Effects of homologues and analogues of palmitoylethanolamide upon the inactivation of the endocannabinoid anandamide

*,[1]Kent-Olov Jonsson, [2]Séverine Vandevoorde, [2]Didier M. Lambert, [1]Gunnar Tiger & [1]Christopher J. Fowler

[1]Department of Pharmacology and Clinical Neuroscience, Umeå University, SE-901 87 Umeå, Sweden and [2]Unité de Chimie pharmaceutique et de Radiopharmacie, Université catholique de Louvain, Avenue Mounier, 73, UCL-CMFA 73.40, B-1200 Brussels, Belgium

1 The ability of a series of homologues and analogues of palmitoylethanolamide to inhibit the uptake and fatty acid amidohydrolase (FAAH)-catalysed hydrolysis of [^{3}H]-anandamide ([^{3}H]-AEA) has been investigated.

2 Palmitoylethanolamide and homologues with chain lengths from 12–18 carbon atoms inhibited rat brain [^{3}H]-AEA metabolism with pI_{50} values of ~5. Homologues with chain lengths ⩽ eight carbon atoms gave <20% inhibition at 100 μM.

3 R-palmitoyl-(2-methyl)ethanolamide, palmitoylisopropylamide and oleoylethanolamide inhibited [^{3}H]-AEA metabolism with pI_{50} values of 5.39 (competitive inhibition), 4.89 (mixed type inhibition) and 5.33 (mixed type inhibition), respectively.

4 With the exception of oleoylethanolamide, the compounds did not produce dramatic inhibition of [^{3}H]-WIN 55,212-2 binding to human CB_2 receptors expressed on CHO cells. Palmitoylethanolamide, palmitoylisopropylamide and R-palmitoyl-(2-methyl)ethanolamide had modest effects upon [^{3}H]-CP 55,940 binding to human CB_1 receptors expressed on CHO cells.

5 Most of the compounds had little effect upon the uptake of [^{3}H]-AEA into C6 and/or RBL-2H3 cells. However, palmitoylcyclohexamide (100 μM) and palmitoylisopropylamide (30 and 100 μM) produced more inhibition of [^{3}H]-AEA uptake than expected to result from inhibition of [^{3}H]-AEA metabolism alone.

6 In intact C6 cells, palmitoylisopropylamide and oleoylethanolamide inhibited formation of [^{3}H]-ethanolamine from [^{3}H]-AEA to a similar extent as AM404, whereas palmitoylethanolamide, palmitoylcyclohexamide and R-palmitoyl-(2-methyl)ethanolamide were less effective.

7 These data provide useful information upon the ability of palmitoylethanolamide analogues to act as 'entourage' compounds. Palmitoylisopropylamide may prove useful as a template for design of compounds that reduce the cellular accumulation and metabolism of AEA without affecting either CB_1 or CB_2 receptors.
British Journal of Pharmacology (2001) **133,** 1263–1275

Keywords: Anandamide; fatty acid amidohydrolase; palmitoylethanolamide

Abbreviations: AEA, anandamide; FAAH, fatty acid amidohydrolase; PTMK, palmitoyl trifluoromethyl ketone

Introduction

It is now well established that the endogenous fatty acid amide anandamide (AEA) has, as a result of its actions upon cannabinoid and vanilloid receptors, a number of interesting pharmacological properties including effects on nociception (Calignano *et al.*, 1998; Jaggar *et al.*, 1998; Richardson *et al.*, 1998; Raffa *et al.*, 1999; Gauldie *et al.*, 2001), memory processes (Terranova *et al.*, 1995; Mallet & Beninger, 1998), lung function (Calignano *et al.*, 2000), spasticity (Baker *et al.*, 2001) and cell proliferation (De Petrocellis *et al.*, 1998; Sarker *et al.*, 2000; MacCarrone *et al.*, 2000) (for a recent review of the therapeutic potential of AEA and related endocannabinoids, see Piomelli *et al.*, 2000).

AEA is metabolised by a fatty acid amide hydrolase (Deutsch & Chin, 1993), and inhibition of this enzyme by

phenylmethylsulphonyl fluoride and palmitylsulphonyl fluoride, have been shown to potentiate the pharmacological actions of AEA both *in vitro* and *in vivo* (Childers *et al.*, 1994; Pertwee *et al.*, 1995; Compton & Martin, 1997; Gifford *et al.*, 1999). This raises the possibility that compounds interfering with the metabolism of AEA may be therapeutically useful. A number of FAAH inhibitors have been described, often based on the structure of AEA itself (see e.g. Koutek *et al.*, 1994; Bisogno *et al.*, 1998; Boger *et al.*, 2000b), although an inherent disadvantage in this approach is that some of the compounds are *per se* active at cannabinoid receptors. An alternative approach is to prevent the metabolism of AEA with compounds related to palmitoylethanolamide, since this fatty acid amide, although a substrate for FAAH (Natarajan *et al.*, 1984) is inactive at cannabinoid receptors (Lambert *et al.*, 1999). Indeed, the sulphonyl fluoride analogue of palmitoylethanolamide is a potent inhibitor of AEA metabolism by rat brain FAAH

*Author for correspondence;
E-mail: Kentolov.Jonsson@pharm.umu.se

(IC_{50} 7 nM) with rather weak effects on the binding of the CB agonist [³H]-CP 55,940 to rat forebrain CB_1 receptors (Deutsch *et al*., 1997).

An alternative to transition state inhibitors such as sulphonyl fluoride analogues of fatty acid amides is to use homologues of palmitoylethanolamide to compete with [³H]-AEA for the FAAH active site. In a recent study, a number of homologues and analogues of palmitoylethanolamide were synthesized and demonstrated not to be active towards either CB_1 or CB_2 receptors at concentrations of 1 μM, (Lambert *et al*., 1999; see also Lang *et al*., 1999 for data with stearoylethanolamide and oleoylethanolamide). In the present study, we have investigated the abilities of these compounds to prevent the FAAH-catalysed metabolism of AEA and their interference with cellular AEA uptake, in the hope of identifying possible therapeutically useful 'entourage' compounds. In addition, we have investigated a novel *N*-methyl analogue of palmitoylethanolamide, *R*-palmitoyl-(2-methyl)-ethanolamide. The structures of palmitoylethanolamide, *R*-palmitoyl-(2-methyl)ethanolamide and the two compounds found to show the best activity, palmitoylisopropylamide (C16:0) and oleoylethanolamide (C18:1), are shown in Figure 1.

Methods

Compounds

Anandamide [ethanolamine-1-³H] ([³H]-AEA; specific activity 60 Ci mmol⁻¹) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.). [³H]-WIN 55,212-2 (specific activity 45.5 Ci mmol⁻¹) and [³H]-CP55,940 (specific activity 101.1 Ci mmol⁻¹) were obtained from New England Nuclear, Boston, MA, U.S.A. Decanoylethanolamide (C10:0), lauroylethanolamide (C12:0), myristoylethanolamide (C14:0), palmitoylethanolamide (C16:0), palmitoylbutylamide (C16:0), palmitoylisopropylamide (C16:0), palmitoylcyclohexamide (C16:0) and oleoylethanolamide (C18:1) were synthesized as earlier described by Lambert *et al*. (1999). Non-radioactive anandamide (arachidonoylethanolamide, AEA), (*R*)-(+)-arachidonyl-1′-hydroxy-2′-propylamide (R1-methanandamide), heptadecanoylethanolamide (C17:0), stearoylethanolamide (C18:0), palmitoyltrifluoromethyl ketone (PTMK) and arachidonyl-serotonin were purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). *R*-palmitoyl-(2-methyl)ethanolamide (C16:0) was custom synthesized by the same company on our request and given the catalogue number 90357. *N*-(4-hydroxyphenyl)arachidonylamide (AM404) was purchased from Tocris Cookson (Bristol, U.K.). Fatty acid free bovine serum albumin (BSA) was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All cell culture media, sera and supplements were from Gibco/Life Technologies.

Synthesis of propanoylethanolamide (C3:0)

In a two-neck flask, 14.06 g of ethanolamine (0.23 mol) were poured into 20 ml of dry methylene chloride. The solution was cooled in an ice bath and magnetically stirred. Propionyl chloride (5.325 g, 57 mmol) was added dropwise. The reaction mixture was stirred for 12 h at room temperature, filtered, and the filtrate was evaporated under reduced pressure. The residue of filtrate was chromatographed on silica gel (eluting with ethyl acetate: methyl alcohol 8 : 2) to give 5.031 g (74%) of a colourless oil. TLC (ethyl acetate: methyl alcohol 8 : 2, v v⁻¹) $R_f = 0.5$; spectroscopic data: ¹H NMR (d^6-DMSO): δ(p.p.m.) 1.11 (t, $J = 24$ Hz, 3H); 2.13-2.6 (m, 6H); 3.72–3.76 (m, NH); 2.64 (OH); ¹³C NMR (d^6-DMSO): δ(p.p.m.) 10.64 (CH_3); 29.37, 42.37, 60.90 (CH_2); 174.28 (C=O) mass spectrometry; [M⁺] = 117; IR v(cm⁻¹): 1648 (C=O); 3304 (OH).

Synthesis of butanoylethanolamide (C4:0)

In a two-neck flask, 11.76 g of ethanolamine (0.192 mol) were poured into 20 ml of dry methylene chloride. The solution was cooled in an ice bath and magnetically stirred. Butyryl chloride (5.11 g, 48 mmol) was added dropwise. The reaction mixture was stirred for 12 h at room temperature,
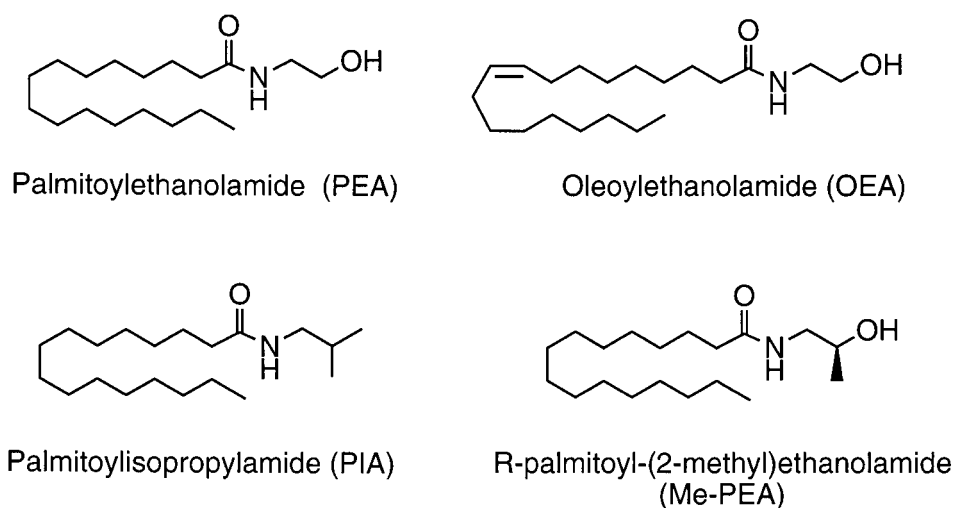
Figure 1 Structures of palmitoylethanolamide (C16:0), oleoylethanolamide (C18:1), palmitoylisopropylamide (C16:0) and *R*-palmitoyl-(2-methyl)ethanolamide (C16:0).

filtered, and the filtrate was evaporated under reduced pressure. The residue of filtrate was chromatographed on silica gel (eluting with ethyl acetate: methyl alcohol 8 : 2, v v^{-1}) to give 4.57 g (73%) of a yellowish oil. TLC (ethyl acetate: methyl alcohol 8 : 2, v v^{-1}) R$_f$ = 0.68; spectroscopic data: ^{1}H NMR (d^6-DMSO): δ(p.p.m.) 0.85 (t, J = 12 Hz, 3H); 1.48 – 1.55 (m, 2H); 2.06 (t, J = 15 Hz, 2H); 3.11 – 3.16 (m, 2H); 3.39 – 3.44 (m, 2H); 4.67 – 4.7 (m, NH); 3.50 (OH); ^{13}C NMR (d^6 - DMSO): δ(p.p.m.) 14.02 (CH$_3$); 19.13, 37.83, 41.90, 60.54 (CH$_2$); 172.79 (C=O); mass spectrometry [M$^+$] = 131; IR v(cm^{-1}): 1648 (C=O); 3302 (OH).

Synthesis of hexanoylethanolamide (C6:0)

In a two-neck flask, 1.294 g of 1,1′-carbonyldiimidazole (7.98 mmol) were poured into 20 ml of dry methylene chloride. The solution was cooled in an ice bath and magnetically stirred. Hexanoic acid (0.92 g, 7.98 mmol) was added dropwise. Ethanolamine (1.95 g, 31.9 mmol) was then added to the reaction mixture, which was stirred for 24 h at room temperature. After this incubation, the reaction mixture was washed with water, 10% citric acid solution and NaCl saturated solution and dried (MgSO$_4$) and the solvent was evaporated under reduced pressure. The residue was chromatographed on silica gel (eluting with ethyl acetate: methyl alcohol 8 : 2, v v^{-1}) to give 1.02 g (80%) of a white solid. Mp: 44 – 45°C (uncorrected); TLC (ethyl acetate: methyl alcohol 8 : 2, v v^{-1}): R$_f$ = 0.68; ^{1}H NMR (d^6-DMSO): δ(p.p.m.) 0.8 (t, J = 12 Hz, 3H); 1.18 – 1.23 (m, 4H); 1.40 – 1.44 (m, 2H); 1.99 (t, J = 15 Hz, 2H); 3.03 – 3.07 (m, 2H); 3.37 – 3.40 (m, 2H); 4.58 – 4.60 (m, NH); 3.63 (OH); ^{13}C NMR (d^6-DMSO): δ(p.p.m.) 13.80 (CH$_3$); 21.86, 24.94, 30.91, 35.34; 41.4; 60 (CH$_2$); 172.31 (C=O); mass spectrometry, [M$^+$] = 159; IR v(cm^{-1}): 1639 (C=O); 3297 (OH).

Synthesis of octanoylethanolamide (C8:0)

In a two-neck flask, 7.35 g of ethanolamine (0.12 mol) were poured into 15 ml of dry methylene chloride. The solution was cooled in an ice bath and magnetically stirred. Octanoyl chloride (1.95 g, 12 mmol) was added dropwise. The reaction mixture was stirred for 12 h at room temperature and then filtered. The solid was recrystallized in ethanol-water to give 2.06 g (92%). TLC (acetone: methylene chloride 9 : 1, v v^{-1}) R$_f$ = 0.67; spectroscopic data: ^{1}H NMR (d-CDCl$_3$): δ(p.p.m.) 0.86(t, J = 7 Hz, 3H); 1.11 – 1.32 (m, 8H); 1.59 – 1.61 (m, 2H); 2.17 – 2.23 (t, J = 15 Hz, 2H); 3.36 – 3.42 (m, 2H); 3.67 – 3.7 (m, 2H); 4.02 (NH); ^{13}C NMR (d-CDCl$_3$): δ(p.p.m.) 14.06 (CH$_3$); 22.62, 25.81, 29.05, 29.29, 31.72, 36.70, 42.42, 61.98 (CH$_2$); 174.74 (C=O); mass spectrometry [M$^+$] = 187; IR v(cm^{-1}): 1639 (C=O); 3307 (OH). m.p. = 57 – 58°C.

Membrane preparation for FAAH assay

Frozen brains (minus cerebellum) stored at −70°C from adult rats were thawed and homogenized at 4°C in 20 mM HEPES buffer, pH 7.0, with 1 mM MgCl$_2$ using a glass homogenizer. The homogenates were centrifuged twice at 36,000 × g for 20 min at 4°C after which the tissue pellets were re-suspended in homogenization buffer and incubated at 37°C for 15 min. After centrifugation at 36,000 × g for 20 min at 4°C, membranes were re-suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 3 mM MgCl$_2$. The protein content in the membrane preparations were determined according to the method of Harrington (1990), using bovine serum albumin as standard, and the preparations were stored at −70°C until used for assay.

FAAH assay

The method used was first described by Omeir *et al.* (1995) who used [^{14}C]-labelled AEA as substrate. In the present study we used [^{3}H]-AEA (Fowler *et al.*, 1997). Briefly, membranes (10 μg protein assay^{-1} unless otherwise stated), test compounds or ethanol carrier (10 μl) and assay buffer (10 mM Tris-HCl, 1 mM EDTA, 1% (w v^{-1}) BSA, pH 7.6) (final assay volume of 200 μl) were preincubated for 0 – 180 min, as indicated, at 37°C. [^{3}H]-AEA (25 μl) (2 μM final concentration unless otherwise stated) was added and the samples were incubated at 37°C for 10 min, unless otherwise stated. Reactions were stopped by placing the tubes in ice and adding 400 μl of chloroform : methanol (1 : 1 v v^{-1}). After vortex mixing of the tubes the phases were separated by centrifugation in a bench centrifuge. Aliquots (200 μl) of the methanol/buffer phase were removed and analysed for radioactivity by liquid scintillation spectroscopy with quench correction. Blanks contained distilled water instead of the homogenate preparations.

Radioligand binding experiments

Human CB$_1$- ('CHO-CB$_1$') and CB$_2$- ('CHO-CB$_2$') transfected CHO cells, kindly donated by Drs Nokin and Detheux (Euroscreen, Belgium) were maintained in culture using Ham's F12 medium containing 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U ml^{-1} penicillin and 200 μg/ml G418. Membranes (40 μg) and test compounds were incubated at 30°C with 1 nM [^{3}H]-CP55,940 (CHO-CB$_1$) or 1 nM [^{3}H]-WIN 55,212-2 (CHO-CB$_2$) for 1 h in 50 mM Tris-HCl with MgCl$_2$ and EDTA (pH 7.4) in the presence of 50 μM PMSF. Non-specific binding was determined with 10 μM HU-210 (CHO-CB$_1$) or 10 μM WIN 55,212-2 (CHO-CB$_2$). After incubation, the membrane suspension was rapidly filtered through 0.5% polyethyleneimine-pretreated GF/B glass fibre filters (Whatman), and the radioactivity trapped on the filters was measured by liquid scintillation spectroscopy. Assays were undertaken in quadruplicate.

Cell cultures

Rat basophilic leukaemia (RBL-2H3) cells (passage range 14 – 22) and rat C6 glioma cells (passage range 52 – 65) were obtained from the American Type Culture Collection, MD, U.S.A. The cells were grown in 75 cm^2 culturing flasks at 37°C, 5% CO$_2$ in air at normal atmospheric pressure. The cells were cultured in Eagle's minimum essential medium, 2 mM L-glutamine supplemented with 15% foetal bovine serum and 100 units ml^{-1} penicillin + 100 μg ml^{-1} streptomycin (RBL-2H3) or Ham's F10 medium, supplemented with 10% foetal bovine serum and 100 units ml^{-1} penicillin + 100 μg ml^{-1} streptomycin (C6). The culture media were changed three times a week.

[^{3}H]-AEA uptake in RBL-2H3 and C6 cells

The method developed by Rakhshan *et al.* (2000) was used. RBL-2H3 or C6 cells were plated on 24 well plates at an initial density of $1.5-2 \times 10^5$ cells.well^{-1} and incubated at 37°C for 18 h under an atmosphere of 5% CO_2 in air at normal atmospheric pressure. After the incubation period, cells were washed once with 0.5 ml warm assay buffer (mM: NaCl 120, KCl 4.7, $CaCl_2$ 2.2, HEPES 10, KH_2PO_4 1.2, $MgSO_4$ 1.2, pH 7.4; gassed with 95% O_2, 5% CO_2) and preincubated in 350 μl buffer with the test compounds or ethanol carrier at 37°C for 10 min. [^{3}H]-AEA (50 μl, final concentration 10 μM) was added to the wells and uptake at 37°C was allowed for 15 min. After the incubation, the plates were placed on ice after which the cells were rinsed three times with ice-cold buffer containing 1% BSA to terminate the transport. The cells were aspirated of the buffer and incubated at 75°C in 0.2 M NaOH (500 μl well^{-1}) for 15 min. Aliquots (300 μl) of the solubilized cells were transferred to scintillation vials and assayed for tritium content by liquid scintillation spectroscopy with quench correction. Test substances and AEA were diluted in ethanol and buffer and the ethanol concentration was constant between the different wells and never exceeded 5%. Previous experiments using RBL-2H3 cells have demonstrated that this concentration of ethanol does not significantly affect the uptake of [^{3}H]-AEA (Jacobsson & Fowler, 2001).

Metabolism of [^{3}H]-AEA in intact C6 glioma cells

The method used was a modification of the [^{3}H]-AEA uptake assay but where [^{3}H]-ethanolamine was collected. C6 cells were seeded in 24-well flat bottom plates (initial density of 0.12×10^6 cells well^{-1}) in Ham's F10 medium, supplemented with 1% foetal bovine serum and 100 units ml^{-1} penicillin + 100 μg ml^{-1} streptomycin. After incubation overnight, the wells were washed once with 400 μl warm assay buffer (mM: NaCl 120, KCl 4.7, $CaCl_2$ 2.2, HEPES 10, KH_2PO_4 1.2, $MgSO_4$ 1.2, pH 7.4; gassed with 95% O_2, 5% CO_2) and pre-incubated for 10 min at 37°C in 350 μl buffer plus 25 μl of either carrier (ethanol), test compound or phenylmethylsulphonyl fluoride (PMSF, 1.5 mM final concentration). [^{3}H]-AEA (25 μl, final concentration 3 μM) was added to the wells. The plates were then incubated for 30 min at 37°C with the lids on, after which they were placed on ice and aliquots (400 μl) of methanol added to each well. The wells were scraped and the contents placed in test tubes. Chloroform (200 μl) was added, the test tubes were vortex mixed and centrifuged to separate the phases. Aliquots (200 μl) of the aqueous phase (containing the [^{3}H]-ethanolamine) were taken and counted for radioactivity by scintillation spectroscopy with quench correction. Blanks were defined as the radioactivity recovered for samples on a separate plate preincubated with PMSF.

Determination of pI_{50}, $K_{i(slope)}$ and $K_{i(intercept)}$ values

pI_{50} values $[-\log_{10}(IC_{50}$ value$)]$ were analysed using the built-in equation 'sigmoid dose-response (variable slope)' of the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, U.S.A.). Two different approaches were used. In order to determine the maximal inhibition produced by the compounds, the data (expressed as per cent of control) were analysed using 'top' (i.e. uninhibited) values fixed at 100. For compounds where the 'bottom' values (i.e. minimum activity remaining) were significantly greater than 0 (i.e. when the 95% confidence intervals of the value were both greater than 0), the pI_{50} values (i.e. reflecting the potency with respect to the inhibitable fraction of the [^{3}H]-AEA metabolism) were taken from these analyses. For compounds where the 95% confidence intervals of the minimum activity remaining straddled zero, the pI_{50} values were taken from analyses where the bottom value was set to 0. This approach was taken to avoid artefactually low pI_{50} values resulting from the use of unrealistic 'bottom values'. An example of this would be decanoylethanolamide (C10:0), where the calculated 'bottom' value was -332 ± 1288 (95% confidence limit -3011 to $+2347$). Apparent K_m and V_{max} values for inhibition of [^{3}H]-AEA metabolism by palmitoy-lisopropylamide (C16:0) and oleoylethanolamine (C18:1) were calculated from the mean data using the Direct Linear Plot analysis (Eisenthal & Cornish-Bowden, 1974) using the Enzyme Kinetics v 1.4 software package, Trinity Software, Campton, NH, U.S.A. These values were then used in secondary replots to determine $K_{i(slope)}$ and $K_{i(intercept)}$ values.

Statistics

ANOVA tests for repeated measures and Bonnferroni-Dunn *post hoc* tests were conducted using the Statview™ computer programme (SAS Institute Inc., Cary, NC, U.S.A.).

Results

Validation of blank value for assay of FAAH

In the FAAH assay used, blanks are defined as the tritium recovered in the aqueous phase following incubation of [^{3}H]-anandamide ([^{3}H]-AEA) in the absence of homogenate (see Methods section). In view of the finding that some of the palmitoylethanolamide analogues and homologues do not produce complete inhibition of [^{3}H]-anandamide metabolism (see below), it was considered to be of importance to confirm that the blanks determined in this way are valid. To this end, three compounds known to inhibit FAAH, namely arachidonoyl-serotonin, AM404 and arvanil (Bisogno *et al.*, 1998; Melck *et al.*, 1999; Jarrahian *et al.*, 2000) and one presumed inhibitor (palmitoyl trifluoromethyl ketone, PTMK; 'presumed' since the arachidonoyl- and oleoyl- trifluoromethyl ketones are highly potent transition-state FAAH inhibitors, Koutek *et al.*, 1994; Patterson *et al.*, 1996). All four compound produced a complete inhibition of [^{3}H]-AEA hydrolysis with calculated minimum activity remaining ('bottom') values that were not different from zero (Figure 2), thereby confirming the validity of the blank. In the case of PTMK, the pI_{50} value was 7.10 ± 0.02.

Inhibition of FAAH catalysed [^{3}H]-AEA hydrolysis by homologues and analogues of palmitoylethanolamide

A series of homologues of palmitoylethanolamide, where the side-chain was reduced in length from C16:0 (palmitoylethanolamide) to C3:0 (propanoylethanolamide) and increased to
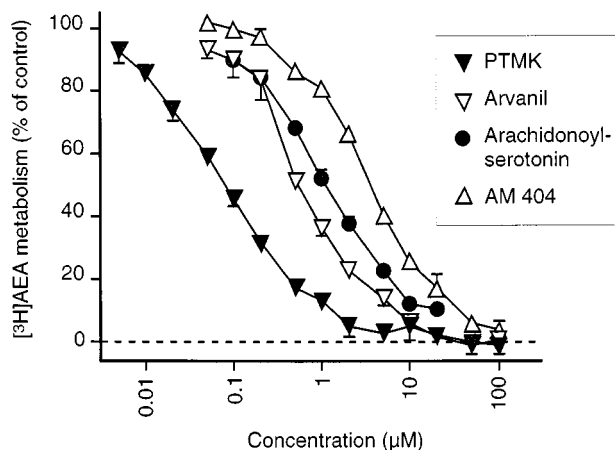
Figure 2 Inhibition of the hydrolysis by rat brain membranes of $2 \mu M$ [^{3}H]-AEA by arachidonoyl-serotonin, AM404, arvanil and PTMK. Data are means$\pm$s.e. mean (when not enclosed by the symbol), $n = 3-6$, with no preincubation with the compounds and membranes prior to addition of [^{3}H]-AEA. Analysis of the data using a constant 'top' value of 100 and variable slope (see Methods) gave 'bottom' values (i.e. the plateau value of the remaining activity not inhibited by the compounds) of: arachidonoyl-serotonin, $3.7 \pm 2.2\%$ (-0.8 to $+8\%$); AM404, $1.3 \pm 2.1\%$ (-3.1 to $+5.7\%$); arvanil, $0.9 \pm 1.9\%$ (-2.9 to $+4.7\%$); PTMK, $0.3 \pm 1.4\%$ (-2.5 to $+3.0\%$) (means$\pm$s.e.mean, with 95% confidence intervals in parentheses). With the 'bottom' values set to zero, the pI_{50} values (means $\pm$s.e.mean) calculated from these data were 5.93 ± 0.02, 5.44 ± 0.02, 6.20 ± 0.03 and 7.10 ± 0.02 for arachidonoyl-serotonin, AM404, arvanil and PTMK, respectively.
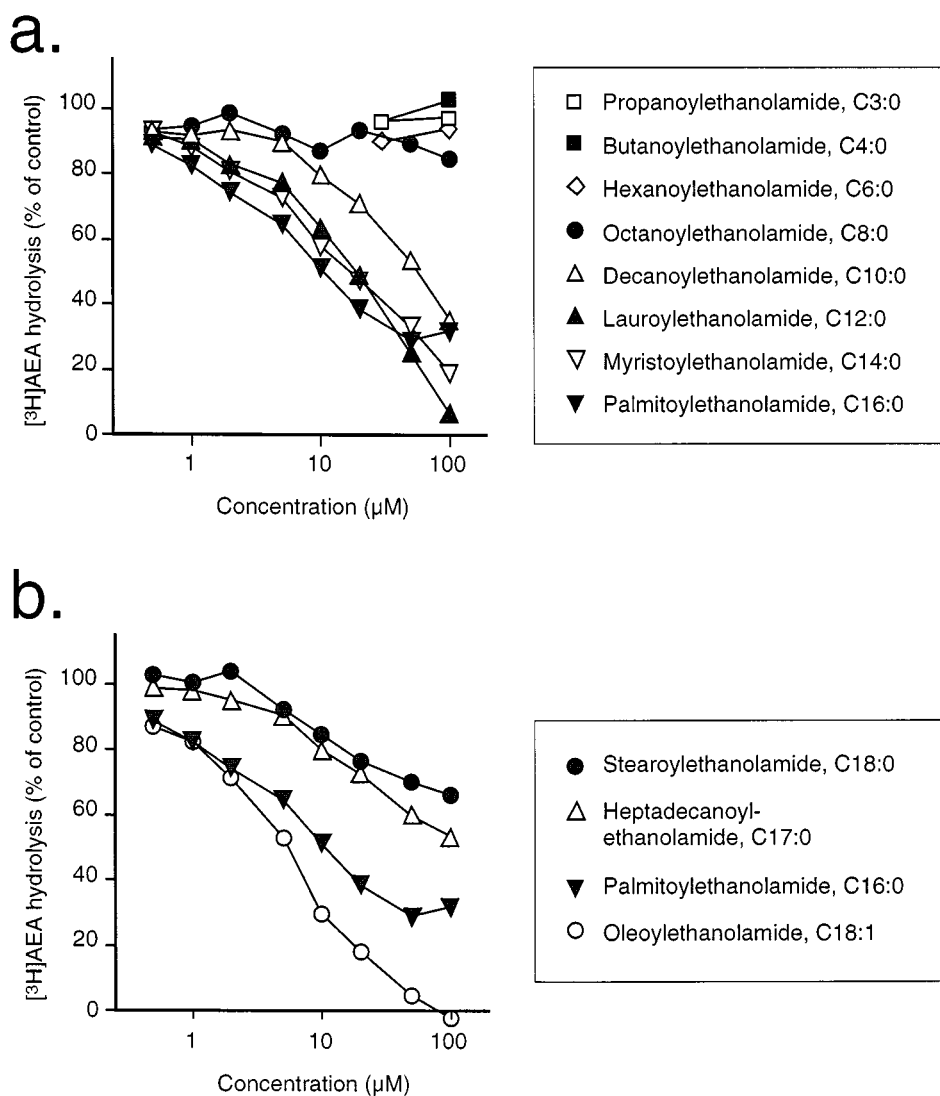
C18:0 (stearoylethanolamide) were tested for their ability to prevent [^{3}H]-AEA hydrolysis (Figure 3a,b). The observed inhibition will reflect both the potency and the solubility of the compound, the latter particularly in the case of the long-chain compounds. Indeed, our experience is that the solubility of these saturated fatty acid amides can vary from preparation to preparation, due to temperature-dependent polymorphism of the pure crystals (J. Wouters, S. Vandevoorde & D. Lambert, unpublished results). In order to calculate potencies, the maximum inhibition was calculated and the pI_{50} values determined for this inhibitable component. Using this approach, it was seen that complete inhibition of [^{3}H]-AEA could be attained for the compounds with side-chain lengths of 10, 12 and 14 carbon atoms, whereas only partial inhibition (78, 55 and 33% for 16, 17 and 18 carbon atoms, respectively) was seen at longer chain lengths (Table 1). This partial inhibition presumably reflects limited solubility of the compounds rather than the presence of an FAAH enzyme subtype with which the compounds do not interact. In the absence of a preincubation phase, the potencies of the compounds towards the inhibitable component of the activity were similar for the compounds with side chains ranging from 18 to 12 carbon atoms (pI_{50} values $4.77-4.95$) with the possible exception of palmitoylethanolamide itself (pI_{50} value of 5.30). This value for palmitoylethanolamide corresponds to an IC_{50} value of $5.1 \mu M$, which is in good agreement with the K_M value of this compound as a substrate for rat brain FAAH using the same assay conditions (range $1.3-8.5 \mu M$, Tiger *et al.*, 2000). For the 10 carbon atom, a lower potency was seen (pI_{50} value of 4.26), and for the four compounds with side chain lengths of 8, 6, 4 and 3 carbon atoms, <20% inhibition of [^{3}H]-AEA

metabolism was seen at the highest concentration tested ($100 \mu M$). Oleoylethanolamide, the mono-unsaturated analogue of stearoylethanolamide, had sufficient solubility and was able completely to inhibit [^{3}H]-AEA metabolism with a pI_{50} value of 5.33.

Four analogues of palmitoylethanolamide where the ethanolamide group was replaced with butylamide, isopropylamide, cyclohexamide and (2-methyl)ethanolamide groups were also investigated for their abilities to prevent [^{3}H]-AEA metabolism (Figure 4). The isopropylamide, butylamide and (2-methyl)ethanolamide analogues were active with pI_{50} values similar to that of palmitoylethanolamide, although complete inhibition of [^{3}H]-AEA metabolism was not seen with any of them, presumably for reasons of solubility (Table 1). The cyclohexamide analogue produced 11% inhibition at the highest concentration tested ($100 \mu M$).

The above experiments were undertaken in the absence of preincubation. Two series of experiments were undertaken to determine whether a preincubation of homogenate with test compound affected the observed inhibition of [^{3}H]-AEA uptake. In the first series, dose-response experiments (analogous to those shown in Figures 3 and 4) for all the compounds except stearoyl- and heptadecanolethanolamide were undertaken using a 60 min preincubation phase. With two exceptions, this treatment did not affect the observed maximum inhibition or the potency of the compounds (data not shown). The two exceptions were for palmitoylethanolamide and palmitoylisopropylamide, where the 95% confidence intervals of the minimum activity remaining straddled zero. In the case of palmitoylethanolamide, the apparent residual activity was $15 \pm 8\%$ [95% confidence intervals -2 to 33] (as opposed to $22 \pm 7\%$ (95% confidence intervals $+7$ to $+37$) in the absence of preincubation). The pI_{50} value for palmitoylethanolamide was calculated to be 5.12 ± 0.13 assuming a residual inhibition and 4.90 ± 0.06 assuming no residual inhibition. This difference underlines the importance of regarding the pI_{50} values as approximate rather than exact estimates of potency. For palmitoylisopropylamide, the apparent pI_{50} value (assuming maximal inhibition) was 4.71 ± 0.05 following a 60 min preincubation period.

In a second experiment, the $10:0-16:0$ ethanolamide compounds, oleoylethanolamide, palmitoylisopropylamide, palmitoylbutylamide and *R*-palmitoyl-(2-methyl)ethanolamide were pre-incubated for 0, 60, 120 and 180 min at 37°C with a stronger homogenate concentration ($50 \mu g$ protein assay^{-1}) before being incubated with [^{3}H]-AEA for a shorter time (3 min). Single concentrations of the compounds, chosen to be on the steep part of the concentration-response curve were tested (data not shown). Only lauroylethanolamide ($20 \mu M$) exhibited a small, but significant reduction in its ability to prevent AEA hydrolysis as the preincubation time was increased. Thus, the per cent activity remaining following 0, 60, 120 and 180 min of incubation with laurylethanolamide were 51 ± 3, 51 ± 5, 57 ± 1 and $62 \pm 4\%$, respectively (means$\pm$s.e.mean, $n = 3$, one-way ANOVA for repeated measures (preincubation time) gave $F_{3,6} = 8.4$ ($P < 0.05$)). For comparison, $15 \mu M$ palmitoylethanolamide gave values of 50 ± 3, 53 ± 1, 58 ± 3 and $53 \pm 5\%$, respectively (means$\pm$s.e.mean, $n = 3$, $P > 0.05$). The same pattern was seen with laurylethanolamide when a 10 min incubation time and a more dilute homogenate was used (data not shown).

a.

Legend for (a):
- □ Propanoylethanolamide, C3:0
- ■ Butanoylethanolamide, C4:0
- ◇ Hexanoylethanolamide, C6:0
- ● Octanoylethanolamide, C8:0
- △ Decanoylethanolamide, C10:0
- ▲ Lauroylethanolamide, C12:0
- ▽ Myristoylethanolamide, C14:0
- ▼ Palmitoylethanolamide, C16:0

b.

Legend for (b):
- ● Stearoylethanolamide, C18:0
- △ Heptadecanoyl-ethanolamide, C17:0
- ▼ Palmitoylethanolamide, C16:0
- ○ Oleoylethanolamide, C18:1

Figure 3 Inhibition of the hydrolysis by rat brain membranes of 2 μM [^{3}H]-AEA by a series of palmitoylethanolamide homologues (**a**) C16:0 – C3:0 (**b**) C16:0 – C18:0 and C18:1. Shown are means of 3 – 6 experiments, with no preincubation with the compounds and membranes prior to addition of [^{3}H]-AEA. The data for palmitoylethanolamide are the same in both graphs.

Mode of inhibition of [^{3}H]-AEA metabolism by palmitoylisopropylamide, oleoylethanolamide and R-palmitoyl-(2-methyl)ethanolamide

The modes of inhibition of [^{3}H]-AEA metabolism by palmitoylisopropylamide and oleoylethanolamide are shown in Figure 5. Both compounds acted as mixed-type inhibitors with $K_{i(slope)}$ and $K_{i(intercept)}$ values, respectively of 15 μM and 87 μM (palmitoylisopropylamide) and 3.9 μM and 11 μM for oleoylethanolamide. *R*-palmitoyl-(2-methyl)ethanolamide was also investigated and found to be a competitive inhibitor with a K_i value of 6.6 μM (data not shown).

Interaction of the analogues and homologues of palmitoylethanolamide with cannabinoid CB receptors expressed on CHO cells

As reported in the introduction, most of the compounds selected for this study have been shown not to interact with

CB receptors (Lambert *et al.*, 1999). However, the highest concentration used in that study was 1 μM, raising the possibility that interactions can occur at higher concentrations of the compounds. In consequence, the effects of the compounds at 10 and 100 μM upon the specific binding of the synthetic cannabinoid receptor agonist radioligand [^{3}H]-WIN 55,212-2 to recombinant human CB_2 receptors expressed on CHO cells was investigated. With the exception of oleoylethanolamide (65% inhibition at 100 μM), all of the compounds produced <15% inhibition of binding at 10 μM and <25% inhibition at 100 μM (Table 2). For the binding of the agonist [^{3}H]-CP55,940 to recombinant human CB_1 receptors expressed on CHO cells, the data was less clear, although <26% inhibition of binding was found at both concentrations for palmitoylethanolamide, *R*-palmitoyl-(2-methyl)ethanolamide and palmitoylisopropylamide and the C4.0 and C10.0-homologues (Table 2). As with the CB_2 receptors, oleoylethanolamide produced a large (63%) inhibition of binding at 100 μM (Table 2).

Table 1 Effect of compounds related to palmitoylethanolamide upon the hydrolysis of [³H]-AEA by rat brain FAAH

Compound	Max. inhibition	pI_{50} value
Propanoylethanolamide (C3:0)	$3\pm1\%$ inhibition at 100 μM[a]	
Butanoylethanolamide (C4:0)	No inhibition at 100 μM[a]	
Hexanoylethanolamide (C6:0)	$6\pm4\%$ inhibition at 100 μM[a]	
Octanoylethanolamide (C8:0)	$15\pm2\%$ inhibition at 100 μM[a]	
Decanoylethanolamide (C10:0)	100%[b]	4.26 ± 0.05
Lauroylethanolamide (C12:0)	100%[b]	4.79 ± 0.04
Myristoylethanolamide (C14:0)	100%[b]	4.77 ± 0.07
Palmitoylethanolamide (C16:0)	78 ± 7[c]	5.30 ± 0.15
Heptadecanoylethanolamide (C17:0)	55 ± 18[c]	4.71 ± 0.36
Stearoylethanolamide (C18:0)	33 ± 2[c]	4.95 ± 0.06
Palmitoylbutylamide (C16:0)	24 ± 3[c]	5.54 ± 0.16
Palmitoylisopropylamide (C16:0)	61 ± 11[c]	4.89 ± 0.27
Palmitoylcyclohexamide (C16:0)	$11\pm3\%$ inhibition at 100 μM[a]	
R-Palmitoyl-(2-methyl)ethanolamide (C16:0)	54 ± 5[c]	5.39 ± 0.12
Oleoylethanolamide (C18:1)	100%[b]	5.33 ± 0.04

For the inhibition of 2 μM [³H]-AEA hydrolysis by rat brain FAAH, pI_{50} values were calculated from the raw data summarized in Figures 1 and 2. [a]Insufficient inhibition was obtained to permit meaningful calculation of pI_{50} values. In consequence, the observed inhibition at the highest concentration tested has been given instead. [b]The calculated bottom value exceeded 100% inhibition (i.e. <0% activity remaining, e.g. $-10\pm35\%$ for myristoylethanolamide). However, since the 95% confidence intervals of the minimum activity remaining straddled zero (e.g. -82 to $+63\%$ for myristoylethanolamide), the pI_{50} values were calculated using a fixed maximum inhibition of 100%. [c]95% confidence intervals of the minimum activity remaining were both greater than zero (e.g. $37-55\%$ for *R*-palmitoyl-(2-methyl)ethanolamide), thus allowing the pI_{50} values to be calculated using the maximum inhibition values given.
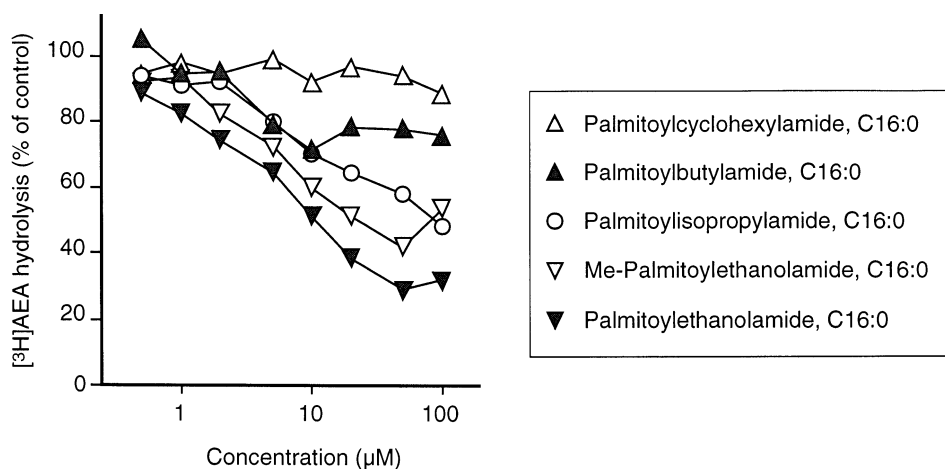

Figure 4 Inhibition of the hydrolysis by rat brain membranes of 2 μM [³H]-AEA by a series of palmitoylethanolamide analogues. Shown are means of 3–4 experiments, with no preincubation with the compounds and membranes prior to addition of [³H]-AEA. The data for palmitoylethanolamide are the same as that shown in Figure 3.

Inhibition of [³H]-AEA uptake in RBL-2H3 and C6 cells

The ability of the compounds to inhibit the uptake of [³H]-AEA into RBL-2H3 and C6 cells is shown in Figure 6. RBL-2H3 cells were chosen since [³H]-AEA transport mechanisms in these cells have been extensively characterized (Bisogno *et al.*, 1997; De Petrocellis *et al.*, 2000; Rakhshan *et al.*, 2000; Jacobsson & Fowler, 2001). Less is known about the uptake of AEA into C6 cells, although there are some apparent differences in the properties of the transporters in the two cell lines (such as sensitivity to nitric oxide donors, Bisogno *et al.*, 2001; Jacobsson & Fowler, 2001). The initial data, shown in Figure 6c, investigated the effects of the palmitoylethanola-mide homologues and analogues at concentrations of 1, 10 and 100 μM, to inhibit the uptake of 10 μM [³H]-AEA into RBL-2H3. [³H]-AEA binds avidly to cell culture wells, and it was proposed to compensate for this by defining non-specific effects as those inhibitable by 100 μM AM 404. However, this assumes that AM 404 at this concentration does not affect the binding of [³H]-AEA to the wells, which turned out to be the case (Figure 6a). The other monounsaturated compounds, oleoylethanolamide and R-methanandamide also affected the binding of [³H]-AEA to the wells, whereas the saturated compounds did not (see Figure 6a–c).

In these subsequent experiments, the percentage of the total apparent uptake in RBL-2H3 cells due to the binding of [³H]-AEA to the wells could be determined ($52\pm6\%$). This
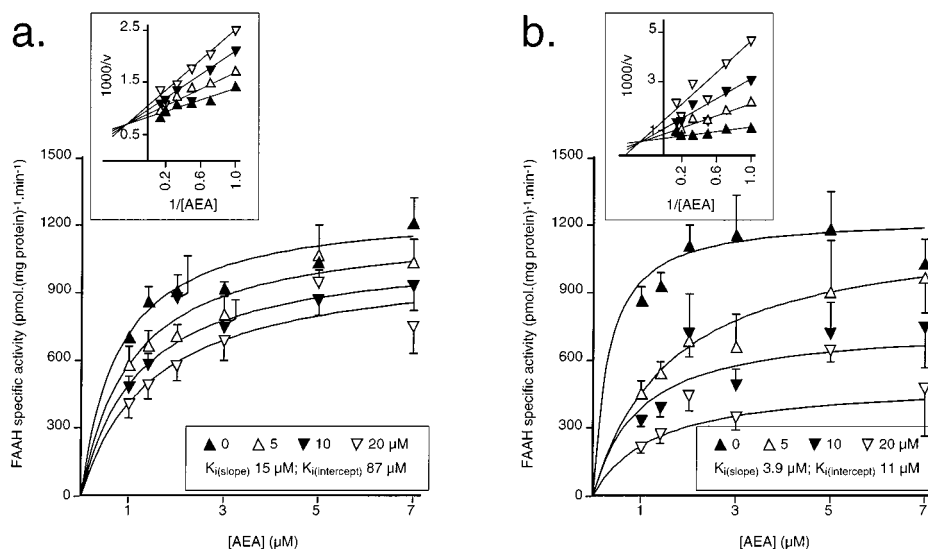
Figure 5 Mode of inhibition of rat brain [³H]-AEA metabolism by (**a**) palmitoylisopropylamide and (**b**) oleoylethanolamide. Shown are means ± s.e. mean of three experiments. Secondary replots of the mean data to illustrate the mixed-type nature of the inhibition are shown as inserts. The $K_{i(slope)}$ and $K_{i(intercept)}$ values were calculated from the mean data as described in Methods.

Table 2 Effect of compounds related to palmitoylethanolamide upon the specific binding of 1 nM [³H]-CP55,940 and 1 nM [³H]-WIN55,212-2 to human CB_1 and CB_2 cannabinoid receptors, respectively, expressed in CHO cells

| | % inhibition of specific binding | | | |
| | Cell line: | CHO-CB_1 | CHO-CB_2 | |
| | Ligand: | [³H]-CP55,940 | [³H]-WIN55,212-2 | |
Compound	10 μM	100 μM	10 μM	100 μM
Propanoylethanolamide (C3:0)	19.5 ± 8.8	7.8 ± 4.3	8.9 ± 0.2	2.9 ± 1.0
Butanoylethanolamide (C4:0)	19.7 ± 5.5	8.0 ± 2.3	4.4 ± 0.5	3.4 ± 2.4
Haxanoylethanolamide (C6:0)	31.6 ± 5.15	8.0 ± 2.3	1.6 ± 0.3	6.2 ± 1.3
Octanoylethanolamide (C8:0)	26.7 ± 2.29	17.6 ± 5.3	5.7 ± 0.8	12.7 ± 0.8
Decanoylethanolamide (C10:0)	11.0 ± 0.98	12.3 ± 2.3	10.0 ± 0.8	17.3 ± 2.6
Lauroylethanolamide (C12:0)	21.9 ± 1.84	37.7 ± 8.3	5.1 ± 0.6	16.0 ± 2.3
Myristoylethanolamide (C14:0)	23.6 ± 6.16	41.1 ± 7.4	5.3 ± 0.6	21.9 ± 3.0
Palmitoylethanolamide (C16:0)	23.8 ± 0.07	25.4 ± 5.3	13.9 ± 1.7	24.5 ± 0.5
Stearoylethanolamide (C18:0)	30.3 ± 9.8	21.9 ± 6.4	3.4 ± 0.6	11.6 ± 1.8
Palmitoylbutylamide (C16:0)	19.6 ± 3.4	35.4 ± 5.3	12.9 ± 0.4	21.8 ± 0.7
Palmitoylisopropylamide (C16:0)	25.4 ± 4.5	14.5 ± 6.0	12.3 ± 0.8	21.1 ± 0.5
Palmitoylcyclohexamide (C16:0)	34.2 ± 1.5	37.5 ± 2.1	8.1 ± 0.2	13.8 ± 0.8
R-Palmitoyl-(2-methyl)ethanolamide (C16:0)	12.6 ± 3.3	25.7 ± 6.7	5.3 ± 0.9	15.5 ± 2.6
Oleoylethanolamide (C18:1)	13.3 ± 2.7	63.4 ± 3.9	14.5 ± 0.5	64.6 ± 1.5

Shown are means ± s.e.mean of quadruplicate determinations.

level of background binding is shown in Figure 6c as a dashed line, and was used, in conjunction with experiments undertaken in the absence of cells, to determine the degree to which the compounds at a concentration of 100 μM affected the binding of the [³H]-AEA to the wells. Although the use of a retroactive measure of binding to the wells prevents quantitative interpretation of these data, the first series of experiments indicated that several of the compounds are inactive (i.e. the homologues of palmitoylethanolamide at C ⩽ 8 atoms) at 10 and 100 μM concentrations. Inhibition of [³H]-AEA uptake was seen at 10 μM concentrations with decanoylethanolamide, palmitoylisopropylamide, lauroylethanolamide and myristoylethanolamide, and at 100 μM con-

centrations with palmitoylethanolamide, palmitoylbutylamide, palmitoylcyclohexamide and oleoylethanolamide (although interpretation of the data with oleoylethanolamide is confounded by its ability to inhibit the binding of [³H]-AEA to the wells). None of the compounds affected the total uptake at concentrations of 1 μM (data not shown).

In the second series of experiments, the effects of palmitoylcyclohexamide and palmitoylisopropylamide (10 and 30 μM) were tested using both C6 and RBL-2H3 cells, and where parallel wells without cells were used to quantify the binding of the [³H]-AEA to the culture wells (Figure 6a). Propylisopropylamide reduced the specific uptake to 66 and 38% of control in the C6 cells at concentrations of 10 and
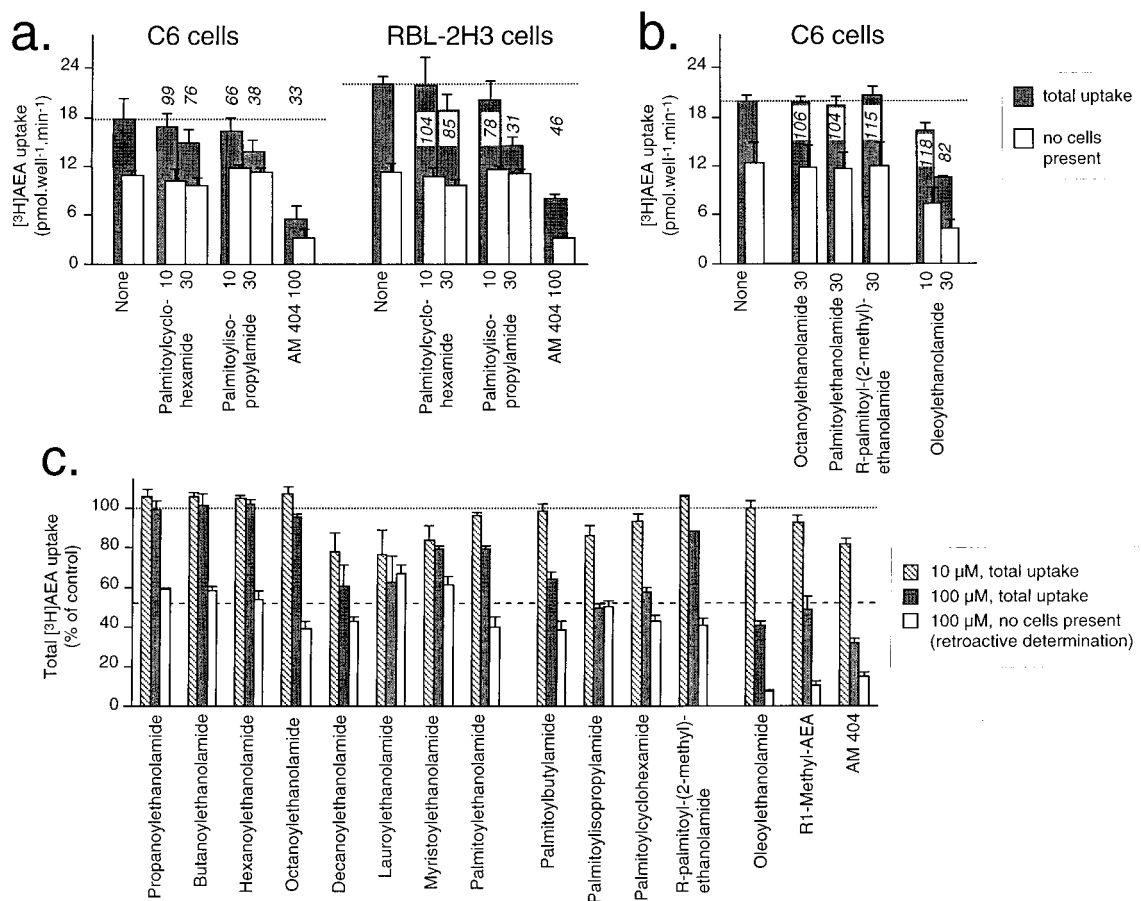
Figure 6 Effect of palmitoylethanolamide and related compounds upon the uptake of 10 μM [^{3}H]-AEA into rat C6 glioma and RBL-2H3 basophilic leukaemia cells (a,b) shown are means$\pm$s.e.mean ($n=3$) of the total uptake in the absence and presence of cells. The numbers given above (or enclosed within) the columns are the specific uptake remaining in the presence of the test compounds, calculated as $100 \times$(mean total uptake - mean uptake, no cells present)/(mean total uptake in the absence of compound - mean uptake, no cells present in the absence of compound). The dotted line shows the mean total uptake in the absence of test compound (i.e. 'none'). (c) effects on the total uptake of [^{3}H]-AEA into RBL-2H3 cells. Shown are means$\pm$s.e.mean, $n=3$. The dotted line shows the mean total uptake in the absence of test compound (i.e. 100%). The dashed line is a retroactive estimation of the mean percentage of the total [^{3}H]-AEA uptake due to binding to the cell culture wells, determined from the data shown as 'none' for the RBL-2H3 cells in panel **a**. The unfilled columns in the experiments shown in panel **c** were calculated from means of three separate experiments where the effects of the compounds upon the binding to [^{3}H]-AEA to culture wells was determined. The observed figure was then normalized to 52% (i.e. the dashed line). Thus, for example, a compound producing a 34% inhibition of the binding to the well would have an unfilled column of $52 \times (0.66) = 34\%$ of total [^{3}H]-AEA uptake.

30 μM, respectively, whereas palmitoylcyclohexamide was less effective (99 and 76%, respectively). Similar results were seen in the RBL-2H3 cells (Figure 6a). Octanoyethanolamide, palmitoyethanolamide and *R*-palmitoyl-(2-methyl)ethanolamide at concentrations of 30 μM had no effect on the uptake of [^{3}H]-AEA into C6 cells, whereas a small inhibition was seen with this concentration of oleoylethanolamide (Figure 6b).

Inhibition of [^{3}H]-ethanolamine production following incubation of [^{3}H]-AEA with intact C6 glioma cells

The six compounds investigated with respect to their effects upon the uptake of [^{3}H]-AEA into C6 glioma cells were also tested for their abilities to inhibit [^{3}H]-ethanolamine production following incubation of [^{3}H]-AEA with intact C6 cells, the rationale being that such inhibition reflects the combination of effects on uptake and FAAH. The data are shown in

Table 3. Oleoylethanolamide and palmitoylisopropylamide were the most potent of the compounds tested, producing $>50\%$ and $>80\%$ inhibition of [^{3}H]-ethanolamine production at 10 and 30 μM, respectively, in both cases. AM404 was slightly less potent (40 and 68% inhibition at 10 and 30 μM, respectively), whereas palmitoylethanolamide, palmitoylcyclohexamide, *R*-palmitoyl-(2-methyl)ethanolamide and octanoylethanolamide produced 42, 45, 30 and 25% inhibition, respectively, at the highest concentration tested (30 μM).

Discussion

The fatty acid amide palmitoylethanolamide has been found to have anti-inflammatory and anti-nociceptive properties (Aloe *et al.*, 1993; Mazzari *et al.*, 1996; Jaggar *et al.*, 1998; Calignano *et al.*, 1998). Given that this compound has a low affinity towards CB receptors *in vitro* and produces no

Table 3 Effect of compounds related to palmitoylethanolamide upon the hydrolysis of [³H]-AEA by intact C6 glioma cells

Compound	% inhibition of [³H]-ethanolamine production		
	3 μM	10 μM	30 μM
Octanoylethanolamide (C8:0)	13 ± 7	14 ± 10	25 ± 7
Palmitoylethanolamide (C16:0)	15 ± 6	22 ± 8	42 ± 4
Palmitoylisopropylamide (C16:0)	24 ± 0.6	53 ± 3	87 ± 1
Palmitoylicyclohexamide (C16:0)	9 ± 5	26 ± 7	45 ± 9
R-palmitoyl-(2-methyl)ethanolamide (C16:0)	-3 ± 16	11 ± 6	30 ± 8
Oleoylethanolamide (C18:1)	24 ± 5	58 ± 2	82 ± 1
AM404	14 ± 9	40 ± 7	68 ± 2

The compounds were preincubated with the C6 glioma cells for 10 min prior to addition of [³H]-AEA (3 μM) and incubation for a further 30 min. Data are means $\pm$ s.e.mean, $n = 3$ (except for *R*-palmitoyl-(2-methyl)-ethanolamide where $n = 6$).

significant effects at concentrations $\leqslant 10$ μM (Felder *et al.*, 1993; Lambert *et al.*, 1999; Ross *et al.*, 1999; Sugiura *et al.*, 2000), it has been suggested that palmitoylethanolamide, by preventing the metabolism of other endocannabinoids, may act as an 'entourage' compound (Lambert & Di Marzo, 1999). The metabolism of AEA is brought about by a process of facilitated diffusion into the cell followed by intracellular FAAH-catalysed metabolism (see Mechoulam *et al.*, 1998). In consequence, we tested both a series of palmitoylethanolamide analogues and homologues, as well as four new homologues (C3:0, C4:0, C6:0 and C8:0) and a 2-methyl analogue, for their ability to affect rat brain FAAH catalysed [³H]-AEA hydrolysis and [³H]-AEA uptake in RBL-2H3 cells. With the exception of oleoylethanolamide, none of the compounds were found at 100 μM concentrations to produce marked inhibition of the binding to [³H]-WIN 55,212-2 to CB_2 receptors expressed on CHO cells. Variable effects upon CB_1 receptors were seen, but for the most interesting compound emanating from this study, palmitoylisopropylamide, no dramatic interaction with this receptor subtype was found.

With respect to the ability of the compounds to inhibit the FAAH-catalyzed metabolism of [³H]-AEA, the chain length proved to be of importance for the potency at lengths of 10 carbon atoms or less. Thus, potencies of the homologues were roughly similar (given the approximate nature of the pI_{50} values as a result of the differences in maximum inhibition obtained) to palmitoylethanolamide as the chain length was reduced to 14 and 12 carbon atoms (or increased to 18 carbon atoms), but was slightly lower for the C10:0 compound (decanoylethanolamide). Compounds with a chain length of $\leqslant 8$ carbon atoms were inactive. This pattern of 'stability' between C12:0 and C18:0 is also seen with saturated fatty acid sulphonyl fluoride compounds, although their potencies are greater than seen here: IC_{50} values of 3, 6 and 7 nM for the inhibition of rat brain AEA metabolism by lauryl- (C12), myristyl- (C14), palmityl- (C16) sulphonyl fluorides were found (Deutsch *et al.*, 1997). In contrast, the C20:0 homologue arachidylsulphonyl fluoride is less potent (IC_{50} value > 48 nM) (Deutsch *et al.*, 1997).

Among the C16:0 analogues, palmitoylisopropylamide, *R*-palmitoyl-(2-methyl)ethanolamide and palmitoylbutylamide showed comparable potency to that of palmitoylethanolamide, although the maximal obtainable inhibition was lower. Oleoylethanolamide (C18:1) was a mixed type inhibitor of [³H]-AEA metabolism with $K_{i(slope)}$ and $K_{i(intercept)}$ values of 3.9 μM and 11 μM, respectively. Although the maximum observed inhibition produced by this compound was greater

than seen with the saturated analogue, stearoylethanolamide, this may reflect solubility differences and the difference in pI_{50} values was not that great. In this respect, the potency of PTMK was very similar to that found previously for its oleoyl-analogue (pI_{50} value 7.24 ± 0.077) in experiments using the same experimental conditions, (Fowler *et al.*, 2000). The potency of PTMK found here is approximately 50 times greater than the potency of this compound towards Ca^{2+}-independent phospholipase A2 (Ackermann *et al.*, 1995), making it worthwhile further to investigate the potential usefulness of this compound as an FAAH inhibitor.

In theory, the palmitoylethanolamide analogues and homologues can inhibit [³H]-AEA metabolism by acting either as inhibitors or as competing substrates. Indeed, amidase activities capable of utilizing C12:0 – C18:0 (but not C8:0) and C18:1 ethandamides as substrates were reported in the literature more than 16 years ago (Natarajan *et al.*, 1984; Schmid *et al.*, 1985). Most available studies in the literature have compared the relative activities of palmitoylethanolamide with AEA with widely varying results (see Tiger *et al.*, 2000), although Lang *et al.* (1999) found a 4 fold greater rate of metabolism of 100 μM oleoylethanolamide than 100 μM stearoylethanolamide using rat brain microsomes as source of FAAH. There is more data on the ability of −amide, rather than −ethanolamide compounds to act as substrates. Thus, palmitoylamide (C16:0), myristoylamide (C14:0) and dodecanoylamide (C12:0) have been shown to act as FAAH substrates with rates of hydrolysis relative to oleamide (C18:1) of 0.72, 0.83 and 0.74, respectively (Boger *et al.*, 2000a; see also Cravatt *et al.*, 1996). These authors also demonstrated that introduction of a single double-bond into the 9-position of the palmitoylamide and myristoylamide molecules did not affect their rates of hydrolysis, whereas the mono-unsaturated oleamide was metabolized at a higher rate than the saturated stearamide equivalent (Boger *et al.*, 2000a). These authors used single high concentrations of substrate (187 μM), thus presumably measuring activities near their V_{max} values. Given (a) that linoleoylethanolamide and linoleoylamide are both substrates for FAAH and are metabolized with similar V_{max} values (Maurelli *et al.*, 1995); and (b) that palmitoylamide inhibits [³H]-AEA metabolism with a pI_{50} value (5.01 ± 0.18, maximum inhibition attainable $51 \pm 7\%$, present authors, unpublished data) similar to that of palmitoylethanolamide, it is reasonable to assume that the C10:0 and C17:0 homologues of palmitoylethanolamide tested here are acting as competing substrates. The mixed-type inhibition of [³H]-AEA found here with oleoylethano-

lamide is perhaps surprising, given that alternate substrates should interact competitively. However, a similar mixed-type inhibition has been seen previously for the ability of 2 μM (but not 1 μM) AEA to inhibit the metabolism of [^{3}H]-palmitoylethanolamide by rat brain FAAH (Tiger *et al.*, 2000). Whether the 'non-competitive component' of the inhibition in these cases represents an additional action of the compounds (such as effects secondary to membrane actions of these fatty acid amides) awaits elucidation.

For the palmitoylethanolamide analogues tested here, it is not known whether they are substrates for FAAH. One way of distinguishing inhibitors from competing substrates is to preincubate the compounds with a strong FAAH preparation prior to addition of [^{3}H]-anandamide: for a competing substrate, the preincubation would be expected to reduce the observed potency of the compound. However, with the exception of lauroylethanolamide, no decreased potency with preincubation time was found when membranes were preincubated with the compounds for up to 3 h. This would suggest that under the conditions used, insufficient amounts of the compounds were metabolized significantly to affect the observed inhibition. Thus, further experiments are required before it can be determined whether these compounds act as substrates or as inhibitors. However, the potencies of the compounds were rather modest, and as a first step it might be more appropriate to investigate further the structure-activity relationships between palmitoylethanolamide analogues and inhibition of [^{3}H]-AEA hydrolysis. Such studies are presently under way.

In addition to studying the effect of the compounds upon [^{3}H]-AEA metabolism by FAAH, their ability to prevent [^{3}H]-AEA accumulation into RBL-2H3 and C6 cells has been investigated. The cellular uptake of AEA is somewhat complicated: it is saturable but sodium- and energy-independent, and is only capable of concentrating the endocannabinoid against a modest gradient (Di Marzo *et al.*, 1994; Beltramo *et al.*, 1997; Hillard *et al.*, 1997, Rakhshan *et al.*, 2000; Deutsch *et al.*, 2001). Thus inhibition of FAAH will result in an indirect reduction in the rate of accumulation of AEA. The compounds tested in the present study were rather modest inhibitors of [^{3}H]-AEA accumulation into RBL-2H3 cells, and in the case of palmitoylethanolamide, the reduction in [^{3}H]-AEA accumulation seen at the highest concentration (100 μM) is presumably a reflection of effects on [^{3}H]-AEA metabolism. Palmitoylcyclohexamide, however, does not affect [^{3}H]-AEA metabolism, suggesting that the ability of this compound to reduce [^{3}H]-AEA

accumulation may reflect a direct effect upon the AEA transport process. Thus, palmitoylcyclohexamide may prove useful as a template for the design of compounds that reduce the cellular accumulation of AEA without having potent effects upon either FAAH or CB_2 receptors. Palmitoyliso-propylamide also produces a greater inhibition of uptake than palmitoylethanolamide, despite having a similar pI_{50} value towards inhibition of [^{3}H]-AEA metabolism, which also suggests a direct action on the AEA uptake process.

At first sight, the present study does not give rise to compounds that are dramatically better than palmitoyletha-nolamide as possible entourage compounds. However, effects on AEA metabolism *in vivo* will be a consequence of the combination of effects on transport and FAAH. As a first step to investigating this combination, we studied the effect of palmitoylisopropylamide, palmitoylcyclohexamide, *R*-palmi-toyl-(2-methyl)ethanolamide and oleoylethanolamide (with palmitoylethanolamide and AM404 for comparison and octanoylethanolamide as a negative control) on the produc-tion of [^{3}H]-ethanolamine following incubation of C6 glioma cells with [^{3}H]-AEA. In this case, the FAAH only has access to the transported AEA, so that a combination of effects upon uptake and FAAH will produce a greater inhibition of [^{3}H]-ethanolamine production than effects on either process alone. Such a result was indeed found: both palmitoyliso-propylamide and oleoylethanolamide were more potent than either palmitoylethanolamide (which competes for FAAH but has no direct effect on AEA uptake) or palmitoylcyclohex-amide (which affects AEA uptake but not FAAH). Interestingly, palmitoylisopropylamide and oleoylethanola-mide were more effective than AM404. Given that AM404 potentiates endogenous AEA *in vivo* (Giuffrida *et al.*, 2000), these data would suggest that palmitoylisopropylamide might be a useful entourage compound that does not have direct effects on CB receptors.

References

ACKERMANN, E.J., CONDE-FRIBOES, K. & DENNIS, E.A. (1995). Inhibition of macrophage Ca^{2+}-independent phospholipase A_2 by bromoenol lactone and trifluoromethyl ketones. *J. Biol. Chem.*, **270,** 445 – 450.

ALOE, L., LEON, A. & LEVI-MOLTALCINI, R. (1993). A proposed autacoid mechanism controlling mastocyte behaviour. *Agents Actions,* **39,** C145 – C147.

BAKER, D., PRYCE, G., CROXFORD, J.L., BROWN, P., PERTWEE, R.G., MAKRIYANNIS, A., KHANOLKAR, A., LAYWARD, L., FEZZA, F., BISOGNO, T. & DI MARZO, V. (2001). Endocannabi-noids control spasticity in a multiple sclerosis model. *FASEB J.,* **15,** 300 – 302.

BELTRAMO, M., STELLA, N., CALIGNANO, A., LIN, S.Y., MAK-RIYANNIS, A. & PIOMELLI, D. (1997). Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science,* **277,** 1094 – 1097.

BISOGNO, T., MACCARRONE, M., DE PETROCELLIS, L., JARRA-HIAN, A., FINAZZI-AGRÒ, A., HILLARD, C. & DI MARZO, V. (2001). The uptake by cells of 2-arachidonoylglycerol, an endogenous agonist of cannabinoid receptors. *Eur. J. Biochem.,* **268,** 1982 – 1989.

BISOGNO, T., MAURELLI, S., MELCK, D., DE PETROCELLIS, L. & DI MARZO, V. (1997). Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. *J. Biol. Chem.*, **272**, 3315 – 3323.

BISOGNO, T., MELCK, D., DE PETROCELLIS, L., BOBROV, M.Y., GRETSKAYA, N.M., BEZUGLOV, V.V., SITACHITTA, N., GERWICK, W.H. & DI MARZO, V. (1998). Arachidonoylserotonin and other novel inhibitors of fatty acid amide hydrolase. *Biochem. Biophys. Res. Commun.*, **248**, 515 – 522.

BOGER, D.L., FECIK, R.A., PATTERSON, J.E., MIYAUCHI, H., PATRICELLI, M.P. & CRAVATT, B.F. (2000a). Fatty acid amide hydrolase substrate specificity. *Bioorg. Med. Chem. Lett.*, **10**, 2613 – 2616.

BOGER, D.L., SATO, H., LERNER, A.E., HEDRICK, M.P., FECIK, R.A., MIYAUCHI, H., WILKIE, G.D., AUSTIN, B.J., PATRICELLI, M.P. & CRAVATT, B.F. (2000b). Exceptionally potent inhibitors of fatty acid amide hydrolase: The enzyme responsible for degradation of endogenous oleamide and anandamide. *Proc. Natl. Acad. Sci. USA.*, **97**, 5044 – 5049.

CALIGNANO, A., KÁTONA, I., DÉSARNAUD, F., GIUFFRIDA, A., LA RANA, G., MACKIE, K., FREUND, T.F. & PIOMELLI, D. (2000). Bidirectional control of airway responsiveness by endogenous cannabinoids. *Nature*, **408**, 96 – 101.

CALIGNANO, A., LA RANA, G., GIUFFRIDA, A. & PIOMELLI, D. (1998). Control of pain initiation by endogenous cannabinoids. *Nature*, **394**, 277 – 281.

CHILDERS, S.R., SEXTON, T. & ROY, M.B. (1994). Effects of anandamide on cannabinoid receptors in rat brain membranes. *Biochem. Pharmacol.*, **47**, 711 – 715.

COMPTON, D.R. & MARTIN, B.R. (1997). The effect of the enzyme inhibitor phenylmethylsulfonyl fluoride on the pharmacological effect of anandamide in the mouse model of cannabimimetic activity. *J. Pharmacol. Exp. Ther.*, **283**, 1138 – 1143.

CRAVATT, B.F., GIANG, D.K., MAYFIELD, S.P., BOGER, D.L., LERNER, R.A. & GILULA, N.B. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature*, **384**, 83 – 87.

DE PETROCELLIS, L., BISOGNO, T., DAVIS, J.B., PERTWEE, R.G. & DI MARZO, V. (2000). Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity. *FEBS Lett.*, **483**, 52 – 56.

DE PETROCELLIS, L., MELCK, D., PALMISANO, A., BISOGNO, T., LAEZZA, C., BIFULCO, M. & DI MARZO, V. (1998). The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 8375 – 8380.

DEUTSCH, D.G. & CHIN, S.A. (1993). Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem. Pharmacol.*, **46**, 791 – 796.

DEUTSCH, D.G., GLASER, S.T., HOWELL, J.M., KUNZ, J.S., PUFFENBARGER, R.A., HILLARD, C.J. & ABUMRAD, N. (2001). The cellular uptake of anandamide is coupled to its breakdown by fatty acid amide hydrolase (FAAH). *J. Biol. Chem.*, **276**, 6967 – 6973.

DEUTSCH, D.G., LIN, S., HILL, W.A., MORSE, K.L., SALEHANI, D., ARREAZA, G., OMEIR, R.L. & MAKRIYANNIS, A. (1997). Fatty acid sulfonyl fluorides inhibit anandamide metabolism and bind to the cannabinoid receptor. *Biochem. Biophys. Res. Commun.*, **231**, 217 – 221.

DI MARZO, V., FONTANA, A., CADAS, H., SCHINELLI, S., CIMINO, G., SCHWARTZ, J.C. & PIOMELLI, D. (1994). Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature*, **372**, 686 – 691.

EISENTHAL, R. & CORNISH-BOWDEN, A. (1974). The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. *Biochem. J.*, **139**, 715 – 720.

FELDER, C.C., BRILEY, E.M., AXELROD, J., SIMPSON, J.T., MACKIE, K. & DEVANE, W.A. (1993). Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 7656 – 7660.

FOWLER, C.J., TIGER, G. & STENSTRÖM, A. (1997). Ibuprofen inhibits rat brain deamidation of anandamide at pharmacologically relevant concentrations. Mode of inhibition and structure-activity relationship. *J. Pharmacol. Exp. Ther.*, **283**, 729 – 734.

FOWLER, C.J., BÖRJESSON, M. & TIGER, G. (2000). Differences in the pharmacological properties of rat and chicken brain fatty acid amidohydrolase. *Br. J. Pharmacol.*, **131**, 498 – 504.

GAULDIE, S.D., MCQUEEN, D.S., PERTWEE, R. & CHESSELL, I.P. (2001). Anandamide activates peripheral nociceptors in normal and arthritic knee joints. *Br. J. Pharmacol.*, **132**, 617 – 621.

GIFFORD, A.N., BRUNEUS, M., LIN, S., GOUTOPOULOS, A., MAKRIYANNIS, A., VOLKOW, N.D. & GATLEY, S.J. (1999). Potentiation of the action of anandamide on hippocampal slices by the fatty acid amide hydrolase inhibitor, palmitylsulphonyl fluoride (AM 374). *Eur. J. Pharmacol.*, **383**, 9 – 14.

GIUFFRIDA, A., RODRIGUEZ DE FONSECA, F., NAVA, F., LOUBET-LESCOULIÉ, P. & PIOMELLI, D. (2000). Elevated circulating levels of anandamide after administration of the transport inhibitor, AM404. *Eur. J. Pharmacol.*, **408**, 161 – 168.

HARRINGTON, C.R. (1990). Lowry protein assay containing sodium dodecyl sulfate in microtiter plates for protein determination on fractions from brain tissue. *Analyt. Biochem.*, **186**, 285 – 287.

HILLARD, C.J., EDGEMOND, W.S., JARRAHIAN, A. & CAMPBELL, W.B. (1997). Accumulation of N-arachidonoylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. *J. Neurochem.*, **69**, 631 – 638.

JACOBSSON, S.O.P. & FOWLER, C.J. (2001). Characterization of palmitoylethanolamide transport in mouse Neuro-2a neuroblastoma and rat RBL-2H3 basophilic leukaemia cells: comparison with anandamide. *Br. J. Pharmacol.*, **132**, 1743 – 1754.

JAGGAR, S.I., HASNIE, F.S., SELLATURAY, S. & RICE, A.S.C. (1998). The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain. *Pain*, **76**, 189 – 199.

JARRAHIAN, A., MANNA, S., EDGEMOND, W.S., CAMPBELL, W.B. & HILLARD, C.J. (2000). Structure-activity relationships among N-arachidonoylethanolamine (anandamide) head group analogues for the anandamide transporter. *J. Neurochem.*, **74**, 2597 – 2606.

KOUTEK, B., PRESTWICH, G.D., HOWLETT, A.C., CHIN, S.A., SALEHANI, D., AKHAVAN, N. & DEUTSCH, D.G. (1994). Inhibitors of arachidonoyl ethanolamide hydrolysis. *J. Biol. Chem.*, **269**, 22937 – 22940.

LAMBERT, D.M. & DI MARZO, V. (1999). The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic? *Curr. Med. Chem.*, **6**, 757 – 773.

LAMBERT, D.M., DIPAOLO, F.G., SONVEAUX, P., KANYONYO, M., GOVAERTS, S.J., HERMANS, E., BUEB, J., DELZENNE, N.M. & TSCHIRHART, E.J. (1999). Analogues and homologues of N-palmitoylethanolamide, a putative endogenous CB₂ cannabinoid, as potential ligands for the cannabinoid receptors. *Biochim. Biophys. Acta*, **1440**, 266 – 274.

LANG, W., QIN, C., LIN, S., KHANOLKAR, A.D., GOUTOPOULOS, A., FAN, P., ABOUZID, K., MENG, Z., BIEGEL, D. & MAKRIYANNIS, A. (1999). Substrate specificity and stereoselectivity of rat brain microsomal anandamide amidohydrolase. *J. Med. Chem.*, **42**, 896 – 902.

MACCARRONE, M., LORENZON, T., BARI, M., MELINO, G. & FINAZZI-AGRÒ, A. (2000). Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors. *J. Biol. Chem.*, **275**, 31938 – 31945.

MALLET, P.E. & BENINGER, R.J. (1998). The cannabinoid CB₁ receptor antagonist SR 141716A attenuates the memory impairment produced by Δ⁹-tetrahydro-cannabinol or anandamide. *Psychopharmacology*, **140**, 11 – 19.

MAURELLI, S., BISOGNO, T., DE PETROCELLIS, L., DI LUCCIA, A., MARINO, G. & DI MARZO, V. (1995). Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma 'anandamide amidohydrolase'. *FEBS Letts.*, **377**, 82 – 86.

MAZZARI, S., CANELLA, R., PETRELLI, L., MARCOLONGO, G. & LEON, A. (1996). N-(2-hydroxyethyl)hexadecamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation. *Eur. J. Pharmacol.*, **300**, 227 – 236.

MECHOULAM, R., FRIDE, E. & DI MARZO, V. (1998). Endocannabinoids. *Eur. J. Pharmacol.*, **359**, 1 – 18.

MELCK, D., BISOGNO, T., DE PETROCELLIS, L., CHUANG, H.H., JULIUS, D., BIFULCO, M. & DI MARZO, V. (1999). Unsaturated long-chain N-acyl-vanillyl-amides (N-AVAMs): vanilloid receptor ligands that inhibit anandamide-facilitated transport and bind to CB1 cannabinoid receptors. *Biochem. Biophys. Res. Commun.*, **262**, 275 – 284.

NATARAJAN, V., SCHMID, P.C., REDDY, P.V. & SCHMID, H.H.O. (1984). Catabolism of N-acylethanolamine phospholipids by dog brain preparations. *J. Neurochem.*, **42**, 1613 – 1619.

OMEIR, R.L., CHIN, S., HONG, Y., AHERN, D.G. & DEUTSCH, D.G. (1995). Arachidonoyl ethanolamide-[1,2-^{14}C] as a substrate for anandamide amidase. *Life Sci.*, **56**, 1999 – 2005.

PATTERSON, J.E., OLLMANN, I.R., CRAVATT, B.F., BOGER, D.L., WONG, C.-H. & LERNER, R.A. (1996). Inhibition of oleamide hydrolase catalyzed hydrolysis of the endogenous sleep-inducing lipid cis-9-octadecenamide. *J. Am. Chem. Soc.*, **118**, 5938 – 5945.

PERTWEE, R.G., FERNANDO, S.R., GRIFFIN, G., ABADJI, V. & MAKRIYANNIS, A. (1995). Effect of phenylmethylsulphonyl fluoride on the potency of anandamide as an inhibitor of electrically evoked contractions in two isolated tissue preparations. *Eur. J. Pharmacol.*, **272**, 73 – 78.

PIOMELLI, D., GIUFFRUDA, A., CALIGNANO, A., DE FONSECA, F.R. (2000). The endocannabinoid system as a target for therapeutic drugs. *Trends Pharmacol. Sci.*, **21**, 218 – 224.

RAFFA, R.B., STONE, D.J. & HIPP, S.J. (1999). Differential cholera-toxin sensitivity of supraspinal antinociception induced by the cannabinoid agonist Δ^9-THC, WIN 55,212-2 and anandamide in mice. *Neuroscience Letts.*, **263**, 29 – 32.

RAKHSHAN, F., DAY, T.A., BLAKELY, R.D. & BARKER, E.L. (2000). Carrier-mediated uptake of the endogenous cannabinoid anandamide in RBL-2H3 cells. *J. Pharmacol. Exp. Ther.*, **292**, 960 – 967.

RICHARDSON, J.D., KILO, S. & HARGREAVES, K.M. (1998). Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB$_1$ receptors. *Pain*, **75**, 111 – 119.

ROSS, R.A., MURPHY, V.L., MCKAY, N.G., ASHFORD, M.L.J. & PERTWEE, R.G. (1999). Characterization of cannabinoid receptors in the spleen and in mast cells. In *Essential Fatty Acids and Eicosanoids: Invited papers from the 4th International Congress*. ed. Riemersma, R.A., Armstrong, R., Kelly, R.W. & Wilson, R. pp. 376 – 379. Champaign, IL, USA, AOCS Press.

SARKER, K.P, OBARA, S., NAKATA, M., KITAJIMA, I. & MARUYAMA, I. (2000). Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3. *FEBS Letts.*, **472**, 39 – 44.

SCHMID, P.C., ZUZARTE-AUGUSTIN, M.L. & SCHMID, H.H.O. (1985). Properties of rat liver *N*-acylethanolamine amidohydrolase. *J. Biol. Chem.*, **260**, 14145 – 14149.

SUGIURA, T., KONDO, S., KISHIMOTO, S., MIYASHITA, T., NAKANE, S., KODAKA, T., SUHARA, Y., TAKAYAMA, H. & WAKU, K. (2000). Evidence that 2-arachidonoylglycerol but not *N*-palmitoylethanolamide or anandamide is the physiological ligand for the cannabinoid CB$_2$ receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. *J. Biol. Chem.*, **275**, 605 – 612.

TERRANOVA, J.-P, MICHAUD, J.-C, LE FUR, G. & SOUBRIÉ, P. (1995). Inhibition of long-term potentiation in rat hippocampal slices by anandamide and WIN55212-2: reversal by SR141716 A, a selective antagonist of CB$_1$ cannabinoid receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **352**, 576 – 579.

TIGER, G., STENSTRÖM, A. & FOWLER, C.J. (2000). Pharmacological properties of rat brain fatty acid amidohydrolase in different subcellular fractions using palmitoylethanolamide as substrate. *Biochem. Pharmacol.*, **59**, 647 – 653.