

Inhibition of Rat C6 Glioma Cell Proliferation by Endogenous and Synthetic Cannabinoids. Relative Involvement of Cannabinoid and Vanilloid Receptors

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Received May 30, 2001; accepted August 28, 2001

ABSTRACT

The effects of the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) upon rat C6 glioma cell proliferation were examined and compared with a series of synthetic cannabinoids and related compounds. Cells were treated with the compounds each day and cell proliferation was monitored for up to 5 days of exposure. AEA time- and concentration-dependently inhibited C6 cell proliferation. After 4 days of treatment, AEA and 2-AG inhibited C6 cell proliferation with similar potencies (IC_{50} values of 1.6 and 1.8 μ M, respectively), whereas palmitoylethanolamide showed no significant antiproliferative effects at concentrations up to 10 μ M. The antiproliferative effects of both AEA and 2-AG were blocked completely by a combination of antagonists at cannabinoid receptors (SR141716A and SR144528 or AM251 and AM630) and vanilloid receptors (capsazepine) as well as by α -tocopherol (0.1

and 10 μ M), and reduced by calpeptin (10 μ M) and fumonisin B₁ (10 μ M), but not by L-cycloserine (1 and 100 μ M). CP 55,940, JW015, olvanil, and arachidonoyl-serotonin were all found to affect C6 glioma cell proliferation (IC_{50} values of 5.6, 3.2, 5.5, and 1.6 μ M, respectively), but the inhibition could not be blocked by cannabinoid + vanilloid receptor antagonists. It is concluded that the antiproliferative effects of the endocannabinoids upon C6 cells are brought about by a mechanism involving combined activation of both vanilloid receptors and to a lesser extent cannabinoid receptors, and leading to oxidative stress and calpain activation. However, there is at present no obvious universal mechanism whereby plant-derived, synthetic, and endogenous cannabinoids affect cell viability and proliferation.

The antimitotic effects of the cannabinoid Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the principal psychoactive component of hashish and marijuana, have been known since the 1970s (Munson et al., 1975). More recent studies have shown that Δ^9 -THC and/or the synthetic cannabinoid (CB) receptor agonist WIN 55,212-2 induces apoptosis in various mouse, rat, and human cells (Sánchez et al., 1998; Zhu et al., 1998; Ruiz et al., 1999; Jacobsson et al., 2000; Sarker et al., 2000) and have been shown to induce regression of malignant gliomas in Wistar rats and mice (Galve-Roperh et al., 2000), a result also seen with the CB₂ receptor-selective agonist JWH-133 (Sánchez et al., 2001). In rat C6 glioma cells, the effect of Δ^9 -THC upon cell viability, which takes ~5 days to become apparent (Sánchez et al., 1998), is brought about by a pathway involving CB receptors followed by sustained ceramide accumulation (Galve-Roperh et al., 2000). Blockade of both

CB₁ and CB₂ receptors by the selective antagonists SR141716A and SR144528 is required to protect the cells against the deleterious effects of Δ^9 -THC upon cell viability (Galve-Roperh et al., 2000). In contrast, in human PC-3 prostate cells, the apoptotic effects of Δ^9 -THC are not mimicked by WIN 55,212-2 and are not prevented by pertussis toxin treatment of the cells, suggestive of a CB receptor-independent mechanism (Ruiz et al., 1999).

The endogenous cannabinoid ("endocannabinoid") arachidonylethanolamide (anandamide, AEA) is a partial agonist with similar affinity at CB₁ and CB₂ receptors but lower efficacy at the CB₂ receptor. In addition, AEA can activate vanilloid receptors (Zygmunt et al., 1999; Smart et al., 2000). AEA has been shown to induce apoptosis in several cell types, but the molecular mechanism behind this action appears to be rather different from that of Δ^9 -THC. Thus, the proapoptotic effects of AEA on human CHP-100 neuroblastoma and U-937 lymphoma cells (Maccarrone et al., 2000) were suggested to be mediated via vanilloid receptors. These authors proposed that AEA induces a rise in intracellular calcium,

This work was supported by MFR Grant 12158 from the Swedish Research Council and research funds of the Medical Odontological Faculty, Umeå University, Umeå, Sweden.

ABBREVIATIONS: Δ^9 -THC, Δ^9 -tetrahydrocannabinol; CB, cannabinoid; AEA, *N*-arachidonylethanolamide (anandamide); 2-AG, 2-arachidonoylglycerol; PEA, palmitoylethanolamide; AA-5-HT, arachidonoyl-serotonin; ACEA, arachidonyl-2'-chloroethylamide/(all *Z*)-*N*-(2-cycloethyl)-5,8,11,14-eicosatetraenamide; ANOVA, analysis of variance; meAEA, *R*-(+)-methanandamide; VR, vanilloid receptor; FAAH, fatty acid amidohydrolase.

mitochondrial uncoupling, and cytochrome *c* release, and activation of the caspase cascade. Furthermore, Sarker et al. (2000) found that AEA induces apoptosis in rat PC-12 pheochromocytoma cells by increasing the superoxide anion formation and caspase-3 activation. AEA has also been shown to inhibit the proliferation of human breast and prostate cancer cell lines in vitro (De Petrocellis et al., 1998) secondary to a CB₁ receptor-mediated inhibition of prolactin action, at the level of the prolactin receptor.

The different mechanisms proposed for the effects of, on the one hand plant-derived and synthetic cannabinoids, and on the other hand AEA, upon cell survival present a somewhat confusing picture. In the present study, we have investigated, in the same cells and under the same conditions, the receptors involved in the antiproliferative effects of synthetic and endogenous cannabinoids, including compounds that are selective for the CB₁ or CB₂ receptors. In addition, we have investigated whether the antiproliferative effects of AEA are mimicked by the endocannabinoid 2-arachidonoylglycerol (2-AG) and the metabolically stable AEA analog *R*-(+)-methanandamide.

Rat glioma C6 cells are ideal for this study because they express both functional cannabinoid and vanilloid receptors (Sánchez et al., 1997; Bíró et al., 1998) and respond to AEA (Maccarrone et al., 2000). These cells, however, do not in our hands show a mitogenic response to prolactin (T. Wallin and S.O.P. Jacobsson, unpublished data), thereby precluding investigation into effects of AEA upon proliferation stimulated by this hormone. We reported previously that single administrations of AEA (≤ 10 μ M) failed to affect C6 glioma cell viability (Jacobsson et al., 2000), although this result presumably reflected the rapid removal of AEA from the culture medium. To reduce the cellular metabolism of AEA, we have used a sensitive assay to minimize the number of cells required per well, and in addition treated the cells daily with AEA (or the other test compounds). Because the effects of Δ^9 -THC upon cell viability are more apparent at low culturing serum contents (Jacobsson et al., 2000), we have elected to investigate the effects of the compounds upon cell proliferation at 1% fetal bovine serum.

Experimental Procedures

Materials. AEA, quinacrine (mepacrine), α -tocopherol, fumonisins B₁, and L-cycloserine were all purchased from Sigma Chemical Co. (St. Louis, MO). Olvanil, palmitoylethanolamide (PEA), arachidonoylserotonin (AA-5-HT), and 2-AG were obtained from the Cayman Chemical Co. (Ann Arbor, MI). Capsaicin, capsazepine, CP 55,940 [(*-*)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol], JWH 015 [(2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone], *R*-(+)-methanandamide [*R*-(all-*Z*)]-*N*-(2-hydroxy-1-methylethyl)-5,8,11,14-eicosatetraenamide; MeAEA], WIN 55,212-2 [(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1 naphthalenylmethanone mesylate]; AM 630 [6-lodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl) methanone]; AM 251 [*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide], and ACEA [arachidonyl-2'-chloroethylamide/(all *Z*)-*N*-(2-cycloethyl)-5,8,11,14-eicosatetraenamide] were obtained from Tocris Cookson (Bristol, UK). CyQUANT cell proliferation assay kits and calcein-AM were bought from Molecular Probes (Eugene, OR). SR141716A (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) and SR144528 (*N*-[1*S*]-*endo*-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-

pyrazole-3-carboxamide) were kind gifts from Sanofi Recherche (Montpellier, France). Arachidonylethanolamide-[1-³H] ([³H]AEA) (specific activity 60 Ci mmol⁻¹) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Tissue culture media and all supplements were obtained from Invitrogen (Sweden). Calpeptin was obtained from Calbiochem (San Diego, CA).

Cell Cultures. Rat C6 glioma cells were obtained from American Type Culture Collection (Manassas, VA) and used over a passage range of 41 to 54. Cells were cultured in 75-cm² culturing flasks in Ham's F10 medium supplemented with 25 mM HEPES buffer, L-glutamine, 10% fetal bovine serum (FBS), and 100 units ml⁻¹ penicillin + 100 μ g ml⁻¹ streptomycin (PEST). The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ in air and the medium was changed every 3 to 4 days.

C6 Cell Proliferation Assay. C6 glioma cells were seeded in 96-well flat bottom microplates at a density of 2500 cells well⁻¹ in Ham's F10 medium, supplemented with penicillin-streptomycin. In all experiments (except in the initial AEA experiments, where serum-free conditions were also tested) the culture medium was also supplemented with 1% FBS. Substances to be tested were introduced 6 h after seeding. The total assay volume was 200 μ l. Test substances were serially diluted in appropriate solvents. AEA, olvanil, AA-5-HT, *R*-(+)-methanandamide, PEA, ACEA, JWH015, arachidonic acid, capsaicin, and capsazepine were dissolved in ethanol. 2-AG was dissolved in acetonitrile, whereas SR141716A, SR144528, AM251, AM630, CP 55,940, and WIN 55,212-2 were dissolved in dimethyl sulfoxide. The total solvent concentration was kept constant at 0.5% in all assays. Prolactin was dissolved in phosphate-buffered saline (pH 7.2). Test substances were administered daily, by replacing 100 μ l of medium with fresh medium containing test substances at the indicated concentration. After incubation for the desired time, the cell density was determined, using the CyQUANT cell proliferation assay kit, which quantifies the total amount of nucleic acid in the sample. The medium was removed by gently inverting the microplate, and blotting it onto a paper towel. The plate was then frozen (-80°C). At the day of analysis, plates were thawed over a period of 30 min at room temperature. Fluorescence reagents were then added. After 5-min incubation at room temperature, the sample fluorescence was measured (excitation/emission: 495/520 nm) in a FLUOstar Galaxy microplate reader (BMG Labtechnologies GmbH, Germany). Sample fluorescence values were converted into cell numbers from a C6 cell reference standard curve.

Statistical Analyses. The concentration-dependent effects of the test compounds upon C6 cell proliferation were analyzed for statistically significant differences using one-way analysis of variance (ANOVA) with post hoc Bonferroni's, or where appropriate Dunnett's, multiple comparisons test between each concentration and the corresponding control data. The time- and concentration-dependent effects of AEA upon C6 cell proliferation were analyzed using two-way ANOVA. Apparent IC₅₀ values were determined by fitting the data to a sigmoidal dose-response equation by using nonlinear regression. All statistical analyses were undertaken using GraphPad Prism 3.00 (GraphPad Software, San Diego, CA). All data are presented as the means $\pm$ S.E.M. of at least three separate experiments.

Results

Inhibition of C6 Cell Proliferation by Synthetic and Endogenous Cannabinoids. In initial experiments, AEA was administered daily and the cell growth was followed during 5 days in either serum-free (Fig. 1A) or 1% serum (Fig. 1B) conditions. When cultured in the presence of 1% FBS, 10 μ M AEA decreased the cell density from $34 \pm 1.3 \times 10^3$ cells/well in the control cultures to $9.4 \pm 1.0 \times 10^3$ cells/well ($p < 0.001$; $n = 4$). Even in serum-free conditions, AEA showed antiproliferative effects (Fig. 1A), but the low growth rate and the general status of the C6 cells when deprived of

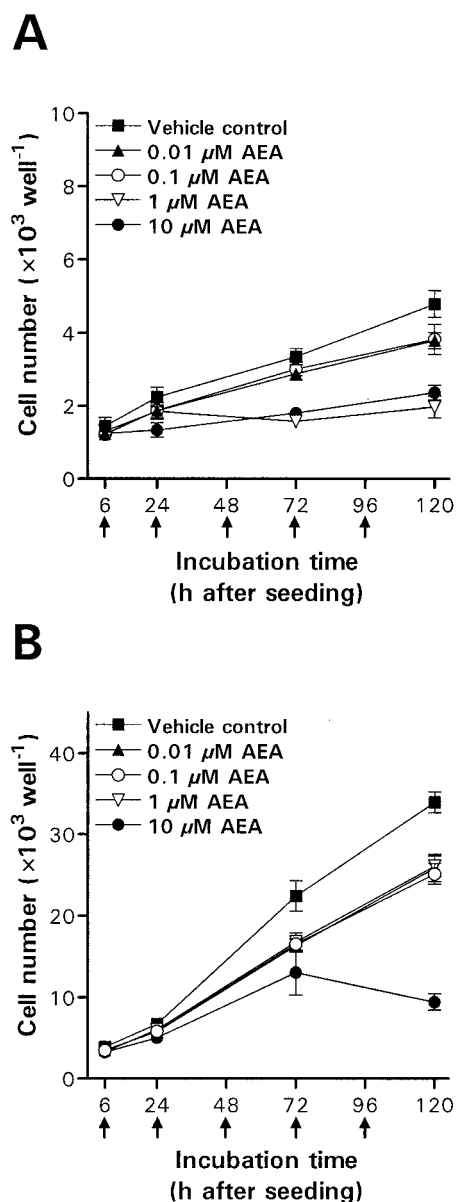


Fig. 1. Time dependence of the inhibition of C6 cell proliferation by AEA. Cells were cultured in the absence (A) or presence (B) of 1% FBS. Shown are means $\pm$ S.E.M. of four different experiments. Arrow bars indicate each administration of AEA. Two-way ANOVA of the results indicated a significant interaction between AEA and time upon the C6 cell proliferation in both serum-free ($F_{12,60} = 5.81$; $p < 0.0001$) and serum-containing ($F_{12,60} = 13.7$; $p < 0.0001$) conditions.

serum during 5 days ruled out further experiments in this extreme condition. In consequence, the remaining experiments were undertaken using 1% FBS in the culture medium.

Concentration-response curves for the effects upon C6 proliferation of 4 days of exposure to a variety of synthetic and endogenous cannabinoids and related compounds are shown in Fig. 2. Both AEA and 2-AG decreased the cell number (Fig. 2A), with IC_{50} values of 1.6 μ M ($n = 8$) and 1.8 μ M ($n = 4$), respectively. This antiproliferative effect could completely be blocked by concomitant incubation of the cells with the antioxidant α -tocopherol (0.1 and 10 μ M), and greatly reduced with the cell-permeable calpain inhibitor calpeptin (10 μ M) (Table 1). In contrast, the phospholipase A_2 inhibitor quina-

crine (1 μ M) did not affect the antiproliferative response to AEA (Table 1). The effects of two inhibitors of cellular ceramide biosynthesis, L-cycloserine and fumonisin B_1 , on the antiproliferative effect of the endocannabinoids are shown in Fig. 3. L-Cycloserine had no effect on the cellular response to endocannabinoids, although a concentration of 100 μ M L-cycloserine caused a significant decrease in cell number per se (Fig. 3). Fumonisin B_1 significantly inhibited the endocannabinoid effect upon C6 cells at a concentration of 10 μ M, whereas 0.1 μ M was without effect (Fig. 3).

PEA, which has a low affinity for CB receptors in vitro (Lambert et al., 1999), showed no significant inhibitory effect upon C6 cell proliferation at concentrations up to 10 μ M (Fig. 2A). Further experiments have, however, found a 40% reduction in cell viability at a PEA concentration of 30 μ M (K.-O. Jonsson, S.O.P. Jacobsson, and C. J. Fowler, unpublished data). The metabolically stable analog of AEA, meAEA, showed no effect on cell proliferation at concentrations up to 3 μ M, but a small decrease was obtained at 10 μ M (Fig. 2B; $n = 4$). In a subsequent experiment (see below), 1 μ M meAEA was without effect, whereas 10 μ M showed a greater degree of inhibition (56%).

Four synthetic CB receptor agonists were investigated. The nonselective agonists CP 55,940 and WIN 55,212-2 both inhibited cell proliferation, although the latter appeared to show a "threshold"-like profile with little or no inhibition ≤ 3 μ M and maximal inhibition at 10 μ M (Fig. 2C). CP 55,940 and the CB₂ receptor-selective agonist JWH015 (Showalter et al., 1996) were roughly equipotent inhibitors of cell proliferation (IC_{50} values of 5.6 and 3.2 μ M, respectively; Fig. 2, B and C; $n = 4$). The CB₁ receptor-selective agonist ACEA (Hillard et al., 1999) was also tested, but inconsistent results were found in different experimental series, precluding conclusions to be made as to its antiproliferative effects (data not shown).

Two vanilloid receptor agonists were investigated. Olvanil produced an inhibition of C6 cell proliferation, with an IC_{50} value of 5.5 μ M ($n = 4$), whereas capsaicin had modest effects (Fig. 2D). AA-5-HT, a synthetic inhibitor of fatty acid amide hydrolase reported as inactive at CB₁ receptors in vivo (Bisogno et al., 1998), inhibited the cell proliferation with an IC_{50} value of 1.6 μ M (Fig. 2B; $n = 7$), a potency also seen with arachidonic acid (Fig. 2A). Representative photomicrographs of the effects of 3 μ M AEA and 3 μ M olvanil upon C6 cell morphology after 4 days of incubation are presented in Fig. 4, A–C.

Effects of Cannabinoid and Vanilloid Receptor Antagonists upon Antiproliferative Effects of Synthetic and Endogenous Cannabinoids. SR141716A (1 μ M), a CB receptor antagonist with marked selectivity for CB₁ receptors (Rinaldi-Carmona et al., 1994), significantly attenuated the antiproliferative effect of 3 μ M 2-AG (Fig. 5), but was less effective in blocking the 3 μ M AEA-induced decrease in cell number. Essentially the same results were obtained when SR144528 (1 μ M), a CB receptor antagonist with a 700-fold higher affinity for the CB₂ receptor than the CB₁ receptor (Rinaldi-Carmona et al., 1998), was used (Fig. 5). However, both the 2-AG- and AEA-mediated effects were significantly reduced by incubating the cultures with a combination of 1 μ M SR141716A and 1 μ M SR144528 (Fig. 5). In contrast, the cannabinoid receptor antagonists had no effect upon the decrease in cell number produced by 3 μ M olvanil (Fig. 5).

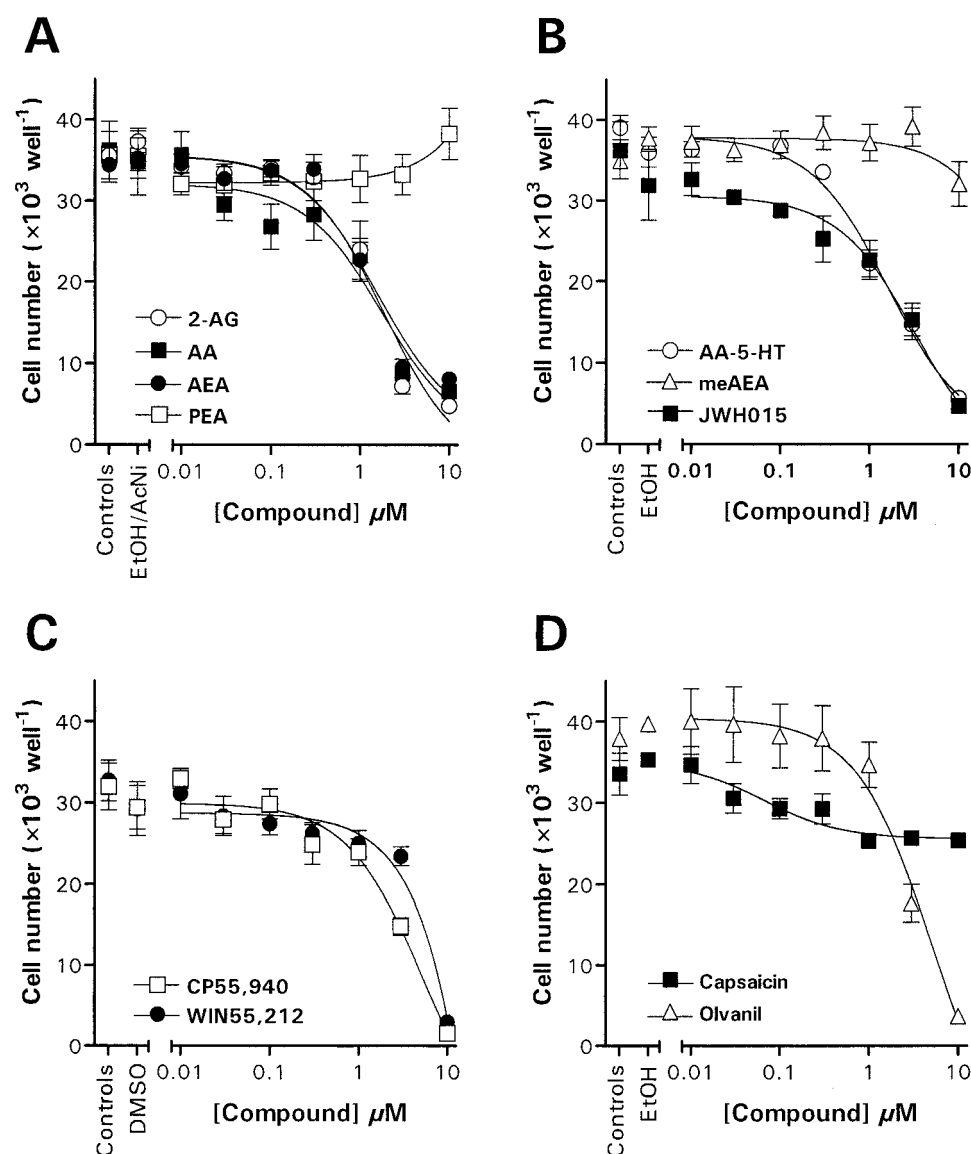


Fig. 2. Concentration-dependent effects of AEA, 2-AG, arachidonic acid (AA), and PEA (A); AA-5-HT, meAEA, and JWH015 (B); CP 55,940 and WIN 55,212-2 (C); and olvanil and capsaicin (D) and upon C6 glioma cells cultured in the presence of 1% FBS were treated daily for 4 days with the concentrations of compounds shown, as described under *Experimental Procedures*. Data are means $\pm$ S.E.M. ($n = 4$ except for AEA where $n = 8$ and AA-5-HT where $n = 3-7$). "Control" indicates untreated cells, whereas EtOH/AcNi (ethanol/acetonitrile) or dimethyl sulfoxide indicates cells treated with the solvent carriers for the cannabimimetic fatty acid derivatives tested.

AA-5-HT (3 μ M) produced a significant ($p < 0.001$) reduction in cell number per se, but this reduction was not affected by the cannabinoid antagonists. Thus, mean ($\pm$ S.E.M., $n = 3$) densities ($\times 10^3$ well $^{-1}$) for cells treated with 3 μ M AA-5-HT in the absence and presence of SR141716A, SR144528, and SR141716A + SR144528 were 20.1 ± 0.40 , 20.6 ± 0.38 , 21.3 ± 0.61 , and 20.6 ± 0.67 , respectively.

At a concentration of 1 μ M, the vanilloid receptor antagonist capsazepine significantly blocked the antiproliferative effects of AEA and almost completely blocked the effect of 2-AG (Fig. 5). Higher concentrations of capsazepine (5 and 10 μ M) showed antiproliferative effects per se although the toxic effects of the combination of AEA (or 2-AG, but not olvanil) and 10 μ M capsazepine were less than for either substance given alone (Table 2). When 1 μ M capsazepine was combined with SR141716A and SR144528, the antiproliferative effects of 3 μ M AEA and 3 μ M 2-AG were almost completely blocked (Fig. 5). In contrast, capsazepine had no effect on the olvanil-mediated decrease in cell numbers either per se or in combination with SR141716A and SR144528 (Fig. 5).

In a second series of experiments, AM251 and AM630 were

TABLE 1

Effects of α -tocopherol, calpeptin, and quinacrine upon the antiproliferative effects of 3 μ M AEA and 2-AG in C6 glioma cells. C6 glioma cells were treated daily for 4 days with the concentrations of compounds shown, as described under *Experimental Procedures*. Shown are means $\pm$ S.E.M., with the number of experiments given in brackets.

Test Compound	Cell Number ($\times 10^3$ well $^{-1}$)		
	Control	AEA (3 μ M)	2-AG (3 μ M)
μ M			
α -Tocopherol			
0	32.6 ± 1.2 [5]	9.5 ± 0.73^a [5]	12.7 ± 1.2^a [4]
0.1	32.3 ± 1.3 [5]	34.1 ± 1.3^b [5]	30.8 ± 1.3^b [4]
10	33.3 ± 1.9 [5]	35.0 ± 1.8^b [5]	33.9 ± 2.0^b [4]
Calpeptin			
0	30.4 ± 0.92 [5]	9.8 ± 0.50^a [5]	13.6 ± 1.1^a [4]
0.1	32.5 ± 1.7 [5]	9.5 ± 1.1 [5]	13.5 ± 1.6 [4]
10	28.6 ± 0.54 [5]	21.1 ± 4.1^b [5]	29.9 ± 1.6^b [4]
Quinacrine			
0	32.6 ± 1.2 [5]	9.5 ± 0.73^a [5]	12.7 ± 1.2^a [4]
0.01	31.6 ± 2.0 [5]	8.2 ± 0.80 [5]	7.3 ± 1.8 [4]
1	27.3 ± 2.7 [3]	6.7 ± 2.1 [5]	10.8 ± 3.3 [4]

^a $p < 0.001$ versus the corresponding control values in the absence of α -tocopherol, quinacrine, or calpeptin.

^b $p < 0.001$ versus the corresponding values for the same AEA/2-AG treatment in the absence of α -tocopherol or calpeptin.

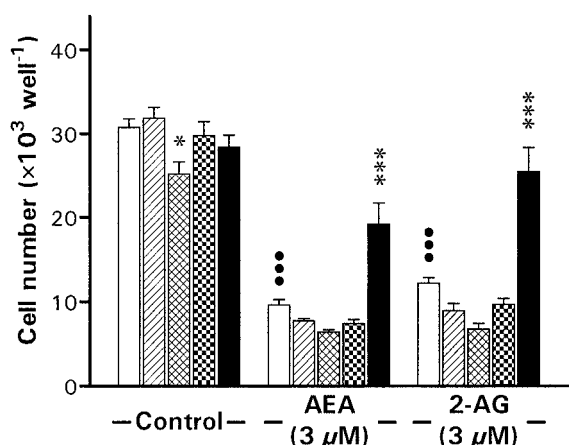


Fig. 3. Effects of L-cycloserine (▨, 1 μ M; ▩, 100 μ M) and fumonisins B₁ (▤, 0.1 μ M; ■, 10 μ M) upon the sensitivity to the antiproliferative effects of AEA and 2-AG. C6 glioma cells were treated daily for 4 days with the concentrations of compounds shown, as described under *Experimental Procedures*. ●●●, $p < 0.001$ (effect of the AEA/2-AG per se compared with control samples in the absence of the antagonists); *, $p < 0.05$; ***, $p < 0.001$ (effect of the test compound compared with the corresponding "none" sample at the same AEA/2-AG concentration). Data are means $\pm$ S.E.M., $n = 4-5$. □, no additions ("none").

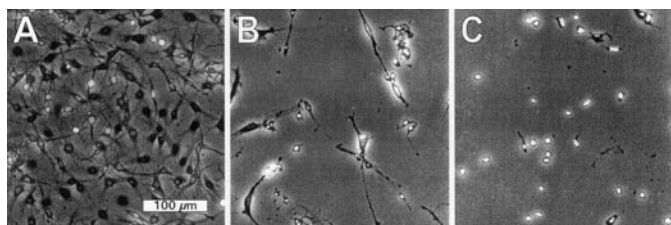


Fig. 4. Phase-contrast photomicrographs of representative experiments showing control (A), AEA- (3 μ M, B), and olvanil- (3 μ M, C)-mediated effects upon C6 glioma cells cultured in 1% FBS (A to C) after 4 days of incubation. The control cultures (A) showed a confluent cell layer with some bright-phase cell bodies and spreading extensions. Upon exposure to AEA (B), the remaining cells revealed swollen soma and fragmented extensions. Olvanil (C) produced detached small bright-phase cell bodies with virtually no extensions.

used at concentrations of 0.3 μ M as selective antagonists of CB₁ and CB₂ receptors, respectively (Pertwee et al., 1995; Lan et al., 1999; Ross et al., 1999). The antiproliferative effect of 3 μ M AEA was not affected by AM630, but was completely blocked by AM251 (and by capsazepine) (Fig. 6). In contrast, the antiproliferative effects of 3 μ M CP 55,940, JWH015, and AA-5-HT were not inhibited by AM251, AM630, and capsazepine either alone or in combination; indeed, the combination appeared to increase the antiproliferative effects of these compounds (Fig. 6).

Combination of Effects of meAEA and Capsaicin upon C6 Glioma Cell Proliferation. The effects of the combination of meAEA (1 and 10 μ M) and capsaicin (1 and 10 μ M) upon the proliferation of C6 cells is shown in Table 3. Essentially additive effects that could not be blocked by the combination of AM251, AM630, and capsazepine were found for the two compounds.

Discussion

In the present study, the effects of endogenous and synthetic cannabinoids upon the proliferation of C6 glioma cells were investigated to resolve some of the conflicting data in the literature concerning the receptor systems involved in

the effects of these compounds. The choice of a glioma cell line was prompted by the finding that cannabinoids reduce their cell proliferation in vivo (Galve-Roperh et al., 2000). Indeed, the recent finding by this group of a high frequency of CB₂ receptor expression in human astrocytomas, and that the expression correlated with tumor malignancy (Sánchez et al., 2001), raises the possibility that modulation of cannabinoid receptors in vivo may provide a possible avenue for treatment of patients with glioma and motivate per se further experimentation in glioma cells. We have previously shown that the effects of Δ^9 -THC upon C6 glioma cell viability are highly dependent upon the assay serum concentration, with no deleterious effects being seen after a 6-day treatment with 1 μ M Δ^9 -THC at a FBS concentration of 10%. In contrast, in serum-free medium, sensitivity to Δ^9 -THC and cannabidiol was seen (Jacobsson et al., 2000). C6 glioma cells, however, proliferate poorly in serum-free medium, and the FBS concentration in the present study was chosen as a balance between sensitivity to cannabinoids and rate of cell proliferation.

Robust effects of AEA and 2-AG upon cell proliferation were seen after their repeated administration over 4 days. The protective effects of α -tocopherol and calpeptin found in the present study implicate oxidative stress and excessive intracellular calcium to be involved in the antiproliferative effects of AEA and 2-AG toward C6 cells. These results are perhaps not surprising given that 1) vanilloid receptors are directly coupled to cation channels, with a high permeability to calcium (Szallasi and Blumberg, 1999); and 2) calpain overexpression and apoptosis is a commonly used pathway for toxic stimuli in C6 glioma cells (Ray et al., 1999). Cannabinoid receptor-mediated activation of the ceramide pathway is well characterized (for review, see Guzmán et al., 2001), and the finding that fumonisins B₁, an inhibitor of ceramide synthase (Wang et al., 1991), reduces the antiproliferative effects of AEA and 2-AG is consistent with the hypothesis described by others (Galve-Roperh et al., 2000) that the proapoptotic actions of ceramide are involved in cannabinoid actions in C6 glioma cells. Although the lack of effect in the present study of the serine palmitoyltransferase inhibitor L-cycloserine is a surprising result, it is not unreasonable to conclude that the antiproliferative effects of AEA and 2-AG in our C6 glioma cells require a combination of cannabinoid- and vanilloid-receptor-mediated mechanisms, including influx of calcium and activation of the ceramide pathway. However, this conclusion must be validated in further experiments.

Stimulation of CB₁ receptors results in arachidonic acid production secondary to phospholipase A₂ activation (see Shivachar et al., 1996, for data with AEA), and this pathway is responsible for the neurotoxic effects of Δ^9 -THC in cultured hippocampal neurons (Chan et al., 1998). Although the lack of effect of SR141716A per se (see below) would argue against such a mechanism being important for the antiproliferative effects of AEA in C6 glioma cells, we investigated the phospholipase A₂ inhibitor quinacrine upon the antiproliferation produced by endocannabinoids. No antagonism of the effects of either AEA or 2-AG was found with either 0.01 or 1 μ M quinacrine. Although higher concentrations of quinacrine are often used in short-term experiments, a concentration of 1 μ M is sufficient to produce a large functional inhibition of phospholipase A₂ in fibroblast cells (Kim et al., 1998). In any

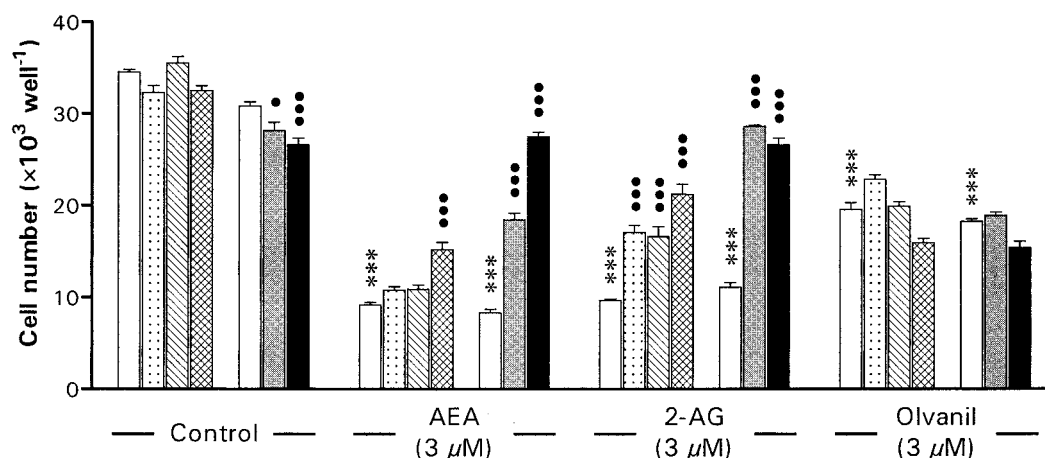


Fig. 5. Effects of concomitant treatment of C6 glioma cells cultured in 1% FBS with SR141716A, SR144528, and/or capsazepine upon the sensitivity to the antiproliferative effects of AEA, 2-AG, and olvanil. C6 glioma cells were treated daily for 4 days with the concentrations of compounds shown, as described under *Experimental Procedures*. The data for capsazepine and their corresponding controls are also given in Table 1. Statistical treatment of each set of data was undertaken using one-way ANOVA with Bonferroni's post hoc multiple comparison (in the case of the capsazepine data, the values also included the 5 and 10 μM concentrations of this compound shown in Table 2): ***, $p < 0.001$ (effect of the treatments per se compared with control samples in the absence of the antagonists); •, $p < 0.05$, ••, $p < 0.01$, •••, $p < 0.001$ (effect of the antagonist treatment compared with the corresponding "none" sample [containing the appropriate combination of solvent vehicles] at the same AEA/2-AG/olvanil concentration). In the absence of any solvents, the cell number was $31.9 \pm 2.7 \times 10^3 \text{ well}^{-1}$. Data are means $\pm$ S.E.M., $n = 3-9$. □, SR141716A (1 μM); ▤, SR144528 (1 μM); ▨, SR141 + SR145 (both 1 μM); ▩, capsazepine (1 μM); ■, SR141 + SR145 + capsazepine (all 1 μM); □, no additions ("none").

TABLE 2

Effects of capsazepine upon the antiproliferative effects of AEA, 2-AG, and olvanil in C6 glioma cells

C6 glioma cells were treated daily for 4 days with the concentrations of compounds shown, as described under *Experimental Procedures*. Shown are means $\pm$ S.E.M., with the number of experiments given in brackets. The data with 0 and 1 μM capsazepine are also shown in Fig. 5. Statistical significance was determined using the whole data set (i.e., including the combination of capsazepine and the SR compounds shown in Fig. 5).

Capsazepine μM	Cell Number ($\times 10^3 \text{ well}^{-1}$)			
	Control	AEA (3 μM)	2-AG (3 μM)	Olvanil (3 μM)
0	30.8 ± 0.47 [8]	8.3 ± 0.42^c [7]	11.1 ± 0.51^c [8]	18.3 ± 0.28^c [3]
1	28.1 ± 0.89^a [7]	18.5 ± 0.70^b [9]	28.6 ± 0.14^b [4]	18.9 ± 0.40 [4]
5	20.5 ± 0.72^b [3]	23.8 ± 1.3^b [3]		
10	13.7 ± 0.46^b [7]	21.2 ± 0.83^b [7]	20.1 ± 0.32^b [4]	15.1 ± 0.33 [4]

^a $p < 0.05$, ^b $p < 0.001$ versus the corresponding values for the same AEA/2-AG/olvanil treatment in the absence of capsazepine.

^c $p < 0.001$ versus the corresponding control values in the absence of capsazepine.

case, higher concentrations of quinacrine were found to produce toxic effects per se on C6 cell proliferation under the long incubation times used (data not shown).

It is well established that AEA (and 2-AG) activates vanilloid receptors (VR, Zygmunt et al., 1999; De Petrocellis et al., 2000; Smart et al., 2000). We found that the VR antagonist capsazepine significantly blocked the effects of AEA and 2-AG. This result is consistent with the effects of this compound upon the proapoptotic actions of AEA on suspensions of several cell lines, including C6 glioma cells (Maccarrone et al., 2000) and suggests involvement of vanilloid receptors. Maccarrone et al. (2000) found that 10 μM capsazepine reduced the number of apoptotic bodies produced by AEA treatment of C6 cell suspensions by 57%. In our hands, this concentration of capsazepine was toxic per se, but intriguingly the toxicity of the combination of 10 μM capsazepine and AEA (and 2-AG) was lower than found for either compound per se.

AEA is removed from the medium by cellular uptake followed by fatty acid amidohydrolase (FAAH)-catalyzed metabolism, raising the possibility that compounds that block FAAH may increase endogenous AEA to a level sufficient to cause effects upon cell proliferation. Indeed, in a recent study, it was demonstrated that the antiproliferative effect of

AEA upon breast cancer cells was enhanced by PEA, which not only competes for FAAH but also produced a down-regulation of this enzyme (Di Marzo et al., 2001). The FAAH inhibitor AA-5-HT did produce an antiproliferative effect in the present study, but this effect is most probably due to a nonspecific cell toxicity rather than an increased cellular level of AEA because it was not prevented by CB + VR antagonists.

The experiments with CB receptor antagonists were more complex. In the first experimental series, significant attenuation of the antiproliferative effect was seen with the combination of the CB₁ receptor antagonist SR141716A and the CB₂ receptor antagonist SR144528, whereas modest (in the case of 2-AG) or no (in the case of AEA) attenuation was seen when the compounds were given separately. In the second series of experiments, the CB₂ receptor antagonist AM630 was without effect upon the antiproliferative effects of AEA, whereas the CB₁ receptor antagonist AM251 completely blocked the effect. In both experiments, however, a combination of CB receptor and VR antagonists completely blocked the antiproliferative effects of AEA. In a recent study, it was reported that although 1 μM SR141716A did not affect the vanilloid receptor-mediated calcium mobilization response to AEA and capsaicin in human embryonic kidney cells trans-

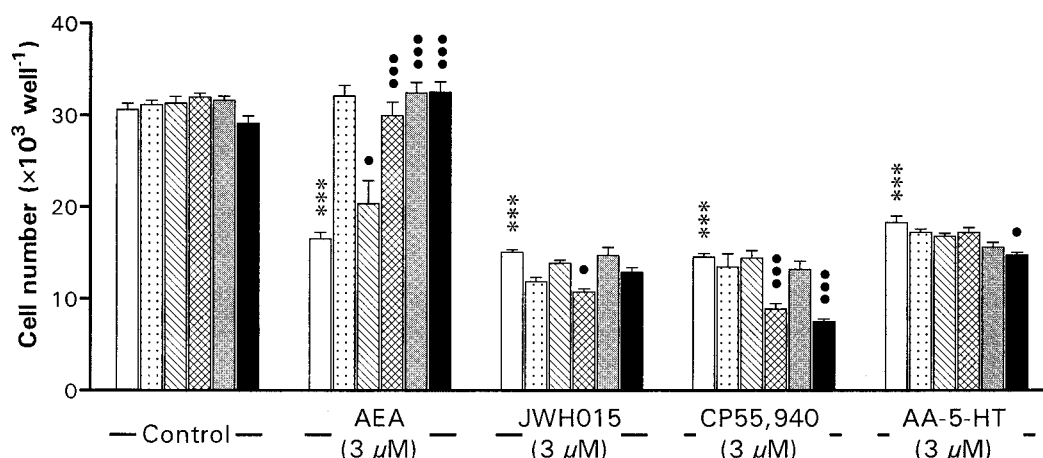


Fig. 6. Effects of concomitant treatment of C6 glioma cells cultured in 1% FBS with AM251, AM630, and/or capsazepine upon the sensitivity to the antiproliferative effects of AEA, JWH015, CP 55,940, and AA-5-HT. C6 glioma cells were treated as described under *Experimental Procedures* and Fig. 5. Statistical analysis was undertaken using one-way ANOVA with with Bonferroni's post hoc multiple comparison: ***, $p < 0.001$ (effect of the treatments per se compared with control samples in the absence of the antagonists); •, $p < 0.05$, ••, $p < 0.01$, •••, $p < 0.001$ (effect of the antagonist treatment compared with the corresponding "none" sample [containing the appropriate combination of solvent vehicles] at the same AEA/2-AG/olvanil concentration). In the absence of any solvents, the cell number was $32.9 \pm 0.7 \times 10^3$ well $^{-1}$. Data are means $\pm$ S.E.M., $n = 4$. □, AM251 (0.3 μ M); ▤, AM630 (0.3 μ M); ▨, AM251 + AM630 (both 0.3 μ M); ▩, capsazepine (1 μ M); ■, AM251 + AM630 + capsazepine (0.3 + 0.3 + 1 μ M); □, no additions ("none").

TABLE 3

Effects of combinations of meAEA and capsaicin upon the proliferation of C6 glioma cells

C6 glioma cells were treated daily for 4 days with the concentrations of compounds shown, as described under *Experimental Procedures*. Shown are means $\pm$ S.E.M., $n = 4$. In the absence of any solvents, the cell number was $33.4 \pm 2.9 \times 10^3$ well $^{-1}$. In the "Antagonists" column, – and + refer to solvent control and the combination of AM251 (0.3 μ M), AM630 (0.3 μ M), and capsazepine (1 μ M), respectively. Two-way ANOVA of the results obtained in the absence of antagonists revealed a significant effect of both capsaicin ($F_{2,27} = 6.26$; $p = 0.006$) and meAEA ($F_{2,27} = 57.47$; $p < 0.0001$) but no interaction between the compounds ($F_{4,27} = 0.53$; $p = 0.72$).

Capsaicin μ M	Antagonists	Cell Number ($\times 10^3$ well $^{-1}$) after [meAEA] (μ M) of:		
		0	1	10
0	–	30.9 ± 0.70	28.0 ± 3.2	13.7 ± 3.7
0	+	30.4 ± 3.9	26.0 ± 3.0	7.3 ± 1.9
1	–	29.7 ± 3.4	27.1 ± 1.6	8.1 ± 1.8
1	+	26.8 ± 3.2	23.4 ± 2.9	4.2 ± 0.67
10	–	23.0 ± 2.8	24.1 ± 1.0	4.8 ± 1.1
10	+	23.6 ± 3.3	19.9 ± 2.9	2.4 ± 0.43

ected with the human VR $_1$ receptor, concentrations of 2.5 and 5 μ M SR141716A antagonized the response (De Petrocellis et al., 2001). Given that AM251 is structurally related to SR141716A (Lan et al., 1999), it is possible that the compound affects the function of vanilloid receptors at the concentration used (0.3 μ M), thereby contributing to its antagonism of the antiproliferative effect of AEA. An alternate explanation for the different results with the CB $_1$ receptor antagonists in the two series of experiments, which were undertaken 4 months apart, is that they reflect the difficulties associated with the use of a heterogeneous population of cells where the cannabinoid receptor component of the antiproliferative effect may vary (Galve-Roperh et al., 2000). However, were this the case, antagonism of the effects of CP 55,940 by the CB antagonists would have been expected.

Taken together, these data would suggest that the antiproliferative effects of AEA and 2-AG toward our C6 cells under the experimental conditions used here are a consequence of a combination of effects on both cannabinoid and vanilloid receptors, and that the effect upon vanilloid receptors predominates. If this result can be extended to the synthetic cannabinoids, it can be predicted that CP 55,940 and WIN 55,212-2 should not affect cell proliferation to any large extent, given that these compounds do not activate vanilloid

receptors (Zygmunt et al., 1999; Smart et al., 2000). Indeed, given the potencies of these compounds functionally to activate CB receptors in intact cells (Hillard et al., 1999; Ross et al., 1999), antiproliferative effects of CP 55,940 and WIN 55,212-2 in the submicromolar range would have been predicted whether effects upon CB receptors alone were sufficient to produce antiproliferative effects. Similarly, the VR receptor activator capsaicin would not be expected to produce as good antiproliferative effects as found with AEA, given its lack of effects at CB receptors (Di Marzo et al., 1998). At these submicromolar concentrations, no effects were seen in our hands, and the antiproliferative effects seen at the higher concentrations of CP 55,940 were presumably nonspecific effects of this compound, because they could not be antagonized by the CB (or VR) antagonists. Such nonspecific effects may also account for the antiproliferative actions of WIN 55,212-2 and JWH015. In this respect it should be noted that the induction of apoptosis produced by high concentrations (200–250 μ M) of capsaicin in human glioblastoma cells is not related to actions upon vanilloid receptors (Lee et al., 2000).

Olvani and meAEA are agonists at both vanilloid and cannabinoid receptors over the concentration ranges used (Di Marzo et al., 1998) and would thus have been expected to produce effects similar to those seen with AEA. In the case of

olvanil, antiproliferative effects were observed but could not be blocked by CB receptor and VR antagonists either alone or in combination, suggestive of a nonspecific mode of action at the relatively high concentrations used here. No antiproliferative effects at low concentrations of olvanil (i.e., those giving a selective activation of VR) were seen, consistent with the data with capsaicin. meAEA produced a weaker inhibition of proliferation than AEA, again despite the fact that meAEA interacts with both receptor systems (Ralevic et al., 2000). Although we have no firm explanation for these anomalous results, one possibility is that a certain balance of agonist effects is required on the two receptor systems for antiproliferative effects to be observed. In the case of AEA, concentrations required for activation of VR receptors are approximately 10-fold higher than required for activation of CB receptors (Zygmunt et al., 1999; Smart et al., 2000), whereas olvanil is highly VR-selective (Di Marzo et al., 1998; De Petrocellis et al., 2000). meAEA is approximately 10 times less potent than AEA at inhibiting the binding of [³H]resiniferatoxin to Chinese hamster ovary cells transfected with rVR1 (Ross et al., 2001). In this assay, capsaicin and AEA are equipotent (Ross et al., 2001), raising the possibility that the combination of meAEA and capsaicin may mimic AEA with respect to antiproliferative effects in C6 cells. Although the antiproliferative effects of meAEA + capsaicin were greater than for meAEA alone, the combination of the two compounds produced additive effects and could not be antagonized by CR receptor + VR receptor antagonists.

In conclusion, the present study suggests that AEA and 2-AG, but not meAEA or olvanil, produce antiproliferative effects upon C6 glioma cells by a mechanism that involves both cannabinoid and vanilloid receptors, followed by oxidative stress and calpain activation. In our hands, compounds activating either CB receptors or VR alone do not produce antiproliferative effects at relevant concentrations. The protective effects of CB receptor antagonists (either SR14116A + SR144528 or AM251) are in contrast to the report by Maccarrone et al. (2000) that the number of apoptotic bodies produced 48 h after incubation of C6 glioma cell suspensions with 10 μ M AEA was increased by 77% in the presence of SR141716A and by 11% in the presence of SR144528 (both 1 μ M). Interestingly, these authors demonstrated that the addition of AM404 further increased the number of apoptotic bodies produced by the combination of AEA and SR141716A (and of AEA alone), a result that is presumably a combination of the ability of this compound to prevent AEA metabolism (Beltramo et al., 1997) and to activate vanilloid receptors (De Petrocellis et al., 2000, 2001; Zygmunt et al., 2000). Such differences between the data of Maccarrone et al. (2000) and the present study may reflect differences in the properties of the C6 cells used in the two laboratories, given that these cells are rather heterogeneous in nature. In this respect, Galve-Roperh et al. (2000) demonstrated that two subclones of C6 cells showed a dramatic difference in the sensitivity to the effects of Δ^9 -THC upon cell viability. Clearly, there is at present no obvious universal mechanism whereby plant-derived, synthetic, and endogenous cannabinoids affect cell viability and proliferation.

Acknowledgments

We thank Ingrid Persson for excellent technical assistance.

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