Biosynthesis, Uptake, and Degradation of Anandamide and Palmitoylethanolamide in Leukocytes*

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Anandamide (arachidonoylethanolamide, AnNH) and palmitoylethanolamide (PEA) have been proposed as the physiological ligands, respectively, of central and peripheral cannabinoid receptors. Both of these receptors are expressed in immune cells, including macrophages and mast cells/basophils, where immunomodulatory and/or anti-inflammatory actions of AnNH and PEA have been recently reported. We now provide biochemical grounds to these actions by showing that the biosynthesis, uptake, and degradation of AnNH and PEA occur in leukocytes. On stimulation with ionomycin, J774 macrophages and RBL-2H3 basophils produced AnNH and PEA, probably through the hydrolysis of the corresponding N-acylphosphatidylethanolamines, also found among endogenous phospholipids. Immunological challenge of RBL-2H3 cells also caused AnNH and PEA release. The chemical structure and the amounts of AnNH and PEA produced upon ionomycin stimulation were determined by means of double radiolabeling experiments and isotope dilution gas chromatography/electron impact mass spectrometry. Both cell lines rapidly sequestered the two amides from the culture medium through temperature-dependent, saturable and chemically inactivable mechanisms. Once uptaken by basophils, AnNH and PEA compete for the same inactivating enzyme which catalyzes their hydrolysis to ethanolamine. This enzyme was found in both microsomal and 10,000 x g fractions of RBL cell homogenates, and exhibited similar inhibition and temperature/pH dependence profiles but a significantly higher affinity for PEA with respect to neuronal “anandamide amidohydrolase.” The finding of biosynthetic and inactivating mechanisms for AnNH and PEA in macrophages and basophils supports the previously proposed role as local modulators of immune/inflammatory reactions for these two long chain acylethanolamides.

In the past 15 years evidence has been accumulating to support the existence of a neuroimmune axis. The presence of neuropeptide receptors in immune cells allows them to respond to peptidergic stimulation with decreased or augmented proliferation, chemotaxis, phagocytosis, degranulation, lymphokine and cytokine release, superoxide radical formation, and leukotriene biosynthesis (see Refs. 1 and 2, for reviews). The concomitant action on vascular permeability of neuropeptides like substance P, calcitonin gene-related peptide, and neurokinins, concurs to the onset of immune/inflammatory reactions, as in the “axon-reflex” model for neurogenic inflammation. These reactions can be modulated through feed-back actions on autonomic and sensory fibers by mediators and neurotransmitters produced by immune cells (3, 4). Some circulating leukocytes can, in fact, synthesize, store, and release neuropeptides such as vasoactive intestinal peptide, endorphins, and substance P (1, 4), and the recent finding of nerve growth factor in mast cells (5) widens the spectrum of potential responses that backfed peripheral neurons can produce during neuroimmune interactions. Among the receptor classes whose expression and function have been described in both the central nervous system and lymphoid tissues, increasing attention has been paid recently to cannabinoid receptors, particularly since members of a class of endogenous lipids, the acylethanolamides (AEs),1 have been proposed as their possible physiological ligands (6, 7). The brain cannabinoid receptor, known as CB1, was cloned and sequenced in 1990 (8), and evidence for the existence, in spleen marginal cells, of a second cannabinoid receptor, christened CB2, was gained in 1993 (9). While CB1 receptors have been found to be expressed also in peripheral tissues and cells, no strong evidence exists to date for the presence of CB2 receptors in the central nervous system.

Several studies have suggested for anandamide (arachidonoylethanolamide, AnNH) and other polyunsaturated AEs a role as endogenous agonists at the CB1 receptor (see Refs. 10 and 11, for reviews). AnNH mimicks most of the pharmacological and behavioral actions known for cannabinoids, and enzymes for its synthesis from and its degradation to ethanolamine and arachidonic acid (AA) have been partially characterized in mammalian brain (12–15). Furthermore, it has been possible: 1) to induce the release of AnNH and other AEs from intact rat central neurons upon stimulation with depolarizing agents (i.e. ionomycin, high potassium, and kainate), through a mechanism different from the condensation of ethanolamine and AA, i.e. the hydrolysis of a preformed membrane phospholipid, N-arachidonoylphosphatidylethanolamine (16); 2) to demonstrate the uptake of AnNH by intact rat central neurons via a rapid, saturable, selective and temperature-dependent mechanism (16). Several studies have been carried out also on the enzyme “anandamide amidohydrolase,” responsible for the hydrolysis of AnNH amide bond (14, 15, 17, 18),

1 The abbreviations used are: AEs, acylethanolamides; AnNH, anandamide; PEA, palmitoylethanolamide; NaPEs, N-acylphosphatidylethanolamines; AACOCF3, arachidonoyltrifluoromethyl ketone; AA, arachidonic acid; PLA2, phospholipase A2; GC-MS, gas chromatography-mass spectrometry; RP-HPLC, high performance liquid chromatography; DMEM, Dulbecco’s modified Eagle’s medium; DNP, 2,4-dinitrophenol; EI, electron impact.
and have led to the suggestion that this enzyme may catalyze also: 1) the hydrolysis of another putative neuroactive amide, the sleep-inducing oleoyl-amide (17); 2) the synthesis of AnNH in the presence of AA and high concentrations of ethanolamine (18).

Although very potent at functionally activating the central CB1 receptor, AnNH binds poorly to the "peripheral" CB2 receptor, where it rather behaves as an antagonist (9, 19). While the discovery of CB1 receptors also on immune cells, like macrophages and lymphocytes (20), explains the regulatory actions on macrophage morphology and NO synthesis (37) and on lymphocyte proliferation and apoptosis (21, 22) that AnNH, under certain conditions, shares with cannabinoids, there remains to find both a physiological function and an endogenous ligand for the CB2 receptor. In a recent report (23), cannabinoids and a congener of AnNH, palmitoylethanolamide (PEA), were shown to 1) bind selectively to membrane preparations from rat basophilic leukemia (RBL-2H3) cells, where the CB2, but not the CB1, receptor was found to be constitutively expressed; and 2) to inhibit the immunogenic activation of RBL-2H3 and mast cells. Interestingly, AnNH did not exert the latter action but antagonized the effect induced by PEA. The authors suggested that PEA, by acting at CB2 receptors, might function as an "autacoid local inflammation antagonist amide" (23). The immunosuppressant and anti-inflammatory effects of PEA and other saturated or monounsaturated AEs, similar in some ways to those ascribed to cannabinoids, were also briefly discussed in a review (24) where data on the accumulation of these lipids, as well as of their phospholipid precursors, during tissue injury and inflammation are also described. However, no data are available yet on the binding of PEA to membranes of CB2 receptor cDNA-transfected cells, nor on the capability of this ethanolamide to elicit a typical CB2-mediated response, i.e. the inhibition of adenylate cyclase (25). Cytotrophic and anti-inflammatory actions by PEA, respectively, against glutamate-evoked excitotoxicity in rat cerebellar granule neurons and substance P-induced plasma extravasation in rodents, have been, instead, described (26, 27).

Despite the well documented immunomodulatory actions of AnNH and PEA, no report exists on the actual availability to leukocytes of molecular mechanisms for the formation and inactivation of these two AEs. Here we report that AnNH and PEA, either radio-labeled or unlabeled, were synthesized by the diisopropylcarbodiimide method described previously (28), starting from either [14C]ethanolamine (Amersham, United Kingdom, 53–57 mCi/mmoyl) or unlabeled ethanolamine plus the corresponding free fatty acids. The resulting synthetic [14C]AEs had a specific radioactivity of 5.3–5.7 mCi/mmol.

RBL-2H3 cells were obtained by Dr. A. Leon, ResearchLife, Italy. RBL-1 and J774A.1 cells were purchased from DSM (Germany). RBL and J774 cells were cultured, respectively, in minimum Eagle’s medium (MEM, Sigma) or in Dulbecco’s modified Eagle’s medium (DMEM, Sigma), both containing 10% fetal bovine serum (Sigma) plus 1% penicillin: streptomycin (Sigma), in 5% CO2 at 37°C. The biosynthesis of AnNH and PEA in the two cell types under study was investigated by employing a methodology analogous to that used previously to demonstrate AnNH biosynthesis in rat central neurons (16) and mouse neuroblastoma cells (29). Cells were prelabeled overnight with 0.1 μCi/ml culture medium of either [14C]ethanolamine, [3H]AA (NEN DuPont de Nemours, Germany, 230 Ci/mmol), or [3H]palmitic acid (Sigma, 50 Ci/mmol), and then rinsed twice with serum-free DMEM before stimulation. Confluent cultures from at least ten 100-mm Petri dishes (0.5–1.5 × 10^7 cells/dish) were stimulated for 20 min with 3 ml/dish of DMEM containing either Streptomycin (NPS, Nunc, Roskilde, Denmark) or ionomycin (Sigma, 5 μM). Control incubations were conducted with the same number of cells treated with DMEM only. RBL-2H3 cells were also stimulated for 15 min with human serum albumin-dinitrophenol (HSA-DNP, Sigma, 0.1 μg/ml), after 2 h sensitization with anti-DNP antibody (Sigma, 0.3 μg/ml) (23). Cell viability after stimulation was checked by trypan blue exclusion and found to be higher than 95% in all cases.

Arachidonoyltrifluoromethyl ketone (AACOCF3, 10 μM, Cascade, UK), an inhibitor of both anandamide amidohydrolase and phospholipase A2 (PLA2) (30, 31), was also introduced in both control and stimulated cells, 10 min before stimulation in order to minimize AnNH degradation and to rule out the participation of endogenous PLA2 in AnNH release. After the incubation, 3 ml of methanol was added to each dish and cells were transferred to Falcon tubes before adding 6 ml of chloroform. The mixtures were then sonicated and the two phases allowed to separate by centrifugation. In order to purify and characterize the AEs and NaPEs therein contained, the organic phase was then submitted to a series of chromatographic steps described previously (28, 29), including SiO2 open bed chromatography, thin layer chromatography (TLC), and reversed-phase high pressure liquid chromatography (HPLC). NaPEs were characterized further by digestion with phospholipase D from S. chromofuscus, which released the corresponding AEs, followed by TLC and RP-HPLC (28, 29). Spots and bands on TLC plates were visualized by exposure to iodine vapors. Radioactivity profiles of TLC analyses were determined by using a radioactivity scanner (Packard).

The radioactivity profiles of HPLC analyses were determined by liquid scintillation counting of each 1-ml fraction. The yields of AEs and their phospholipid precursors after the extraction and purification procedure have been described previously (28, 29).

For conclusive identification and quantitation of AnNH and PEA, cells from 60 100-mm Petri dishes (about 5 × 10^6 cells, corresponding approximately to 4 g wet tissue weight) were stimulated with 5 μM ionomycin and the lipids extracted and purified as described above. 250 pmol of [H]AnNH and [H]PEA (a generous gift by Dr. C. Felder, National Institute of Mental Health, Bethesda, MD) as well as of hexadecanoyl ethanolamide and cis-eicosadienoylethanolamide, which are not synthesized by the cells under study, were introduced before extraction. TLC fractions with the same Rf as synthetic AnNH and PEA were scraped off preparative TLC plates and acetylated with 100 μl acetic anhydride in 400 μl of anhydrous pyridine. The acetylation product was analyzed by gas chromatography-mass spectrometry (GC-MS) carried out on a Hewlett-Packard instrument consisting of a HP-GC 5890 series II apparatus equipped with a HP-5MS capillary column (30 mm x 0.25 mm, 0.25-μm film thickness, cross-linked 5% HP ME siloxane), and of a HP-MS 5989B quadrupole mass analyzer equipped with an electron impact (EI) source operating at 70 eV and 250°C in order to improve sensitivity, acquired in the selected ion monitoring mode which consists in monitoring the presence in the GC elute of only few selected ion fragments. GC was then carried out using a 3-min isothermal step at 200°C followed by a temperature gradient from 200 to 300°C at a rate of 5°C/min (elium flow 1 ml/min, injector and transfer line temperature 200°C). The quantities of AnNH and PEA produced by cells were determined by constructing standard curves for stable isotope dilution GC-MS measurement of the two AEs, 250 pmol of either [3H]AnNH or [3H]PEA plus varied amounts (0, 50, 100, 250, 500, and 1000 pmol) of either deuterated compound were dissolved in methanol/water, extracted, purified by preparative TLC, derivatized, and analyzed by GC-MS as described above. Ion current ratios, which represent the ion current peak area for the unlabeled derivative divided by that of the deuterated derivative, were calculated for the molecular ions as well as the fragment ions corresponding to the loss of acetic acid (see "Results"). Standard curves were obtained by reporting on the abscissa axis the amount of AE and on the ordinate axis the ion current ratios. The amounts, in GC-MS measurements of cell extracts, of acetoxyl-AnNH and -PEA, which, under the above elution conditions, are eluted after 18.1 and 13.6 min, respectively, are not synthesized by the cells under study, were introduced before extraction. TLC fractions with the same Rf as synthetic AnNH and PEA were scraped off preparative TLC plates and acetylated with 100 μl of acetic anhydride in 400 μl of anhydrous pyridine. The acetylation product was analyzed by gas chromatography-mass spectrometry (GC-MS) carried out on a Hewlett-Packard instrument consisting of a HP-GC 5890 series II apparatus equipped with a HP-5MS capillary column (30 mm x 0.25 mm, 0.25-μm film thickness, cross-linked 5% HP ME siloxane), and of a HP-MS 5989B quadrupole mass analyzer equipped with an electron impact (EI) source operating at 70 eV and 250°C in order to improve sensitivity, acquired in the selected ion monitoring mode which consists in monitoring the presence in the GC elute of only few selected ion fragments. GC was then carried out using a 3-min isothermal step at 200°C followed by a temperature gradient from 200 to 300°C at a rate of 5°C/min (elium flow 1 ml/min, injector and transfer line temperature 200°C). The quantities of AnNH and PEA produced by cells were determined by constructing standard curves for stable isotope dilution GC-MS measurement of the two AEs, 250 pmol of either [3H]AnNH or [3H]PEA plus varied amounts (0, 50, 100, 250, 500, and 1000 pmol) of either deuterated compound were dissolved in methanol/water, extracted, purified by preparative TLC, derivatized, and analyzed by GC-MS as described above. Ion current ratios, which represent the ion current peak area for the unlabeled derivative divided by that of the deuterated derivative, were calculated for the molecular ions as well as the fragment ions corresponding to the loss of acetic acid (see "Results"). Standard curves were obtained by reporting on the abscissa axis the amount of AE and on the ordinate axis the ion current ratios. The amounts, in GC-MS measurements of cell extracts, of acetoxyl-AnNH and -PEA, which, under the above elution conditions, are eluted after 18.1 and 13.6 min, respectively, are calculated by calculation of the current ratios on the standard curves. Using the selected ion monitoring acquisition mode, amounts of acetoxyl-AnNH and -PEA, as little as 5 pmol, can be detected (28), which starting from 5.0 × 10^7 cells, corresponds to about 0.1 pmol/10^6 cells.

Uptake and Degradation of Radiolabeled AnNH and PEA by Cells—The uptake and degradation of [3H]AnNH and [3H]PEA by intact cells
was studied as described previously (16) by incubating for different time intervals confluent cells, in 6-well dishes (0.1–0.3 x 10^5 cells/well), with 1 ml of serum-free DMEM containing 10,000 cpm (1.2 μM) of either AE. The rate of disappearance of [1^4]C]AEs from incubation mixtures and of appearance of [1^4]C]AEs in cell extracts (after having washed three times the cells with 3 ml of DMEM, 1% bovine serum albumin) was taken as a measure of AE uptake, while the rate of formation of [1^4]C]ethanolamine in the incubation liquid plus cell extracts, determined as described previously (16), was taken as a measure of AE degradation. The contribution to carrier-mediated transport of non-carrier-mediated partitioning of AEs into membranes was determined by conducting incubations at 4°C. Incubations (20 min) were also carried out with different concentrations of [1^4]C]AEs for K_m and V_{max} determination (in this case the uptake at 4°C was subtracted from that at 37°C), or with 50,000 cpm of either AE in the presence of 0.1 mM phenylmethylsulfonyl fluoride (100 μM) or 0.1 mM N-ethylmaleimide (100 μM).

Characterization of RBL-2H3 Cell Amidohydrolase—Confluent RBL-2H3 cells were harvested and homogenized in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, using a Dounce homogenizer. The homogenate was centrifuged sequentially at 800 g, 10,000 g (15 min), and 100,000 g (30 min). Pellets from the last two centrifugations (in aliquots of 100–200 μg of total proteins) were incubated, unless otherwise stated, in 0.5 ml of 50 mM Tris-HCl, pH 7.4, at 37°C for 30 min in the presence of 100,000 cpm (24 μM) of either [1^4]C]anandamide, [1^4]C]PEA, or other [1^4]C]AEs (5.3 μCi/mmol, labeled on the ethanolamine moiety). Incubations were terminated by adding 1 ml of chloroform/methanol (1:1, v/v), and lowering the temperature to 4°C. [1^4]C]Ethanolamine produced by the enzymatic hydrolysis of [1^4]C]AEs was determined by open bed chromatography of the aqueous phase on Porapak mini-columns (16), followed by liquid scintillation counting. Experiments were carried out also with different amounts of total membrane proteins (0–400 μg) or with different concentrations of [1^4]C]AEs, for K_m and V_{max} determination, or in different buffers at different pH values, as described previously (17). Thermal stability of the enzymatic activities was determined by keeping identical aliquots of the same enzyme preparation (100–200 μg of total proteins) at various temperatures for 5 min, and then conducting the incubation as usual (17). The effect of ACOCCF_3 (10 μM), PEA (50 μM), or AnNH (50 μM) was also determined by conducting the assay in identical aliquots of the same 10,000×g pellet suspension and incubating as usual with and without the inhibitors. The effect of 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM p-bromophenacyl bromide, 0.1 mM p-hydroxymercurobenzoate, 0.1 mM N-ethylmaleimide, 0.1 mM o-phenanthroline, and 0.1 mM benzamidine, all purchased from Sigma, was likewise studied.

RESULTS

Biosynthesis of Acylethanolamides in J774 Macrophages and RBL-2H3 Cells—When stimulated with either S. chromofuscus phospholipase D (10 units/ml) or 5 μM ionomycin, J774 macrophages and RBL-2H3 basophils, prelabeled with [1^4]C]ethanolamine, produced a radioactive lipid component (Fig. 1 and data not shown) which was absent in extracts from unstimulated cells and co-chromatographed with AE standards in TLC analyses (carried out in conditions (28) that do not discriminate among different AEs). The same component, albeit in smaller amounts, was found also upon stimulation with albumin-DNP of RBL-2H3 cells previously sensitized with an antibody against this antigen (Fig. 1). A period of 20 min was employed for stimulation with ionomycin since pilot experiments conducted in J774 cells (Ref. 29 and data not shown) had shown that a 15–20 min incubation was necessary to observe maximal formation of AnNH. When the AE-like material (which was present in negligible amounts if ACOCCF_3 was not introduced in the incubation medium) was analyzed by RP-HPLC, a series of radiolabeled metabolites co-eluting with synthetic standards of AEs with different fatty acid chains was found in both cell types (Table I). The identity of PEA and AnNH was confirmed

![Radioactivity profiles of TLC analyses of lipids from stimulated RBL-2H3 cells. RBL-2H3 basophils, prelabeled with [1^4]C]ethanolamine, were treated for 20 min with either ionomycin (5 μM in 3 ml of MEM), or 3 ml of MEM with no other substance (control). Alternatively, cells were activated with an anti-DNP antibody for 2 h and then stimulated for 15 min with human serum albumin-DNP (antigen (23)). Following the incubation, lipids were extracted and pre-purified as described, and analyzed by TLC (developing system: chloroform/methanol, 95:5, v/v). The R_P = 0.40–0.44, under the developing conditions used, was the same for all synthetic AEs, and is shown by a line. Data are representative of two separate experiments. Similar profiles were obtained with samples from ionomycin-stimulated and unstimulated J774 macrophages.](image)

### Table I

| Substance                     | J774 macrophages (% of total acylethanolamides released) | RBL-2H3 basophils (% of total acylethanolamides released) |
|------------------------------|--------------------------------------------------------|---------------------------------------------------------|
|                              | Ionomycin (5 μM) | PLD digestion of purified NaPEs | Ionomycin (5 μM) | Antigen challenge | PLD digestion of purified NaPEs |
| Palmitylolethanolamide (C_{16:0}) | 56.2 | 43.7 | 55.2 | 38.5 | 45.2 |
| Stearoylolethanolamide (C_{18:0}) | 14.8 | 47.6 | 7.8 | ND | 25.8 |
| Oleoylolethanolamide (C_{18:1}) | 6.3 | 0.8 | 7.1 | ND | 3.4 |
| Linolenoylolethanolamide (C_{18:3}) | 2.4 | 10.2 | 7.2 | ND | 16.3 |
| Arachidonylolethanolamide (C_{20:4}) | 12.1 | 0.8 | 15.7 | 37.5 | 4.0 |

| Total (cpm) | 810 ± 38 | 1210 ± 543 | 482 ± 42 | 285 ± 18 | 4174 ± 688 |

ND, not detectable. Total radioactivity (subtracted of background) associated with NaPEs in unstimulated cells and with AEs in stimulated cells is also shown, while total acyl-ethanolamine radioactivity in unstimulated cells was 108 ± 25 cpm and 168 ± 50 cpm (n = 2), respectively, in RBL-2H3 and J774 cells.
by labeling both cell types with either \[^{3}H\]palmitic or \[^{3}H\]AA, respectively, and by stimulating the cells with 5 \(\mu\)M ionomycin. Radiolabeled components, absent from extracts of unstimulated cells, and with the same chromatographic behavior as PEA and AnNH authentic standards in TLC and RP-HPLC analyses, were found in both macrophages (318 \pm 34 and 280 \pm 26 cpm, respectively, means \pm S.D. of two experiments, subtracted of background) and basophils (155 \pm 35 and 128 \pm 16 cpm, respectively). Finally, conclusive identification of PEA and AnNH produced by ionomycin stimulation of either RBL-2H3 or J774 cells was achieved by GC-MS analyses of acetylated samples. These analyses were carried out using the selected ion monitoring mode of acquisition, which revealed the presence of GC peaks eluting with the same retention time and yielding the same electron impact MS fragment ions as synthetic acetylated AnNH and PEA (Fig. 2 and data not shown).

The most abundant fragment for both compounds was that at \(m/z = 85\) due to \(\beta\)-cleavage followed by McLafferty rearrangement of the fragments at, respectively, \(m/z = 329\) and 281, which, in turn, are due to loss of acetic acid from the molecular ion peaks at \(m/z = 389\) and 341. Other fragments were found at \(m/z = 346\) and 298 (loss of an acetyl-group), respectively, for AnNH and PEA, \(m/z = 244\) (\(\beta\)-cleavage) for AnNH, \(m/z = 239\) (amide bond cleavage with loss of acetylenolamine) and 256 (aliphatic fragmentation, loss of \(-(CH_{2})_{5}CH_{3}\)) for PEA (for typical electron impact MS spectra of acylethanolamides, see also Ref. 28). GC peaks with the same retention time and electron impact MS fragment ions as synthetic acetylated stearoyl-, oleoyl-, linolenoyl- and \(\gamma\)-linolenoylthanolamides were also found (data not shown). The construction of appropriate stand-
ard curves for the stable isotope dilution GC-EIMS measurement allowed to calculate AnNH and PEA amounts, which were, respectively, 4.5 ± 0.1 and 10.9 ± 3.0 pmol/10^7 cells in ionomycin-stimulated RBL-2H3 cells, and 5.1 ± 1.5 and 12.9 ± 1.3 pmol/10^7 cells in ionomycin-stimulated J774 cells (means ± S.D., n = 2). AnNH and PEA amounts in unstimulated cells were below the detection limit of the GC-MS technique used in this study.

The two cell lines under study, prelabeled with [14C]ethanolamine, [3H]palmitic acid, or [3H]AA, were also shown to contain radioactive lipids which co-chromatographed (in TLC analyses) with synthetic standards of N-palmitoyl or N-arachidonoyl phosphatidylethanolamine (not shown), the two proposed phospholipid precursors, respectively, of PEA and AnNH. The identity of these lipids as NaPEs was confirmed when, upon digestion with S. chromofuscus phospholipase D, they released a family of AEs whose percent composition was determined by RP-HPLC (Table I).

Uptake and Degradation of Palmitoylethanolamide and Anandamide—We studied the rate of clearance of either [14C]AnNH or [14C]PEA from the culture medium by J774 and RBL-2H3 cells. Both cell types rapidly sequestered the two metabolites, AnNH being uptaken more quickly than PEA (t_1/2 = 5.2 min versus 6 min, in J774 macrophages, and 7 min versus 15 min, in RBL cells). However, while in RBL-2H3 cells uptake of both AnNH and PEA was immediately followed by their hydrolysis to [14C]ethanolamine (respectively, 40.3 and 29.7% of total radioactivity, after 20 min incubation, Fig. 3, A and B), J774 macrophages seemed to degrade much more slowly the AEs that had been cleared from the incubation medium (7 and 3%, respectively, for AnNH and PEA, after 30 min incubation, not shown). Uptake of [14C]AnNH or [14C]PEA by RBL-2H3 basophils was saturable (K_m = 33 and 40 μM, V_max = 0.6 and 0.4 nmol min^{-1}, respectively), and was reduced when the incubation temperature was lowered to 4 °C (Fig. 3, C and D), or when incubations were carried out in the presence of either of

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**FIG. 3. Disappearance from incubation medium and uptake and degradation by RBL-2H3 cells of [14C]AnNH (A-C) and [14C]PEA (B-D).** 10,000 cpm of either [14C]AnNH or [14C]PEA were incubated with RBL-2H3 cells in 6-well dishes for 0, 3, 5, 10, 15, and 20 min. After each incubation, the amount of radioactivity found to be associated with the AEs in the incubation medium (open triangles) or cell extracts (open circles) and the total radioactivity bound to ethanolamine (open squares) were determined as described previously (16), and are shown as a function of incubation time in (A) for [14C]AnNH and (B) for [14C]PEA. The effect of 50 μM PEA or AnNH (open squares) and of lowering the temperature to 4 °C (full squares) on the radioactivity found to be associated with AnNH (open rhombs) or PEA (full rhombs) in cell extracts, is shown, respectively, in C and D. Data are representative of two separate experiments. EtNH₂, ethanolamine. Similar results, except for the formation of [14C]ethanolamine, were obtained with J774 cells incubated with [14C]AnNH or [14C]PEA under the same conditions.
the two alkylating agents phenylmethylsulfonyl fluoride and N-ethylmaleimide (100 μM, 48.2 or 53.7% and 41.3 or 40.7% inhibition of the 20-min uptake of [14C]AnNH and [14C]PEA, respectively). Co-incubation with either PEA or AnNH (50 μM) only negligibly affected the uptake, respectively, of [14C]AnNH and [14C]PEA (Fig. 3, C and D). Similar results were obtained with J774 cells (not shown).

Characterization of a Fatty Acid Ethanolamide Hydrolase in RBL Cells—We examined 10,000 x g, microsomal and cytosolic fractions of RBL-2H3 cells, as well as of their cognate cell line RBL-1, for the presence of anandamide amidohydrolase and found high levels of [14C]AnNH hydrolizing activity in the two former fractions (49.0 and 15.4% of total specific activity in RBL-2H3 cells, respectively, and 79.8% and 10.5% of total specific activity in RBL-1 cells). Less than 5% of total specific activity was detected in cytosolic fractions. The 10,000 x g fractions from either RBL cell line were used for the first preliminary characterization of a “non-neuronal anandamide amidohydrolase.” In these fractions the same inhibition and protein amount/pH/temperature dependence profiles for the hydrolysis of either [14C]AnNH or [14C]PEA were found. With both substrates, optimal pH was observed at 10.0 (Fig. 4A) and a linear correlation between rate of hydrolysis and total protein amount was found in the range 0–50 μg, with maximal rate at 384 μg; inactivation of enzymatic hydrolysis was achieved at 72 °C (Fig. 4B) or in the presence of any of the alkylating agents, phenylmethylsulfonyl fluoride, N-ethylmaleimide, p-bromophenacyl bromide, and p-hydroxymercuribenzoate (Table II), while EDTA (5 mM) and the peptidase inhibitors benzamidine and o-phenanthroline (100 μM) were ineffective (Table II). Palmitoyl trifluoromethyl ketone, synthesized as described in Ref. 30, also significantly inhibited the hydrolysis of both AEs (Table II). The conversion of [14C]AnNH and [14C]PEA to [14C]ethanolamine was also inhibited in a competitive fashion by co-incubation with: (a) AACOCF₃ or palmitoyl trifluoromethyl ketone, and (b) unlabeled PEA or AnNH, respectively (Fig. 4, C and D, and data not shown). AACOCF₃ produced the same shift in the apparent Kₘ values of each substrate (respectively, from 67 to 130 μM and from 31 to 62 μM). Unlike neuronal anandamide amidohydrolase (Table III), the “basophilic” enzyme exhibited for PEA an affinity higher than for AnNH (apparent Kₘ values for the two amides in RBL-2H3 cells were 31 ± 3 and 67 ± 10 μM, respectively, means ± S.E., n = 4). Moreover, when other [14C]AEs were tested, the enzyme displayed a catalytic efficiency (expressed as the Vₐₘₙ/Kₘ ratio, which in turn is directly proportional to the kₐₜₐₜ/Kₘ ratio) that was the higher, the shorter, and the less saturated the fatty acid chain (compare the Vₐₘₙ/Kₘ values of
PEA and stearoyl ethanolamide, linoleoyl- and cis-eicosadienoyl ethanolamide, and those of the C16, C20, and C22 ethanolamide series, in Table III). In RBL-2H3, RBL-1, and N18TG2 cells, the V_{max}/K_m ratio for [14C]AnNH was, respectively, 2.1-, 2.4-, and 9.4-fold higher than that for [14C]PEA, in 10,000 × g fractions, and 1.5-, 2.2-, and 35-fold higher than that for [14C]PEA, in microsomal fractions (legend to Table III).

### DISCUSSION

Several recent reports, starting from the well-known immunomodulatory effects of Δ⁹-tetrahydrocannabinol (11), have dealt with the pharmacological actions of AnNH and PEA in immune cells (21–23, 37), while some earlier literature (32, 24) had already described the immunosuppressant effects of AEs. However, no evidence for the existence of biosynthetic and inactivating mechanisms for this family of lipids had been obtained in blood cells before the present study. The choice of RBL-2H3 cells as experimental model for auxiliary leukocytes was urged by the recent report (23) of the effects of PEA and those of the C18, C20, and C22 ethanolamide series, in Table III. In RBL-2H3, RBL-1, and N18TG2 cells, the V_{max}/K_m ratio for [14C]AnNH was, respectively, 2.1-, 2.4-, and 9.4-fold higher than that for [14C]PEA, in 10,000 × g fractions, and 1.5-, 2.2-, and 35-fold higher than that for [14C]PEA, in microsomal fractions (legend to Table III).

#### TABLE II

**Effect of typical esterase inhibitors and of previously reported anandamide amidohydrolase inhibitors on acylethanolamide hydrolysis by RBL-2H3 cell membrane preparations**

Data (means ± S.D., n = 3) are expressed as percent of the rate of [14C]AnNH and [14C]PEA hydrolysis by 10,000 × g pellet preparations with no substance added, assayed as described under “Materials and Methods.” See also legend to Table III. In J774 cells, only very little [14C]AnNH hydrolyzing activity was detected and was not characterized.

| Substance (concentration) | Rate of [14C]AnNH hydrolysis | Rate of [14C]PEA hydrolysis |
|---------------------------|-------------------------------|----------------------------|
| None                      | 100                           | 100                        |
| Phenylmethylsulfonyle     | 12.7 ± 0.6                    | 43.4 ± 6.3                 |
| fluoride (100 μM)         |                               |                            |
| p-Bromophenacylbromide    | 11.2 ± 0.7                    | 41.5 ± 1.7                 |
| (100 μM)                  |                               |                            |
| p-Hydroxymercuribenzoate  | 12.3 ± 0.3                    | 56.5 ± 10.1                |
| (100 μM)                  |                               |                            |
| N-Ethylmaleimide (100 μM)| 34.0 ± 0.3                    | 48.1 ± 6.5                 |
| Arachidonoyltrimethylketone (50 μM) | 7.2 ± 0.2 | 36.7 ± 1.6 |
| Palmitoyltrimethylketone (50 μM) | 11.8 ± 0.6 | 32.6 ± 1.3 |
| o-Phenanthroline (100 μM) | 100.0 ± 3.1                   | 120.0 ± 9.8                |
| Benzanidin (100 μM)       | 104.0 ± 1.5                   | 104.0 ± 4.2                |
| EDTA (5 mM)               | 104.0 ± 2.0                   | 113.8 ± 7.4                |
| Dithiothreitol (1 mM)     | 94.4 ± 0.4                    | 99.4 ± 0.5                 |

**TABLE III**

**Substrate specificity of the fatty acid ethanolamide hydrolyase (amidohydrolase) activity from RBL-2H3, RBL-1, and N18TG2 cells**

Apparent K_m (μM) and V_{max} (nanomoles min⁻¹ mg of total proteins⁻¹) values were calculated from Lineweaver-Burk profiles obtained using different concentrations (1.2, 2.4, 12.0, 24.0, and 48.0 μM) of different [14C]acyl ethanolamides. In RBL-1 cells, experiments were conducted only with AnNH and PEA. Apparent K_m and V_{max} values for AnNH and PEA were calculated also using proteins from the 100,000 × g pellet (microsomal) fraction and were, respectively, in RBL-2H3 cells, K_m = 57 and 12.5 μM, V_{max} = 0.34, and 0.05 nmol min⁻¹ mg total proteins⁻¹, K_{N18TG2} = 0.006 and 0.004; in RBL-1 cells, K_m = 29 and 22 μM, V_{max} = 0.05 and 0.13 nmol min⁻¹ mg total proteins⁻¹, K_{N18TG2} = 0.013 and 0.006; in N18TG2 cells, K_m = 6.9 and 67 μM, V_{max} = 0.95 and 0.27 nmol min⁻¹ mg total proteins⁻¹, K_{N18TG2} = 0.14 and 0.004. Data are means of at least three separate experiments. Standard errors are not shown for the sake of clarity and were never higher than 15%.

| Substrate                        | RBL-2H3 | RBL-1 | N18TG2* |
|----------------------------------|---------|-------|---------|
| Palmitoyl ethanolamide (C₁₈:₀)   | 31      | 20    | 80      |
| Stearoyl ethanolamide (C₁₈:₀)    | 50      | 100   | 20      |
| Linoleoyl ethanolamide (C₁₈:₂)   | 100     | 97.9  | 1.41    |
| Eicosanoyl ethanolamide (C₂₀:₁) | 110     | 42.4  | 1.41    |
| cis-Eicosadienoyl ethanolamide (C₂₀:₂) | 100 | 39.2  | 4.09    |
| Arachidonoyl ethanolamide (C₂₀:₄) | 67      | 1.29  | 2.06    |
| Erucoyl ethanolamide (C₂₂:₁)     | 200     | 0.14  | 0.09    |
| Docosahexaenoyl ethanolamide (C₂₂:₅) | 200 | 0.84  | 0.09    |

* Data for N18TG2 amidohydrolase are from Ref. 17. In J774 cells, only very little [14C]AnNH hydrolyzing activity was detected and was not characterized.
dishes containing 1–2 ml of culture medium, concentrations which are not below those required, for example, to activate cannabinoid receptors (6, 23). However, due to the nature of the cells under study and the agents used to stimulate them, further studies will be required in order to assess whether AnNH and PEA are produced by basophils/macrophages in vivo in sufficient amounts to exert, for example, an immunomodulatory effect on other leukocytes or a neuromodulatory action on peripheral neurons.

Apart from the capability of biosynthesizing AnNH and PEA, both J774 macrophages and RBL-2H3 cells were found in this study to possess also the biochemical means for the inactivation of the two bioactive AEs by efficiently sequestering radio-labeled AnNH and PEA from the cell culture medium. This uptake mechanism may be partly mediated by carrier protein(s), inasmuch as it was rapid, saturable, and greatly reduced at 4 °C or by treatment of cells with alkylating agents. Since high concentrations of unlabeled PEA and AnNH did not prevent the uptake, respectively, of [14C]AnNH and [14C]PEA, the presence of distinct clearance mechanisms for the two AEs may be suggested. Intact RBL-2H3 cells, but not J774 macrophages, were also shown here to degrade both AnNH and PEA through hydrolysis of their amide bond with subsequent production of ethanolamine (and, presumably, of the corresponding fatty acids), as previously shown for rat central neurons (16). The enzymatic activity responsible for AnNH and PEA hydrolysis in two different lines of RBL cells was characterized in this study, and found to have properties similar but not identical to those of rat and porcine brain anandamide amidohydrolase (14–18). In particular, we compared substrate specificity, sensitivity to inhibitors, and pH/temperature dependence profiles of [14C]AnNH hydrolyzing activities from membranes of either RBL-2H3 or N18TG2 neuroblastoma cells. The latter provide the only example of anandamide amidohydrolase from a single neuronal cell line so far characterized (17). The enzyme optimal pH and temperature of inactivation in RBL cells were slightly higher than those observed in neuroblastoma cells, but the major difference between basophilic and “neuronal” amidohydrolase activities lay in their substrate specificities, which may reflect the different responsiveness of neurons and basophils to different AEs. While the neuroblastoma enzyme displayed, respectively, a very low and a relatively high affinity for PEA and AnNH, the enzymatic activity from RBL cells exhibited the lowest apparent \( K_m \) value for PEA (Table III). This difference may confer to basophils, which are more responsive to PEA (IC\(_{50} = 270 \mu M\) for RBL-2H3 cell down-regulation [23]) than to AnNH (EC\(_{50} = 18.5 \mu M\)) for the induction of AA release\(^2\) the capability to terminate the action also of the former AE. Conversely, the amidohydrolase from neuroblastoma cells, which respond to nanomolar concentrations of AnNH (with inhibition of the N-type Ca\(^{2+}\) channels (35) through the CB1 receptor), and to micromolar concentrations of PEA (with blockade of the fast Na\(^+\) channel (36)), hydrolyzes AnNH at concentrations lower than those required for PEA. Accordingly, the catalytic efficiency of the amidohydrolase (measured by calculating the \( V_{max}/K_m \) ratios) was either much higher with AnNH than with PEA or comparable with either substrate, respectively, for the neuronal or the basophilic enzyme (Table III). In RBL-2H3 cells, the \( V_{max}/K_m \) ratios increased with increasing unsaturation and decreasing length of the substrate fatty acid chain, thus explaining why the catalytic efficiency of the basophilic enzyme was highest with AnNH, PEA, and linolenoyl ethanolamide as substrates.

The presence, in RBL cells, of different isozymes with different affinities for AnNH and PEA appears unlikely. In fact, when the inhibition and pH/temperature dependence profiles for the hydrolysis of either [\(^{14}\)C]AnNH or [\(^{14}\)C]PEA were compared, identical optimal pH and inactivation temperature, and the same response to alkylating agents, peptidase inhibitors and EDTA were found. Moreover, both activities were inhibited competitively by AACOCF\(_3\), which produced the same shift in the apparent \( K_m \) values of each substrate. More importantly, the hydrolysis of either [\(^{14}\)C]AnNH or [\(^{14}\)C]PEA was competitively inhibited by either PEA or AnNH, respectively. This last finding provides evidence for the fact that the same enzyme catalyzes the hydrolysis of both AEs, and suggests that a reciprocal control of AnNH levels by PEA and PEA levels by AnNH can be potentially effected through competition at the catalytic site of the same amidohydrolase.

In conclusion, the present study, by describing at once the biosynthesis, uptake, and degradation of AnNH and PEA in J774 macrophages and RBL-2H3 basophils, has provided biochemical grounds to the previous hypothesis (21–24, 26, 32, 37) that these AEs may function as endogenous immunomodulators. Studies aimed at clarifying further the molecular mode of action of AnNH and PEA on blood cells and investigating the regulation of the metabolic processes leading to leukocyte AE formation and degradation are now recommended in order to proceed toward a complete evaluation of this hypothesis. Moreover, our findings may open the way to future investigations on the involvement of AnNH and PEA in the chemical signaling between basophils/macrophages and other cannabinoid receptor-containing and AE-synthesizing cells under physiopathological conditions. It will be tempting to investigate the possible association of dysfunctions of the AE biosynthetic and inactivating systems described herein with immunological disorders such as hypersensitivity, immunodeficiency, and autoimmune diseases.

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**Bioactive Acylethanolamides in Leukocytes**

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