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Inhibition of C6 glioma cell proliferation by anandamide, 1-arachidonoylglycerol, and by a water soluble phosphate ester of anandamide: variability in response and involvement of arachidonic acid

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Abstract

It has previously been shown that the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) inhibit the proliferation of C6 glioma cells in a manner that can be prevented by a combination of capsazepine (Caps) and cannabinoid (CB) receptor antagonists. It is not clear whether the effect of 2-AG is due to the compound itself, due to the rearrangement to form 1-arachidonoylglycerol (1-AG) or due to a metabolite. Here, it was found that the effects of 2-AG can be mimicked with 1-AG, both in terms of its potency and sensitivity to antagonism by Caps and CB receptor antagonists. In order to determine whether the effect of Caps could be ascribed to actions upon vanilloid receptors, the effect of a more selective vanilloid receptor antagonist, SB366791 was investigated. This compound inhibited capsaicin-induced Ca^{2+} influx into rVR1-HEK293 cells with a pK_B value of 6.8 ± 0.3. The combination of SB366791 and CB receptor antagonists reduced the antiproliferative effect of 1-AG, confirming a vanilloid receptor component in its action. 1-AG, however, showed no direct effect on Ca^{2+} influx into rVR1-HEK293 cells indicative of an indirect effect upon vanilloid receptors. Identification of the mechanism involved was hampered by a large inter-experimental variation in the sensitivity of the cells to the antiproliferative effects of 1-AG. A variation was also seen with anandamide, which was not a solubility issue, since its water soluble phosphate ester showed the same variability. In contrast, the sensitivity to methanandamide, which was not sensitive to antagonism by the combination of Caps and CB receptor antagonists, but has similar physicochemical properties to anandamide, did not vary between experiments. This variation greatly reduces the utility of these cells as a model system for the study of the antiproliferative effects of anandamide. Nevertheless, it was possible to conclude that the antiproliferative effects of anandamide were not solely mediated by either its hydrolysis to produce arachidonic acid or its CB receptor-mediated activation of phospholipase A_2 since palmitoyltrifluoromethyl ketone did not prevent the response to anandamide. The same result was seen with the fatty acid amide hydrolase inhibitor palmitoylethylamide. Increasing intracellular arachidonic acid by administration of arachidonic acid methyl ester did not affect cell proliferation, and the modest antiproliferative effect of umbelliferyl arachidonate was not prevented by a combination of Caps and CB receptor antagonists.

Keywords: 2-Arachidonoylglycerol; 1-Arachidonoylglycerol; Anandamide; Vanilloid receptors; Cannabinoid receptors; Cell proliferation; SB366791

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Abbreviations: 2-AG, 2-arachidonoylglycerol; 1-AG, 1-arachidonoylglycerol; SB366791, *N*-(3-methoxyphenyl)-4-chlorocinnamanilide; Caps, capsazepine; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-ethyl-1*H*-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-*H*-indol-3-yl-(4-methoxyphenyl) methanone; PTMK, palmitoyltrifluoromethyl ketone; VDM11, (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide.

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1. Introduction

Δ^9-Tetrahydrocannabinol exerts a variety of biological actions in the body as a result of its ability to activate a family of G-protein coupled CB receptors [1,2]. The discovery of CB receptors led to a search for endogenous substances that could activate these receptors. In 1992 arachidonoylethanolamide (anandamide) was isolated when porcine brains were extracted with organic solvents and the extracts screened for cannabinoid activity [3]. Subsequently, anandamide was shown to activate not only CB receptors but also vanilloid type 1-receptors (VR1) [4,5]. Over the last few years, several compounds have been identified as ''endocannabinoids'' such as 2-AG [6] and 2-arachidonoylglyceryl (noladin ether) [7].

A potentially important function of endogenous cannabinoids is their ability to modulate cell proliferation [8,9]. In 2000, Maccarrone *et al.* [10] found that treatment of a variety of cell lines with anandamide led to apoptosis that could be prevented by treatment of the cells with the VR antagonist Caps [10]. Jacobsson *et al.* [11] showed further in C6 glioma cells, which produce functional responses to both cannabinoid and capsaicin stimulation [12,13] and express CB_1 receptors [10,14,15], that anandamide produced a time-dependent reduction in cell proliferation that was blocked by a combination of Caps and antagonists for CB_1 and CB_2 receptors. The inhibition of cell proliferation was also prevented by the antioxidant α-tocopherol, and reduced by calpeptin, suggesting an antiproliferative mechanism of anandamide involving oxidative stress and calpain activation secondary to the combined activation of VR and CB receptors [11].

Some anomalies were, however, seen in the study of Jacobsson *et al.* [11]. Firstly, the stable anandamide analogue methanandamide, which interacts with both CB and VR receptors [4,5,16–18] did not inhibit cell proliferation as well as with anandamide, and that the inhibition seen was not prevented by a combination of Caps and CB_1 and CB_2 receptor antagonists. Secondly, 2-AG treatment of the cells produced inhibition of cell proliferation with a similar potency to anandamide. The antiproliferative effect of 2-AG was also blocked by combination of Caps + antagonists for CB_1 and CB_2 receptors, by α-tocopherol, and reduced by calpeptin [11]. However, 2-AG is a rather modest activator of VR1 receptors [19] which would suggest a different mechanism of antiproliferative action. Finally, in a follow-up study [20], we found that the ability of anandamide to inhibit cell proliferation varied between experimental series, a finding that was not seen in our initial study [11].

A number of explanations for these anomalies can be made. It is possible that Caps-sensitivity is not an adequate index of VR-mediated activity, and that other properties of this compound (see e.g. [21]), may account for its efficacy in preventing the antiproliferative effects of anandamide and/or 2-AG. Secondly, 2-AG rapidly undergoes rearrangement to form 1-AG in biological conditions [22,23], and the effects of 2-AG may therefore be mediated by 1-AG. Thirdly, the time-dependent effects of anandamide, whereby no antiproliferative effects are seen until ≥72 hr of incubation, may indicate that this endocannabinoid is in fact acting as a precursor that cannot be mimicked by methanandamide. An obvious candidate molecule in this respect would be arachidonic acid (or a downstream arachidonate metabolite), since methanandamide is enzymatically hydrolysed much more slowly than anandamide [24]. Finally, the inter-experimental variation in anandamide sensitivity [20] may reflect variations in the expression of target proteins or metabolising enzymes, or alternatively be a consequence of the difficulties associated with the use of highly lipophilic compounds in an aqueous environment.

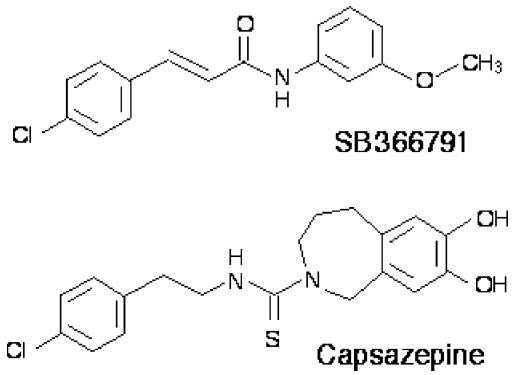

Fig. 1. Structures of capsazepine (Caps) and SB366791.

In the present study we have investigated these possibilities further, by (a) comparing the efficacy of Caps with SB366791, a potent and more selective VR1 antagonist than Caps ([25], structure, see Fig. 1), (b) comparing the effects of 1-AG with 2-AG both with respect to antiproliferative effects and direct effects upon VR1, (c) determining whether compounds preventing production of arachidonic acid from anandamide, or alternatively producing an intracellular increase in arachidonate levels affect cell proliferation, and (d) determining whether water-soluble phosphate esters of anandamide and methanandamide [26,27] behave in a similar way to the parent compounds.

2. Materials and methods

2.1. Materials

SB366791 was synthesised at GlaxoSmithKline, but is now commercially available from Sigma RBI and Tocris Cookson. Anandamide phosphate [26] (phosphoric acid mono-[2-((5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenoylamino)-ethyl] ester) and methanandamide phosphate [27] (phosphoric acid mono-[2-((5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenoylamino)-propyl] ester) were synthesised

at the laboratory of co-authors Järvinen and Juntunen. Palmitoylethylamide was synthesised at the laboratory of co-authors Vandevoorde and Lambert. 2-AG (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid), 1-AG, umbelliferyl arachidonate (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, 2-oxo-2*H*-1-benzopyran-7-yl ester), arachidonic acid methyl ester (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, methyl ester) and palmitoyltrifluoromethyl ketone were obtained from the Cayman Chemical Co. Capsaicin, AM630, VDM11, AM251, AM630, and *N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothioamide (Caps) were purchased from Tocris Cookson. CyQuant® cell proliferation assay kits were bought from Molecular Probes. Indomethacin was obtained from the Sigma Chemical Co. All cell culture media and supplements were obtained from Life Technologies (for the rVR1-HEK293 cells) or from Invitrogen (for the C6 cells).

2.2. Measurement of intracellular Ca^{2+} concentrations in rVR1-HEK293 cells

Intracellular Ca^{2+} concentrations was monitored using FLIPR™ (Molecular Devices) as described previously [28]. Briefly, rat vanilloid VR1 receptor-HEK293 cells, seeded into 96-well plates (25,000 cells/well), were incubated with culture medium containing the cytoplasmic Ca^{2+} indicator, Fluo-3 (4 μM; Teflabs) at 25° for 120 min. The cells were then washed four times with Tyrode's medium, before being incubated for 30 min at 25° in the presence or absence of various antagonists (0.1 nM–10 μM). The plates were then placed into a FLIPR™ to monitor cell fluorescence ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 540$ nm) [29] before and after the addition of capsaicin (0.1 nM–10 μM).

2.3. C6 cell proliferation assay

The method described by Jacobsson *et al.* [11] was used. Rat C6 glioma cells (passage range: 41–68), obtained from the American Type Culture Collection, were cultured in 75 cm^2 flasks in Ham's F10 medium, supplemented with 10% foetal bovine serum and 100 units/mL penicillin & 100 μg/mL streptomycin (1% PEST) at 37°, 5% CO_2 in air at normal atmospheric pressure. Culture media were changed three times a week. For each assay, the cells were plated on flat-bottomed 96-well plates at an initial density of 2500 cells/well in cell culture media supplemented with 1% FBS and 1% PEST. After a 6 hr incubation at 37° under an atmosphere of 5% CO_2 in air, the compounds were incubated with the cells at different concentrations. The compounds to be tested were mixed with Ham's F-10, 1% FBS and 1% PEST and introduced by adding 100 μL of the mixture to each well, giving a total volume of 200 μL/well. The compounds were administered daily for 4 days by replacing 100 μL of medium with fresh medium-substance mixture. The carrier (ethanol, ethanol/DMSO or acetonitrile) concentration was kept constant for the different concentrations of the test substances and was in the range of 0.5–1% for ethanol, 0.1% for DMSO and 0.5% for acetonitrile. These concentrations did not affect cell proliferation *per se*. In general, each experiment consisted of determinations on three to four 96-well plates that were in some cases run concomitantly, sometimes separately. For the experiments run concomitantly, each plate used cells derived from a different 75 cm^2 flask. Triplicate determinations were undertaken for each condition on each plate. The *n* given in the figures thus refers to the number of plates with separate cell preparations used, not the number of replicates/plate.

After 5 days of incubation, the medium was removed by inverting the plates and gently shaking them after which they were placed upside down on top of a paper towel. After about 30 min, the plates were frozen in −80° for at least 24 hr until assayed for cell density using the CyQuant® cell proliferation assay kit, which measures the nucleic acid content in the test samples. Briefly, the frozen micro plates were thawed at room temperature, after which the fluorescence reagents were added and incubated for 5 min in room temperature. Fluorescence was measured (excitation/emission; 495/520 nm) in a FLUOstar Galaxy microplate reader (BMG Labtechnologies GmbH) and the values, after subtraction of blanks, were used directly to determine the effects of the compounds upon cell proliferation. *Post-hoc* calibration curves indicated that the relationship between cell density and fluorescence deviated very slightly from linear at high cell densities. However, for ease of analysis, this small deviation is not taken into account when the data are simply expressed as % of control. This means, however, that the degree of inhibition of cell proliferation is slightly underestimated, i.e. that the effects of the compounds upon cell proliferation, if anything, are slightly greater than shown in the figures.

2.4. Data analysis

Curve-fitting and parameter estimation were carried out using GraphPad Prism (GraphPad Software Inc.). In the case of the antiproliferative effects of the compounds, the data was analysed using the ''sigmoid dose response (variable slope)'' built-in analysis of the programme, with the ''top'' (i.e. uninhibited) value set to 100. The programme returned the ''bottom'' (i.e. minimum value) value together with confidence limits. When the confidence limits straddled zero (i.e. the maximum attainable inhibition of cell proliferation was not significantly different from 100%), the data was reanalysed using a bottom value fixed at zero to avoid biases due to unrealistic bottom values. For 1-AG, the bottom values were significantly greater than 0, and so the pI_{50} values refer to the maximal attainable inhibition (i.e. 100%—bottom value). Statistical comparisons were made where appropriate using either one

way factorial ANOVA with Bonnferoni–Dunn's *post-hoc* test or with Student's *t*-test.

3. Results

3.1. SB366791 is a competitive antagonist at rVR1

In order to be able to interpret effects of the selective VR1 antagonist SB366791 component of 1-AG inhibition, it is important to know the potency of this compound towards rat VR1 receptors, in particular given the fact that human and rat VR show different pharmacological properties [30]. Both the classical VR1 antagonist, Caps (1 nM–10 μM), and SB366791 (0.1 nM–10 μM) (structures, see Fig. 1), inhibited the capsaicin (100 nM)-induced calcium response in rVR1-HEK293 cells (Fig. 2), with pK_B values of 6.6 ± 0.2 (N = 3) and 6.8 ± 0.3 (N = 5), respectively. SB366791 acted as a competitive antagonist at rVR1 as it caused a parallel rightward shift of the capsaicin concentration–response relationship (Fig. 2). SB366791 was used at concentrations of 1 and 3 μM in the experiments described below.

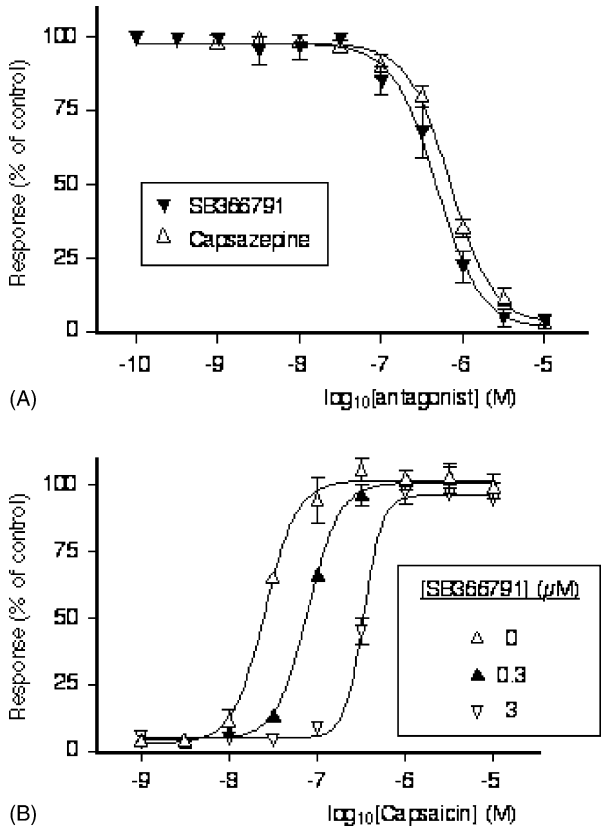

Fig. 2. SB366791 is a competitive antagonist at rat VR1. (A) Intracellular Ca^{2+} concentration was monitored using Fluo-3 in rat vanilloid VR1 receptor-HEK293 cells before and after the addition of capsaicin (100 nM), in the presence or absence of Caps (1 nM–10 μM) or SB366791 (0.1 nM–10 μM). (B) Effects of 0.3 and 3 μM SB366791 upon the concentration response curves to capsaicin. Data are mean ± SEM where N = 5.

3.2. Effects of 1-AG, 2-AG and related compound upon Ca^{2+} responses in rVR-HEK293 cells

In order to determine whether observed antiproliferative effects of the arachidonoyl compounds could be ascribed to direct or indirect effects at VR, 1-AG, 2-AG, umbelliferyl arachidonate and arachidonic acid methyl ester were investigated for their ability to activate VR1 in rVR-HEK293 cells. No large Ca^{2+} responses were seen. Thus responses of 6.4, 11.4, 6.5 and 4.6 (as % of the corresponding responses seen with 10 μM anandamide) were found with 10 μM concentrations of 1-AG, 2-AG, umbelliferyl arachidonate and arachidonic acid methyl ester, respectively. These data would argue against any direct VR1-mediated effects of these compounds upon cell proliferation.

3.3. Comparison of the effects of 1-AG and 2-AG upon C6 glioma cell proliferation

The effects of 1-AG and 2-AG upon C6 glioma cell proliferation are shown in Fig. 3. Both compounds inhibited the proliferation with very similar potencies. In the case of 2-AG, the maximum inhibition of proliferation ($86 \pm 7\%$) was not significantly different from 100%, and the pI_{50} value was 5.51 ± 0.04. In the case of 1-AG, the maximum inhibitable inhibition of cell proliferation was similar ($88 \pm 5\%$) but in this case was significantly different from 100%, and the pI_{50} value of the inhibitable fraction was 5.73 ± 0.06.

3.4. Variation in the antiproliferative effect of 1-AG

Similar to the situation for anandamide [16], the ability of 1-AG to inhibit cell proliferation varied between experimental series. Thus, in the first, second and fourth series of experiments, a robust inhibition was found, whereas in the third and fifth series, the inhibition of cell proliferation was very modest (Fig. 4). Analysis of the fourth series of experiments gave a pI_{50} value of 5.72 ± 0.05 (maximum attainable inhibition: $68 \pm 5\%$). In these experiments, parallel wells were incubated with 1-AG in the presence of 1 μM VDM11. This concentration of VDM11 produced a slight inhibition *per se*, but when this was compensated for by expressing the data as % of controls containing the same concentration of VDM11, 1-AG inhibited cell proliferation with a pI_{50} value of 5.91 ± 0.17 (maximum attainable inhibition: $68 \pm 12\%$) (data not shown). Thus, co-incubation with VDM11 does not affect the potency of 1-AG in this assay.

3.5. The effect of CB receptor and VR1 blockade upon the antiproliferative effect of 1-AG

The similar potencies of 1-AG and 2-AG (Section 3.2 above) would suggest that 2-AG exerts its antiproliferative effects due to its spontaneous rearrangement to 1-AG. If

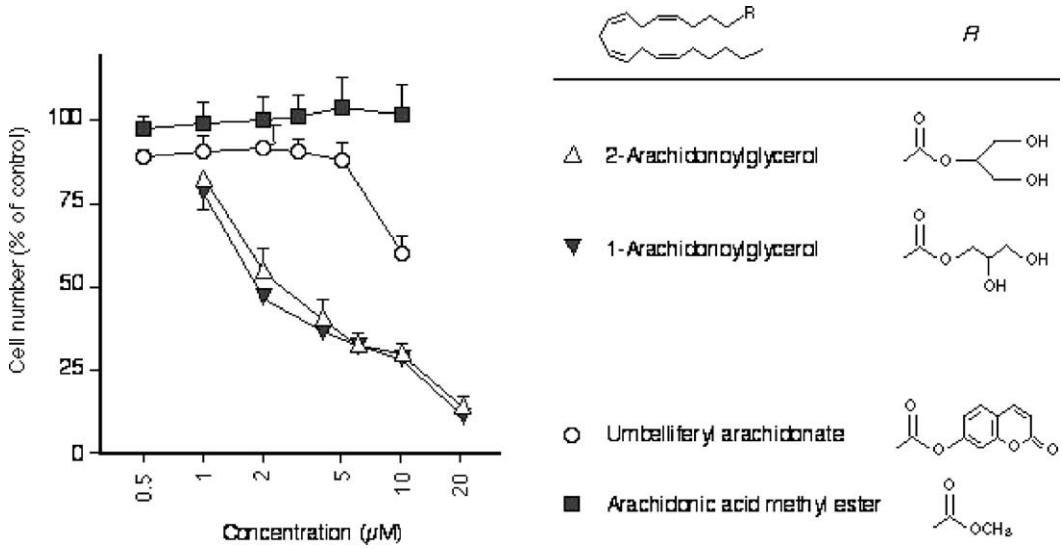

Fig. 3. Inhibition of C6 glioma cell proliferation by 2-AG and related compounds. The compounds were added daily for 4 days, as described in detail in Section 2. Shown are means ± SEM, N = 3–6. It should be noted that the concentrations given in this and subsequent figures and tables are the nominal concentrations added, and that metabolism and/or accumulation of the compounds upon repeated administration will affect the actual assay concentrations.

this is the case, the antiproliferative effects of 1-AG should also be sensitive to the combination of Caps and the combination of CB_1 and CB_2 cannabinoid receptor antagonists. This was found to be the case. Thus, in the second series of experiments (described in Section 3.4), parallel wells were treated with either Caps or SB366791 either *per se* or in combination with the CB_1 and CB_2 cannabinoid receptor antagonists AM251 and AM630 (Table 1). Caps alone was not sufficient to block the antiproliferative effect of 1-AG. However, the combination of Caps and the cannabinoid antagonists AM251 and AM630 completely blocked the effects of 1-AG. The effects of Caps were essentially mimicked by 3 μM SB366791, whereas no significant effects of 1 μM SB366791 were seen.

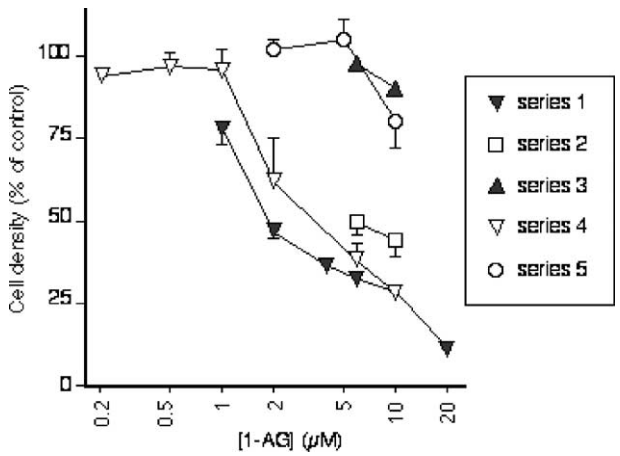

Fig. 4. Comparison of the variation in the ability of 1-AG to inhibit cell proliferation in different experimental series. The data for Series 1 are the same as shown in Fig. 3, and the data for Series 2 are shown as the controls in Fig. 5. Shown are means ± SEM (when not enclosed within the symbols) of: Series 1, N = 6; Series 2, N = 4; Series 4, N = 3; Series 5, N = 5. For Series 3, the symbols represent means, N = 2.

3.6. Effect of palmitoyltrifluoromethyl ketone and palmitoylethylamide on C6 cell proliferation

Arachidonic acid can be produced following anandamide treatment of cells both as a result of its hydrolysis (by fatty acid amide hydrolase) [31] and by CB receptor-mediated activation of phospholipase A_2 [32]. In order to determine whether these processes could account for the antiproliferative effects of anandamide, cells were treated with anandamide and the fatty acid amide hydrolase/phospholipase A_2 inhibitor PTMK (3 μM) [33,34]. The data are shown in Fig. 5. In the first experiment, concentrations of AEA ≤1 μM were not significantly affected by PTMK, whereas a potentiation (rather than an inhibition) of the

Table 1
Effects of Caps, SR366791 alone and in combination with CB receptor antagonists upon the antiproliferative effects of 1-AG in C6 glioma cells

	Cell density (% of control)	
	6 μM 1-AG	10 μM 1-AG
Vehicle (EtOH/DMSO)	50 ± 4	44 ± 5
Caps (1 μM)	79 ± 8	70 ± 13
Caps (1 μM + AM/AM)	92 ± 4*	93 ± 5*
SB366791 (1 μM)	53 ± 4	43 ± 4
SB366791 (1 μM + AM/AM)	67 ± 9	78 ± 15
SB366791 (3 μM)	82 ± 11	83 ± 13
SB366791 (3 μM + AM/AM)	91 ± 10*	83 ± 8

Abbreviation: AM/AM, 0.3 μM AM251 + 0.3 μM AM630. Data are means ± SEM, N = 4, expressed as % of the corresponding control sample treated with the same combination of CB receptor and VR1 antagonist in the absence of 1-AG. None of the antagonist treatments produced changes *per se* in the level of cell proliferation (data not shown). One way ANOVA with *post-hoc* Bonnferoni–Dunn tests were used to assess significance at each 1-AG concentration.

* $P < 0.05$ vs. corresponding samples not exposed to the CB receptor and VR antagonists.

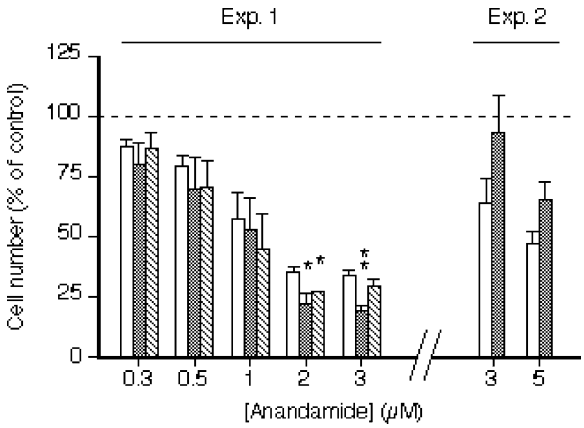

Fig. 5. Effect of palmitoyltrifluoromethyl ketone and palmitoylethylamide upon the antiproliferative effects of anandamide. Anandamide was administered daily for 4 days in the absence (unfilled columns) or presence of either palmitoyltrifluoromethyl ketone (filled columns, added concentration: 3 μM) or palmitoylethylamide (hatched columns, added concentration: 30 μM) and cell proliferation was measured on day 5. Shown are means ± SEM, N = 3 for two separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the corresponding value in the absence of palmitoyltrifluoromethyl ketone or palmitoylethylamide (two-tailed *t*-test).

effects of 2 and 3 μM anandamide were seen. However, in the second experiment, no such potentiation was seen. When the data for the two experiments were taken together, the cell proliferation, as % of control, seen with 3 μM anandamide was 49 ± 8 and 56 ± 18% in the absence and presence of PTMK, respectively (means ± SEM, N = 6).

In the first experiment in this section, the effects of the fatty acid amide hydrolase inhibitor palmitoylethylamide [35] (30 μM) were also investigated. The compound produced a slight, but significant, potentiation of the antiproliferative effect of 2 μM anandamide (from 35 to 27% of control) but did not affect any of the other concentrations of anandamide (Fig. 5).

3.7. Effects of umbelliferyl arachidonate and arachidonic acid methyl ester on C6 cell proliferation

In order to determine whether the actions of 1-AG (and anandamide) could be mimicked by other arachidonic acid precursors, cells were treated with umbelliferyl arachidonate and arachidonic acid methyl ester. A modest inhibition of cell proliferation was seen with umbelliferyl arachidonate, although the data was not analysed further since <50% inhibition was attained at the highest concentration tested (10 μM). Arachidonic acid methyl ester was without effect on cell proliferation over the concentration range tested (Fig. 3). In contrast to the situation for 1-AG, the combination of Caps and AM251/AM630 did not prevent the antiproliferative effects of umbelliferyl arachidonate. Thus, the cell proliferation (% of control) following treatment with 10 μM umbelliferyl arachidonate was 52 ± 6 and 46 ± 3% in the absence and presence of Caps + AM251/AM630, respectively (means ± SEM, N = 3).

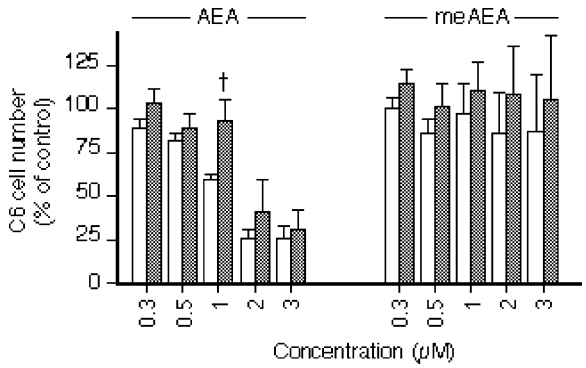

Fig. 6. Comparison of the effects of anandamide and methanandamide (meAEA) (unfilled columns) and their water-soluble phosphate analogues (filled columns) on C6 glioma cell proliferation. The compounds were administered daily for 4 days at the concentrations shown in the figure and cell proliferation was measured on day 5. Data are presented as % of carrier control. Shown are means ± SEM, N = 3. $^{\dagger}P = 0.052$, otherwise $P > 0.2$, two-tailed *t*-test vs. the corresponding anandamide or meAEA-treated cells.

3.8. Inhibition of cell proliferation by the phosphate esters of anandamide and methanandamide

Anandamide is a highly lipophilic compound, and it is possible that the variation in its effects from experiment to experiment reflect solubility issues. If this was the case, no such variation should be seen with the water-soluble phosphate ester of anandamide. This compound and the corresponding phosphate ester of methanandamide were tested and compared with the parent compounds in the range 0.3–3 μM (Fig. 6). The potencies of anandamide and anandamide phosphate were very similar, with pI_{50} values of 5.90 ± 0.04 and 5.71 ± 0.06, respectively, being found. Neither methanandamide nor its phosphate ester produced marked inhibition when assayed concomitantly at these concentrations. However, higher concentrations of these compounds did reduce cell proliferation (Table 2).

In two subsequent series of experiments, the ability of Caps and CB receptor antagonists to block the antiproliferative effects of the phosphate esters and their parent compounds were investigated (Table 2). The combination of Caps and CB receptor antagonists completely blocked the antiproliferative effects of anandamide and anandamide phosphate, but not the methanandamide compounds. In the final series of experiments, however, the ability of anandamide phosphate to inhibit cell proliferation was lost. Thus, the cell proliferation (as % of control) for 2 and 3 μM anandamide phosphate was 102 ± 4 and 100 ± 5%, respectively. In these experiments, 3 μM anandamide was also without effect, whereas 5 μM anandamide produced a small reduction in cell proliferation (data not shown). Interestingly, methanandamide and methanandamide phosphate retained their effects in these experiments, cell proliferation rates of 60 ± 1 and 40 ± 5% of control, respectively, being found for 10 μM concentrations of the compounds.

Table 2
Effect of treatment with Caps and cannabinoid receptor antagonists upon the antiproliferative effects of anandamide, methanandamide and their phosphate esters

	Cell density (% of control)	
	Vehicle	Caps + AM251 + AM630
Anandamide (3 μM)	63 ± 10	125 ± 19*
Anandamide (5 μM)	47 ± 5.1	92 ± 20†
Anandamide phosphate (2 μM)	57 ± 5.8	101 ± 4.0**
Anandamide phosphate (3 μM)	51 ± 5.6	103 ± 11*
Methanandamide (5 μM)	62 ± 6.1	63 ± 7.5
Methanandamide (10 μM)	51 ± 5.5	49 ± 7.6
Methanandamide phosphate (10 μM)	29 ± 6.3	17 ± 5.2

Data are means ± SEM, N = 3 of the cell densities expressed as % of the corresponding values with the same antagonist concentrations but in the absence of the anandamide analogue. Anandamide and methanandamide were dissolved in ethanol, whereas the phosphate esters were dissolved in water. The corresponding controls contained the same concentrations of solvent carrier. Caps: 1 μM capsazepine. The concentrations of AM251 and AM630 were 0.3 μM.

* $P < 0.05$, two-tailed t-test vs. the corresponding vehicle-treated cells.

** $P < 0.01$, otherwise $P > 0.2$ two-tailed t-test vs. the corresponding vehicle-treated cells.

† $P = 0.098$, two-tailed t-test vs. the corresponding vehicle-treated cells.

4. Discussion

In the present study, the abilities of anandamide, 1-AG and water soluble analogues of anandamide to affect the proliferation of C6 glioma cells have been studied in a series of experiments. 1-AG is rapidly formed from 2-AG under biological conditions [22,23], and the finding here that 1-AG and 2-AG have the same pI_{50} values and sensitivities to the combination of Caps and CB receptor antagonists (present study for 1-AG, [11] for 2-AG) are consistent with the suggestion that the effects of 2-AG in this model system are secondary to its spontaneous chemical rearrangement to 1-AG.

The most important finding of the present study, given the considerable interest in the antiproliferative properties of endocannabinoids, is that the C6 model system as used here is by no means ideal, in view of the unpredictable variation in the sensitivity of the cells to 1-AG and anandamide between experiments. C6 glioma cells are somewhat heterogeneous in nature, and different subclones have been found to show different sensitivities to the antiproliferative effects of Δ^9-tetrahydrocannabinol, which are mediated by CB receptor mobilisation of the ceramide pathway [36]. It is possible that use of "1-AG-sensitive and anandamide-sensitive subclones" may prove more useful (not the least in order to determine whether the two compounds act via the same or different mechanisms, and whether the reason(s) for the variabilities in response are the same for 1-AG and anandamide), should such subclones be identified, isolated, and found to be stable. It is also possible that other factors, such as passage number, may be of importance. In this respect, β-adrenoceptor sensitivity of C6 cells has been shown to be sensitive to the passage number [37] and the same situation may apply here. However, no obvious relation between passage number and 1-AG sensitivity was seen in the present study. A number of explanations for the variation in sensitivity to anandamide and 1-AG can be considered.

4.1. Involvement of vanilloid and CB receptors in the antiproliferative effects of 1-AG: variation in expression

A key question is the relative roles played by vanilloid and CB receptors in the antiproliferative effects of anandamide and 1-AG, since inter-experimental variations in receptor expression could provide a simple explanation for the variability seen here, regardless as to whether it is the parent compounds or downstream metabolites that are responsible for the effects on cell proliferation (see below). The combination of Caps and $CB_1 + CB_2$ receptor antagonists entirely blocked the antiproliferative effects of these compounds ([11] and present study). However, the role of the cannabinoid receptors is somewhat unclear, since Maccarrone *et al.* found that CB_1 and CB_2 receptor antagonists alone increased apoptotic body formation in C6 cells following a 48 hr incubation with 10 μM AEA [10], whereas a reduction in the antiproliferative effects of AEA was seen with these inhibitors using the experimental conditions used in the present study [11]. In those experiments, significant antagonism of the antiproliferative response to 3 μM 2-AG was seen by the CB_1-selective antagonist SR141716A (rimonabant, 1 μM) alone, by the CB_2-selective antagonist SR144528 (1 μM) alone, and by the combination of the two compounds. Thus, the rates of proliferation (as % of the corresponding control, calculated from the mean values from Fig. 5 of [11]) for 3 μM 2-AG were 28, 53, 47 and 65% for no antagonists, 1 μM SR141716A, 1 μM SR144528 and the combination of the two compounds, respectively [11]. Given that these effects of 2-AG are in all likelihood mediated by 1-AG, these data indicate that both CB receptors and a Caps-sensitive component mediate the antiproliferative effect of 1-AG, but that a combination is required for maximal effects.

The complete antagonism of the effects of 1-AG by the combination of Caps and the CB antagonists would at first sight implicate only VR1 and CB receptors in the response. Caps, however, is far from specific for VR1 (see e.g. [21]) raising the possibility that this Caps-sensitivity is not an adequate index of VR-mediated activity, and that other actions of 1-AG (and in theory also anandamide, which has effects at other ion channels or receptors, see e.g. [38]) may be involved. To this end, we investigated the effects of SB366791, which has been reported to be a more selective VR1 antagonist than Caps [25]. However, the reported data for this compound was for the human VR1 [25]. Since human and rat VR1 show different sensitivities to

inhibition by compounds such as Caps [30], it is important to know the antagonist potency of SB366791 towards the rat VR1. The observed pK_B value, 6.8 ± 0.3 ($N = 5$) for SB366791 at the rat VR1 was found to be almost an order of magnitude lower than for the human VR1, where a pK_B value of 7.6 was found [25]. Thus the concentrations of SB366791 used in this study to assess antagonism of the antiproliferative effects of 1-AG are appropriate. The combination of 3 μM SB366791 and the CB receptor antagonists antagonised the effect of 1-AG, which would support strongly the involvement of the VR1 in the antiproliferative effects produced by treatment with this compound. However, SB366791 was less potent than Caps in this regard, despite similar pK_B values towards the rat VR1 seen in the experiments using transfected cells, so the possibility remains that the effects of Caps in this model have both VR1-dependent and VR1-independent components. If this is also the case for anandamide, a VR1-independent component would be consistent with the relative ethanol insensitivity of its antiproliferative effect [20], given that ethanol potentiates effects at VR1 [39]. A likely candidate for a VR1-independent component would be the potent antioxidant properties of Caps [21] since the antiproliferative effects of both 2-AG and anandamide can be prevented by α-tocopherol [11].

4.2. Do anandamide and 1-AG exert their effects by increasing the production of arachidonic acid?

The long incubation times used in the present study raise the distinct possibility that metabolites of anandamide and 1-AG may be ultimately responsible for their antiproliferative effects, and that variations in these metabolic processes may account for the variations in efficacy seen here. A direct effect of anandamide would in addition be expected to be mimicked by its stable analogue methanandamide, or by the combination of capsaicin and methanandamide, which is not the case ([11], present study). This is further supported by the finding that 1-AG *per se* does not produce a VR1-mediated Ca^{2+} response in rVR-HEK293 cells. Possibilities range from simple non-enzymatic oxidation of anandamide and 1-AG to bioactivation. Whilst the two compounds may produce their effects via different metabolic pathways, variation in non-enzymatic oxidation rates of the acyl side chain seem unlikely for either of them, since other arachidonoyl compounds like methanandamide, AM404 and VDM11 do not show the same variation as 1-AG and anandamide (see Section 4.4). In the present study, the possibility that the antiproliferative effects, and variations thereof, are due to the production and further metabolism of arachidonic acid has been investigated, given that exogenous arachidonic acid reduces C6 cell proliferation under the same conditions as are used here [11].

In the case of anandamide, arachidonic acid can be produced as a result of its hydrolysis by fatty acid amide hydrolase [31]. In addition, a pathway whereby activation of CB receptors leads to an activation of cytosolic phospholipase A_2 and thereby production of arachidonic acid has been described in WI-38 fibroblasts [32,40]. Under such conditions, blockade of fatty acid amide hydrolase and/or phospholipase A_2 should produce a dramatic reduction in the effect of anandamide. This was investigated using PTMK, which potently inhibits both enzymes [33,34] and palmitoylethylamide, which inhibits fatty acid amide hydrolase [35]. No large or consistent blockade of the effects of anandamide were seen. This is consistent with our previous finding that the phospholipase A_2 inhibitor quinacrine, does not block the antiproliferative effects of anandamide or 2-AG in the present model system [11]. This would argue against arachidonic acid as an important mediator of anandamide actions here.

Whilst arachidonic acid formation does not mediate the effects of anandamide, it may mediate the effects of 2-(1-)AG. Both fatty acid amide hydrolase and monoacylglycerol lipase can catalyse the production of arachidonic acid from 2-AG [41–43]. At present, there are no selective inhibitors of monoacylglycerol lipase available, and it is perhaps unwise to compare the effects of exogenous arachidonic acid (which is in any case rapidly cleared from the medium by C6 glioma cells [44]) with intracellular arachidonic acid produced by enzymatic cleavage of 1-AG. However, the methyl ester of arachidonic acid, which is cleaved intracellularly by esterases to produce arachidonic acid, was totally inactive towards C6 cell proliferation. Umbelliferyl arachidonate is also a useful compound in this regard, since it is a substrate for phospholipase A_2 [45]. Thus, treatment of the cells with umbelliferyl arachidonate will also generate arachidonic acid intracellularly. However, umbelliferyl arachidonate was a relatively weak inhibitor of C6 glioma cell proliferation, and, more importantly, the inhibition seen was not prevented by the combination of Caps and CB antagonists. Although the possibility that arachidonic acid produced from 1-AG and from umbelliferyl arachidonate and arachidonic acid methyl ester may be compartmentalised differently within the cell should be considered as an important caveat, these data would argue against an antiproliferative effect of 1-AG resulting solely from the generation of arachidonic acid.

4.3. Intracellular transport of endocannabinoids

In a recently completed study, we were able to demonstrate that VDM11 and other compounds affecting the cellular accumulation of anandamide, at concentrations that had modest effects *per se* upon cell proliferation, could prevent its antiproliferative effect [20], consistent with the interaction of anandamide at the intracellular face of the VR and the predominant role of this receptor in this response [46,47]. Although the nature of the transport processes for endocannabinoids and related compounds,

is a matter of some debate [48–50], this raises the possibility that variations in the ability of the cells to accumulate anandamide may account for its experimental variability. Certainly, it has been argued that the number of subculturing passages can affect the rate of anandamide accumulation into C6 cells [15]. In contrast, we found here that VDM11 did not affect the antiproliferative potency of 1-AG, ruling out this explanation for this compound, unless, of course, the balance of opposing effects of the uptake inhibitor to potentiate actions at CB receptors and reduce effects at VR1 exactly negate each other. The alternative explanation, that 1-AG accumulates in the cells primarily by a mechanism of passive diffusion would suggest that the structural requirements of the uptake are very stringent, since AM404 (which has a very similar structure to VDM11) is able to block anandamide and 2-AG uptake into C6 cells with similar potencies [15].

4.4. Solubility issues

One of the great difficulties associated with the endocannabinoids is that their lipophilicity affects their effective concentrations at the biophase. A good example of this is the interaction of anandamide with fatty acid amide hydrolase, where K_m values ranging from 0.8 to 180 μM have been reported [51]. However, solubility issues with these very lipophilic compounds can be ruled out as an explanation here for this variability, at least for anandamide, since the variation in sensitivity of anandamide was mirrored by its water-soluble phosphate ester. Furthermore, compounds like methanandamide (as well as its phosphate ester) VDM11 and AM404, which inhibit C6 cell proliferation under the conditions used here by a Caps and CB receptor-insensitive mechanism, but which share the lipophilic character of anandamide, do not show this variation in sensitivity between experiments ([20] and present study).

In conclusion, the present study has suggested that 2-AG inhibits C6 glioma cell proliferation as a result of its rapid biotransformation to 1-AG, and that the antiproliferative effects of 1-AG are mediated at least in part by activation of VR. It is far from clear, however, how 1-AG and anandamide compounds produce their antiproliferative effects, or indeed whether similar mechanisms operate for the two. In the case of anandamide, a direct effect is possible since the compound activates VR1, whereas the lack of direct effect of 1-AG upon VR1 indicates a requirement for a biotransformation of this compound. Arachidonic acid does not appear to be a mediator for anandamide, whereas it cannot be ruled out for 1-AG. Other possibilities for both anandamide and 1-AG include lipoxygenase-derived compounds. Indeed, in the guinea-pig bronchus and vagus nerve, pharmacological evidence was reported to suggest that lipoxygenase-derived metabolites of anandamide contribute to the Caps-sensitive actions of this compound [52,53]. 2-AG is also a substrate for lipoxygenases [54]. Although such a possibility is worth further investigation, the large intra-experimental variation in sensitivity to 1-AG, anandamide and its water-soluble analogue would suggest that C6 cells are not a suitable model system for such investigations.

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