Astrocytes in Culture Produce Anandamide and Other Acylethanolamides*

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Anandamide (arachidonylethanolamide) is an endocannabinoid that belongs to the acylethanolamide lipid family. It is produced by neurons in a calcium-dependent manner and acts through cannabinoid CB1 receptors. Other members of the acylethanolamide lipid family are also produced by neurons and act through G-protein-coupled receptors: homo-γ-linolenylethanolamide (HEA) and docosatetraenylethanolamide (DEA) act through CB1 receptors, palmitylethanolamide (PEA) acts through CB2-like receptors, and oleylthanolamide (OEA) acts through receptors that have not yet been cloned. Although it is clear that anandamide and other acylethanolamides play a major role in neuronal signaling, whether astrocytes also produce these lipids is unknown. We developed a chemical ionization gas chromatography/mass spectrometry method that allows femtomole detection and quantification of anandamide and other acylethanolamides. Using this method, we unambiguously detected and quantified anandamide, HEA, DEA, PEA, and OEA in mouse astrocytes in culture. Stimulation of mouse astrocytes with ionomycin, a calcium ionophore, enhanced the production of anandamide, HEA, and DEA, whereas PEA and OEA levels were unchanged. Endothelin-1, a peptide known to act on astrocytes, enhanced the production of anandamide, without affecting the levels of other acylethanolamides. These results show that astrocytes produce anandamide, HEA, and DEA in a calcium-dependent manner and that anandamide biosynthesis can be selectively stimulated under physiologically relevant conditions. The relative levels of acylethanolamides in astrocytes from rat and human were different from the relative levels of acylethanolamides in mouse astrocytes, indicating that the production of these lipids differs between species. Because astrocytes are known to express CB1 receptors and inactivate endocannabinoids, our finding strongly suggests the existence of a functional endocannabinoid signaling system in these cells.

Acylethanolamides (acyl-EAs)1 are structurally related lipids that contain a saturated or unsaturated fatty acid moiety linked to ethanolamine (1). Several studies have shown that members of the acyl-EA lipid family may act as signaling molecules. For example, the prototypic endocannabinoid anandamide (arachidonylethanolamide, AEA) is produced by neurons in a stimulus-dependent manner and is released toward the extracellular space where it diffuses to activate CB1 receptors (2–6). Uptake into either neurons or astrocytes with subsequent hydrolysis inactivates AEA (2, 7). Other acyl-EAs, including HEA, DEA, PEA, and OEA, are also present in brain and may act as signaling molecules (2, 8, 9). HEA and DEA act on CB1 receptors with potencies and efficacies similar to that of AEA (10–12), PEA acts on CB2-like receptors (13, 14), whereas OEA acts on a target that does not belong to the cannabinoid receptor family (15–17).

The molecular mechanism that underlies acyl-EA production is starting to be understood. Cultured neurons analyzed under basal conditions produce low amounts of AEA, but this production is dramatically increased after a stimulus such as a rise in intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) (2, 5, 18). This calcium-dependent increase in production is likely mediated through the calcium-dependent increase in acyltransferase activity, the enzyme that gives rise to the precursor of AEA, arachidonylphosphatidylethanolamide, which is in turn directly cleaved by phospholipase D (1, 2, 9, 8). AEA production is not restricted to neurons, because many different cell types have been shown to produce this lipid (13, 19–22).

Whether astrocytes, the major glial cell type of the central nervous system, produce AEA was previously unknown, mainly because its presence in these cells has only been assessed with radioactive labeling, a method that has a poor detection limit (2, 23). Also, it is unclear if an increase in [Ca\(^{2+}\)]\(_i\) enhances the production of all acyl-EAs concomitantly. To address these two questions, we developed a chemical ionization gas chromatography/mass spectrometry (CI-GC/MS) method that allows the simultaneous and femtomole quantification of AEA, HEA, DEA, PEA, and OEA and determined their levels in astrocytes in culture under basal conditions and after ionomycin-induced increases in [Ca\(^{2+}\)]\(_i\). Furthermore, we measured acyl-EA levels in response to endothelin-1, a peptide that has been shown to

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1 The abbreviations used are: acyl-EA, acylethanolamide; AEA, arachidonylethanolamide; HEA, homo-γ-linolenylethanolamide; DEA, docosatetraenylethanolamide; PEA, palmitylethanolamide; OEA, oleylthanolamide; [Ca\(^{2+}\)]\(_i\), intracellular calcium; CI-GC/MS, chemical ionization gas chromatography/mass spectrometry; PBSglc, phosphate-buffered saline containing high glucose; GFAP, glial fibrillary acidic protein; DAPI, 4',6-diamidino-2-phenylindole; HPLC, high performance liquid chromatography; ANOVA, analysis of variance; LDH, lactate dehydrogenase; MEM, minimal essential medium; BStF, bis(tri-methylsilyl)trifluoracetamide; TMS, trimethylsilyl; FBS, fetal bovine serum.
stimulate phospholipase D in astrocytes (24), and determined if the production of AEA and other acyl-EA differs between species.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ethanolamine, poly-t-ornithine (M, 30,000–70,000), poly-t-lysine (M, 70,000–150,000), endothelin-1, and EGTA were purchased from Sigma Chemical Co. Deuterated ethanolamine (chemical purity 98%+) was from Cambridge Isotope Laboratories. Fatty acid chlorides were from Nu-Chek Prep. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and micro-reaction borosilicate vials were from Supelco. Filter cap 75-cm² flasks and 13-mm coverslips (Thermanox) were from Nalge Nunc. 100- and 35-mm culture dishes were from Corning. Dulbecco’s modified Eagle’s medium (#11995-037, MEM (#51200-038), F-12 (#21700-075), horse serum, and heat-inactivated fetal bovine serum (FBS) were from Invitrogen. CellGro (Complete serum free cell culture media) was from Mediatech. Ionomycin (free acid) was from Calbiochem.

**Synthesis and Handling of Unlabeled and ²H₄-Labeled Acylethanolamide Standards**—Unlabeled and ²H₄-labeled acylethanolamide (²H₄-labeled EA) standards were synthesized as previously described (25, 26), diluted in chloroform, and stored for a maximum of 6 months at −20°C. Flasks pre-coated with 1 cm² flasks or coverslips pre-coated with 1 cm² flasks were from Corning. Dulbecco’s modified Eagle’s medium (#11995-037, MEM (#51200-038), F-12 (#21700-075), horse serum, and heat-inactivated fetal bovine serum (FBS) was from Invitrogen. CellGro (Complete serum free cell culture media) was from Mediatech. Ionomycin (free acid) was from Calbiochem.

**Quantitative Limit of Isotope Dilution Calibration Curves**—The quantification limits of our isotope dilution assay were determined by spiking the sample with increasing concentrations of ³H₂₄-labeled EA, and then measuring the peak ratio at different concentrations. The standard deviation gave the ratio that established the quantification limit of our isotope dilution assay.

**GC/MS Analysis**—For GC/MS analysis, acyl-EA TMS derivatives were dried, recovered in 2–4 µl of hexane, and injected into a Varian CP3800 GC (split loss mode) equipped with a 30-m column (fused-silica, CP-Sil 8 CB, low bleed). The capillary injector (model 1079) contained a Silico liner (5.4 mm, OD 3.4 mm) with a carborfit to improv...
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Retention times for [H4]acyl-EA were ~0.03 min shorter than their corresponding acyl-EA, because of GC isotope discrimination (not shown). Thus, this method allowed the concomitant, yet individual, detection and analysis of AEA, HEA, DEA, PEA, and OEA.

The mass spectrum of AEA is shown in Fig. 2a. There were two predominant ions with high diagnostic value: the ion at m/z 420, corresponding to the protonated TMS molecule ([M+H]+) and the ion at m/z 330 (base peak), which was produced by the neutral loss of the TMS alcohol ([M+H–90]+). An additional informative ion was present at m/z 404, which corresponded to the neutral loss of methane ([M+H–16]+). A minor ion species at m/z 492 was present, corresponding to the protonated di-TMS molecule (the second TMS being added onto the nitrogen of the ethanolamine).

A similar mass spectrum pattern (i.e. a mass spectrum with predominant ions at [M+H]+ and [M+H–90]+) was obtained when we analyzed [H4]AEA (Fig. 2b), HEA (Fig. 3a), [H4]HEA (not shown), DEA (Fig. 3b), and [H4]DEA (not shown). The mass spectra of PEA and OEA are shown in Fig. 3, c and d, respectively. The predominant ions in these spectra are also [M+H]+ and [M+H–90]+, but here the base peaks are the protonated TMS molecules.

From the knowledge acquired by analyzing the mass spectra of synthetic acyl-EAs and [H4]acyl-EAs, it is possible to use CI-GC/MS in the selected ion monitoring mode to quantify the amount of AEA and other acyl-EA in a biological matrix.

**Calibration Curves and Quantification Limits**—CI does not cause extensive fragmentation of analytes but, rather, produces ions with high m/z that affords excellent structural identification and improved detection limits (31). We took advantage of the improved detection limit to develop an isotope dilution method. Calibration curves were built by injecting a fixed amount of each [H4]acyl-EA (200 pmol) together with decreasing amounts of each acyl-EA (200, 100, 20, 10, 2, 1, and 0 pmol). When selectively monitoring the base peaks: i.e. [M+H–90]+ for AEA, HEA, and DEA, and [M+H]+ for PEA and OEA, we found that the MS responses of each calibration curve were linear (r² ≥ 0.98) and that their quantification limits were below 1 pmol (Table I). This result indicates that decreasing amounts of acyl-EA produce a linear decrease in the signals of the area ratio down to the femtomole range, which allows the unambiguous quantification of AEA, HEA, DEA, PEA, and OEA in the low picomole range in a biological matrix such as astrocytes in culture.

**Immunocytochemical Characterization of Mouse Astrocytes in Culture**—It is well known that cultures of astrocytes contain small amounts of other types of cells, such as microglial cells and fibroblasts (27). Microglial cells are macrophage-like cells that invade the central nervous system during development and are therefore present in the brain parenchyma of the pups used to prepare astrocytes in culture (32). Furthermore, microglial cells proliferate in the presence of serum (33, 34). Remnants of meninges contain fibroblasts that also proliferate in the presence of serum (27). We sought to determine the relative amount of microglial cells and fibroblasts in cultures of mouse astrocytes that were grown for 5–6 weeks and serum-deprived. Using an antibody against GFAP, a protein specific to astrocytes, we determined that 94 ± 2% of the cells in the cultures were astrocytes (Fig. 4A). An antibody against the macrophage marker MAC1 (Fig. 4b) stained 2 ± 1% of the cells (Fig. 4d), whereas an antibody against the fibroblast marker fibronectin (Fig. 4c) stained 3 ± 1% of the cells (Fig. 4e). Approximately 2% of the cells were not stained by these antibodies. Thus, the cultures used in this study were highly enriched in astrocytes.
Mouse, Rat, and Human Astrocytes in Culture Produce Anandamide and Other Acylethanolamides—Lipids of mouse astrocytes in culture were extracted and purified by HPLC, and the amounts of AEA and other acyl-EAs were quantified by CI-GC/MS. Under basal conditions, these cells produced detectable amounts of each acyl-EA, with the following relative amounts: PEA >> OEA > AEA > HEA > DEA (Table I).

Astrocytes in culture from different species have different phenotypes (35). To determine whether astrocytes from different species vary in their acyl-EA content, we prepared rat and
human astrocytes in culture, analyzed their basal levels of AEA and other acyl-EAs, and compared it to that of mouse astrocytes in culture. Under basal conditions, rat astrocytes produced a different pattern of acyl-EA: PEA ≫ AEA ≫ OEA ≈ HEA ≈ DEA (Table II). Under basal conditions, human astrocytes in culture produced a pattern of acyl-EA that was reminiscent of the one found in rat astrocytes, with PEA ≫ AEA ≫ DEA ≈ HEA ≈ OEA (Table II). Thus, the relative levels of anandamide and other acyl-EAs in astrocytes vary between species.

C6 rat glioma cells had yet a different profile of acyl-EA amounts under basal conditions: PEA ≫ DEA ≫ AEA ≫ OEA. HEA was not detectable.

**Rise in Intracellular Calcium Increases the Production of Anandamide, HEA, and DEA in Mouse Astrocytes**—We assessed whether increasing intracellular calcium with the calcium ionophore ionomycin increases the production of AEA and other acyl-EAs. To limit the impact of the variability between cell culture preparations on data interpretation, the effects of ionomycin were compared with the basal (i.e. vehicle-treated) within the same cell culture preparation (5). Ionomycin increased the production of AEA, HEA, and DEA, without affecting the amounts of PEA and OEA. The effect of ionomycin was prevented when calcium was chelated by EGTA (Fig. 5).

Because ionomycin is known to disrupt cell membranes, we treated mouse astrocytes with ionomycin for increasing time periods and assessed the release of lactate dehydrogenase (LDH). Addition of ionomycin to mouse astrocytes for 2.5, 5, or 10 min did not significantly affect the basal release of LDH (Fig. 6). Yet, a 4- to 5-fold increase in LDH activity was measured after 20 min of ionomycin treatment, as well as after 20 min of Triton treatment (Fig. 6). This result shows that cell membranes were intact after 5 min of ionomycin, which is the stimulus time point that we used to study acyl-EA production.

**Endothelin-1 Selectively Increases the Production of Anandamide in Mouse Astrocytes**—To address whether relevant stim-

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**Table I**

**Amount of anandamide and other acylethanolamides in mouse astrocytes analyzed under basal conditions**

| Acyl-EA | Quantification limit | Amounts in mouse astrocytes |
|---------|----------------------|-----------------------------|
|         | pmol                 | pmol/dish | pmol/mg | %     |
| AEA     | 0.46                 | 1.25 ± 0.16 | 1.02 ± 0.19 | 16.9  |
| HEA     | 0.50                 | 1.13 ± 0.28 | 0.87 ± 0.30 | 14.4  |
| DEA     | 0.48                 | 0.86 ± 0.28 | 0.71 ± 0.26 | 11.8  |
| PEA     | 0.27                 | 2.57 ± 0.58 | 2.17 ± 0.49 | 35.9  |
| OEA     | 0.39                 | 1.87 ± 0.84 | 1.27 ± 0.46 | 21.0  |
| Total   |                      | 6.04      | 100.0    |

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**Fig. 4. Immunocytochemical characterization of cultures of mouse astrocytes.**

- **a**, cultures of astrocytes were immunostained with anti-GFAP.
- **b**, cultures of microglial cells were immunostained with anti-MAC1.
- **c**, cultures of meninges cells were immunostained with anti-fibronectin. All cells were also counterstained with DAPI (blue) to identify the cell’s nuclei. **Insets** in **a**, **b**, and **c** show immunostaining performed in the absence of the respective primary antibodies. Cultures of astrocytes were immunostained with **d** anti-MAC1 and **e** anti-fibronectin. The arrows show that a small number of **d** microglial cells and **e** fibroblasts are present in the culture of astrocytes. **Bars** are 100 μm.
uli can elicit the changes observed using ionomycin, we measured the production of acyl-EAs in mouse astrocytes in culture that were stimulated with endothelin-1, a peptide that is present in brain and is known to stimulate phospholipase D activity in astrocytes (24). Endothelin-1 significantly increased the production of AEA, without affecting the amounts of HEA, DEA, PEA, and OEA. This effect was prevented when calcium was chelated by EGTA (Fig. 5).

**DISCUSSION**

The endocannabinoid signaling system is composed of 1) endocannabinoid ligands, with their biosynthesis pathways...
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and release mechanisms; 2) cannabinoid receptors, with their signal transduction mechanisms; and 3) endocannabinoid inactivation mechanisms, which encompass uptake and subsequent hydrolysis (36). In the central nervous system, each of these components has been identified in neurons (2, 37). Thus, neurons can signal between each other by using the endocannabinoid signaling system without involving other cell types of the central nervous system. It has been shown that astrocytes express CB1 receptors and inactivate endocannabinoids by uptake and subsequent hydrolysis (2, 7, 38, 39). However, their ability to produce endocannabinoids remained unknown. In the present study we demonstrate that cultured astrocytes produce, in a calcium-dependent manner, the acyl-EAs AEA, HEA, and DEA, three endocannabinoids known to act on CB1 receptors with high potency and moderate efficacy (10–12, 25, 37). Our results underscore the existence of a complete endocannabinoid signaling system in astrocytes, thus their ability to use this system to signal between each other or with surrounding neurons.

Many of the mediators in the body, including eicosanoids, are present not as single entities but as large families of structurally related substances that act on different receptors. It seemed reasonable to expect that AEA was only the first representative of a larger family of bioactive lipids that might act on either CB1 receptors or different targets (10, 11, 25). However, whether acyl-EAs are synthesized through a common calcium-dependent pathway is unclear (1). Are different acyl-EAs produced by one type of phospholipase D that cleaves different precursors produced by one type of acyltransferase? Here, we show that an increase in [Ca$^{2+}$], in mouse astrocytes enhances the production of AEA, HEA, and DEA, whereas the amount of PEA and OEA are unchanged. These results are consistent with recent studies showing the independent production of AEA and PEA/OEA in different cell types. Specifically, in mouse epidermal JB6 P+ cells in culture, UVB irradiation and serum deprivation increase the production of PEA/OEA, while slightly decreasing the basal amount of AEA (28). In rat cortical neurons in culture, co-activation of N-methyl-D-aspartate (NMDA) receptors and nicotinic receptors increases AEA production, without affecting the basal amounts of PEA/OEA (5).

Furthermore, in the present study, we show that C6 glioma cells produce AEA and DEA, whereas HEA is undetectable. Finally, we demonstrate that endothelin-1 selectively increases the production of AEA. Together, these results emphasize the existence of independent biosynthesis pathways for the production of different acyl-EA. Because phospholipase D activity is thought to be independent of calcium and to directly cleave the precursors of acyl-EAs, we hypothesize that multiple isomers of acyltransferase with distinct calcium-sensitivities exist and that each of these isomers establishes independent biosynthesis pathways for different acyl-EA.

Astrocytes in culture prepared from different species may vary in their phenotype (35). For example, rat astrocytes in culture express CB1 receptors (40, 41), whereas mouse astrocytes in culture do not (42). Here, we quantified the basal levels of acyl-EA in astrocytes from mouse, rat, and humans and found that PEA is abundantly produced in each species. However, although AEA, HEA, and DEA are equally abundant in mouse astrocytes, AEA is ~4–5 times more abundant than HEA and DEA in both rat and human astrocytes. Although the first occurrence of these differences is not known, our finding does suggest that AEA may be a predominant endocannabinoid in rat and human astrocytes.

What is the role of the endocannabinoid signaling system in astrocytes? The majority of the perivascular astrocytes in adult rat brain express CB1 receptors, with more than 70% of these receptors being localized on the plasmalemma of either their cell body or their filamentous glial processes (38). Activation of CB1 receptors on rat astrocytes in culture increases the rate of glucose oxidation and ketogenesis, two mechanisms involved in the energy supply of the brain (43, 44). Because perivascular astrocytes are pivotally located and involved in supplying energy from blood to neurons in a stimulus-dependent manner (45), one hypothesis is that the endocannabinoid signaling system in astrocytes regulates the energy supply from blood to neurons. In agreement with this hypothesis, rat brain energetic metabolism is increased after exposure to AEA or Δ$^2$-tetrahydrocannabinol (46).

It has been shown that AEA produces biological effects through CB1 receptors as well as through receptors that are distinct from CB1 receptor, i.e. “anandamide” receptors. Specifically, AEA inhibits gap junctions and intercellular calcium signaling between cultured mouse striatal astrocytes, an effect that is not prevented by CB1 receptor antagonists (47). It is thus possible that astrocytes produce AEA in a calcium-dependent manner to modulate energy metabolism by acting through CB1 receptors and regulate intercellular calcium signaling by acting through anandamide receptors.

Rat glioma cells express CB1 and CB2 receptors, whereas rat astrocytes only express CB1 receptor (40, 48). The kinetic of AEA uptake into C6 cells is different from that measured with rat astrocytes in culture (7, 49). Here, we show that C6 cells have a different pattern of acyl-EA amounts compared with rat astrocytes in culture. Together, these results suggest that transformation of astrocytes into glioma cells is likely associated with a profound change in their endocannabinoid signaling system.

In summary, our study shows that astrocytes in culture produce AEA and other acyl-EAs under basal conditions. Production of AEA, HEA, and DEA is increased by a calcium stimulus, whereas the levels of PEA and OEA are unaffected. Endothelin-1 selectively increases the production of AEA. This indicates that the production of different acyl-EAs can be independently increased and that independent biosynthesis pathways are involved in the production of different acyl-EAs. Finally, the acyl-EA production in astrocytes varies between species.

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