

# Palmitoylethanolamide inhibits the expression of fatty acid amide hydrolase and enhances the anti-proliferative effect of anandamide in human breast cancer cells

Vincenzo DI MARZO<sup>\*1</sup>, Dominique MELCK<sup>\*</sup>, Pierangelo ORLANDO<sup>†</sup>, Tiziana BISOGNO<sup>\*</sup>, Orna ZAGOORY<sup>‡</sup>, Maurizio BIFULCO<sup>§</sup>, Zvi VOGEL<sup>‡</sup> and Luciano DE PETROCELLIS<sup>||</sup>

<sup>\*</sup>Istituto per la Chimica di Molecole di Interesse Biologico, Via Toiano 6, 80072, Arco Felice, Napoli, Italy, <sup>†</sup>Istituto di Biochimica delle Proteine ed Enzimologia, Via Toiano 6, 80072, Arco Felice, Napoli, Italy, <sup>‡</sup>Department of Neurobiology, Weizmann Institute of Science, Rehovot 76100, Israel, <sup>§</sup>Centro di Endocrinologia e Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, and Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli Federico II, 80131, Napoli, Italy, and <sup>||</sup>Istituto di Cibernetica, Consiglio Nazionale delle Ricerche, Via Toiano 6, 80072, Arco Felice, Napoli, Italy

Palmitoylethanolamide (PEA) has been shown to act in synergy with anandamide (arachidonylethanolamide; AEA), an endogenous agonist of cannabinoid receptor type 1 (CB<sub>1</sub>). This synergistic effect was reduced by the CB<sub>2</sub> cannabinoid receptor antagonist SR144528, although PEA does not activate either CB<sub>1</sub> or CB<sub>2</sub> receptors. Here we show that PEA potently enhances the anti-proliferative effects of AEA on human breast cancer cells (HBCCs), in part by inhibiting the expression of fatty acid amide hydrolase (FAAH), the major enzyme catalysing AEA degradation. PEA (1–10  $\mu$ M) enhanced in a dose-related manner the inhibitory effect of AEA on both basal and nerve growth factor (NGF)-induced HBCC proliferation, without inducing any cytostatic effect by itself. PEA (5  $\mu$ M) decreased the IC<sub>50</sub> values for AEA inhibitory effects by 3–6-fold. This effect was not blocked by the CB<sub>2</sub> receptor antagonist SR144528, and was not mimicked by a selective agonist of CB<sub>2</sub> receptors. PEA enhanced AEA-evoked inhibition of the expression of NGF Trk

receptors, which underlies the anti-proliferative effect of the endocannabinoid on NGF-stimulated MCF-7 cells. The effect of PEA was due in part to inhibition of AEA degradation, since treatment of MCF-7 cells with 5  $\mu$ M PEA caused a ~30–40% down-regulation of FAAH expression and activity. However, PEA also enhanced the cytostatic effect of the cannabinoid receptor agonist HU-210, although less potently than with AEA. PEA did not modify the affinity of ligands for CB<sub>1</sub> or CB<sub>2</sub> receptors, and neither did it alter the CB<sub>1</sub>/CB<sub>2</sub>-mediated inhibitory effect of AEA on adenylate cyclase type V, nor the expression of CB<sub>1</sub> and CB<sub>2</sub> receptors in MCF-7 cells. We suggest that long-term PEA treatment of cells may positively affect the pharmacological activity of AEA, in part by inhibiting FAAH expression.

**Key words:** 2-arachidonoylglycerol, arvanil, cell proliferation, endocannabinoids, receptors.

## INTRODUCTION

Anandamide (arachidonylethanolamide; AEA) was the first endogenous substance to be proposed as an agonist for the cannabinoid receptor type 1 (CB<sub>1</sub> receptor) [1,2]. AEA belongs to a family of lipids, the *N*-acylethanolamines (NAEs) (see [3] for a review), whose most well known component, palmitoylethanolamide (PEA), was described as early as 1957 as a potent anti-inflammatory compound in egg yolk [4]. More recently, the anti-inflammatory properties of PEA and other saturated NAEs have been revisited [5], and it was proposed [6] that this lipid could act as an agonist for the CB<sub>2</sub> receptor [7]. Since these findings, PEA has been seen as a rather enigmatic molecule (see [8] for a review), in as much as it is capable of inducing numerous cannabinoid-like pharmacological actions both *in vitro* [6,9] and *in vivo* [10–12], in some cases in a manner that is sensitive to a CB<sub>2</sub> receptor antagonist, SR144528 [11]. Yet PEA exhibits very little, if any, affinity for the cloned CB<sub>1</sub> and CB<sub>2</sub> receptors from rat, mouse or human [13–15]. While some authors have proposed the existence of CB<sub>2</sub>-like cannabinoid receptors for this com-

pound [11], this hypothesis has not found any molecular support to date. Other authors have suggested that PEA may act as an ‘entourage’ compound, i.e. inhibiting the inactivation of endogenous cannabinoids such as AEA, thereby increasing their levels [8]. Indeed, PEA can be hydrolysed by the enzyme that is mostly responsible for AEA degradation [16,17], fatty acid amide hydrolase (FAAH) (see [18], and [19] for a recent review). However, PEA is not a very efficacious inhibitor of AEA hydrolysis [20,21], possibly because FAAH appears to prefer as substrates unsaturated rather than saturated NAEs. The recent discovery of another enzyme catalysing the hydrolysis of AEA and PEA at similar rates [22] re-opens the possibility that the latter compound acts as an ‘enhancer’ of the activity of the former, as shown for another endocannabinoid, 2-arachidonoylglycerol (2-AG) and some of its congeners [23]. PEA is co-synthesized with AEA in most of the cells analysed so far, and in amounts 5–10-fold higher than those of the endocannabinoid [8,16,24], and could thus also play a role as an entourage substance for AEA when the two substances are produced endogenously.

Abbreviations used: AC, adenylate cyclase; AEA, arachidonylethanolamide (anandamide); 2-AG, 2-arachidonoylglycerol; CB<sub>1</sub>/CB<sub>2</sub> receptor, cannabinoid receptor type 1/2; FAAH, fatty acid amide hydrolase; HBCC, human breast cancer cell; NAE, *N*-acylethanolamine; NGF, nerve growth factor; PEA, palmitoylethanolamide; RT-PCR, reverse transcriptase-PCR.

<sup>1</sup> To whom correspondence should be addressed (e-mail vdimarzo@icmib.na.cnr.it)

We have reported previously that 2–4 days of treatment with AEA and 2-AG, but not PEA, can potentially inhibit the proliferation of human breast cancer cells (HBCCs) via CB<sub>1</sub>-like receptors [25]. This effect was mediated by inhibition of cAMP production and protein kinase A, and by dis-inhibition of mitogen-activated protein kinase [26], and was due to suppression of the expression of the receptor for prolactin, a hormone used by HBCCs as an autacoid mitogen [25]. In fact, AEA and 2-AG, but not PEA, also inhibit the proliferation of human prostate cancer cells induced by exogenous prolactin [27]. We also found that AEA and 2-AG, but not PEA, inhibit the nerve growth factor (NGF)-induced proliferation of MCF-7 cells, a HBCC line, by blocking, through the same intracellular signalling pathways, expression of the high-affinity NGF receptors Trk [27]. In the present study we have investigated whether PEA enhances the anti-proliferative effects of AEA in MCF-7 cells, and, if so, through which mechanism. We report that chronic treatment with PEA, at concentrations 5–10-fold higher than those of AEA (which reflect the PEA/AEA ratio of concentrations in tissues), significantly enhances the inhibitory effect of the endocannabinoid on the basal and NGF-induced proliferation of MCF-7 cells, as well as on Trk expression. We demonstrate that this entourage effect of PEA is not due to direct interaction with CB<sub>1</sub>, CB<sub>2</sub> or FAAH, or to interference with cannabinoid-receptor-coupled signalling, but can be explained in part by inhibition of the expression of FAAH.

## MATERIALS AND METHODS

### Materials

MCF-7, T-47D and DU-145 cells were purchased from A.T.C.C. (Manassas, VA, U.S.A.), and EFM-19 cells were from DSM; cells were cultured as advised by the manufacturers. COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere consisting of 5% CO<sub>2</sub> and 95% air, at 37 °C. [<sup>14</sup>C]AEA (5 mCi/mmol), AEA and PEA were synthesized as described in [1,16]. Arvanil [*N*-(3-methoxy-4-hydroxybenzyl)arachidonoyl-amide] was synthesized as described in [28]. The synthetic cannabinoid HU-210 and linoleylethanolamide were kindly donated by Professor R. Mechoulam (Hebrew University of Jerusalem, Israel). [2-<sup>3</sup>H]Adenine (18.0 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). The phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine and RO-20-1724 were from Calbiochem (La Jolla, CA, U.S.A.). Forskolin and cAMP were from Sigma (St. Louis, MO, U.S.A.). SR144528 was a gift from Sanofi Recherche (Montpellier, France). BML-190 was purchased from Biomol (Plymouth Meeting, PA, U.S.A.), and human prolactin and recombinant β-NGF were from Sigma. [<sup>3</sup>H]SR141716A (55 Ci/mmol) was purchased from Amersham. For cell transfection, the plasmid consisting of rabbit adenylate cyclase type V (AC-V) cDNA in the pXMD1 vector [29] was as described previously [30]. Human CB<sub>2</sub> cDNA was kindly provided by Dr S. Munro (University of Cambridge, U.K.), and human CB<sub>1</sub> cDNA was kindly provided by Dr M. Parmentier (University of Bruxelles, Belgium).

### Cell proliferation assays

Cell proliferation assays were carried out according to the method described previously [25–27] in six-well dishes containing subconfluent cells (at a density of approx. 50 000 cells/well). With MCF-7, EFM-19 and T-47D cells, test substances were introduced 3 h

after cell seeding and then daily at each change of medium. Cells were trypsinized and counted in a haemocytometer 4 days after the addition of test substances. No significant decrease in cell viability (as assessed by Trypan Blue exclusion) was observed with up to 100 µM AEA. With DU-145 cells the effect was studied after stimulation with prolactin, as AEA does not inhibit the basal proliferation of these cells. Prolactin (1 m-unit/ml) or vehicle was added 24 h after seeding with the change of medium, in the presence of the test substances or vehicle. After 72 h, cells were trypsinized and counted in a haemocytometer. In order to study the effect of NGF on MCF-7 cell proliferation, we used a different procedure [27]. At 24 h after cell seeding (50 000 cells/well) the medium was changed to serum-free medium and cells were starved for 24 h. Cells were then treated with serum-free medium containing β-NGF (100 ng/ml) plus test substances or vehicle, and trypsinized and counted after 48 h. Means were compared using the unpaired Student's *t* test, with *P* < 0.05 as the threshold for statistical significance.

### Western immunoblotting

After treatment with test substances, which was carried out under the same conditions as described above for cell proliferation assays, but in 100 mm Petri dishes, cells were washed twice with buffer containing 137 mM NaCl, 3 mM KCl, 12 mM Na<sub>2</sub>HPO<sub>4</sub> and 2 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.4), and then lysed with a lysis buffer consisting of 50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1% Nonidet P40, 0.25% sodium deoxycholate, 1 mM PMSF, 1% Triton X-100 and 1 µg/ml each of aprotinin, leupeptin and pepstatin A. Lysates were loaded on to gels containing 7.5% (w/v) polyacrylamide for blotting of Trk. Proteins were transferred to nitrocellulose membranes, which were then incubated first for 1 h at room temperature with the first antibody, i.e. anti-(mouse Trk) monoclonal antibody (B-3; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, U.S.A.; diluted 1:500), and then with the appropriate horseradish peroxidase-labelled second antibody conjugates (1:5000; Bio-Rad, Hercules, CA, U.S.A.). Bands were visualized by the enhanced chemiluminescence technique (Bio-Rad). The anti-Trk antibody cross-reacts with human Trk.

### Binding assays

Binding assays were carried out as described in [25,27] on membranes prepared from either MCF-7 cells or male CD rat brains, as described therein. The binding of increasing concentrations (0.1–10 nM) of [<sup>3</sup>H]SR141716A to aliquots (0.4 mg of total protein) of these membranes, and the displacement of a fixed concentration (300 pM) of [<sup>3</sup>H]SR141716A by increasing concentrations (0.025, 0.1, 0.5, 1.0, 5.0) of AEA in the presence or absence of PEA, were measured in equilibrium assays. SR141716A (10 µM) was used to determine non-specific binding. Receptor binding results were analysed with GraphPad software (San Diego, CA, U.S.A.). Scatchard curves for the binding of [<sup>3</sup>H]SR141716A were used to calculate *B*<sub>max</sub> and *K*<sub>d</sub> values for this ligand by using non-linear regression, and one- and two-site analyses were compared to determine better-fit values (*r*<sup>2</sup> = 0.88 for one-site binding). Displacement curves (calculated by means of Pharm/PCS software) were used to calculate the *K*<sub>1</sub> values for AEA by inserting the corresponding IC<sub>50</sub> values from the best-fitting curves into the Cheng–Prusoff equation.

### COS cell transfection with AC-V and cAMP assay

At 24 h before transfection, a 10 cm plate of confluent COS-7 cells was trypsinized and cells were divided into five 10 cm plates.

The cells were transfected, using the DEAE-dextran chloroquine method [30], with 2 µg/plate human CB<sub>1</sub> or CB<sub>2</sub> cDNA and 2 µg/plate AC-V cDNA. The cells were trypsinized after 24 h and re-cultured in 24-well plates, and after an additional 24 h the cells were assayed for AC activity.

The cAMP assay was performed as described previously [30]. In brief, cells cultured in 24-well plates were incubated for 2 h with 0.25 ml/well fresh growth medium containing 5 µCi/ml [2-<sup>3</sup>H]adenine. This medium was replaced with Dulbecco's modified Eagle's medium containing 20 mM Hepes (pH 7.4) and the phosphodiesterase inhibitors RO-20-1724 (0.5 mM) and 3-isobutyl-1-methylxanthine (0.5 mM). Substances diluted in 20 mg/ml fatty-acid-free BSA were then added. AC activity was stimulated in the presence or absence of test compounds by the addition of forskolin. After 10 min at 37 °C the medium was removed, and the reaction was terminated by adding to the cell layer 1 ml of 2.5% perchloric acid containing 0.1 mM unlabelled cAMP. Aliquots of 0.9 ml of the acidic extract were neutralized with 100 µl of 3.8 M KOH/0.16 M K<sub>2</sub>CO<sub>3</sub> and applied to a two-step column separation procedure, following which the [<sup>3</sup>H]-cAMP was eluted into scintillation vials and radioactivity was counted. Unless otherwise described, background levels (cAMP accumulation in the absence of stimulant) were subtracted from all values. AEA or PEA, alone or in combination, were added together with the forskolin for the 10-min assay period (acute treatment) or incubated with the cells for 18 h (or for the times indicated) prior to the 10-min assay (started by the addition of forskolin) (chronic treatment). All experiments were performed in triplicate. In MCF-7 cells, the cAMP assay was performed using a kit (Amersham), as described previously [26].

#### Reverse transcriptase-PCR (RT-PCR) amplification of CB<sub>1</sub>/CB<sub>2</sub> mRNA

Total RNA was prepared from MCF-7 cells by the Trizol<sup>®</sup> method (Life Technologies) according to the manufacturer's instructions. To remove contaminating DNA, 12 µg RNA samples were digested with DNase according to the DNA-free (Ambion) protocol. Retro-transcription of mRNA into cDNA was performed in a 20 µl reaction mixture containing 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 1 mM dNTPs, 50 mM Tris/HCl, pH 8.3, 5 µg of total RNA, 20 units of RNase inhibitor (Boehringer-Roche), 0.125 A<sub>260</sub> unit of hexanucleotide mixture (Boehringer-Roche) for random priming and 200 units of Moloney murine leukaemia virus reverse transcriptase (Superscript; GIBCO). The reaction mixture was incubated at room temperature for 10 min and then at 37 °C for 90 min, and was stopped by heating at 98 °C for 5 min, cooled in ice, and stored at -20 °C. Control samples were prepared by omitting reverse transcriptase from the retro-transcription mixture.

DNA amplification was performed in a 50 µl PCR reaction mixture containing 0.5–2 µl of the retro-transcription mixture, 1 × PCR buffer (supplied as a component of the DNA polymerase kit), 2 mM MgCl<sub>2</sub>, 250 µM dNTPs, 0.5 µM each of 5' and 3' primers and 2.5 units of *Platinum*<sup>™</sup> Taq DNA polymerase (Life Technologies). The mixtures were amplified in a PE Gene Amp PCR System 2400 thermocycler (Perkin Elmer). The primers used were: CB<sub>1</sub> sense primer, 5'-CGCAAAGATAGCCGCAA-CGTGT-3'; CB<sub>1</sub> antisense primer, 5'-CAGATTGCAGTT-TCTCGCAGTT-3'; CB<sub>2</sub> sense primer 5'-TTTCCCCTGA-TCCCCAATG-3'; CB<sub>2</sub> antisense primer, 5'-AGTTGATGAG-GCACAGCATG-3'; FAAH sense primer, 5'-GCCTGGGAA-GTGAACAAAGGGACC-3'; FAAH antisense primer, 5'-CCACTACGCTGTCGCACTCCGCCG-3'; β<sub>2</sub>-microglobulin sense primer, 5'-CCAGCAGAGAATGGAAAGTC-3'; β<sub>2</sub>-

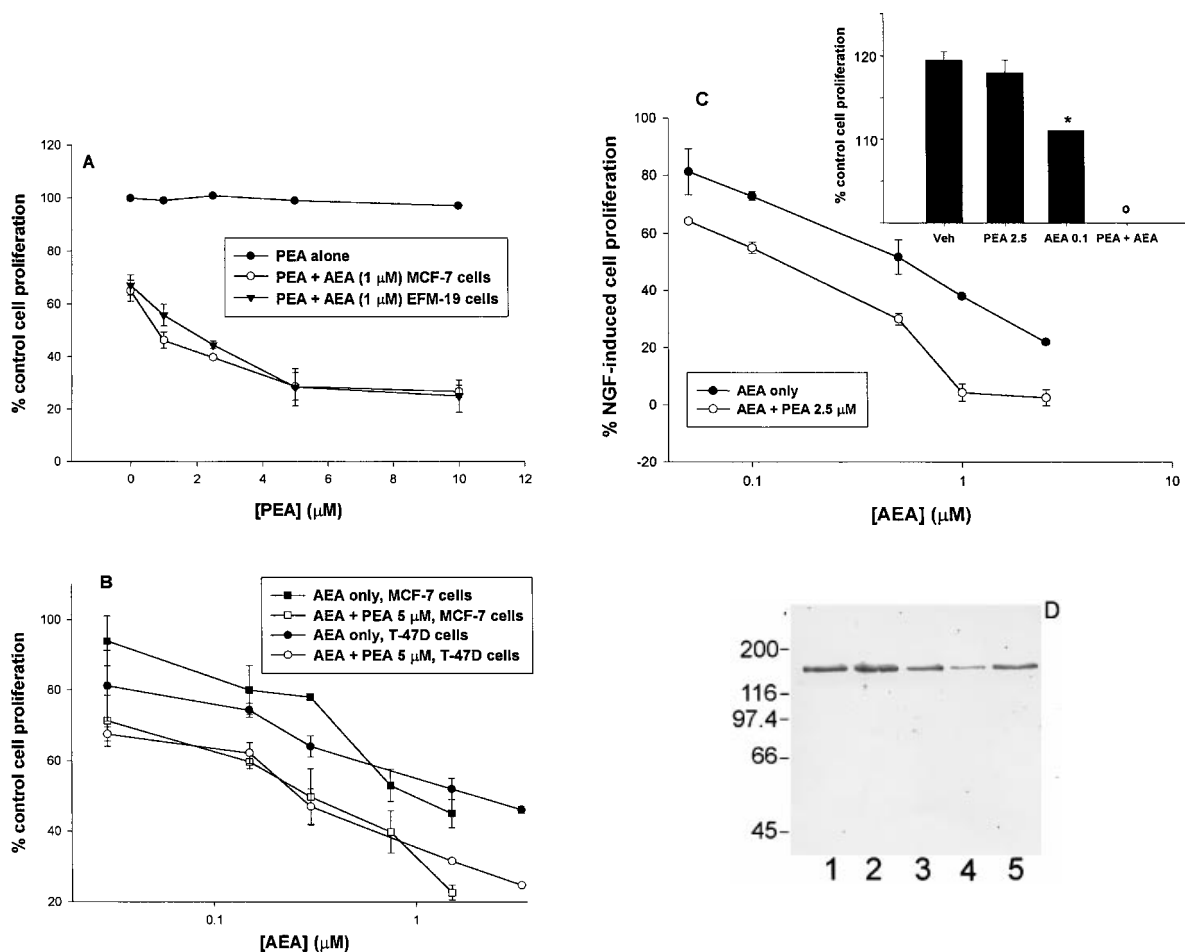
microglobulin antisense primer, 5'-GATGCTGCTTACATGT-CTCG-3'. The amplification profile consisted of an initial denaturation of 2 min at 95 °C, and then 20–35 cycles of 30 s at 95 °C, annealing for 30 s at 55 °C (CB<sub>1</sub> and β<sub>2</sub>-microglobulin) or at 60 °C (CB<sub>2</sub> and FAAH) and elongation for 1 min at 72 °C. A final extension of 10 min was carried out at 72 °C. The expected sizes of the amplicons were 244 bp for CB<sub>1</sub>, 337 bp for CB<sub>2</sub>, 202 bp for FAAH and 268 bp for β<sub>2</sub>-microglobulin. β<sub>2</sub>-Microglobulin was used as a housekeeping gene in order to evaluate variations in mRNA quality and content and to monitor cDNA synthesis in the different preparations. Furthermore, the PCR primers for FAAH and β<sub>2</sub>-microglobulin were selected by including an intron sequence; subsequently, in the presence of contaminating genomic DNA, the expected sizes of the amplicon would be 425 bp and 886 bp for FAAH and β<sub>2</sub>-microglobulin respectively. Quantification of expression levels was performed in the exponential phase of amplification determined, for a fixed quantity of retro-transcription mixture, by analysing the amount of amplicon synthesized at different numbers of amplification cycles. Aliquots of 10–20 µl of PCR products were electrophoresed on a 2% (w/v) agarose gel (MS agarose; Boehringer-Roche) in 1 × TAE buffer at 4 V/cm for 4 h. Ethidium bromide (0.1 µg/ml) was included in both the gel and electrophoresis buffer, and PCR products were detected by UV visualisation.

#### AEA hydrolysis in intact cells

After 4 days of treatment with either vehicle or 5 µM PEA, MCF-7 cells in 10 cm Petri dishes were washed with serum-free medium and incubated with [<sup>14</sup>C]AEA (10000 c.p.m./ml) in 10 ml of serum-free medium for up to 1 h. After various intervals of time, aliquots were taken and extracted with chloroform/water (2:1, v/v), and the radioactivity of the aqueous phase, due uniquely to [<sup>14</sup>C]ethanolamine produced from [<sup>14</sup>C]AEA hydrolysis, was measured by scintillation spectrometry.

#### RESULTS AND DISCUSSION

We have shown previously that PEA, up to a concentration of 10 µM, is not capable of mimicking the anti-proliferative effect of AEA on HBCCs and human prostate cells [25,27]. This early finding was not surprising, since this anti-proliferative effect was shown to be due to interaction with CB<sub>1</sub> receptors, for which PEA has very little, if any, affinity [13–15]. Based on the proposed role of PEA as an entourage substance for AEA [23], on the previously synergistic action of this compound on the anti-hyperalgesic effects of AEA [11], and on the observation that MCF-7 cells do synthesize PEA [31], we wanted to investigate whether this compound could enhance the AEA-induced inhibition of HBCC proliferation. As PEA is usually 5–10 times more abundant than AEA in HBCCs [8], and since the IC<sub>50</sub> values for the anti-proliferative effects of AEA are in the 0.2–2 µM concentration range [25–27], we tested the effects of 1–10 µM PEA. We found that co-incubation of cells with PEA in this concentration range enhanced the AEA-evoked inhibition of EFM-19 and MCF-7 cell basal proliferation with an EC<sub>50</sub> of ~ 2 µM (Figure 1A). When 5 µM PEA was tested with increasing concentrations of AEA, the IC<sub>50</sub> for the inhibition of cell proliferation was lowered from 1.1 ± 0.2 to 0.3 ± 0.1 µM in MCF-7 cells, and from 2.3 ± 0.3 to 0.3 ± 0.1 µM in T-47D cells (Figure 1B). These differences were significant (*P* < 0.01 by ANOVA). When MCF-7 cell proliferation was stimulated by NGF, we found that PEA (5 µM) again enhanced the anti-proliferative effect of AEA, the IC<sub>50</sub> of which was reduced from 0.55 ± 0.11 to 0.15 ± 0.05 µM (Figure 1C; *P* < 0.01). We have shown previously



**Figure 1** Effects of PEA on inhibition of HBCC and prostate cancer cell proliferation by AEA

(A) Dose-dependent potentiation by PEA of the cytostatic effect of AEA in two different HBCC lines. (B) Effect of 5 μM PEA on the dose-dependent inhibition of HBCC proliferation by AEA. (C) Effect of 2.5 μM PEA on the dose-dependent inhibition by AEA of the NGF-induced proliferation of MCF-7 cells. The inset shows the effect of 2.5 μM PEA on the inhibitory action of AEA on the prolactin-induced proliferation of DU-145 prostate cancer cells. (D) Western immunoblot of proteins from MCF-7 cells treated for 2 days with vehicle (lane 1), PEA (5 μM; lane 2), AEA (0.5 μM; lane 3), AEA (0.5 μM) + PEA (5 μM) (lane 4) or AEA (0.5 μM) + BML-190 (5 μM) (lane 5); BML-190 is a selective agonist of CB<sub>2</sub> receptors. A monoclonal antibody against mouse Trk was used for the blotting. Molecular mass markers are also shown, and indicate that the only band detected had the same molecular mass (140 kDa) as human TrkA and TrkB proteins. In (A), (B) and the inset of (C), the effects are expressed as a percentage of basal cell proliferation in the presence of vehicle. In (C) the effects are expressed as a percentage of NGF-induced proliferation (see [27] for further details). In (A)–(C), data are means ± S.D. of *n* = 3 experiments. In (C) significance is indicated by: \**P* < 0.01 compared with vehicle (Veh); <sup>o</sup>*P* < 0.01 compared with AEA only (by ANOVA). The gel in (D) is representative of three experiments with similar results. The same amount of protein (10 μg) was loaded in each lane. Values (arbitrary units) from densitometric scans of the five bands were: 111.0 ± 3.2, 119.2 ± 5.0, 103.0 ± 2.1, 67.0 ± 5.1 and 93.9 ± 4.9 for lanes 1–5 respectively (means ± S.D.; *n* = 3). The bands in lane 4 and 5 were significantly less intense than that in lane 1 (*P* < 0.05 by unpaired Student's *t* test), whereas the band in lane 4, but not that in lane 5, was significantly less intense than that in lane 3 (*P* < 0.01 by unpaired Student's *t* test).

that the inhibitory effect of AEA on the NGF-induced proliferation of MCF-7 cells is due to suppression of the high-affinity Trk receptors for NGF [27]. Here we found that PEA (5 μM) also significantly enhanced the down-regulatory action of AEA on Trk expression, as assessed by Western immunoblotting of MCF-7 cell total proteins carried out with a monoclonal antibody against Trk (Figure 1D). Finally, we ran an experiment in DU-145 human prostate cancer cells, in which AEA blocks prolactin-induced proliferation [27]. Again, PEA (2.5 μM) significantly enhanced the inhibitory action of AEA (0.1 μM), from 42.6% to 100% inhibition (Figure 1C, inset). These effects of PEA were not due to prevention of AEA adhesion to the plasticware used for the assays. In fact, when the plates were incubated with [<sup>14</sup>C]AEA in the absence or presence of PEA (5 μM), no difference was found in the recovery of radioactivity in the incubation medium after up to 1 h of incubation (results not shown).

PEA was suggested previously to act as an agonist at CB<sub>2</sub>-like receptors [6,11], although this hypothesis is not supported by more recent data [13–15]. A CB<sub>2</sub> receptor RNA transcript was indeed identified in HBCCs by RT-PCR [27]. However, here we found that the effect of PEA on the inhibitory actions of AEA in basal or NGF-induced HBCC proliferation was not mimicked by BML-190, a selective agonist of CB<sub>2</sub> receptors (Table 1). Furthermore, this effect of PEA was not influenced by SR144528, a selective antagonist of CB<sub>2</sub> receptors (Table 2). Finally, the stimulatory effect of PEA on the AEA-induced down-regulation of Trk receptors was not mimicked by use of BML-190 (Figure 1D). These findings indicate that CB<sub>2</sub> receptors are not involved in the effects of PEA on HBCC proliferation described here.

We next investigated whether the stimulatory effect of PEA on the cytostatic actions of AEA could indeed be due to an entourage effect, i.e. to inhibition of intracellular AEA degradation by cells.

**Table 1** Effects of an agonist selective for the CB<sub>2</sub> receptor on the cytostatic action of AEA in MCF-7 cells

The effects of BML-190 (2.5 and 5  $\mu$ M), a selective CB<sub>2</sub> receptor agonist, on the inhibitory action of AEA on the basal and NGF-induced proliferation of MCF-7 cells were assessed. The concentration of AEA was 1 and 0.5  $\mu$ M in experiments with basal and NGF-induced proliferation respectively. The effects are expressed as a percentage of basal or NGF-induced cell proliferation in the presence of vehicle. Data are means  $\pm$  S.D. of  $n = 3$  experiments; \* $P < 0.05$  compared with control (ANOVA).

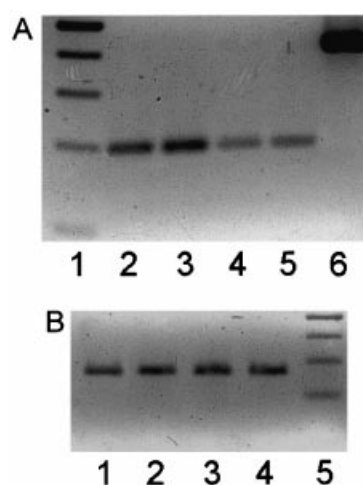
Additions	Proliferation (%)	
	Basal	NGF-induced
BML-190 (2.5 $\mu$ M)	100.7 $\pm$ 1.0	98.1 $\pm$ 3.0
BML-190 (5.0 $\mu$ M)	98.0 $\pm$ 4.1	98.9 $\pm$ 4.0
AEA	67.2 $\pm$ 3.8*	51.1 $\pm$ 7.0*
AEA + BML-190 (2.5 $\mu$ M)	68.7 $\pm$ 2.0*	48.1 $\pm$ 4.0*
AEA + BML-190 (5.0 $\mu$ M)	66.1 $\pm$ 1.0*	41.3 $\pm$ 6.0*

**Table 2** Effect of an antagonist selective for the CB<sub>2</sub> receptor on the cytostatic effect of AEA in MCF-7 cells

The effects of SR144528, a selective CB<sub>2</sub> receptor antagonist, on the potentiation of the inhibitory action of AEA on the basal and NGF-induced proliferation of MCF-7 cells by 5  $\mu$ M PEA were assessed. The concentration of AEA was 1 and 0.5  $\mu$ M in experiments with basal and NGF-induced proliferation respectively. The effects are expressed as a percentage of basal or NGF-induced cell proliferation in the presence of vehicle. Data are means  $\pm$  S.D. of  $n = 3$  experiments; \* $P < 0.05$  compared with control; † $P < 0.05$  compared with AEA only (ANOVA).

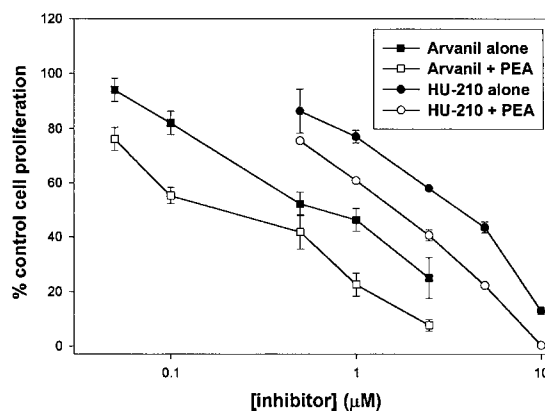
Additions	Proliferation (%)	
	Basal	NGF-induced
SR144528 (0.2 $\mu$ M)	103.8 $\pm$ 3.8	100.0 $\pm$ 0.3
SR144528 (0.5 $\mu$ M)	100.0 $\pm$ 2.5	87.8 $\pm$ 9.1
AEA	47.5 $\pm$ 2.3*	49.0 $\pm$ 3.5*
AEA + PEA (5.0 $\mu$ M)	20.4 $\pm$ 0.2†	26.1 $\pm$ 1.6†
AEA + PEA (5.0 $\mu$ M) + SR144528 (0.2 $\mu$ M)	21.4 $\pm$ 0.4†	28.6 $\pm$ 1.2†
AEA + PEA (5.0 $\mu$ M) + SR144528 (0.5 $\mu$ M)	20.3 $\pm$ 2.5†	20.4 $\pm$ 2.5†

In fact, HBCCs express FAAH [31], the enzyme mostly responsible for AEA degradation in cells, which is also capable of recognizing PEA as a substrate. However, we have shown previously that PEA does not effectively inhibit the hydrolysis of [<sup>14</sup>C]AEA by intact EFM-19 cells during short (0–30 min) incubations of cells with both compounds [31], in agreement with previous observations that this lipid is a worse FAAH substrate than AEA and is not capable of inhibiting AEA uptake by cells [16,20,21,31]. Therefore we investigated here whether a 4-day treatment of MCF-7 cells with PEA (i.e. the same conditions necessary to observe potentiation of AEA cytostatic effects) would lead to modulation of FAAH expression, as assessed by quantitative RT-PCR of a FAAH RNA transcript. We found that PEA (5  $\mu$ M) significantly decreased (by approx. 30–40%) the expression of FAAH at the transcriptional level (Figures 2A and 2B) (the values from densitometric scans of the bands decreased from 1590  $\pm$  51 to 1093  $\pm$  59 arbitrary units; means  $\pm$  S.E.M.;  $n = 8$ ;  $P < 0.01$  by ANOVA). This effect was accompanied by a corresponding decrease in FAAH activity, as assessed by the decreased ability of intact MCF-7 cells to hydrolyse exogenous [<sup>14</sup>C]AEA (from 4099  $\pm$  331 to 3006  $\pm$  250 c.p.m./h per 10<sup>6</sup> cells; mean  $\pm$  S.E.M.;  $n = 8$ ;  $P < 0.05$  by ANOVA). These data indicate that the stimulatory action of PEA on the cytostatic effects of AEA is due, at least in part, to inhibition of

**Figure 2** Effects of PEA on the expression of FAAH in MCF-7 cells

Cells were treated for 4 days with 5  $\mu$ M PEA and then either analysed for their ability to hydrolyse [<sup>14</sup>C]AEA or extracted for RNA preparation and RT-PCR analysis. (A) Agarose gel showing the size of the FAAH mRNA transcript from MCF-7 cells treated with either vehicle (lanes 2 and 3) or PEA (lanes 4 and 5). The FAAH transcript obtained when using DNA instead of cDNA is shown in lane 6. A 100 bp DNA ladder (starting from 100 bp) is shown in lane 1. (B) Agarose gel showing the size of the  $\beta_2$ -microglobulin mRNA transcript from MCF-7 cells treated with either vehicle (lanes 1 and 2) or PEA (lanes 3 and 4). A 100 bp DNA ladder (starting from 200 bp) is shown in lane 5. Data are representative of four separate experiments with similar results carried out in duplicate.

AEA degradation by FAAH, potentially resulting in higher levels of AEA during the proliferation experiments and, subsequently, in a potentiation of AEA activity. We then challenged this hypothesis by examining the effects of PEA in the presence of: (1) an AEA analogue, arvanil [28], which is more stable than AEA to enzymic hydrolysis, and (2) HU-210, a synthetic CB<sub>1</sub> receptor agonist and cytostatic agent for HBCCs, which cannot be hydrolysed by FAAH. We found that PEA (5  $\mu$ M) also potentiated the anti-proliferative effects of both arvanil and HU-210 in all cell lines under study, and under all conditions used (Figure 3 and results not shown), although less potently than

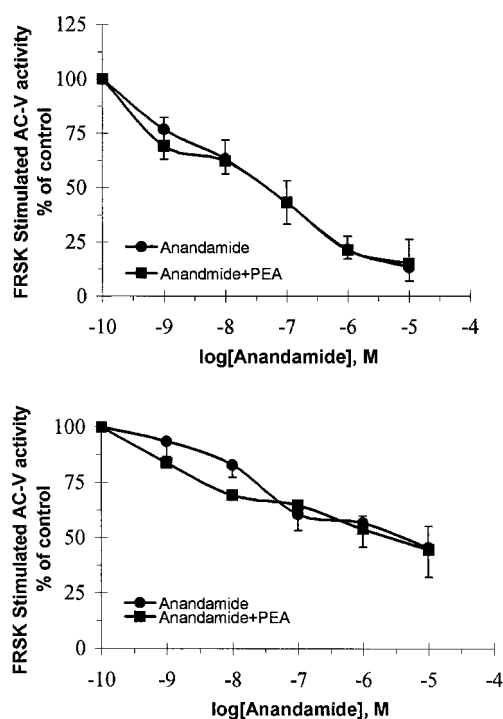
**Figure 3** Effects of PEA on the inhibition of MCF-7 cell proliferation by arvanil and HU-210

Results are expressed as percentage of basal cell proliferation in the presence of vehicle, and are means  $\pm$  S.D. of  $n = 3$  experiments.

with AEA. In MCF-7 cells, for example, the  $IC_{50}$  values were decreased only 2-fold (from  $0.6 \pm 0.1$  to  $0.3 \pm 0.1 \mu\text{M}$  with arvanil and from  $3.6 \pm 0.3$  to  $1.8 \pm 0.2 \mu\text{M}$  with HU-210;  $n = 3$ ;  $P < 0.05$  by ANOVA), compared with 3–6-fold with AEA (see above). These data suggest that PEA is also capable of enhancing the cytostatic action of AEA through mechanisms other than inhibition of its enzymic hydrolysis.

Since the anti-proliferative effects of AEA are mediated by  $CB_1$  receptors and through the inhibition of AC [25–27], we studied the possibility that PEA enhances AEA action by exerting either short- or long-term stimulatory actions on the expression, affinity for ligands or coupling to AC of  $CB_1$  receptors. We started by examining the possibility of an allosteric effect of PEA on [ $^3\text{H}$ ]SR141716A binding to rat brain cell membranes, or on displacement of [ $^3\text{H}$ ]SR141716A binding by AEA. We found no stimulatory action on [ $^3\text{H}$ ]SR141716A binding ( $B_{\text{max}}$   $1805 \pm 121$  and  $2082 \pm 150$  fmol/mg of protein;  $K_d$   $1.45 \pm 0.18$  and  $1.69 \pm 0.20$  nM; without and with  $5 \mu\text{M}$  PEA respectively; means  $\pm$  S.E.M.;  $n = 3$ ). We found a non-statistically significant effect on the  $K_i$  values for displacement of [ $^3\text{H}$ ]SR141716A by AEA ( $1.0 \pm 0.2$  and  $0.6 \pm 0.2 \mu\text{M}$  without and with  $5 \mu\text{M}$  PEA respectively; means  $\pm$  S.E.M.;  $n = 3$ ). Likewise, no significant effect of PEA was found when using membrane preparations from MCF-7 cells (results not shown), which contain much less specific [ $^3\text{H}$ ]SR141716A binding sites than rat brain [27]. The effect on cannabinoid receptor expression of long-term treatment of cells with PEA was studied by means of RT-PCR, as described above for FAAH. We found that the same 4-day treatment of MCF-7 cells with PEA ( $5 \mu\text{M}$ ) that led to inhibition of FAAH expression did not significantly affect the expression of RNA transcripts for either  $CB_1$  or  $CB_2$  receptors (results not shown). Finally, we examined whether either short- or long-term treatment of cells with PEA could influence CB receptor coupling to AC. We found no effect of PEA ( $10 \mu\text{M}$ ) on AEA-induced inhibition of forskolin-stimulated cAMP production in MCF-7 cells (results not shown). As we reasoned that the level of expression of either  $CB_1$  receptors or AC in these cells might depend on several factors, such as the number of subculturing passages [27], we repeated these experiments under more controlled and reproducible conditions, i.e. in COS cells co-transfected with  $CB_1$  receptors and the AC-V isoform. We found no effect of either acute (Figure 4) or chronic (results not shown) treatment with PEA on the AEA-induced inhibition of cAMP formation in COS cells transfected with AC-V and either  $CB_1$  or  $CB_2$  cDNAs.

In summary, we have found that PEA is capable of inhibiting the expression of FAAH in HBCCs, and that this regulatory effect is responsible, at least in part, for the PEA-induced enhancement of the anti-proliferative effects of AEA. Although there has been previous evidence for effects of fatty acid amides on FAAH activity [32,33], this is, to the best of our knowledge, the first time that a regulatory action on FAAH expression (and at the transcriptional level) has been reported for a member of this class of bioactive lipids. Our findings have several potentially important implications. Firstly, our data strengthen the previous hypothesis [31] that NAEs might play a role as tumour growth suppressors in HBCCs. While members of this family of lipids that activate  $CB_1$  receptors, such as AEA, might inhibit proliferation directly, the saturated and monounsaturated NAEs, such as PEA, which are usually more abundant than AEA, might enhance this effect. Secondly, our present findings might be exploited pharmaceutically by leading to the development of tumour-suppressing 'cocktails' whose potency might be greater than that of simple  $CB_1$  receptor agonists. In fact, there is increasing evidence that substances that activate cannabinoid receptors can also be used as anti-neoplastic drugs *in vivo*



**Figure 4** Effects on forskolin-induced stimulation of cAMP formation of AEA, alone or co-incubated with PEA ( $10 \mu\text{M}$ ), in COS cells transfected with either  $CB_1$  (upper panel) or  $CB_2$  (lower panel) and AC-V cDNAs

Data are expressed as a percentage of the effects observed with forskolin (FRSK) alone, and are means  $\pm$  S.E.M. of  $n = 3$  experiments.

([34,35]; M. Bifulco and V. Di Marzo, unpublished work). However, this therapeutic application might be limited by the undesired psychotropic side effects expected from these drugs. This limitation might be overcome by the administration of endocannabinoids, which seem to have much lower potential for dependence [36], in combination with a non-psychotropic substance, such as PEA, which would lower the concentrations necessary to observe the tumour-suppressing effect. Finally, the present study improves our knowledge of the molecular mechanisms through which PEA acts synergistically with AEA [8]. It is possible that these synergistic effects, at least when they are observed after chronic PEA treatment, may be due in part to modulation of the expression of FAAH and a subsequent increase in the amount of AEA available for cannabinoid receptor activation. However, since PEA is also capable of increasing the anti-proliferative effects of HU-210, additional molecular mechanisms are likely to underlie the synergistic effects of this NAE on cannabinoid-receptor-mediated biological actions. Indeed, we have preliminary data showing that PEA, independently of FAAH, can significantly enhance acute effects of AEA that are mediated by vanilloid  $VR_1$  receptors [37] (L. De Petrocellis and V. Di Marzo, unpublished work). Here we have presented data that argue against possible regulatory effects of PEA on  $CB_1$ / $CB_2$  receptor expression, ligand affinity and functional activity. Future studies will aim at finding other molecular targets involved in the biochemical and pharmacological actions of PEA.

We are grateful to Ms Ines Brandi for technical support, and to Mr Salvatore Piantedosi for some of the artwork. This work was supported by MURST (3933, to V. D. M.), INTAS (97-1297, to V. D. M.) and AIRC (to M. B.).

## REFERENCES

- 1 Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D. and Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**, 1946–1949
- 2 Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C. and Bonner, T. I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature (London)* **346**, 561–564
- 3 Schmid, H. H. O., Schmid, P. C. and Natarajan, V. (1990) N-acylated glycerophospholipids and their derivatives. *Prog. Lipid Res.* **29**, 1–43
- 4 Kuehl, Jr, F. A., Jacob, T. A., Ganley, O. H., Ormond, R. E. and Meisinger, M. A. P. (1957) The identification of N-(2-hydroxyethyl)-palmitamide as a naturally occurring anti-inflammatory agent. *J. Am. Chem. Soc.* **79**, 5577–5578
- 5 Aloe, L., Leon, A. and Levi-Montalcini, R. (1993) A proposed autocoid mechanism controlling mastocyte behavior. *Agents Actions* **39**, C145–C147
- 6 Facci, L., Dal Toso, R., Romanello, S., Buriani, A., Skaper, S. D. and Leon, A. (1995) Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3376–3380
- 7 Munro, S., Thomas, K. L. and Abu-Shaar, M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature (London)* **365**, 61–65
- 8 Lambert, D. M. and Di Marzo, V. (1999) The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic? *Curr. Med. Chem.* **6**, 757–773
- 9 Ross, R. A., Brockie, H. C. and Pertwee, R. G. (2000) Inhibition of nitric oxide production in RAW264.7 macrophages by cannabinoids and palmitoylethanolamide. *Eur. J. Pharmacol.* **401**, 121–130
- 10 Mazzari, S., Canella, R., Petrelli, L., Marcolongo, G. and Leon, A. (1996) N-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation. *Eur. J. Pharmacol.* **300**, 227–236
- 11 Calignano, A., La Rana, G., Giuffrida, A. and Piomelli, D. (1998) Control of pain initiation by endogenous cannabinoids. *Nature (London)* **394**, 277–281
- 12 Jaggar, S. I., Hasnie, F. S., Sellaturay, S. and Rice, A. S. (1998) The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain. *Pain* **76**, 189–199
- 13 Lambert, D. M., DiPaolo, F. G., Sonveaux, P., Kanyonyo, M., Govaerts, S. J., Hermans, E., Bueb, J., Delzenne, N. M. and Tschirhart, E. J. (1999) Analogues and homologues of N-palmitoylethanolamide, a putative endogenous CB(2) cannabinoid, as potential ligands for the cannabinoid receptors. *Biochim. Biophys. Acta* **1440**, 266–274
- 14 Griffin, G., Tao, Q. and Abood, M. E. (2000) Cloning and pharmacological characterization of the rat CB(2) cannabinoid receptor. *J. Pharmacol. Exp. Ther.* **292**, 886–894
- 15 Sheskin, T., Hanus, L., Slager, J., Vogel, Z. and Mechoulam, R. (1997) Structural requirements for binding of anandamide-type compounds to the brain cannabinoid receptor. *J. Med. Chem.* **40**, 659–667
- 16 Bisogno, T., Maurelli, S., Melck, D., De Petrocellis, L. and Di Marzo, V. (1997) Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. *J. Biol. Chem.* **272**, 3315–3323
- 17 Tiger, G., Stenstrom, A. and Fowler, C. J. (2000) Pharmacological properties of rat brain fatty acid amidohydrolase in different subcellular fractions using palmitoylethanolamide as substrate. *Biochem. Pharmacol.* **59**, 647–653
- 18 Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A. and Gilula, N. B. (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature (London)* **384**, 83–87
- 19 Ueda, N., Puffenberger, R. A., Yamamoto, S. and Deutsch, D. G. (2000) The fatty acid amide hydrolase. *Chem. Phys. Lipids* **108**, 107–121
- 20 Katayama, K., Ueda, N., Katoh, I. and Yamamoto, S. (1999) Equilibrium in the hydrolysis and synthesis of cannabimimetic anandamide demonstrated by a purified enzyme. *Biochim. Biophys. Acta* **1440**, 205–214
- 21 Maccarrone, M., Fiorucci, L., Erba, F., Bari, M., Finazzi-Agrò, A. and Ascoli, F. (2000) Human mast cells take up and hydrolyze anandamide under the control of 5-lipoxygenase and do not express cannabinoid receptors. *FEBS Lett.* **468**, 176–180
- 22 Ueda, N., Yamanaka, K., Terasawa, Y. and Yamamoto, S. (1999) An acid amidase hydrolyzing anandamide as an endogenous ligand for cannabinoid receptors. *FEBS Lett.* **454**, 267–270
- 23 Ben-Shabat, S., Frider, E., Sheskin, T., Tamiri, T., Rhee, M. H., Vogel, Z., Bisogno, T., De Petrocellis, L., Di Marzo, V. and Mechoulam, R. (1998) An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. *Eur. J. Pharmacol.* **353**, 23–31
- 24 Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C. and Piomelli, D. (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature (London)* **372**, 686–691
- 25 De Petrocellis, L., Melck, D., Palmisano, A., Bisogno, T., Laezza, C., Bifulco, M. and Di Marzo, V. (1998) The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8375–8380
- 26 Melck, D., Rueda, D., Galve-Roperh, I., De Petrocellis, L., Guzman, M. and Di Marzo, V. (1999) Involvement of the cAMP/protein kinase A pathway and of mitogen-activated protein kinase in the anti-proliferative effects of anandamide in human breast cancer cells. *FEBS Lett.* **463**, 235–240
- 27 Melck, D., De Petrocellis, L., Orlando, P., Bisogno, T., Laezza, C., Bifulco, M. and Di Marzo, V. (2000) Suppression of nerve growth factor Trk receptors and prolactin receptors by endocannabinoids leads to inhibition of human breast and prostate cancer cell proliferation. *Endocrinology* **141**, 118–126
- 28 Melck, D., Bisogno, T., De Petrocellis, L., Chuang, H., Julius, D., Bifulco, M. and Di Marzo, V. (1999) Unsaturated long-chain N-acyl-vanillyl-amides (N-AVAMs): vanilloid receptor ligands that inhibit anandamide-facilitated transport and bind to CB1 cannabinoid receptors. *Biochem. Biophys. Res. Commun.* **262**, 275–284
- 29 Wallach, J., Droste, M., Kluxen, F. W., Pfeuffer, T. and Frank, R. (1994) Molecular cloning and expression of a novel type V adenylyl cyclase from rabbit myocardium. *FEBS Lett.* **338**, 257–263
- 30 Rhee, M.-H., Bayewitch, M. L., Avidor-Reiss, T., Levy, R. and Vogel, Z. (1998) Cannabinoid receptor activation differentially regulates the various adenylyl cyclase isozymes. *J. Neurochem.* **71**, 1525–1534
- 31 Bisogno, T., Katayama, K., Melck, D., Ueda, N., De Petrocellis, L., Yamamoto, S. and Di Marzo, V. (1998) Biosynthesis and degradation of bioactive fatty acid amides in human breast cancer and rat pheochromocytoma cells – implications for cell proliferation and differentiation. *Eur. J. Biochem.* **254**, 634–642
- 32 van der Stelt, M., Paoletti, A. M., Maccarrone, M., Nieuwenhuizen, W. F., Bageeta, G., Veldink, G. A., Finazzi Agrò, A. and Vliegenterthart, J. F. (1997) The effect of hydroxylation of linoleoyl amides on their cannabimimetic properties. *FEBS Lett.* **415**, 313–316
- 33 Maccarrone, M., Salvati, S., Bari, M. and Finazzi-Agrò, A. (2000) Anandamide and 2-arachidonoylglycerol inhibit fatty acid amide hydrolase by activating the lipoxygenase pathway of the arachidonate cascade. *Biochem. Biophys. Res. Commun.* **278**, 576–583
- 34 Galve-Roperh, I., Sanchez, C., Cortes, M. L., del Pulgar, T. G., Izquierdo, M. and Guzman, M. (2000) Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat. Med. (N.Y.)* **6**, 313–319
- 35 De Petrocellis, L., Melck, D., Bisogno, T. and Di Marzo, V. (2000) Endocannabinoids and fatty acid amides in cancer, inflammation and related disorders. *Chem. Phys. Lipids* **108**, 191–209
- 36 Aceto, M. D., Scates, S. M., Razdan, R. K. and Martin, B. R. (1998) Anandamide, an endogenous cannabinoid, has a very low physical dependence potential. *J. Pharmacol. Exp. Ther.* **287**, 598–605
- 37 De Petrocellis, L., Bisogno, T., Davis, J. B., Pertwee, R. G. and Di Marzo, V. (2000) Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: first inhibitors of anandamide uptake with negligible capsaicin-like activity. *FEBS Lett.* **483**, 52–56