Anandamide Hydrolysis by Human Cells in Culture and Brain*

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Anandamide (arachidonylethanolamide; AnNH) has important neuromodulatory and immunomodulatory activities. This lipid is rapidly taken up and hydrolyzed to arachidonate and ethanolamine in many organisms. As yet, AnNH inactivation has not been studied in humans. Here, a human brain fatty-acid amide hydrolase (FAAH) has been characterized as a single protein of 67 kDa with a pI of 7.6, showing apparent $K_i$ and $V_{max}$ values for AnNH of 2.0 ± 0.2 $\mu$M and 800 ± 75 pmol·min$^{-1}$·mg of protein$^{-1}$, respectively. The optimum pH and temperature for AnNH hydrolysis were 9.0 and 37°C, respectively, and the activation energy of the reaction was 43.5 ± 4.5 kJ·mol$^{-1}$. Hydro(pero)xides derived from AnNH or its linoleoyl analogues by lipoxynase nase action were competitive inhibitors of human brain FAAH, with apparent $K_i$ values in the low micromolar range. One of these compounds, linoleoylanethanolamide, is the first natural inhibitor ($K_i = 9.0 ± 0.9$ $\mu$M) of FAAH as yet discovered. An FAAH activity sharing several biochemical properties with the human brain enzyme was demonstrated in human neuroblastoma CHP100 and lymphoma U937 cells. Both cell lines have a high affinity transporter for AnNH, which had apparent $K_m$ and $V_{max}$ values for AnNH of 0.20 ± 0.02 $\mu$M and 30 ± 3 pmol·min$^{-1}$·mg of protein$^{-1}$ (CHP100 cells) and 0.13 ± 0.01 $\mu$M and 140 ± 15 pmol·min$^{-1}$·mg of protein$^{-1}$ (U937 cells), respectively. The AnNH carrier of both cell lines was activated up to 170% of the control by nitric oxide.

Anandamide (arachidonylethanolamide; AnNH)$^1$ is an endogenous lipid that binds to cannabinoid CB1 and CB2 receptors, which are mainly found in the central nervous system and in peripheral immune cells. It mimics the pharmacological effects of $\Delta^9$-tetrahydrocannabinol, the active principle of hashish and marijuana (1, 2). AnNH formation occurs mainly through phosphodiesterase-mediated cleavage of N-arachidonylphosphatidylethanolamine (3, 4), although a direct synthesis from arachidonic acid and ethanolamine has also been described (5, 6). AnNH can be released from depolarized neurons (3). Upon binding to CB1 receptors, AnNH induces inhibition of forskolin-induced cAMP accumulation, inhibition of N-type Ca$^{2+}$ channels, and activation of mitogen-activated protein kinase signal transduction pathway (reviewed in Ref. 7) and increases protein tyrosine phosphorylation (8). Activation of the CB2 receptor leads to inhibition of adenylate cyclase and activation of the mitogen-activated protein kinase signaling (9). Interestingly, AnNH binding to cannabinoid receptors is coupled to nitric oxide (NO) release in the central nervous system of invertebrates and in peripheral immune cells of both invertebrates and humans (10).

The pharmacological effects of AnNH on CB1 and CB2 receptors depend on the life span of the lipid in the extracellular space, which is limited by a rapid and selective process of cellular uptake, followed by intracellular degradation of AnNH to ethanolamine and arachidonic acid by the enzyme fatty-acid amide hydrolase (FAAH). Both components of the inactivation process of AnNH are the subject of active investigation. AnNH uptake has been characterized in rat neuronal cells (3, 11, 12) and rat basophilic leukemia (RBL-2H3) cells (13). FAAH has been demonstrated and partially characterized in rat, porcine, and dog brains (14–16). Furthermore, FAAH activity has been shown in one "neuronal" cell line, namely mouse neuroblastoma N18TG2 (17), and in one "non-neuronal" cell line, namely RBL-2H3 (13). The FAAH gene has recently been cloned from rat, mouse, and human liver cDNAs, allowing molecular mass determination and substrate specificity analysis of the enzyme (18, 19). As yet, no information is available on the activity of human FAAH or on AnNH uptake in human cells. This prompted us to investigate some biochemical properties of FAAH from human brain and human neuronal and immune cells, i.e. neuroblastoma CHP100 and lymphoma U937 cells. AnNH uptake was characterized in these two cell types to gain information on the AnNH inactivation process in humans. The cell lines chosen are widely used as experimental models for neuronal (20) and immune (21) tissues. In these two cell types, AnNH uptake was demonstrated and characterized. Taken together, the results reported here represent the first biochemical characterization of human brain FAAH. Most properties of this enzyme are shared by FAAH found in human neuronal and immune cells in culture. Remarkably, both cell lines seem to inactivate AnNH in the same way, which strengthens the concept of a neuroimmune axis in humans, which is evident, for instance, in the "axon-reflex" model for neurogenic inflammation (13). Possible implications of FAAH activity and expression in brain pathologies are also discussed.
EXPERIMENTAL PROCEDURES

Materials—Chemicals were of the purest analytical grade. Anandamide (arachidonylthanolamide), arachidonic acid, ethanolamine, phenylmethylsulfonyl fluoride (PMSF), iodoacetic acid, N-ethylmaleimide, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and sodium nitroprusside (SNP) were purchased from Sigma. S-Nitroso-N-acetylpenicillamine was from Research Biochemicals International, and spermine NONOate (Z7-1-L-argininooctyl)-N,N,N′,N′-tetramethylammonium (Z7-1-acetyl-1,2-dioleoylamide) was from Alexis Corp. (Laufenfingen, Switzerland). Leukotriene B4 and prostaglandin E2 were from Cayman Chemical Co., Inc. [1-14C]AnNH was synthesized from ethanolamine and [1-14C]arachidonic acid (52 mCi/mmol; NEN DuPont de Nemours, Köln, Germany) as reported (22). Linoleoylthanolamide ([OZ,12Z]-octadeca-9,12-dienoylthanolamide; ODNHETOH), linoleoylamine ([OZ,12Z]-octadeca-9,12-dienoylamine; ODNDHE), linoleoylamide ([OZ,12Z]-octadeca-9,12-dienoylamide; ODNDHM), and prostaglandin E2 were from Cayman Chemical Co., Inc. [1-14C]AnNH, 9% 8/9-H(P)AnNH, and 4% 12-H(P)AnNH by reversed-phase high performance liquid chromatography (HPLC) as described (23). 15-Hydro(peroxy)xyeicosa-(5,8,11,12)-tetraenoylthanolamide; ODNHEtOH, and 13-hydroxy derivatives (13-HODNHEtOH, 13-HODNHe, and 13-HODNHMe) were synthesized and characterized (purity >96% by gas-liquid chromatography) as reported (23). 15-Hydro(peroxy)xy-anandamide (15-hydro(peroxy)xyeicosa-(5Z,8Z,11Z,13E)-tetraenoylthanolamide; 15-H(P)AnNH, purity >96%) and 11-hydro(peroxy)xyanandamide (11-H(P)AnNH, a mixture of 55% 11-H(P)AnNH, 24% 5-H(P)AnNH, 18% 15-H(P)AnNH, 9% 9-H(P)AnNH, and 4% 12-H(P)AnNH) by reversed-phase high performance liquid chromatography were a gift from Guus van Zadelhof (Bijvoet Center for Biomolecular Research, Utrecht University).

Biological Material—Human brain specimens were obtained from five different male patients (aged 73–77) undergoing surgical operations to remove meningiomas tumors. Brain tissues were removed and donated by Prof. R. Giuffre and Dr. G. De Caro (Neurosurgery Division, University of Rome Tor Vergata, Sant’Eugenic Hospital, Rome, Italy). In four cases, the perilesional white matter surrounding the tumor area was removed (1 g of fresh tissue in total) and used for FAAH characterization. In one case, both meningioma and perilesional white matter (0.1 g of each fresh tissue) were removed and used to compare FAAH activity and expression in meningioma and healthy brain.

Human neuroblastoma CHP100 cells were cultured as reported (20) in a mixture of Eagle’s minimal essential medium plus Earle’s salts and Ham’s F-12 medium (Flow Laboratories Ltd., Irvine, United Kingdom) supplemented with 15% heat-inactivated fetal bovine serum, 1.2 g/liter sodium bicarbonate, 15 mM Hepes, 2 mM l-glutamine, and 1% nonessential amino acids. Human lymphoma U937 cells, a gift from Dr. E. Faggioni (Department of Public Health and Cell Biology, University of Rome Tor Vergata), were cultured in RPMI 1640 medium (Gibco, Paisley, United Kingdom) supplemented with 25 mM Hepes, 2.5 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (21). Both CHP100 and U937 cells were maintained at 37 °C in a humidified 5% CO2 atmosphere.

Assay of FAAH—Immediately after surgical removal, human brain specimens were washed in phosphate-buffered saline and homogenized with an UltraTurrax T25 in 50 mM Tris-HCl and 1 mM EDTA, pH 7.4. The homogenate was then centrifuged at 10,000 × g for 10 min. The dry pellet was resuspended in 30 μl of ice-cold buffer A at a protein concentration of 1 mg/ml and stored at −80 °C until use. Both CHP100 and U937 cells (3 × 106/sample) were collected in phosphate-buffered saline and centrifuged at 1000 × g for 10 min. The dry pellet was resuspended in 30 μl of ice-cold buffer A and sonicated on ice three times for 10 s, with 10-s intervals, using a Vibratecell sonifier (Sonics & Materials Inc.) with a microtip at maximum power. The homogenate was then centrifuged sequentially as described above for the human brain, and the final pellet was stored at −80 °C in buffer B at a protein concentration of 1 mg/ml until use.

The assay of FAAH (arachidonylthanolamide amidohydrolase, EC 3.5.1.4) activity was performed by reversed-phase high performance liquid chromatography (HPLC) as recently described (22). Thermal stability and pH dependence of FAAH activity were studied as described (17). Activation energy values were calculated as reported (25). Kinetic and inhibition studies were performed using different concentrations of [1-14C]AnNH (in the 0–21 μM range) and two different concentrations of 10 and 20 μM of each inhibitor to calculate the kinetic parameters. Fitting of the experimental points to a Lineweaver-Burk plot by a linear regression program (Kaleidagraph Version 3.0) yielded straight lines with r values >0.95.

The assay of the FAAH synthase activity was performed by measuring the formation of [1-14C]AnNH from [1-14C]arachidonic acid and ethanolamine as reported (5). Tissue or cell homogenates (20 μg of proteins/test) were incubated for 15 min at 37 °C in 200 μl of 50 mM Tris-HCl, pH 9.0, containing 10 μM [1-14C]arachidonic acid (52 mCi/mmol) and 2 mM ethanolamine. The reaction was stopped, and the products were extracted and analyzed by reversed-phase HPLC following the same procedure as described above for the hydrolyase activity. FAAH synthase activity is expressed as picomoles of AnNH formed per min/mg of protein. The effect of various compounds on the hydrolyse or synthase activity of FAAH was determined by adding each substance directly to the assay buffer at the indicated concentrations.

Immunochimical Analysis—SDS-polyacrylamide gel electrophoresis (12%) was performed under reducing conditions in a Mini-Protean II apparatus (Bio-Rad) with 0.75-mm spacer arms (26). Rainbow molecular mass markers (Amersham International, Buckinghamshire, United Kingdom) were phosphorylase b (97.4 kDa), bovine serum albumin (66.0 kDa), and ovalbumin (46.0 kDa). Native isoelectric focusing was performed in the Mini-Protein II apparatus using a 5% polyacrylamide gel containing ampholytes in the pH range 5.0–9.0 (Sigma) as described (27). Isoelectric focusing was calibrated by running the following pI markers (Sigma): lentil (Lens culinaris) lectin (pI 8.8, 8.6, and 8.2), myoglobin from horse heart (pI 7.2 and 6.8), carbonic anhydrase II from bovine erythrocytes (pI 12%), was phosphorylase a (pI 97.4 kDa), bovine serum albumin (pI 6.6), and carbonic anhydrase I from bovine erythrocytes (pI 9.4). Human brain homogenates (20 μg/lane), prepared as described above for FAAH assay, were subjected to either SDS-
polyacrylamide gel electrophoresis or isoelectric focusing, and then slab gels were electroblotted onto 0.45-μm nitrocellulose filters (Bio-Rad) using a Mini-TransBlot apparatus (Bio-Rad) as reported (26). Immuno-detection of FAAH on nitrocellulose filters was performed with specific anti-FAAH polyclonal antibodies (diluted 1:200), raised in rabbits against the conserved FAAH sequence VGYYETDNYTMPSPAMR (19), conjugated to ovalbumin. This peptide antigen and the anti-FAAH polyclonal antibodies were prepared by Primm s. r. l. (Milan, Italy). Goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad; diluted 1:2000) was used as secondary antibody, and immunoreactive bands were stained with the alkaline phosphatase staining solution according to the manufacturer’s instructions (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA) was performed by coating the plate with human brain homogenate (20 μg/well), prepared as described above for the FAAH assay. Anti-FAAH polyclonal antibodies were used as primary antibody (diluted 1:300), and goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad; diluted 1:2000) was used as secondary antibody, and immunoreactive bands were stained with the alkaline phosphatase staining solution according to the manufacturer’s instructions (Bio-Rad).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Sequencing—2–5 × 10⁶ cells or 20 mg of tissue were used to isolate total RNA by means of the S.N.A.P. TM total RNA isolation kit (Invitrogen). Control reactions were carried out to ensure complete removal of genomic DNA. RT-PCRs were performed using the EZ rTth RNA PCR kit (Perkin-Elmer) following the manufacturer’s instructions. The amplification parameters were as follows: 2 min at 95 °C, 45 s at 95 °C, 30 s at 55 °C, and 30 s at 60 °C. Linear amplification was observed after 20 cycles. The primers were as follows: (1) 5'-TGGAAGTCCTCCAAAAGCCCAG and (2) 5'-TGTCATAGACACAGCCCTTCAG for FAAH and (1) 5'-AGTTGCTGCATTTAAAAAGC and (2) 5'-CCTCAGTTCCCCAAAACAC for 18 S rRNA. Five μl of the reaction mixture were electrophoresed on a 6% polyacrylamide gel, which was then dried and subjected to autoradiography. Products were validated by size determination and sequencing.

### Table I

Inhibition of human brain FAAH activity by different anandamide products and analogues

| Structure of the compound | Inhibition constant (K_i, μM) | Total activity (%) |
|---------------------------|-------------------------------|--------------------|
| (AnNH)                    | None                          | 100⁴ |
| (15-HPAnNH)               | 4.8±0.5                       | 80±8               |
| (15-HAnNH)                | 3.2±0.3                       | 82±8               |
| (11-HPAnNH)¹               | 5.2±0.5                       | 83±8               |
| (11-HAnNH)¹               | 4.0±0.4                       | 85±9               |
| (ODNHeOH)                 | 9.0±0.9                       | 76±8               |
| (ODNH₂)                   | 14.1±1.3                      | 70±7               |
| (ODNMe)                   | 24.5±2.1                      | 72±7               |
| (13-HODNHeOH)             | 3.0±0.3                       | 56±6               |
| (13-HODNH₂)               | 5.3±0.5                       | 49±5               |
| (13-HODNMe)               | 9.3±0.9                       | 50±5               |

*¹ 100% = 750 ± 70 pmol ⋅ min⁻¹ ⋅ mg of protein⁻¹.

b 11-HPAnNH was a mixture of 11-HPAnNH (45%), 5-HPAnNH (24%), 15-HPAnNH (18%), 8/9-HPAnNH (9%), and 12-HPAnNH (4%).

c 11-HAnNH was the same mixture as 11-HPAnNH, reduced with NaBH₄.
quantitation of the RT-PCR products, bands were excised from the gel and counted in an LKB1214 Rackbeta scintillation counter (Amersham Pharmacia Biotech, Uppsala, Sweden). Linear amplification sequencing was performed using a Cyclist™ DNA sequencing kit (Stratagene) according to the manufacturer’s instructions. RT-PCR products for sequencing were prepared without [α-32P]dCTP and sequenced with the same primers used for amplification after labeling them with [γ-32P]dATP (3000 Ci/mmol; Amersham International).

**Determination of Anandamide Uptake**—The uptake of [1-14C]AnNH (52 mCi/mmol) in intact CHP100 or U937 cells was studied essentially as described (13). CHP100 and U937 cells were suspended in their own medium at a density of 1 x 10⁶ cells/ml. Cell suspensions (2 ml/test) were incubated for different time intervals at 37 °C with 100 nm [14C]AnNH in the presence of each compound. Activity and uptake values are expressed as percentage of the untreated controls, arbitrarily subtracted from that at 37 °C. The Qₑ value was calculated as the ratio of AnNH uptake at 30 and 20 °C (11). AnNH uptake is expressed as picomoles of AnNH taken up per min/mg of protein. The effect of different compounds on AnNH uptake was determined by adding each substance directly to the incubation medium at the indicated concentrations. In the case of CCCP, cells were preincubated with 50 μm CCCP for 15 min at 37 °C before the addition of [1-14C]AnNH to abolish mitochondrial transmembrane potential (29). Cell viability after each treatment was checked with trypan blue and found to be higher than 90% in all cases. It is noteworthy that no specific binding of 3HCP55940, a potent cannabinoid, was obtained with plasma membranes of CHP100 cells. Consequently, CHP100 and U937 cells express hardly detectable levels of CB1 mRNA and very low levels of CB2 mRNA (21); thus, [1-14C]AnNH binding to CB receptors is not likely to interfere in the uptake experiments (11, 13).

**Data Analysis**—Data reported in this paper are the means ± S.D. of at least three independent determinations, each performed in duplicate. Statistical analysis was performed by the Student’s t test, elaborating experimental data by means of the InStat program (GraphPAD Software for Science).

### RESULTS

**Characterization of FAAH in Human Brain and Human Cells**—Pilot experiments indicated that human brain FAAH activity was linearly dependent on the amount of tissue homogenate (in the range 0–30 μg of protein) and the incubation time of the reaction (in the range 0–30 min), whereas it depended on AnNH concentration according to Michaelis-Menten kinetics (Fig. 1A) (data not shown), yielding an apparent Kₘ of 2.0 ± 0.2 μM and a Vₘₐₓ of 800 ± 75 pmol·min⁻¹·mg of protein⁻¹. The activity of FAAH was assayed in the pH range 5.0–11.0 and in the temperature range 20–65 °C, showing an optimum pH and temperature at 9.0 and 37 °C, respectively. Arrhenius diagrams of AnNH hydrolysis by FAAH in the temperature range 20–45 °C allowed us to calculate an activation energy of 43.5 ± 4.5 kJ·mol⁻¹.

Hydroxylated AnNH derivatives and the linoleoyl analogues of AnNH were competitive inhibitors of human brain FAAH, with apparent Kᵢ values ranging from 3.2 to 24.5 μM (Table I).

### Table II

**Inhibition of FAAH activity and [14C]Anandamide uptake in human brain and human CHP100 and U937 cells**

FAAH activity was determined using 10 μM AnNH as substrate. For uptake experiments, cells (2 x 10⁶) were incubated for 15 min at 37 °C with 100 nM [14C]AnNH in the presence of each compound. Activity and uptake values are expressed as percentage of the untreated controls, arbitrarily set to 100 (see below for absolute values). Results on FAAH activity in CHP100 and U937 cells were superimposable; thus, FAAH activity in CHP100 cells was omitted for the sake of clarity.

| Compound          | FAAH activity | Anandamide uptake |
|-------------------|---------------|-------------------|
|                   | Brain (%)     | U937 (%)          | CHP100 (%) | U937 (%) |
| None              | 100           | 100               | 100        | 100      |
| Arachidonic acid  | 100           | 100               | 100        | 100      |
| (100 μM)          | 100           | 100               | 100        | 100      |
| Ethanolamine      | 83 ± 8        | 80 ± 8            | 95 ± 10    | 88 ± 9   |
| (100 μM)          | 33 ± 3        | 50 ± 5            | 90 ± 9     | 87 ± 9   |
| 15-HAnNH (10 μM)  | 56 ± 6        | 62 ± 6            | 89 ± 9     | 85 ± 9   |
| ODNHETeOH (10 μM) | 26 ± 3        | 43 ± 4            | 80 ± 8     | 82 ± 8   |
| 13-HODNHHetOH (10 μM) | ND² | 105 ± 10         | 100 ± 10  | 100 ± 10 |
| Leukotriene B₄ (1 μM) | ND³ | 105 ± 10         | 100 ± 10  | 100 ± 10 |
| Prostaglandin E₂ (10 μM) | ND | 105 ± 10        | 100 ± 10  | 100 ± 10 |
| PMSF (100 μM)     | 6 ± 1         | 8 ± 1             | 50 ± 5     | 52 ± 5   |
| Iodoacetic acid   | 10 ± 1        | 12 ± 1            | 50 ± 5     | 48 ± 5   |
| (100 μM)          | 15 ± 2        | 18 ± 2            | 55 ± 5     | 50 ± 5   |
| N-Ethylmaleimide  | 85 ± 9        | 85 ± 9            | 170 ± 17   | See Fig. 5B |
| (100 μM)          | 85 ± 9        | 87 ± 9            | 175 ± 18   | See Fig. 5B |
| CCP (50 μM)       | 88 ± 9        | 84 ± 9            | 172 ± 17   | See Fig. 5B |

¹ 100% = 750 ± 70 pmol·min⁻¹·mg of protein⁻¹.
² 100% = 390 ± 40 pmol·min⁻¹·mg of protein⁻¹.
³ 100% = 7.0 ± 0.7 pmol·min⁻¹·mg of protein⁻¹.
⁴ 100% = 53.0 ± 5.5 pmol·min⁻¹·mg of protein⁻¹.
⁵ ND, not determined; SNAP, S-nitroso-N-acetylpenicillamine; SPER-NO, spermine NONOate.
Anandamide Degradation by Human Tissues

FIG. 3. Quantitation of FAAH in human brain and human CHP100 and U937 cells. A, tissue or cell homogenates (20 µg/well) were subjected to ELISA using specific anti-FAAH polyclonal antibodies (white bars). Antigen competition ELISA (hatched bars) was performed by preincubating anti-FAAH polyclonal antibodies with a 1000-fold molar excess of peptide antigen. Absorbance values are expressed as percentage of the maximum, arbitrarily set to 100 (100% corresponds to 0.760 ± 0.080 absorbance units at 405 nm). FAAH mRNA levels (dotted bars) were quantitated by liquid scintillation counting and are expressed as percentage of the maximum, arbitrarily set to 100 (100% = 20,000 ± 2000 cpm). The radioactivity of the bands corresponding to 18 S rRNA (see B) was identical in all samples (5000 ± 500 cpm). B, FAAH mRNA (50 ng/lane) and 18 S rRNA (0.2 ng/lane) were amplified so that the amidase consensus sequence (amino acids 215–246) typical of all FAAHs as yet known.

These AnNH congeners were also alternate substrates of FAAH, yielding total activities that ranged from 85% (11-HAnNH) to 49% (13-HODNH2) of the activity obtained with AnNH itself (Table I). The substrate specificity of FAAH from human brain resembled that of the enzyme from mouse or rat brain (18, 19, 22).

Western blotting showed that anti-FAAH polyclonal antibodies specifically recognized a single immunoreactive band in brain homogenates, corresponding to a molecular mass of ~67 kDa and an isoelectric point of ~7.6 (Fig. 2).

Human neuronal (CHP100) and immune (U937) cells in culture also showed FAAH activity, with pH and temperature profiles superimposable to those observed with the human brain enzyme (data not shown). Both cell lines showed an FAAH activity (Fig. 1, A and B) characterized by apparent $K_m$ and $V_{max}$ values of 6.5 ± 0.6 µM and 32 ± 3 pmol-min⁻¹-mg of protein⁻¹ (CHP100) and 6.5 ± 0.6 µM and 520 ± 50 pmol-min⁻¹-mg of protein⁻¹ (U937) for AnNH. The activation energy of AnNH hydrolysis by FAAH in CHP100 or U937 cells (45.0 ± 4.5 kJ-mol⁻¹ in either case) was the same as the human brain enzyme. Moreover, 15-HAnNH, ODNH2, and 13-HODNH2 competitively inhibited FAAH activity in both cell lines, with apparent $K_i$ values of 4.5 ± 0.4, 11.1 ± 0.9, and 6.1 ± 0.5 µM (CHP100) and 3.8 ± 0.4, 10.5 ± 1.0, and 4.5 ± 0.4 µM (U937), respectively. Excess (100 µM) arachidonic acid, but not ethanolamine, strongly inhibited FAAH activity in all human sources tested, in line with previous findings on mouse FAAH (17).

A comparison of FAAH activity and expression in human healthy brain and meningioma. FAAH activity (white bars) was measured using 10 µM AnNH as substrate. FAAH protein content (hatched bars) was determined by ELISA using 20 µg of proteins/well. Antigen competition ELISA (dotted bars) was performed by preincubating anti-FAAH polyclonal antibodies with a 1000-fold molar excess of peptide antigen. FAAH activity and content are expressed as percentage of the control (healthy brain), arbitrarily set to 100 (100% = 750 ± 70 pmol-min⁻¹-mg of protein⁻¹ for the activity; 100% = 0.760 ± 0.080 absorbance units at 405 nm for the protein content).

Expression of FAAH in Human Brain and CHP100 and U937 Cells—The analysis of FAAH expression in human brain and human cells was performed at the protein (by ELISA) and mRNA (by RT-PCR) levels. The amount of FAAH protein in human brain was ~2- or 10-fold higher than the observed in U937 or CHP100 cells, respectively (Fig. 3A). This quantitation was validated by antigen competition experiments (18), showing that immunoreaction of the anti-FAAH polyclonal antibodies with the enzyme protein in human homogenates was specific (Fig. 3A). RT-PCR analysis showed similar differences in the mRNA levels (Fig. 3, A and B). Sequencing of
the FAAH mRNA, amplified by RT-PCR from human brain or human CHP100 or U937 cells, showed that human FAAH possesses a completely conserved sequence between amino acids 208 and 272, which contains a typical amidase consensus sequence (Fig. 3C).

FAAH activity and expression were measured also in human meningioma and were compared with those found in the perilesional white matter (healthy brain). AnNH hydrolysis by meningioma FAAH followed Michaelis-Menten kinetics, with apparent \( K_m \) and \( V_{max} \) values of 4.0 ± 0.4 \( \mu \)M and 370 ± 40 pmol\( \cdot \)min\(^{-1}\)\cdot mg of protein\(^{-1}\), respectively. Interestingly, the specific activity of FAAH in human meningioma was 50% compared with that in healthy brain, a value that was paralleled by the amount of FAAH protein in the same tissues (Fig. 4).

Characterization of AnNH Uptake in Human CHP100 and U937 Cells—Neuroblastoma CHP100 and lymphoma U937 cells were able to accumulate \(^{14}\)CAnNH, a process that was temperature-dependent (\( Q_{10} = 1.5 \) for both cell lines), time-dependent (\( t_{1/2} = 5 \) min for both cell lines), and concentration-dependent (Fig. 5A) (data not shown). \(^{14}\)CAnNH uptake in CHP100 and U937 cells was saturable (\( K_m = 0.20 \pm 0.02 \) and 0.13 ± 0.01 \( \mu \)M and \( V_{max} = 30 \pm 3 \) and 140 ± 15 pmol\( \cdot \)min\(^{-1}\)\cdot mg of protein\(^{-1}\), respectively); was enhanced when incubations were carried out in the presence of the NO donors SNP, S-nitroso-N-acetylpenicillamine, and spermine NONOate (Table II and Fig. 5B); and was reduced in the presence of PMSF, iodoacetic acid, or \( N \)-ethylmaleimide, each used at a 100 \( \mu \)M final concentration (Table II). Enhancement of \(^{14}\)CAnNH uptake by 5 mM SNP was prevented by co-incubation with either 20 \( \mu \)M hemoglobin, a typical NO scavenger (20), or 100 \( \mu \)M PMSF (data not shown). SNP and PMSF affected the uptake kinetics by changing the \( V_{max} \) value, but not the \( K_m \), thus changing the catalytic efficiency (i.e. the \( V_{max}/K_m \) ratio) of the transporter (Table III). On the other hand, 100 \( \mu \)M arachidonic acid or ethanolamine and 10 \( \mu \)M 15-HetE, ONDNEtOH, or 13-LODNEtOH did not significantly influence AnNH uptake in either cell type, nor did 1 \( \mu \)M leukotriene B\(_4\), 10 \( \mu \)M prostaglandin E\(_2\), or 50 \( \mu \)M CCCP (Table II).

**DISCUSSION**

Meningioma is a histologically benign tumor that is brain-invasive only in 4% of cases (33). Thus, perilesional white matter is not the same as the surrounding normal brain tissue. The meningioma FAAH followed Michaelis-Menten kinetics, with apparent \( K_m \) and \( V_{max} \) values of 4.0 ± 0.4 \( \mu \)M and 370 ± 40 pmol\( \cdot \)min\(^{-1}\)\cdot mg of protein\(^{-1}\), respectively. Interestingly, the specific activity of FAAH in human meningioma was 50% compared with that in healthy brain, a value that was paralleled by the amount of FAAH protein in the same tissues (Fig. 4).

**Characterization of AnNH Uptake in Human CHP100 and U937 Cells**

Neuroblastoma CHP100 and lymphoma U937 cells were able to accumulate \(^{14}\)CAnNH, a process that was temperature-dependent (\( Q_{10} = 1.5 \) for both cell lines), time-dependent (\( t_{1/2} = 5 \) min for both cell lines), and concentration-dependent (Fig. 5A) (data not shown). \(^{14}\)CAnNH uptake in CHP100 and U937 cells was saturable (\( K_m = 0.20 \pm 0.02 \) and 0.13 ± 0.01 \( \mu \)M and \( V_{max} = 30 \pm 3 \) and 140 ± 15 pmol\( \cdot \)min\(^{-1}\)\cdot mg of protein\(^{-1}\), respectively); was enhanced when incubations were carried out in the presence of the NO donors SNP, S-nitroso-N-acetylpenicillamine, and spermine NONOate (Table II and Fig. 5B); and was reduced in the presence of PMSF, iodoacetic acid, or \( N \)-ethylmaleimide, each used at a 100 \( \mu \)M final concentration (Table II). Enhancement of \(^{14}\)CAnNH uptake by 5 mM SNP was prevented by co-incubation with either 20 \( \mu \)M hemoglobin, a typical NO scavenger (20), or 100 \( \mu \)M PMSF (data not shown). SNP and PMSF affected the uptake kinetics by changing the \( V_{max} \) value, but not the \( K_m \), thus changing the catalytic efficiency (i.e. the \( V_{max}/K_m \) ratio) of the transporter (Table III). On the other hand, 100 \( \mu \)M arachidonic acid or ethanolamine and 10 \( \mu \)M 15-HetE, ONDNEtOH, or 13-LODNEtOH did not significantly influence AnNH uptake in either cell type, nor did 1 \( \mu \)M leukotriene B\(_4\), 10 \( \mu \)M prostaglandin E\(_2\), or 50 \( \mu \)M CCCP (Table II).
matter surrounding the meningoïda can be considered an essentially healthy brain area and was chosen in this study to characterize FAAH. Human brain showed a remarkable FAAH activity, and anti-FAAH antibodies recognized a single protein of 67 kDa with an isoelectric point of 7.6, characterized here for the first time (Fig. 2). These values were in good agreement with the size of the full-length human liver FAAH cDNA (19) and the isoelectric point predicted from FAAH sequence by the GCG Sequence Analysis Software Package (46). Moreover, human brain FAAH cDNA had the same amidase consensus sequence (Fig. 3C) as FAAH cloned from human, mouse, and rat livers (18, 19). It is noteworthy that the activation energy of the AnNH hydrolysis catalyzed by FAAH from all three sources was identical. Furthermore, the FAAH activity in human CHP100 and U937 cells shared several other biochemical properties, such as pH and temperature dependence and inhibition profile, with the enzyme from human brain. In addition, the enzymes contained an identical amidase sequence. This might indicate that the same enzyme was present in all human samples, although the participation of other enzymes cannot be ruled out.

Human brain FAAH was further characterized with respect to its interaction with inhibitors. Here, linoleoyl analogues of AnNH and hydro(per)oxides generated thereof, which are likely to be produced in vivo by brain lipoxigenases (16, 22, 23, 34), were shown to be competitive inhibitors of FAAH activity, with apparent $K_i$ values in the low micromolar range (Table I). Interestingly, linoleoylthanolamide is a physiological constituent of rat neurons (3) and has recently been reported to displace $[^{3}H]$CP55940, a potent cannabinoid, only at high concentrations ($K_i > 1 \mu M$) from cannabinoid receptors in rat brain membranes (22). This compound might be the first natural inhibitor of FAAH as yet discovered. It has recently been shown, however, that oleamide, a sleep-inducing lipid, inhibited FAAH activity, but as high as 100 $\mu M$ oleamide was needed to inhibit it by 50% in mouse neuroblastoma N1E115TG2 cells (24).

It is noteworthy that the apparent $V_{max}$ values of human brain FAAH was 2- or 25-fold higher than that of U937 or CHP100 cells, respectively. The presence of different amounts of FAAH in the cells could explain this observation. Indeed, the amount of FAAH protein was 2- or 10-fold higher in human brain than in U937 or CHP100 cells, respectively (Fig. 3A), and similar differences were observed in the level of FAAH mRNA (Fig. 3B). Therefore, it can be suggested that a different expression (both at the transcriptional and translational level) of the same enzyme might be responsible for the different apparent $V_{max}$ values of FAAH from the different human sources. A differential expression of FAAH might also be involved in human brain pathology, as suggested by comparison of meningioma and the surrounding (healthy) white matter (Fig. 4). This seems of interest if one recalls that a neurotrophic effect of AnNH has been proposed (8) and that AnNH might act as growth factor for hematopoietic cell lines (35, 36). Therefore, a lower expression of the AnNH-hydrolyzing enzyme FAAH might be instrumental in prolonging AnNH-associated growth stimulus, ultimately leading to cell immortalization.

To be inactivated by FAAH, AnNH has to be transported into the cell. Recent experiments performed on rat neuronal cells (3, 11, 12), rat basophilic leukemia (RBL-2H3) cells, and mouse J774 macrophages (13) clearly showed the presence of a high affinity AnNH transporter in the outer cell membranes. A similar methodology was used here to characterize, for the first time, the AnNH uptake in human neuronal (CHP100) and immune (U937) cells. Both cell types rapidly took up AnNH ($t_{1/2} = 5 \text{ min}$) in a temperature-dependent ($Q_{10} = 1.5$) and saturable way (Fig. 5A and data not shown). $[^{14}C]$$\text{AnNH}$ was taken up by CHP100 and U937 cells with similar high affinity, but remarkably different velocity (Table III). Interestingly, U937 cells, which possessed higher FAAH activity than CHP100 cells, showed also a more efficient AnNH uptake. The affinity of the AnNH transporter in human cells was comparable to that in rat astrocytes ($K_m = 0.32 \mu M$) (12) and was almost an order of magnitude higher than the affinity reported for dopamine ($K_m = 1 \mu M$) or glutamate ($K_m = 1-5 \mu M$) carriers in rat brain (37, 38). Furthermore, the uptake of AnNH in human cells was affected by AnNH hydrolysis products, leukotriene B$_4$, prostaglandin E$_2$, and alkylating agents (Table II) in much the same way as reported for rat neuronal and non-neuronal cells (11–13). This suggests that AnNH accumulation is selective and mediated by a transporter other than the long chain fatty acid transporter protein (39) or the prostaglandin transporter (40), in keeping with recent data on the AnNH carrier of rat neurons and astrocytes (12). AnNH uptake in human CHP100 and U937 cells was independent of mitochondrial energy metabolism because the uncoupling agent CCCP (29) hardly affected AnNH accumulation (Table II). These results indicate that AnNH is accumulated by a carrier-mediated facilitated diffusion, as recently reported for rat cells (11). The enhancement of AnNH uptake by the NO donor SNP (Table II) was due to increased apparent $V_{max}$ values (up to 170% of the control value), without changes in the apparent $K_m$.

Conversely, the alkylating agent PMSF reduced the apparent $V_{max}$ to 50% of the control, without changing the apparent $K_m$ (Table III). It is tempting to suggest that the active site of the transporter may contain a cysteine residue, which could be the target of both NO donors and alkylating agents. The effect of co-incubation with PMSF strengthens this hypothesis.

Altogether, the results reported here form the first characterization of human brain FAAH. In addition, the observations highlight the possible role of linoleoyl analogues of AnNH (and hydro(per)oxides generated thereof and from AnNH itself by lipoxigenase activity) as inhibitors of human brain FAAH. The AnNH transporter also has been characterized for the first time in human cells, showing that it was not affected by the AnNH derivatives/analogues that inhibited FAAH, but was sensitive to NO donors.

These findings give rise to a general picture of the inactivation process of AnNH in human neuronal and immune cells (Scheme 1). AnNH is brought into the cell by a transporter protein and is rapidly cleaved by intracellular FAAH. Lipoxigenase-generated products of AnNH can competitively inhibit FAAH, which affords an elevated intracellular AnNH concentration. The resulting dissipation of the AnNH gradient potentiates the transporter protein, which clears AnNH from the extracellular space. The NO-stimulated accumulation of AnNH might be further enhanced by the fact that short pulses of NO are able to inhibit lipoxygenase activity (30), thus preventing inhibition of FAAH by lipoxigenase-generated hydroperoxides of AnNH and congeners. Interestingly, any signaling pathway leading to NO release, either coupled or not coupled to cannabinoid receptors, might affect AnNH metabolism by activating AnNH (re)uptake. In this perspective, CB1 and/or CB2 receptors might reside on the same cell bearing the sequestration machinery or on different cells. The autocoid local inflammation antagonism (41) and the glutamate excitotoxicity on neurons (42), where AnNH exerts an(anti)gonistic effects on cannabinoid receptors and nitric oxide is released (10, 43), might be two relevant processes in which the proposed...
sequestration scheme is operational. It is noteworthy that lipoxigenase activity is found in processes such as lymphocyte activation and neuronal cell death, where lipoxigenase activation (44, 45) might prolong the effects of AnNH (13).

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