FEBS Letters 468 (2000) 176–180 FEBS 23367

Human mast cells take up and hydrolyze anandamide under the control of 5-lipoxygenase and do not express cannabinoid receptors

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Received 4 January 2000; received in revised form 27 January 2000

Edited by Guido Tettamanti

Abstract Human mast cells (HMC-1) take up anandamide (arachidonoyl-ethanolamide, AEA) with a saturable process ($K_m = 200 \pm 20$ nM, $V_{max} = 25 \pm 3$ pmol min^{-1} mg protein^{-1}), enhanced two-fold over control by nitric oxide-donors. Internalized AEA was hydrolyzed by a fatty acid amide hydrolase (FAAH), whose activity became measurable only in the presence of 5-lipoxygenase, but not cyclooxygenase, inhibitors. FAAH ($K_m = 5.0 \pm 0.5$ μM, $V_{max} = 160 \pm 15$ pmol min^{-1} mg protein^{-1}) was competitively inhibited by palmitoylethanolamide. HMC-1 cells did not display a functional cannabinoid receptor on their surface and neither AEA nor palmitoylethanolamide affected tryptase release from these cells.

Key words: Anandamide; Endocannabinoid; Inflammation; Lipoxygenase; Nitric oxide; Tryptase

1. Introduction

Mast cells are multifunctional bone marrow-derived cells found in mucosal and connective tissues and in the nervous system, where they play essential roles in fibrosis, angiogenesis, inflammation and neuroimmune interactions [1,2]. As yet, little is known about endogenous modulators and mechanisms of mast cell activation. Anandamide (arachidonoylethanolamide, AEA) and palmitoylethanolamide (PEA) belong to an emerging class of endogenous lipids including amides and esters of long chain polyunsaturated fatty acids, termed collectively 'endocannabinoids' [3]. These compounds mimic the pharmacological effects of Δ^9-tetrahydrocannabinol, the active principle of hashish and marijuana, by binding to cannabinoid receptors (CBR) [4]. Two types of CBR have been characterized, which differ for their ligand selectivity and affinity, and for their distribution: type 1 receptors (CB1R) are present in the central nervous system and in peripheral tissues, whereas type 2 receptors (CB2R) are present only in peripheral cells [4]. Recently, rat mast cells were reported to express a functional CB2R, which could bind AEA and PEA [5]. Only binding of PEA to CB2R was found to down-regulate mast cell activation and, thus, inflammation [5]. However, the ability of PEA to activate CB2R is under debate (reviewed by Lambert and Di Marzo [6]) and AEA has been shown to reduce inflammation in whole rats via interaction with CB1R [7]. On the other hand, the peripheral endocannabinoid system is activated during human shock conditions, such as hemorrhagic and septic shock [8] and mediates also the response to endotoxins [9]. Therefore, attention is being focused on the ability of peripheral cells to degrade endocannabinoids, by a two-step process including (i) cellular uptake by a specific AEA transporter and (ii) intracellular degradation by the enzyme fatty acid amide hydrolase (FAAH). This degradation machinery has been shown in peripheral rat cells [10], human lymphoma cells [11] and human platelets [12]. The identity of physiological regulators of the endocannabinoid degradation remains elusive [11,12]. Also the contribution of mast cells in regulating the peripheral actions of endocannabinoids is still unknown. This prompted us to investigate the ability of the HMC-1 human mast cell line to take up and hydrolyze AEA and how degradation of this lipid might be controlled. Also the presence of a functional cannabinoid receptor in HMC-1 cells was assessed. Moreover, the possible role of AEA and PEA in tryptase release from HMC-1 cells was investigated. Tryptases are trypsin-like proteinases stored in the cytoplasmic granules of mast cells from several organisms [13,14]. Their release upon degranulation is a marker of mast cell activation [1,2].z

2. Materials and methods

2.1. Materials

Chemicals were of the purest analytical grade. Anandamide (arachidonoylethanolamide, AEA), arachidonic acid, A23187, 5,8,11,14-eicosatetraynoic acid (ETYA), indomethacin, ibuprofen, hydroxocobalamin, sodium nitroprusside (SNP) and *N*-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide (TGPL) were purchased from Sigma (St. Louis, MO, USA). Arachidonoyl-trifluoromethyl-ketone (ATFMK), *S*-nitroso-*N*-acetylpenicillamine (SNAP) and *N*-(4-hydroxyphenyl)-arachidonoylamide (AM404) were from Research Biochemicals International (Natick, MA, USA). Leukotriene B_4 and prostaglandin E_2 were from Cayman Chemical Company (Ann Arbor, MO, USA). [^{3}H]-CP55.940 (5-(1,1′-dimethyheptyl)-2-[1*R*,5*R*-hydroxy-2*R*-(3-hydroxy-

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Abbreviations: AEA, anandamide (arachidonoylethanolamide); AM404, *N*-(4-hydroxy-phenyl)-arachidonoylamide; ATFMK, arachidonoyl-trifluoromethyl-ketone; CB1/2R, type 1/2 cannabinoid receptor; CP55.940, 5-(1,1′-dimethyheptyl)-2-[1*R*,5*R*-hydroxy-2*R*-(3-hydroxy-propyl)cyclohexyl]phenol; ETYA, 5,8,11,14-eicosatetraynoic acid; FAAH, fatty acid amide hydrolase; GAR-AP, goat anti-rabbit alkaline phosphatase conjugate; HMC, human mast cells; MES, 2-[*N*-morpholino]ethanesulfonic acid; MK886, 3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*tert*-butylthioindol-2-yl]-2,2-dimethyl propanoic acid; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PEA, palmitoylethanolamide; RP-HPLC, reversed phase high performance liquid chromatography; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; TGPL, *N*-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide

PII: S0014-5793(00)01223-0

propyl)cyclohexyl]phenol, 126 Ci/mmol) and [^{3}H]AEA (223 Ci/mmol) were purchased from NEN Dupont de Nemours (Köln, Germany). 5-Lipoxygenase specific inhibitor 3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*tert*-butylthioindol-2-yl]-2,2-dimethyl propanoic acid (MK886) was a kind gift from Dr. A.W. Ford-Hutchinson (Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Canada). Palmitoylethanolamide (PEA) was synthesized and characterized (purity > 96% by gas–liquid chromatography) as reported [15]. Rabbit anti-human CB1R polyclonal antibodies were from Calbiochem (La Jolla, CA, USA).

2.2. Cell culture

The human mast cell line HMC-1 was kindly provided by Dr. J.H. Butterfield (Division of Allergic Diseases, Mayo Clinic, Rochester, MN, USA). HMC-1 cells were cultured as described [16]. Cells at the fourth–fifth passage were harvested, washed in phosphate-buffered saline and used for biochemical assays, as detailed below. Cell viability was assessed in a Neubauer chamber by trypan blue dye exclusion.

2.3. Determination of anandamide uptake

The uptake of [^{3}H]AEA by intact HMC-1 cells (2×10^6) was studied essentially as described [11]. To discriminate non-carrier-mediated from carrier-mediated transport of AEA through cell membranes, control experiments were carried out at 4°C [11]. The Q_{10} value was calculated as the ratio of AEA uptake at 30 and 20°C [17]. Apparent K_m and V_{max} of the uptake were determined by Lineweaver–Burk analysis [11]. The effect of different compounds on AEA uptake (15 min) was determined by adding each substance directly to the incubation medium, at the indicated concentrations. The cell viability after each treatment was higher than 90% in all cases.

2.4. Assay of fatty acid amide hydrolase (FAAH)

Fatty acid amide hydrolase (EC 3.5.1.4; FAAH) activity was assayed at pH 9.0 in HMC-1 cell homogenates, prepared as reported [18], by reversed phase high performance liquid chromatography [18]. Kinetic studies were performed as described [11].

2.5. Analysis of cannabinoid receptors

Membrane fractions were prepared from HMC-1 cells (2×10^8) as reported [15]. These membranes and those prepared from the brain of Wistar rats (male, weighing 250–280 g) were used in rapid filtration assays with the synthetic cannabinoid [^{3}H]CP 55.940 (400 pM) [15]. Unspecific binding was determined in the presence of 10 μM AEA [15].

SDS–polyacrylamide gel electrophoresis (12%) was performed under reducing conditions [11] on cell homogenates (25 μg/lane), prepared as described [18] and was followed by electroblotting onto 0.45 μm nitrocellulose filters (Bio-Rad, Hercules, CA, USA) [11]. Prestained molecular weight markers (Bio-Rad) were bovine serum albumin (84 kDa) and ovalbumin (47 kDa). Human brain specimens were homogenized as reported [11]. Immunodetection of CB1R was performed with specific anti-human CB1R polyclonal antibodies, diluted 1:400, and goat anti-rabbit immunoglobulins conjugated with alkaline phosphatase (GAR-AP, Bio-Rad) at 1:2000 dilution [11].

2.6. Tryptase release

Tryptase secretion from HMC-1 cells (6×10^6) was stimulated for 8 h with 500 ng/ml A23187 at 37°C [2], in the presence or absence of AEA or PEA (10 μM). Cell suspensions were centrifuged at $1000\times g$ for 10 min and tryptase was assayed in the cell-free supernatants [2]. Residual tryptase in the packed cells was released by sonication in 10 mM MES buffer (pH 6.1), containing 1 M NaCl. Tryptase activity was measured by adding each sample (100 μl) to 1 ml of 0.1 mM TGPL substrate in 50 mM Tris–HCl, pH 7.4, and by monitoring product formation at 405 nm [2]. The release of tryptase was calculated by the equation: (released tryptase/(released tryptase+cell-associated tryptase))$\times$100.

2.7. Statistical analysis

Data reported in this paper are the mean (±S.D.) of at least three independent determinations, each 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, CA, USA).

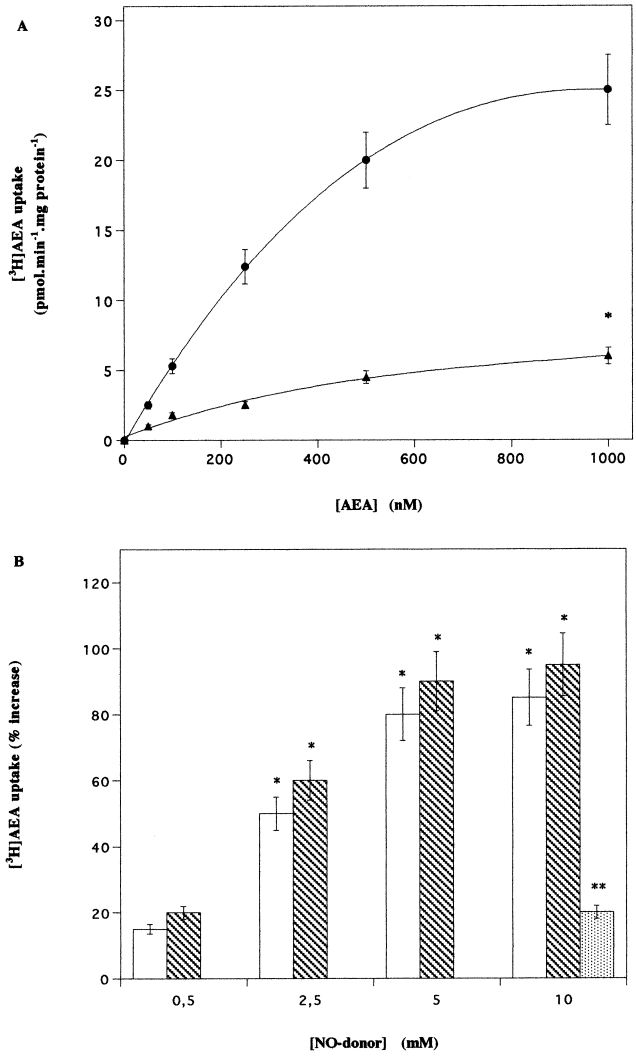

Fig. 1. A: Dependence of [^{3}H]AEA uptake by intact HMC-1 cells (15 min) on AEA concentration, at 37 (circles) or 4°C (triangles). The asterisk indicates the effect of 10 μM AM404 on the uptake of 1 μM AEA at 37°C. B: Effect of nitric oxide-donors SNP (empty bars) or SNAP (hatched bars) on the uptake of 200 nM [^{3}H]AEA at 37°C. The effect of 0.5 mM hydroxocobalamin on the [^{3}H]AEA uptake in the presence of 10 mM SNP is shown by the dotted bar. Uptake was expressed as percentage increase over the untreated control (100% = 10 ± 1 pmol min^{-1} mg protein^{-1}). *Denotes $P<0.01$ compared with control; **denotes $P<0.01$ compared with 10 mM SNP.

3. Results

Intact HMC-1 cells were able to accumulate [^{3}H]AEA, in a temperature- ($Q_{10}=1.7$), time- ($t_{1/2}=4$ min) and concentration-dependent manner (Fig. 1A and data not shown). [^{3}H]AEA uptake at 37°C was saturable (apparent $K_m = 200\pm20$ nM, $V_{max} = 25\pm3$ pmol min^{-1} mg protein^{-1}) and was almost completely inhibited by the specific AEA transport inhibitor AM404 [19]. The uptake was enhanced dose-dependently when incubations were carried out in the presence of NO donors SNP or SNAP (Fig. 1B). These donors were used at millimolar concentrations which release physiological nanomolar concentrations of NO in solution [20]. Enhancement of [^{3}H]AEA uptake by SNP was prevented by hydroxocobalamin (Fig. 1B), a typical NO scavenger [21].

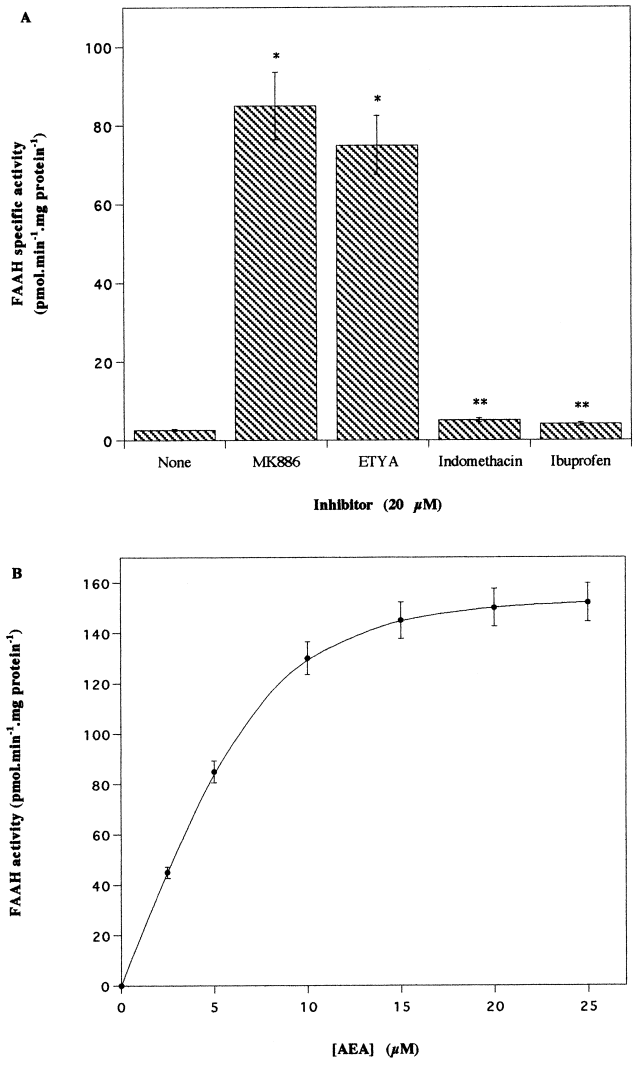

Fig. 2. A: Effect of 5-lipoxygenase inhibitors MK886 and ETYA, or cyclooxygenase inhibitors indomethacin and ibuprofen, on FAAH activity in HMC-1 cells. B: Dependence of HMC-1 FAAH activity, assayed in the presence of 20 μM ETYA, on substrate concentration. *Denotes $P < 0.01$ compared with control; **denotes $P > 0.05$ compared with control.

Moreover, uptake of 200 nM [^{3}H]AEA in the presence of arachidonic acid, PEA, leukotriene B_4 or prostaglandin E_2 (each used at 2 μM) was approximately 90% of the control in all cases, suggesting that AEA accumulation was selective.

HMC-1 cells did not show basal FAAH activity at pH 9.0, which became measurable upon addition of 20 μM MK886 or ETYA, both 5-lipoxygenase inhibitors [22], to the FAAH assay buffer (Fig. 2A). Under the same experimental conditions, 20 μM indomethacin or ibuprofen, both cyclooxygenase inhibitors [23], were ineffective (Fig. 2A). In the presence of 20 μM ETYA, FAAH hydrolyzed AEA in a Michaelis–Menten way (Fig. 2B), showing an apparent K_m of 5.0 ± 0.5 μM and a V_{max} of 160 ± 15 pmol min^{-1} mg protein^{-1}. HMC-1 FAAH was completely inhibited by 10 μM ATFMK, a specific FAAH inhibitor [24] and was inactive at pH 5.0. Interestingly, PEA inhibited FAAH-catalyzed hydrolysis of AEA with an IC_{50} of 20 ± 2 μM, acting as a competitive inhibitor with an inhibition constant of 10 ± 1 μM.

Unlike rat brain membranes, used as a positive control, HMC-1 cells were unable to bind [^{3}H]CP55.940 (Fig. 3), a synthetic cannabinoid which binds with high affinity both CB1R and CB2R [4]. Western blot analysis showed that HMC-1 cells, unlike human brain used as a positive control, did not react with specific anti-human CB1R antibodies (Fig. 3, inset).

Tryptase release from HMC-1 cells stimulated with calcium ionophore A23187 reached approximately 15%, compared to a spontaneous level $< 2\%$ (Fig. 4). AEA or PEA did not induce tryptase release from HMC-1 cells, neither affected the release triggered by A23187, when used at 10 μM (Fig. 4) or up to 50 μM (not shown).

4. Discussion

HMC-1 cells have a specific AEA transporter and a FAAH, characterized here for the first time. The affinity of the transporter for AEA in HMC-1 was very close to that of human lymphoma (130 nM) [11] and identical to that of human platelets [12]. Therefore, the same carrier might be present on the surface of peripheral human cells. As reported for human lymphoma [11] and platelets [12], AEA uptake by HMC-1 cells was increased by NO-donors SNP or SNAP (Fig. 1B). This suggests an inverse relationship between NO and AEA, which is noteworthy if one recalls that NO is a pro-inflammatory agent [25], whereas AEA exerts an opposite action in rats [7].

A major finding of this investigation is that HMC-1 cells have negligible basal levels of FAAH activity, which becomes remarkable in the presence of specific 5-lipoxygenase inhibitors (Fig. 2A). This is noteworthy, because hydroperoxides generated from AEA by lipoxygenase have been recently shown to be potent (K_i as low as 3 μM) inhibitors of FAAH [11]. Since HMC-1 cells express an active 5-lipoxygenase [26], it can be suggested that AEA used as a substrate to assay FAAH is converted instead by 5-lipoxygenase into hydroperoxides, which inhibit FAAH activity. It must be mentioned that MK886 can exert also lipoxygenase-unrelated ef-

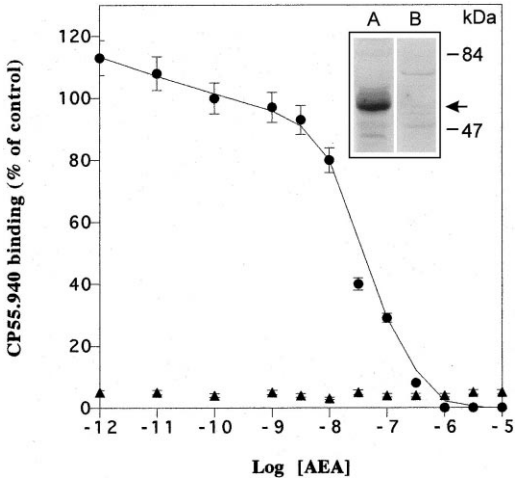

Fig. 3. Displacement of the synthetic cannabinoid [^{3}H]CP55.940 by AEA in rat brain (circles) or HMC-1 (triangles) cell membrane preparations. Inset: Western blot analysis of human brain (A) or HMC-1 cells (B) extracts (25 μg/lane), reacted with specific anti-human CB1R polyclonal antibodies. Molecular weight markers are shown on the right-hand side and arrow indicates the expected molecular mass of CB1R.

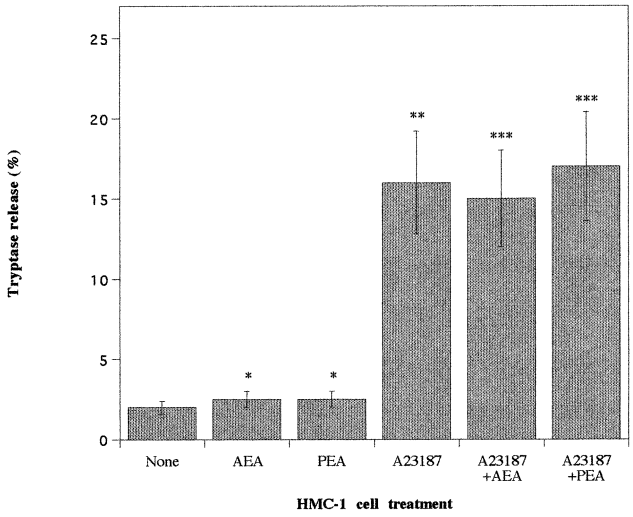

Fig. 4. Tryptase release by HMC-1 cells, stimulated for 8 h at 37°C with 500 ng/ml A23187, alone or in combination with 10 μM AEA or PEA. *Denotes $P > 0.05$ compared with control; **denotes $P < 0.01$ compared with control; ***denotes $P > 0.05$ compared with A23187-treated cells.

fects on mammalian cells [27]. However, the observation that ETYA and MK886 yielded the same effects on HMC-1 FAAH seems to rule out lipoxygenase-independent pathways. In this context it is worth reminding that NO inhibits lipoxygenase activity [28]. Therefore, it can be proposed that NO reduces hydroperoxide formation from AEA, thus preventing inhibition of FAAH. This would result in a faster dissipation of the AEA gradient, leading to enhanced uptake of this lipid. The interplay between NO, 5-lipoxygenase and FAAH might contribute to activation of AEA transport, together with a direct effect of NO on the carrier, e.g. through nitrosylation of cysteine residues [11]. AEA can be metabolized also by cyclooxygenase activity [29], which is abundant in HMC-1 cells [26]. However, these AEA metabolites do not seem to inhibit FAAH, because cyclooxygenase inhibitors were unable to 'disclose' its activity (Fig. 2A). In the presence of ETYA, HMC-1 FAAH showed catalytic properties comparable to those of human lymphoma [11] and human platelets [12]. Remarkably, PEA acted as a competitive inhibitor of FAAH, which favors the hypothesis that it is an 'entourage compound' for endocannabinoids, i.e. an enhancer of their activity through inhibition of their inactivation [6]. Finally, the lack of FAAH activity at pH 5.0 rules out the possibility that HMC-1 FAAH might be the new AEA-hydrolyzing enzyme recently found in lysosomes and mitochondria [30].

Unlike rat mast cells [5], human mast cells do not express functional cannabinoid receptors on their surface (Fig. 3). The availability of anti-human CB1R antibodies allowed to corroborate the binding data for this receptor subtype (Fig. 3, inset). The lack of cannabinoid receptors in HMC-1 cells seems interesting, because CB2R in rat mast cells [5] and peripheral CB1R in whole rats [7] have been proposed to down-regulate inflammation. However, endocannabinoids can act also independently of cannabinoid receptors [3], therefore the possibility that AEA or PEA might modulate HMC-1 activation was investigated. Tryptase release was used as a marker [2]. Unlike rat mast cells [5], human mast cells were not down-modulated by AEA or PEA, which did not interfere with spontaneous or A23187-triggered degranulation (Fig. 4). Taken together, these findings suggest major differences between rats and humans in mast cell activation and do not favor the hypothesis of an autacoid local inflammation antagonism (ALIA) mechanism [5] in humans. On the other hand, the ability of HMC-1 to take up AEA, and to hydrolyze it under 5-lipoxygenase control, suggests that mast cells can contribute to regulate the peripheral endocannabinoid system, hence the actions of these lipid mediators in inflammation, vascular tone and neuroimmune interactions.

Acknowledgements: The authors wish to thank Dr. J.H. Butterfield (Division of Allergic Diseases, Mayo Clinic, Rochester, MN, USA) for kindly donating HMC-1 cells, Professor R. Giuffrè (Neurosurgery Division, University of Rome Tor Vergata, Sant'Eugenio Hospital, Rome, Italy) for human brain specimens and Dr. A.W. Ford-Hutchinson (Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Canada) for MK886. This investigation was supported by Ministero dell'Università e della Ricerca Scientifica e Tecnologica and Consiglio Nazionale delle Ricerche (MURST-CNR Biotechnology program L. 95/95), Rome, by Consiglio Nazionale delle Ricerche (Target Project on Biotechnology), Rome, and by Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST-PRIN 1997), Rome.

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