Biosynthesis and degradation of bioactive fatty acid amides in human breast cancer and rat pheochromocytoma cells

Implications for cell proliferation and differentiation

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The endogenous cannabinoid, anandamide (arachidonoyl ethanolamide), and the sleep-inducing factor, oleamide (cis-9-octadecenoicamide), represent two classes of long-chain fatty acid amides with several neuronal actions and metabolic pathways in common. Here we report that these two compounds are present in human breast carcinoma EFM-19 cells and rat adrenal pheochromocytoma PC-12 cells, together with the enzyme responsible for their degradation, fatty acid amide hydrolase, and the proposed biosynthetic precursors for arachidonoyl ethanolamide and related acylethanolamides, the N-acyl-phosphatidylethanolamines. Lipids extracted from cells labelled with [14C]ethanolamine contained radioactive compounds with the same chromatographic behaviour as arachidonoyl ethanolamide and acyl-PtdEtns. The levels of these compounds were not influenced by either stimulation with ionomycin in EFM-19 cells or two-week treatment with the nerve growth factor in PC-12 cells. The chemical nature of arachidonoyl ethanolamide, related acylethanolamides and the corresponding acyl-PtdEtns was confirmed by gas chromatographic/mass spectrometric analyses of the purified compounds, which also showed the presence of higher levels of oleamide. The latter compound, which does not activate the central CB1 cannabinoid receptor, exhibited an anti-proliferative action on EFM-19 cells at higher concentrations than arachidonoyl ethanolamide (IC50 = 11.3 µM for oleamide and 2.1 µM for arachidonoyl ethanolamide), while at a low, inactive dose it potentiated an arachidonoyl ethanolamide cytostatic effect. The CB1 receptor selective antagonist SR 141716A (0.5 µM) reversed the effect of both arachidonoyl ethanolamide and oleamide. EFM-19 cells and PC-12 cells were found to contain a membrane-bound [14C]arachidonoyl ethanolamide-hydrolysing activity with pH dependency and sensitivity to inhibitors similar to those previously reported for fatty acid amide hydrolase. This enzyme was inhibited by oleamide in both intact cells and cell-free preparations. The presence of transcripts of fatty acid amide hydrolase in these cells was shown by northern blot analyses of their total RNA. The rate of [14C]arachidonoyl ethanolamide hydrolysis by intact cells, the kinetic parameters of arachidonoyl ethanolamide enzymatic hydrolysis and the amounts of the fatty acid amide hydrolase transcript, were not significantly influenced by a two-week treatment with nerve growth factor and subsequent transformation of PC-12 cells into neuron-like cells. These data show for the first time that: (a) induction by nerve growth factor of a sympathetic neuronal phenotype in PC-12 cells has no effect on arachidonoyl ethanolamide/oleamide metabolism, (b) arachidonoyl ethanolamide and oleamide are autacoid suppressors of human breast cancer cell proliferation. Moreover these data lend conclusive support to the previous hypothesis that oleamide may act as an enhancer of arachidonoyl ethanolamide actions through competitive inhibition of its degradation.

Keywords: anandamide; oleamide; cannabinoid; PC12 cells; breast cancer.

Long-chain fatty acid amides have been recently suggested to have a physiological role in the mammalian nervous system. The anandamides are polyunsaturated fatty acid ethanolamides capable of binding to central (CB1) cannabinoid receptors, and of eliciting typical cannabimimetic responses (for a review see [1]). Cis-9-octadecenoicamide (oleamide) is a primary fatty acid amide isolated from the cerebrospinal fluid of sleep-deprived mammals and shown to induce sleep in rats [2]. The first member of the former compounds to be discovered [3] was arachidonoyl ethanolamide (AEA), whose cannabinoid-like pharmacological effects and possible physiological roles in both central and peripheral tissues have been investigated most thor-
oughly (for reviews see [4–7]). Another long-chain ethanolamide, palmitoylethanolamide (PEA), which cannot be considered an anandamide since it does not bind CB1 receptors, was known from previous studies to behave as a possible anti-inflammatory agent (reviewed in [8]). More recently, PEA was shown to inhibit serotonin release from mast cells [9], as well as mast cell-mediated degranulation and their participation in nociceptive transduction [10], and to prevent glutamate-induced excitotoxicity in cerebellar granule cells [11]. Both these actions were suggested to be mediated by non-CB1 cannabinoid receptors, possibly of the peripheral (CB2) subtype [9, 11]. AEA and PEA, together with other acylethanolamines, were found to: (a) be co-produced upon ionomycin stimulation of several cell types, e.g. rat cortical and striatal neurons [12], rat basophilic leukaemia (RBL-2H3) cells and mouse J774 macrophages [13, 14], probably through the phospholipase-D-catalysed hydrolysis of the corresponding N-acyl-phosphatidylethanolamines (acyl-PtdEtns) [8]; (b) to co-exist, together with the acyl-PtdEtns, in all mammalian and invertebrate tissues examined so far (for example see [15–20]), and (c) to be recognised, in N18TG2, RBL-2H3 and RBL-1 cells, by the same hydrolytic enzyme, AEA amidohydrolase, albeit with significantly different affinities [14]. AEA amidohydrolase displays an alkaline optimal pH, and is localised on microsomal and mitochrondrial membranes from several mammalian tissues, including brain, liver and kidney [20–23]. Due to these properties, this enzyme is likely to be the same amidase previously shown to catalyse the hydrolysis of saturated and monounsaturated fatty acid ethanolamides [24]. Moreover, AEA amidohydrolase has been suggested to be identical to fatty acid amide hydrolase, the enzyme catalysing oleamide hydrolysis, which was cloned from rat liver [25], over-expressed by transfection into host cells, and found to recognise AEA as the preferential substrate as well as other fatty acid primary amides and methyl esters [25, 26]. Evidence that AEA and oleamide are substrates for the same hydrolytic enzyme had already been gained in previous studies conducted in N18TG2 cells [27], and was provided also for the recently cloned mouse and human fatty acid amide hydrolase [28]. However, the existence of other amidohydrolases selective for either AEA or oleamide has not been ruled out [25], even though a recent study, by showing that rat tissues contain amounts of AEA amidohydrolase comparable to their fatty acid amide hydrolase mRNA levels, provided further evidence that these two are the same enzyme [29]. No data are available on the possible regulation of fatty acid amide hydrolase during cell differentiation into neurons.

Apart from inducing sedation in rats, oleamide was also shown to mimic AEA by inhibiting lymphocyte proliferation [30] and exhibiting a weak but significant activity in the tetrad of mouse behavioural tests (hypothermia, antinociception, inhibition of locomotor activity), which are highly indicative of cannabinoid compounds [31]. However, oleamide does not bind to either cannabinoid receptor subtype [31]. Due to the fact that oleamide can serve as a substrate for the same enzyme catalysing the hydrolysis of AEA [25–28], it was hypothesised that some of the pharmacological actions of oleamide, including sleep induction, were due to an enhancement of endogenous AEA levels [31]. Accordingly, oleamide, which is present in N18TG2 cells in amounts up to 100 times higher than AEA [32], was found to inhibit AEA degradation by both cell-free and whole cell preparations and to potentiate AEA effects in the tetrad of tests [31]. However, due to the lack of efficacy of the only selective CB1 receptor antagonist developed to date, SR 141716A [33], against AEA actions in these in vivo tests in mice [42], it was not possible to confirm this hypothesis conclusively.

In the present study we have addressed three open questions concerned with bioactive fatty acid amide research. Is the production and inactivation of these compounds influenced by cell differentiation into sympathetic-like neurons? Are these metabolites synthesised and degraded by human breast cancer cells, where a strong and selective anti-proliferative action by AEA has been recently discovered [34]? Is oleamide present also in peripheral cells, and does it function as an endogenous enhancer of AEA actions also in these cells? Therefore, we examined the occurrence of AEA, PEA, the corresponding acyl-PtdEtns, and oleamide, as well as the expression of AEA amidohydrolase/fatty acid amide hydrolase, in: (a) rat adrenal pheochromocytoma PC-12 cells, either naïve or differentiated into neuron-like cells by a two-week treatment with the nerve growth factor (NGF), and (b) human breast cancer EFM-19 cells, and studied the anti-proliferative effect of oleamide in these latter cells.

MATERIALS AND METHODS

Cell culturing. EFM-19, RBL-1, J774 and N18TG2 cells were purchased from Deutsche Sammlung von Mikroorganismen and cultured according to the intructions of the manufacturer. PC-12 cells were donated by Dr A. Leon (Research & Innova- tion, Vicenza) and grown in 85% RPMI 1640, 15% horse serum and 5% foetal calf serum in the presence of 25 U/ml penicillin, 25 µg/ml streptomycin and 2 mM glutamine. PC-12 cells, seeded on petri dishes treated with 200 µg/dish collagen (Boehringer Mannheim), were differentiated into neuron-like cells (as indicated by the formation of a net of neurites upon observation under an inverted light microscope) by daily treatment with 50 ng/ml mouse NGF 7 S (Alomone Labs) for two weeks. Undifferentiated PC-12 cells were grown on collagen-treated dishes as a control for differentiated cells.

Extraction, purification, characterisation and quantitation of AE, PEA, oleamide and acyl-PtdEtns. AEA, PEA and oleamide were synthesised in large amounts and purified as previously described [2, 3]. Lipids, from cells either unlabelled or labelled by overnight treatment with 0.1 µCi/ml [3H]ethanolamine (52 mCi/mmol, Amersham) or [3H]arachidonic acid (230 Ci/mmol, NEN), were extracted and purified by a sequence of chromatographic steps described previously [13, 14, 35] and including open chromatography on silica gel. TLC, normal-phase and reverse-phase HPLC. In some experiments cells were stimulated with 5 µM ionomycin (Sigma) for 30 min at 37°C prior to lipid extraction [14]. Briefly, chloroform/methanol (2:1, by vol.) extracts of cells were loaded onto silica gel columns. Two fractions, eluted with chloroform/methanol (9:1, by vol. and 1:1, by vol.), were purified by normal-phase HPLC with n-hexane/2-propanol as described previously [13, 14], and TLC developed with chloroform/methanol/NH4OH (85:15:1, by vol.) respectively. The NAPE-like components were scraped from TLC plates and digested with Streptomyces chromofuscus phospholipase D as described [13, 14]. The digests were purified by normal-phase HPLC and fractions with the same retention time (27–28 min) as acylethanolamide standards, from either the 9:1 silica column fractions or the digestion of acyl-PtdEtns-like lipids, were purified further by reverse-phase HPLC with methanol/water as described previously [13, 14], under conditions allowing the separation between different acylethanolamides. Experiments aiming at the full characterisation of AEA, PEA, oleamide and acyl-PtdEtns were carried out with unlabelled confluent cells scraped from ten 100-mm petri dishes (approximately 10 6 cells). The normal-phase HPLC fractions with the same retention time as acylethanolamide and oleamide (26 min) standards were submitted to gas chromatography/electron-impact mass spectrometry (GC/EIMS) as described previously [14], using, alternatively, an isotope dilution methodology for...
the quantitation of acetoxy-AEA in the selected-ion monitoring mode, or the full mass spectrum acquisition mode for structure determination of other acetoxy-acyl ethanolamides and characterisation and quantitation of underivatised oleamide [32]. In the former case, measurements of AEA in the 0.05–10-nmol range in mixture with 1 nmol of [3H]AEA are possible [14], while in the latter case, standard curves for oleamide were constructed with 0.1–5 nmols of the synthetic compound which ran immediately after the unknown samples [32].

Inactivation of AEA by intact EFM-19 and PC-12 cells. Experiments were carried out with intact confluent cells in either 100-mm collagen-treated petri dishes (as in the case of PC-12 cells) or 6-well dishes (as in the case of EFM-19 cells). In the former case, cells were washed three times with 5 ml serum-free medium and then incubated with [14C]AEA (5 Ci/mol), prepared from [14C]ethanolamine and arachidonic acid as described [35], and diluted to 50 μM (10 000 cpm/ml in 6 ml). 500-μl aliquots were then taken after various intervals of time and [14C]ethanolamine produced from [14C]AEA hydrolysis measured as described previously [14]. In the second case, cells, after three washes with 1 ml serum-free medium, were incubated with [14C]AEA (10 000 cpm/ml in 1 ml) for increasing periods of time. The amounts of [14C]AEA and [14C]ethanolamine in the incubation media, as well as the amount of [14C]AEA retained by cells after three washes with 2 ml medium containing 1% BSA, were measured as described previously [14].

Partial characterisation of AEA amidohydrolase. Partial characterisation of AEA amidohydrolase in EFM-19 and PC-12 cells was performed as described previously for N18TG2, RBL-1 and RBL-2H3 cells [14, 27] using synthetic [14C]AEA as the substrate. Assays were performed at 37°C with 50 μM [14C]AEA in 500 μl Tris/HCl pH = 7.5 with 0.05–0.1 mg protein from different subcellular fractions, prepared as described [14, 27], or with proteins (0.05–0.1 mg) from the 10000×g pellet at different pH values or with different concentrations of [14C]AEA or in the presence of various inhibitors, i.e. phenylmethyisulphonyl fluoride (PMSE; 100 μM, Sigma), p-hydroxy-mercuribenzoate (p-HMB; 100 μM, Sigma), EDTA (5 mM), arachidonoylthromethane (Δ6-AchCOMeF, 50 μM), arachidonoyldiazemethane (Δ9-AchCOCHN, 50 μM), arachidonoylchloromethane (Δ6-AchCOCMeCl, 50 μM) and methyl-arachidonoyl flourophosphate (MeΔ6-AchPOF, 50 nM, Bio- mol, UK), or of oleamide and PEA (100 μM). Δ6-AchCOMeF, Δ6-AchCOCHN, and Δ6-AchCOCMeCl are synthetised as described previously [36, 37].

Northern blot analyses. A cDNA fragment for rat fatty acid amid hydrolase was prepared by reverse transcriptase/polymerase chain reaction using rat liver poly(A)-rich RNA as a template. The primers used were an upstream primer 5'-GCTCGAAGCTCTCTGTTGCAGC-3' and a down-stream primer 5'-GCTCTAGAGTCTAGGTGCTGG-3'. The cDNA fragment was then digested with XbaI resulting in the formation of a 781-bp fragment containing 1041–1787 (as from [25]). The latter fragment was labelled with [α-32P]dCTP by the random primer DNA labelling system. Total RNA was extracted from rat liver and EFM-19, PC-12, RBL-1, J774 and N18TG2 cells using a mixture of guanidium isothiocyanate and phenol (Isogen, Nippon Gene). The RNA (25 μg) was denatured, subjected to electrophoresis on a 1% agarose formaldehyde gel, transferred to a Hybond-N+ membrane and hybridised with the [32P]cDNA probe. Distribution of the radioactivity on the membrane was visualised by a BAS 2000 imaging analyser. Staining of 28S and 18S rRNA bands with ethidium bromide confirmed that the same amount of RNA had been applied on each lane.

EFM-19 cell proliferation assay. Cell proliferation assays were carried out in triplicate in 6-well dishes containing subconfluent cells (at a density of about 50,000 cells/well) [34]. Substances to be tested were introduced 24 h after cell seeding and then daily with each change of medium. Various doses of the substances were assayed and cells trypsinised and counted at confluence after 6 days by a hemocytometer. This also allowed cell viability to be checked by the addition of trypan blue to aliquots of trypsinised cells. No significant decrease in cell viability was observed with up to 100 μM AEA, PEA or oleamide.

In separate sets of experiments, various doses of AEA were tested in the presence of 0.5 μM oleamide, and 10 μM oleamide was tested in the presence of the CB1 antagonist, SR 141716A (0.5 μM), which was kindly donated by Sanoﬁ Recherche. Statistically significant differences between different treatments were evaluated by using the unpaired Student’s t-test with P<0.05 as the threshold for significance.

RESULTS

Occurrence of AEA, PEA, oleamide and acyl-PtdEtns in EFM-19 cells. When EFM-19 cells were labelled overnight with [14C]ethanolamine and extracted, lipid components with the same chromatographic behaviour as AEA, PEA and stearoyl-ethanolamide in both normal-phase and reverse-phase HPLC were found in the silica column fraction eluted with chloroform/methanol (9:1, by vol.) (Fig. 1A). The presence of AEA was confirmed when extracts from EFM-19 cells labelled with [3H]arachidonic acid were analysed. When the silica column fraction eluted with chloroform/methanol (1:1, by vol.) was analysed by TLC, a radioactive component with the same Rf as synthetic acyl-PtdEtns could be detected in extracts of cells labelled with either [14C]ethanolamine or [3H]arachidonic acid (data not shown). When this component was substituted to digestion with S. chromofuscus phospholipase D, it released high amounts of AEA, PEA and stearoyl ethanolamide-like compounds, thus suggesting the presence of N-arachidonoyl-, N-palmitoyl- and N-stearoyl-PtdEtn in EFM-19 cells (Fig. 1B). Conclusive evidence for the presence of AEA, PEA and stearoyl ethanolamide, as well as of the corresponding acyl-PtdEtns, in these cells was obtained by GC/EIMS analysis (data not shown) of the acetylated normal-phase HPLC peaks with the retention time of synthetic AEA from both the 9:1 silica column fraction and the digestion of the acyl-PtdEtns-like TLC fraction. The 9:1 fraction also contained a GC peak with a EIMS fragment pattern indistinguishable from that of synthetic oleamide (data not shown). EIMS spectra and fragmentation patterns of underivatised synthetic oleamide and of the acetoxy-derivatives of both synthetic and natural AEA, PEA, and stearoyl ethanolamide have been published previously [14, 19, 32]. Oleamide levels were 160 ± 40 pmol/107 cells (mean + SEM, n = 3). The amounts of AEA in the 9:1 fraction were below the level measurable with the isotope dilution procedure used here (50 pmol), although well above the detection limit (5 pmol). Experiments carried out with cells labelled with [14C]ethanolamine and treated with 5 μM ionomycin showed that the levels of the endogenous radioactive acyl ethanolamides and acyl-PtdEtns were not sensitive to ionomycin stimulation (Fig. 1C).

Occurrence of AEA, PEA, oleamide and acyl-PtdEtns in PC-12 cells. PC-12 cells, either untransformed or differentiated into neuron-like cells upon two-week treatment with NGF and labelled with [14C]ethanolamine, contain two radioactive lipid components which co-elute with either synthetic acyl ethanolamides, in normal-phase HPLC or acyl-PtdEtns in TLC analyses
Fractions, were acetylated and submitted to GC/EIMS analyses other AEA amidohydrolase activities [1]. Acylethanolamides in normal-phase HPLC fractions (Fig. 1) were identified in three separate experiments. NAEs, acyl-Etns; iono, ionomycin; 20:4, arachidonoyl; 18:0, oleoyl.

The levels of the acylethanolamides and acyl-PtdEtns-like compounds were not significantly altered upon cell differentiation cell types contained an enzymatic activity capable of catalysing the hydrolysis of [14C]AEA. EFM-19 and PC-12 cells contain an enzymatic activity catalysing the corresponding increase of [14C]ethanolamine (EtNH2).

When confluent, intact EFM-19 and PC-12 cells were incubated with [3H]arachidonic acid (AA) or [3H]ethanolamine (EtNH2). Cells were extracted and lipids purified as described in Materials and Methods. (A, D) Normal-phase (NP) HPLC analyses of AEA-like silica column fractions from EFM-19 (A) and PC-12 (D) cells; (B, E) normal-phase HPLC analyses of acyl-PtdEtns-like lipids from EFM-19 (B) and PC-12 (E) cells, following digestion with S. chromofuscus phospholipase D. (A, B) Reverse-phase (RP) HPLC analysis of the NAE peak from normal-phase HPLC is shown in the upper traces. The retention times of NAEs with different fatty acid moieties (indicated by the length and degree of unsaturation) are shown. (C, F) Histograms for the amounts of NAEs and acyl-PtdEtns from (C) either unstimulated or ionomycin-stimulated EFM-19 cells or (F) either undifferentiated or NGF-treated PC-12 cells (see legend to Table 1) (F) The effect of ionomycin stimulation on NAE levels is also shown. A, B, D and E are representative of three different experiments. Data in C and F) are means ± SEM of three separate experiments. NAEs, acyl-Etns; iono, ionomycin; 20:4, arachidonoyl; 16:0, palmitoyl; 18:0, stearoyl.

(See Fig. 1D and data not shown). When submitted to digestion with S. chromofuscus phospholipase D, the acyl-PtdEtns-like TLC microsomal fractions) (Table 1) (Fig. 1E). The levels of the acylethanolamides and acyl-PtdEtns-like compounds were not significantly altered upon cell differentiation into neuron-like cells, nor on cell stimulation with 500 µM ionomycin (Fig. 1F). The AEA-like HPLC fractions, from either the 9:1 column fraction or the digested acyl-PtdEtns-like TLC fractions, were acetylated and submitted to GC/EIMS analyses which showed the presence of very little, albeit clearly detectable, amounts of compounds with retention times and fragmentation patterns identical to those of AEA, PEA and stearylethanolamide standards (data not shown); oleamide was present also in this case, in amounts much higher than AEA and PEA and not sensitive to NGF treatment (683 ± 166 pmol/107 cells, mean ± SEM, n = 3). The levels of AEA in the 9:1 fraction from both naive and differentiated PC-12 cells were below the level measurable with the isotope dilution procedure used here (50 pmol) and just above the detection limit (5 pmol).

EFM-19 and PC-12 cells contain an enzymatic activity catalysing the hydrolysis of [14C]AEA. When confluent, intact EFM-19 and PC-12 cells were incubated with [14C]AEA, the level measurable with the isotope dilution procedure used here (50 pmol) and just above the detection limit (5 pmol). The rate of [14C]AEA hydrolysis was not significantly changed by PC-12 differentiation into neuron-like cells (Fig. 2A). 100 µM oleamide significantly inhibited [14C]AEA hydrolysis by both EFM-19 (Fig. 2B) and PC-12 (data not shown) intact cells, thus leading to a corresponding increase of [14C]AEA in the incubation medium. Oleamide did not significantly affect [14C]AEA uptake by EFM-19 cells (Fig. 2B). Cell-free homogenates from both cell types contained an enzymatic activity capable of catalysing [14C]AEA hydrolysis. This was mostly found associated with particulate fractions (10000×g mitochondrial and 100000×g microsomal fractions) (Table 1), as previously described for other AEA amidohydrolase activities [14, 19, 21—29]. Moreover, the amidohydrolase activity displayed apparent active-site pKα values at around 5.5—6.0 and 9.5—10.0 (Fig. 2C), similar to those previously observed both with AEA amidohydrolase enzymes and fatty acid amide hydrolases [14, 19, 22, 25, 27, 28]. The enzymatic hydrolysis of [14C]AEA was also significantly counteracted by typical inhibitors of these enzymes [36, 37], as well as by 100 µM oleamide but not by 100 µM PEA (Table 2). In EFM-19 cells, apparent Kα and Vmax values for [14C]AEA hydrolysis were, 12.8 ± 8.0 µM and 0.79 ± 0.30 nmol min⁻¹ mg protein⁻¹ respectively, (means ± SEM, n = 3) (Fig. 2D). In PC-12 cells, NGF-induced cell differentiation did not sensibly modify the subcellular distribution, pH profile, sensitivity to AEA amidohydrolase inhibitors, and apparent Kα and Vmax values (36.4 ± 7.1 µM and 0.66 ± 0.14 nmol min⁻¹ mg protein⁻¹ in normal cells and 33.4 ± 3.0 µM, and 0.98 ± 0.18 nmol min⁻¹ mg protein⁻¹ in transformed cells; means ± SEM, n = 3) of the enzyme (Fig. 2C, D, Tables 1, 2).
Fig. 2. Degradation of [14C]AEA by EFM-19 and PC-12 intact cells and mitochondrial fractions. (A) [14C]AEA hydrolysis, measured through the formation of [14C]ethanolamine (EtNH₂), by intact confluent untransformed or NGF-treated PC-12 cells. (B) [14C]AEA hydrolysis and uptake by intact confluent EFM-19 cells in the presence and absence of 100 µM oleamide (OA). (C) pH-dependency and (D) Lineweaver-Burk profiles for the enzymatic hydrolysis of [14C]AEA by EFM-19 and PC-12 mitochondrial fractions. In (D) the enzyme activity is measured as the radioactivity (cpm) of [14C]ethanolamine released from [14C]AEA hydrolysis. y is the order of magnitude of the cpm and is 4 for PC-12 untreated, and 5 for PC-12+NGF and EFM-19. Representative of two different experiments carried out in duplicates.

Table 1. Distribution of AEA amidohydrolase activity in different subcellular fractions from EFM-19 and PC-12 cells. Results are means±SD of three separate experiments carried out in duplicate. The concentration of [14C]AEA and of total proteins used were not saturating and, therefore, the activities found were lower than the calculated Vₐₘₐₜ values PC-12 cells were grown on collagen-treated petri dishes and either left untransformed or differentiated into cells with neuronal phenotype by two-week treatment with 50 ng/ml NGF.

<table>
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<tr>
<th>Cell fraction</th>
<th>AEA amidohydrolase activity pmol min⁻¹ mg protein⁻¹</th>
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</thead>
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<tr>
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<td>Debris (800 × g)</td>
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<tr>
<td>Mitochondria (100000 × g)</td>
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<td>Microsomes (100000 × g)</td>
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<tr>
<td>Cytosol (supernatant)</td>
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Fatty acid amide hydrolase mRNA in EFM-19 and PC-12 cells. Total RNAs from EFM-19 cells, untransformed or differentiated PC-12 cells, and RBL-1, N18TG2 and J774 cells, were submitted to agarose gel electrophoresis and northern blot analyses using a [%P]cDNA probe for rat fatty acid amide hydrolase (FAAH). Rat liver RNA was used as a positive control [26]. Apart from EFM-19 and PC-12 cells, of the tested cell lines, RBL-1 and N18TG2 cells, but not J774 cells, have been described to contain an AEA amidohydrolase activity [14, 27]. Accordingly, bands with the same mobility as rat FAAH mRNA were detected in RNA preparations from rat PC-12 and RBL-1 cells (Fig. 3). Fainter mRNA bands were detected in RNA preparations from mouse N18TG2 and human EFM-19 cells, in the latter case with an apparent molecular size lower than rat liver FAAH mRNA. No detectable FAAH transcript was observed in J774 cells. Human FAAH mRNA has been reported to be of a smaller size than rat FAAH mRNA [28], whereas mouse FAAH may not have been efficiently detected by the cDNA probe used here. No reproducible difference was observed between the intensity and electrophoretic mobility of FAAH mRNA bands in either undifferentiated or differentiated PC-12 cells (Fig. 3).

Oleamide, AEA, PEA and EFM-19 cell proliferation. The effects of AEA and oleamide on EFM-19 cell proliferation are shown in Fig. 4. In agreement with previous findings [34], AEA exerted a potent inhibition of cell proliferation, with an estimated IC₅₀ of 2.1 µM. Oleamide, which had not been tested before, also exerted a significative cytotatic action, although at higher concentrations than AEA (estimated IC₅₀ = 11.3 µM,
Fig. 3. Northern blot analysis of total RNA (25 µg) from rat liver and various human and murine tumoral cell lines. RNA was extracted from rat liver (lane 1, positive control), undifferentiated and NGF-differentiated rat PC-12 cells (lanes 2 and 3), rat RBL-1 basophils (lane 4), mouse neuroblastoma N18TG2 cells (lane 5), mouse 3T3 macrophages (lane 6), and human EFM-19 cells (lane 7). Representative of four different blottings. In some other experiments the intensity of the band in lane 2 was slightly higher than that in lane 3. The mobility of 28S and 18S rRNA is shown.

Fig. 4. Dose-related effects of oleamide (OA) and AEA on EFM-19 cell proliferation. (A) Effect of oleamide, with or without 0.5 µM oleamide. 0.5 µM SR 141716A alone did not show any significant effect on cell proliferation. Data are expressed as percent of control cell proliferation and are means ± SEM of three separate experiments. In (B) the antiproliferative effect exerted by OA + AEA was always significantly different from that exerted by AEA alone (unpaired Student’s t-test, P < 0.05), except for [AEA] = 0. In (A) the effect of 0.5 µM SR 141716A + 10 µM oleamide was significantly different from that of 10 µM oleamide alone (P < 0.005).

Table 2. Effect of various substances on the hydrolysis of [14C]AEA by mitochondrial fractions prepared from EFM-19 and either untransformed or NGF-treated PC-12 cells. For details see legend to Table 1. Results are means ± SD of three separate experiments carried out in duplicate. Abbreviations are defined in the first-page footnote.

<table>
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<tr>
<th>Inhibitor</th>
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<th>EFM-19</th>
<th>PC-12 untreated</th>
<th>PC-12 + NGF</th>
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<td>H2O2 (100 µM)</td>
<td>0</td>
<td>11.5 ± 0.7</td>
<td>4.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>HO-BzHgOH (100 µM)</td>
<td>0</td>
<td>9.3 ± 0.2</td>
<td>3.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>EDTA (5 mM)</td>
<td>117.0 ± 0.7</td>
<td>111.0 ± 4.4</td>
<td>105 ± 1.4</td>
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</table>

DISCUSSION

In this study, starting from the lack of data on the effect of cell differentiation on acylethanolamide metabolism, we wanted to assess whether the biosynthesis and degradation of AEA, PEA and congeners in PC-12 cells are subject to regulation following NGF stimulation and subsequent transformation into cells with a phenotype typical of sympathetic neurons. Next, we wanted to investigate whether human breast cancer cells, where AEA exerts a potent anti-proliferative action [34], have the means to biosynthesize and inactivate this compound, and, therefore, to assess whether the function of autacoid suppressor of mammary cell proliferation could be listed among the possible physiopathological roles proposed so far for AEA [4–7]. Finally, we wanted to find out whether oleamide, previously shown to exert some cannabimimetic properties [30, 31], shared with AEA also the cytostatic action on breast cancer cells and, if so, through what mechanism. In the attempt to achieve these three tasks, we have provided evidence for the presence of AEA and PEA in gland-derived peripheral cell types, as well as unprecedented data on the presence of oleamide outside the nervous system. In both EFM-19 and PC-12 cells, AEA and PEA were accompanied by higher levels of their putative biosynthetic precursors, the corresponding acyl-PtdEtns. The biosynthesis of AEA and PEA in EFM-19 and PC-12 cells, unlike that in rat central neurons and murine tumoral leukocytes [12–14], was not induced by ionomycin stimulation (Fig. 1). This may be due to low sensitivity of cells to ionomycin, as previously observed for undifferentiated neuroblastoma x glioma NG 108×15 hybrid cells [13, 35], or to the occurrence of biochemical pathways for acylethanolamide formation that are not dependent on extracellular calcium, as recently suggested for another endocrine gland,
rat tests [38]. Our data, however, do not rule out the possibility that acylethanolamide biosynthesis in EFM-19 and PC-12 cells may be induced by stimuli causing the mobilisation of calcium from intracellular stores. More importantly, we found that the levels of acylethanolamides and the corresponding acyl-PtdEtns did not vary following NGF-induced PC-12 cell transformation into sympathetic-like neurons (Fig. 1). This indicates that the enzymes so far suggested to be involved in acylethanolamidine generation are not subject to up-regulation following prolonged NGF treatment of PC-12 cells. According to the two biosynthetic pathways proposed so far for the acylethanolamides, these enzymes are: (a) a calcium-dependent trans-acylase catalysing the biosynthesis of acyl-PtdEtns through the transfer of acyl moieties from the sn-1 position of phospholipids to the N-position of phosphatidylethanolamine and an acyl-PtdEtn-selective phospholipase D [8, 12, 39], or (b) a synthase catalysing the energy-free condensation of fatty acids and ethanolamine [8, 22, 39–41]. Our data may also suggest that sympathetic neurons may differ from central neurons by their capability of producing AEA. This suggestion may be relevant to the recent finding that macrophage-derived (and not necessarily sympathetic-neuron-derived) anandamide may be responsible for hypotension in haemorrhaged or endotoxin-stimulated rats [43, 44]. Also the amounts of oleamide, whose biosynthetic pathway in the central nervous system has not yet been clarified [32], did not change upon PC-12 cell differentiation. As previously observed for N18TG2 cells [32], oleamide levels in both EFM-19 and PC-12 cells, as measured by GC/MS, were much higher than the amounts estimated for AEA. The latter were below the lower limit of measurement (50 pmol, 5 pmol×10² cells) and above the upper detection limit (5 pmol, 0.5 pmol×10² cells) of the GC/MS method used here, and therefore comparable to those measured by GC/MS in rat and human brain [15] (10² cells correspond to about 0.1 g wet tissue). As to the low levels of AEA in adenral PC-12 cells, it is worthwhile noting that comparable amounts (about 8 pmol/g wet tissue) of this compound have been recently described in rat kidney and mesangial cells [20]. An AEA amidohydrolase activity, identified on the basis of its typical subcellular distribution, pH dependency profile and sensitivity to inhibitors, was also found in both PC-12 and EFM-19 cells (Fig. 2, Tables 1 and 2). RNA transcripts for FAAH, the enzyme catalysing the hydrolysis of both long-chain fatty acid ethanolamides and primary amines [25, 28], were also detected in EFM-19 and PC-12 cells, as well as in two other cell lines previously described to express AEA amidohydrolase activity, i.e. N18TG2 and RBL-1 cells, but were not present in J774 cells, which contain only negligible amounts of this activity [14, 27] (Fig. 3). The amounts of AEA amidohydrolase in either transformed or transformed PC-12 cells were approximately the same and so were the intensities of the bands of the FAAH transcripts in northern blot analyses of RNA from both types of cells. These findings indicate that the expression of this protein is not altered following NGF-induced transformation of PC-12 cells, which, moreover, did not modify the enzyme subcellular distribution, pH dependency profile and sensitivity to inhibitors, nor its affinity for AEA (in terms of apparent Kₘ values) (Fig. 2 C, D, Tables 1, 2).

Due to their capability of synthesising FAAH/AEA amido-hydrolase, both EFM-19 and PC-12 intact cells rapidly hydrolyse [¹³C]AEA to [¹³C]ethanolamine and arachidonic acid (Fig. 2A, B). In agreement with the data discussed above, the ability to degrade [¹³C]AEA was not changed in differentiated PC-12 cells. The presence, in the incubation medium, of oleamide caused a significant inhibition of [¹³C]AEA hydrolysis by intact cells, and, subsequently, a marked enhancement of intact [¹³C]AEA available, for example, for receptor activation (Fig. 2B). This was to be expected from the fact that FAAH/ AEA amidohydrolase recognises as substrates both AEA and oleamide, and is therefore inhibited by oleamide when using [¹³C]AEA as a substrate (Table 2). A similar phenomenon was recently described also for N18TG2 cells, and was suggested to be at the basis of the weak cannabinimetic activity exerted by oleamide, and of the facilitatory action of the latter compound on AEA action, in the tetrad of mouse behavioural tests [31]. We have previously shown that AEA, by acting at CB1-like receptors, can potently inhibit EFM-19 cell proliferation [34]. Here, we wanted to see whether oleamide, which does not bind to CB1 receptors but affects AEA levels at these receptors and shares with AEA several pharmacological actions [30, 31], would exhibit a similar cytostatic action. We found that oleamide can exert an anti-proliferative action on EFM-19 cells at fivefold higher concentrations than AEA (Fig. 4A) and that a low, ineffective, concentration of the primary amide could significantly potentiate AEA anti-proliferative effect (Fig. 4B). PEA, which does not significantly inhibit the enzymatic hydrolysis of [¹³C]AEA by cell particulate fractions (Table 2), was inactive (data not shown). These findings may indicate, as suggested previously for some of its in vivo effects [31], that oleamide, at high concentrations such as those present in EFM-19 cells, but not PEA, might inhibit cell proliferation through raised levels of endogenous AEA, whose hydrolysis is minimised by the primary amide, but not by the saturated acylethanolamide. Accordingly, the CB1 antagonist SR 141716A (which inhibits AEA antiproliferative effect in EFM-19 cells [34]), blocked oleamide cytostatic action (Fig. 4A), suggesting that the latter is mediated by an endogenous cannabinoid substance, possibly AEA.

In conclusion, in this study we have shown that the sleep-inducing factor oleamide is present in non-nervous tumoral cell lines and that the levels of acylethanolamides, acyl-PtdEtns and oleamide, on the one hand, and of FAAH, on the other hand, do not vary upon NGF-induced differentiation of one such cell line (PC-12 cells) into sympathetic neuron-like cells. In EFM-19 cells, where we have shown here that oleamide, like AEA, inhibits cell proliferation, the presence of AEA, its putative precursor N-arachidonoyl-PtdEtn, oleamide and the enzyme FAAH, indicate that these cells have the means to synthesize and inactivate both bioactive fatty acid amides. This, in turn, suggests that oleamide and AEA may function as autocrine, protective signals here, we wanted to see whether oleamide, which does not bind to CB1 receptors but affects AEA levels at these receptors and shares with AEA several pharmacological actions [30, 31], would exhibit a similar cytostatic action. We found that oleamide can exert an anti-proliferative action on EFM-19 cells at fivefold higher concentrations than AEA (Fig. 4A) and that a low, ineffective, concentration of the primary amide could significantly potentiate AEA anti-proliferative effect (Fig. 4B). PEA, which does not significantly inhibit the enzymatic hydrolysis of [¹³C]AEA by cell particulate fractions (Table 2), was inactive (data not shown). These findings may indicate, as suggested previously for some of its in vivo effects [31], that oleamide, at high concentrations such as those present in EFM-19 cells, but not PEA, might inhibit cell proliferation through raised levels of endogenous AEA, whose hydrolysis is minimised by the primary amide, but not by the saturated acylethanolamide. Accordingly, the CB1 antagonist SR 141716A (which inhibits AEA antiproliferative effect in EFM-19 cells [34]), blocked oleamide cytostatic action (Fig. 4A), suggesting that the latter is mediated by an endogenous cannabinoid substance, possibly AEA.

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REFERENCES


