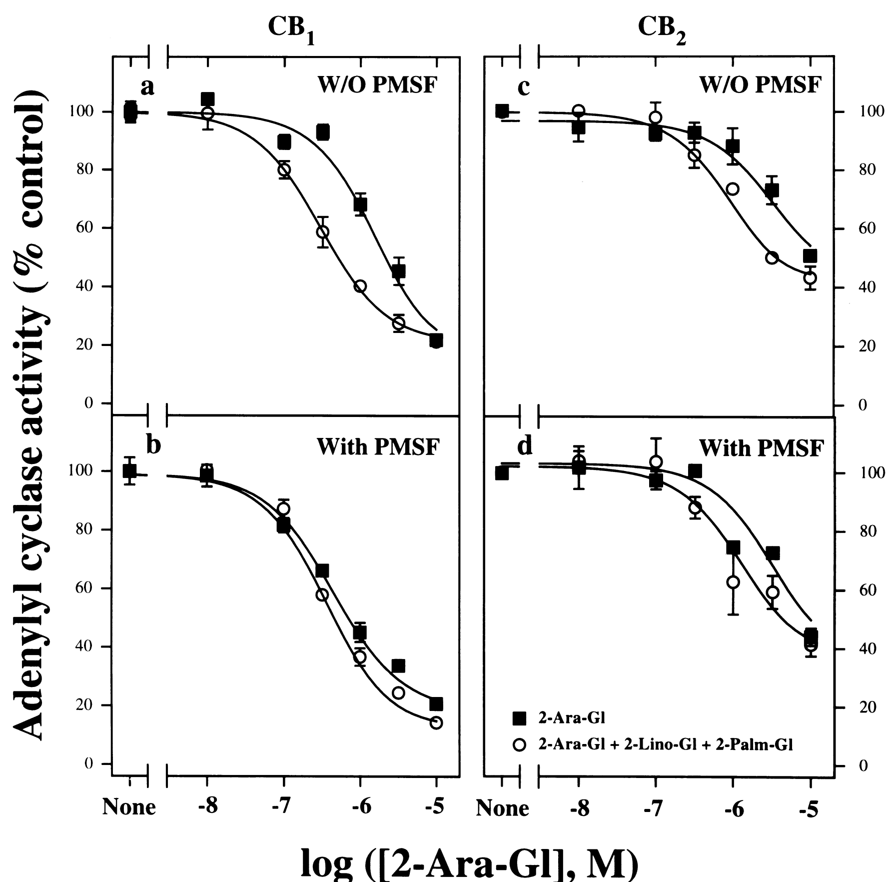


Fig. 3. Inhibition by 2-Ara-Gl of adenylyl cyclase activity. COS-7 cells were transfected with plasmids encoding adenylycl cyclase type V and either $CB_1$ (a,b) or $CB_2$ (c,d) receptors. Two days later, the effect of 2-Ara-Gl on forskolin-stimulated adenylyl cyclase activity was determined in the absence or presence of the acyl glycerols 2-Lino-Gl and 2-Palm-Gl. These glycerol esters had no effect by themselves on adenylyl cyclase activity in the range tested (up to 10 μM). In a second experiment, the cells were preincubated with 0.2 mM PMSF for 5 min before being assayed for adenylyl cyclase activity in the presence of the same concentration of PMSF. PMSF treatment by itself did not affect adenyl cyclase activity. Data presented are from a representative experiment out of three experiments performed in triplicates.

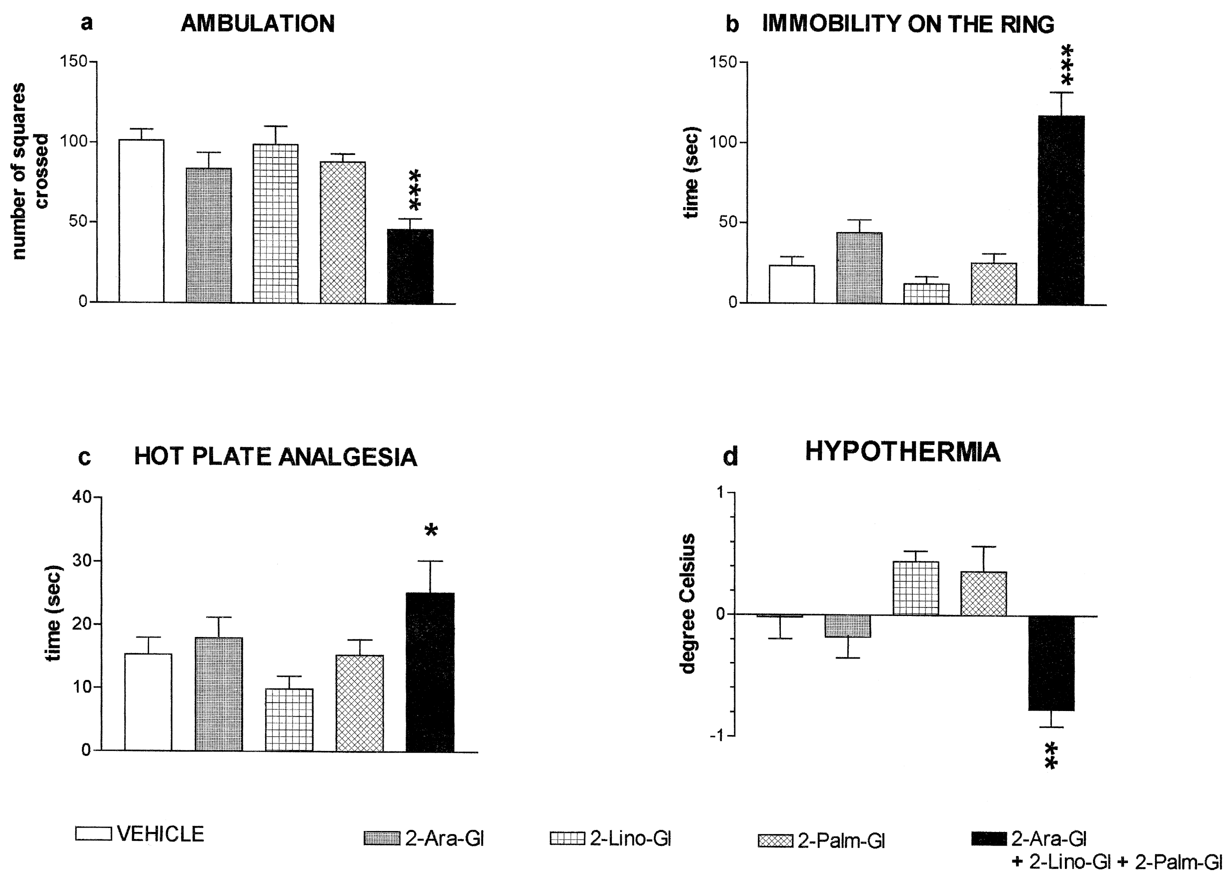


Fig. 4. Mice were tested on the tetrad of tests for cannabinoid-induced effects including ambulation in the open field (a), immobility on a ring (b), analgesia on a hot place (c) and hypothermia (d). Data were analysed by one-way analyses of variance. Individual treatment groups were compared with post hoc Newman–Keuls multiple comparison tests. Injections of 2-Ara-Gl, 1 mg/kg ($n = 10$), 2-Lino-Gl, 10 mg/kg ($n = 5$) or 2-Palm-Gl, 5 mg/kg ($n = 5$) by themselves did not produce any significant effect. However, when 2-Ara-Gl, 2-Lino-Gl and 2-Palm-Gl were injected together, significant effects were obtained in each of the four tests. Moreover, the effects induced by these triple injections differed significantly from each single injection (vehicle, 2-Ara-Gl alone, 2-Palm-Gl alone, 2-Lino-Gl alone). $* \, p < 0.05$, $** \, p < 0.01$, $*** \, p < 0.001$.

cannabinoid-induced activity (Mechoulam et al., 1995; Fride and Mechoulam, 1993; Martin et al., 1991). We investigated whether, similarly to the above described in vitro findings, the presence of 2-Lino-Gl and/or 2-Palm-Gl, potentiates the effects of 2-Ara-Gl. A dose of 2-Ara-Gl (1 mg/kg) at the low end of the dose–response curve was administered, yielding barely observable effects. This low dose was chosen in order to be able to discern any potentiation effects, if present. Administration of 2-Lino-Gl (10 mg/kg) or 2-Palm-Gl (5 mg/kg) alone produced no significant effects (Fig. 4). No effect was observed when the two esters were administered together, at the above dose levels (not shown). Further, injections of 2-Ara-Gl with either 2-Lino-Gl or 2-Palm-Gl at the above indicated doses, produced no significant effects (data not shown). However, injections of 2-Ara-Gl together with both 2-Lino-Gl and 2-Palm-Gl (1, 10, 5 mg/kg, respectively) produced significant inhibition of motor behavior, immobility on the ring, hypothermia and analgesia (Fig. 4). The potentiating effect was especially pronounced in the ring immobility test (Fig. 4b). Since a high dose (20 mg/kg) of either 2-Lino-Gl or 2-Palm-Gl did not produce significant effects, except for reduction in motor activity in the open-field by 2-Palm-Gl (data not shown), we conclude that the potentiation of 2-Ara-Gl induced effects by combined injections of 2-Lino-Gl and 2-Palm-Gl is not the result of a cumulative effect of the low doses of these compounds.

### 3.5. *Inhibition of 2-Ara-Gl enzymatic hydrolysis by 2-Lino-Gl and 2-Palm-Gl*

We assessed whether the facilitatory action of the inactive 2-Lino-Gl and 2-Palm-Gl could be due to protection of 2-Ara-Gl against metabolic inactivation. The possible inhibitory action of the two monoglycerides on 2-Ara-Gl hydrolysis was tested by using two cell lines, i.e., mouse neuroblastoma N18TG2 and rat basophilic leukaemia (RBL-2H3) cells, previously shown to express selectively $CB_1$ and $CB_2$ cannabinoid receptors, respectively (Howlett, 1995; Facci et al., 1995). Subcellular fractions from both cell types have been shown to contain enzymatic

activities for catalyzing 2-Ara-Gl hydrolysis to arachidonic acid (AA) (Bisogno et al., 1997b; Di Marzo et al., 1998). Fig. 5a,b show the effect of 2-Palm-Gl and 2-Lino-Gl on $[^3H]$2-Ara-Gl hydrolysis by the RBL-2H3 and N18TG2 cell particulate fractions. Significant inhibitory effects were obtained with increasing doses of the two compounds.

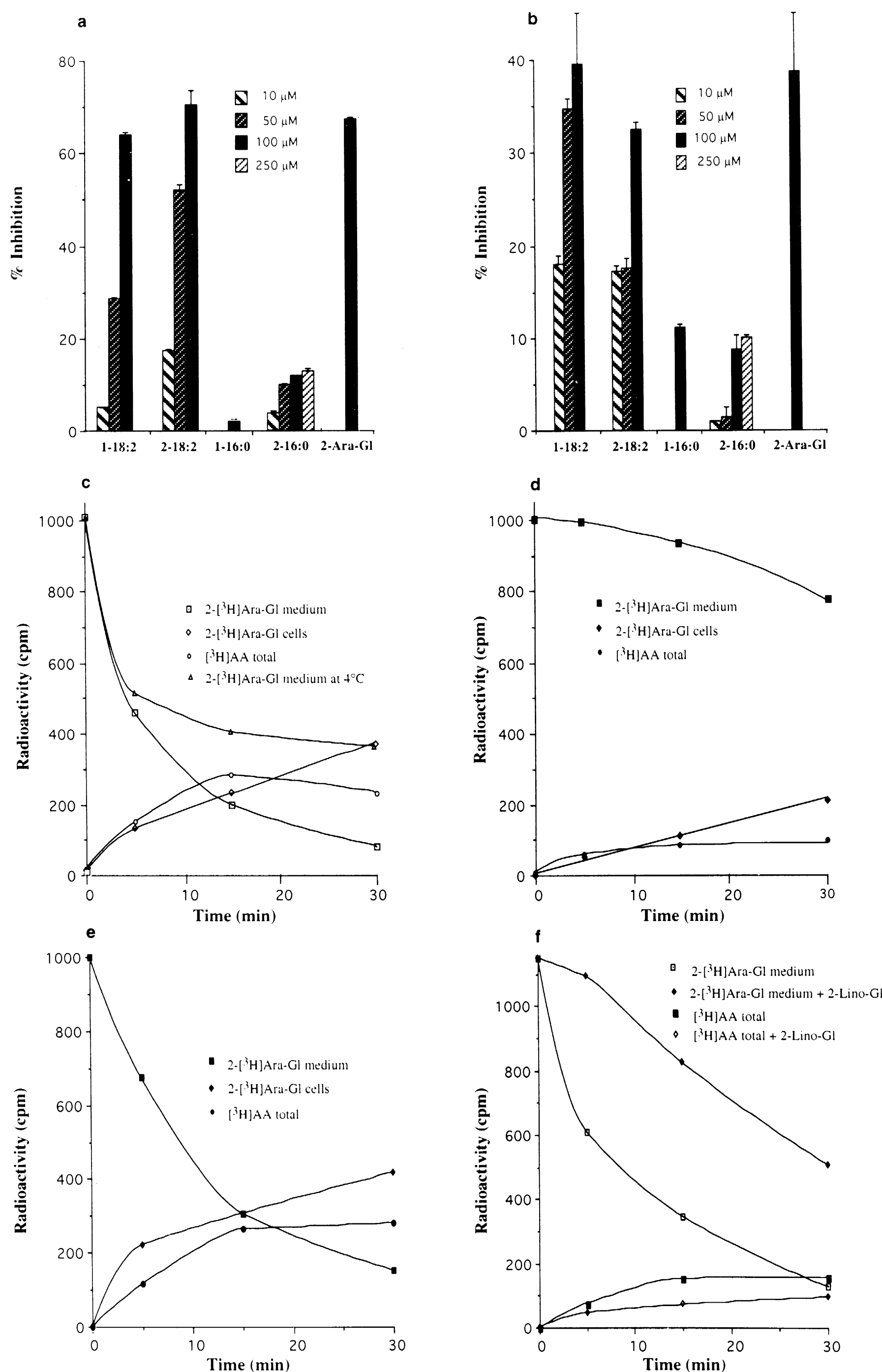


Fig. 5. Effect of monoacylglycerols on 2-Ara-Gl inactivation by RBL-2H3 and/or N18TG2 cells. (a) and (b): effect of 1-Lino-, 2-Lino-, 1-Palm-, 2-Palm- and 2-Ara-Gl on the hydrolysis of $[^3H]$2-Ara-Gl by $10,000 \times g$ pellet fractions from RBL-2H3 (a) and N18TG2 (b) cells. In some cases only the effects of the doses exerting the maximal inhibition are shown. Data are means ± S.D. of three experiments. (c–e) Clearance and hydrolysis of $[^3H]$2-Ara-Gl by intact RBL-2H3 cells, at 37°C or 4°C (c), and in the absence (c) or presence of either 100 μM 2-Lino-Gl (d) or 100 μM 2-Palm-Gl (e) in the incubation media. (f) Clearance and hydrolysis of $[^3H]$2-Ara-Gl by intact N18TG2 cells in the absence or presence of 100 μM 2-Lino-Gl. No significant inhibitory effect was obtained with 100 μM 2-Palm-Gl (not shown for the sake of clarity). Data in (c–f) are means of duplicates and representative of two separate experiments. Occasional differences between the radioactivity cleared from media and that found associated with cells or AA was due to non-specific binding of $[^3H]$-2-Ara-Gl to plastic, and found in the 1% bovine serum albumin washes (see Section 2).

However, only the effects of 2-Lino-Gl were comparable with the inhibitory action observed with 2-Ara-Gl, whereas 2-Palm-Gl was much less effective. A significant inhibition of [$^3$H]2-Ara-Gl hydrolysis by RBL-2H3 membranes was also exerted by a mixture of 2-Palm-Gl, 2-Lino-Gl and 2-Ara-Gl in a molar ratio identical to that observed in the spleen (5:12:1, i.e., 20, 48 and 4 μM, respectively). The inhibitory effect in this case ($42.7 \pm 3.3\%$, means ± S.E.M., $n = 3$), however, was not higher than that exerted by 2-Lino-Gl alone, suggesting that the active component of the mixture responsible for the inhibition was the latter monoglyceride.

### 3.6. *Inhibition of 2-Ara-Gl inactivation by living intact cells*

It was of interest to determine if the protection of 2-Ara-Gl levels by 2-Lino-Gl could also be observed in living intact cells. In RBL-2H3 cells, we found that, without the addition of 2-Lino-Gl and 2-Palm-Gl, 2-Ara-Gl disappeared from the culture media in a temperature-dependent fashion with a half life at 37°C of $6 \pm 2$ min (means ± S.E.M., $n = 4$) (Fig. 5c). This clearance was mostly the result of hydrolysis by and of diffusion into intact cells, as shown by the time-dependent formation of AA and by the finding of increasing amounts of cell-associated [$^3$H]2-Ara-Gl with increasing incubation times (Fig. 5c). Some 2-Ara-Gl was also incorporated into phospholipids (Di Marzo et al., 1998). 2-Lino-Gl, but not 2-Palm-Gl, prevented this loss of 2-Ara-Gl from the medium (Fig. 5d,e). An eight-fold increase of 2-Ara-Gl levels was observed after 30 min incubation. This was accompanied by a reduction in the amount of 2-Ara-Gl associated with the cell fraction as well as with a reduction in the amount of [$^3$H]-arachidonic acid derived from the hydrolysis of [$^3$H]2-Ara-Gl. A similar result was obtained with N18TG2 cells (Fig. 5f and data not shown). A mixture of 2-Palm-, 2-Lino- and 2-Ara-Gl in a 5:12:1 molar ratio (20, 48 and 4 μM, respectively) also inhibited the clearance of the latter from the incubation medium, as well as its inactivation by intact cells, leading to an overall elevation of 2-Ara-Gl levels ($260 \pm 40\%$, after 30 min incubations, means ± S.D., $n = 2$). This value however, was not significantly higher than that observed with 2-Lino-Gl alone ($210 \pm 30\%$). 1-Lino-Gl and 1-Palm-Gl did not differ significantly in their inhibition of the inactivation of 2-Ara-Gl, from 2-Lino-Gl and 2-Palm-Gl (Fig. 5a,b and data not shown).

## 4. Discussion

The above results indicate that in spleen, as in canine gut and rat brain (Mechoulam et al., 1995; Sugiura et al., 1995), 2-Ara-Gl is present together with additional 2-acyl-glycerols, two of which, 2-Lino-Gl and 2-Palm-Gl, showed neither binding activity to $CB_1$ or $CB_2$ cannabinoid receptors in membranes of CHO and/or COS-7 cells nor in vivo cannabinoid effects in mice. However, both 2-Lino-Gl and 2-Palm-Gl separately or together (in the ratio present in the spleen) potentiated the apparent binding of 2-Ara-Gl to $CB_1$ and $CB_2$. The mixture of the three monoglycerides is also more potent than 2-Ara-Gl in the inhibition of adenylyl cyclase in COS-7 cells transfected for either $CB_1$ or $CB_2$ cannabinoid receptors. The same type of 'entourage' effect was observed in several in vivo tests which are commonly used with cannabinoids.

The 'entourage' effects reported above are only in part due to inhibition of 2-Ara-Gl inactivation by cells. In fact, of the two monoacylglycerols tested, only 2-Lino-Gl efficiently inhibited 2-Ara-Gl inactivation by either neuronal or leukocyte cell types used in this study, thus increasing the amounts of 2-Ara-Gl available for cannabinoid receptor activation. 2-Palm-Gl, which was more active than 2-Lino-Gl in the potentiation of 2-Ara-Gl binding to $CB_2$ receptors, did not significantly counteract the inactivation of 2-Ara-Gl by whole cells. These observations suggest that endogenous, inactive 2-acyl-glycerols enhance 2-Ara-Gl activity through inhibition of 2-Ara-Gl inactivation and possibly through other, as yet unknown, mechanisms such as potentiation of binding to CB receptors or inhibition of binding to plasma proteins. Previously, we and others have shown that fatty acid amides, which have no affinity for $CB_1$ receptors, inhibit anandamide metabolism by several types of intact cells (Maurelli et al., 1995; Di Tomaso et al., 1996; Bisogno et al., 1997a; Mechoulam et al., 1997), thus potentially leading to increased levels of endogenous anandamide available for $CB_1/CB_2$ cannabinoid receptor activation. This inhibitory effect may underlie the in vivo cannabimimetic actions observed by us for the fatty acid amide oleamide (Mechoulam et al., 1997), a putative sleep factor. Competition for the binding site of metabolic enzymes also explains, for example, why $\omega - 3$ fatty acids inhibit some of the actions of $\omega - 6$ fatty acids by counteracting the formation of $\omega - 6$ fatty acid derivatives (Okuyama et al., 1997). These results may have considerable specific biological importance. The observation that the potency of 2-Ara-Gl can be modified by related 2-acyl-glycerols present with it could represent a novel route for molecular regulation of endogenous cannabinoid activity.

The results reported now may also be of general importance. Biologically active natural products, from either plant or animal origin, are in many instances accompanied by chemically related, though biologically inactive, constituents. Very seldom is the biological activity of the active constituent assayed together with the inactive 'entourage' compounds. In view of the results described above investigations of the effect of the active component in the presence of its 'entourage' compounds may lead to observations of effects closer to those in Nature than investigations with the active component only.

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