Effects of pH on the inhibition of fatty acid amidohydrolase by ibuprofen

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1 The pharmacological properties of fatty acid amidohydrolase (FAAH) at different assay pH values were investigated using [³H]-anandamide ([³H]-AEA) as substrate in rat brain homogenates and in COS-7 cells transfected with wild type and mutant FAAH.

2 Rat brain hydrolysis of [³H]-AEA showed pH dependency with an optimum around pH 8-9. Between pH 6.3 and 8.2, the difference in activity was due to differences in the V_{max}, rather than the K_M values.

3 For inhibition of rat brain [³H]-AEA metabolism by a series of known FAAH inhibitors, the potencies of the enantiomers of ibuprofen and phenylmethylsulphonyl fluoride (PMSF) were higher at pH 5.28 than at pH 8.37, whereas the reverse was true for oleyl trifluoromethylketone (OTMK) and arachidonoylserotonin. At both pH values, (−)ibuprofen was a mixed-type inhibitor of FAAH. The $K_{i(slope)}$ and $K_{i(intercept)}$ values for (−)ibuprofen at pH 5.28 were 11 and 143 μM, respectively. At pH 8.37, the corresponding values were 185 and 3950 μM, respectively.

4 The pH dependency for the inhibition by OTMK and (−)ibuprofen was also seen in COS-7 cells transiently transfected with either wild type, S152A or C249A FAAH. No differences in potencies between the wild type and mutant enzymes were seen.

5 It is concluded that the pharmacological properties of FAAH are highly pH-dependent. The higher potency of ibuprofen at lower pH values raises the possibility that in certain types of inflamed tissue, the concentration of this compound following oral administration may be sufficient to inhibit FAAH.

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Abbreviations: AA-5HT, arachidonoyl-serotonin; AEA, anandamide; FAAH, fatty acid amidohydrolase, NSAID, non-steroidal anti-inflammatory drug; OTMK, oleyl trifluoromethylketone; PMSF, phenylmethylsulphonyl fluoride

Introduction

The fatty acid amides are a class of endogenous compounds that have attracted considerable recent interest for their possible therapeutic usefulness. Two of these compounds are anandamide (AEA) and palmitoylethanolamide. AEA, which interacts with both cannabinoid and vanilloid receptors, has been shown to affect a number of processes including nociception (antinociceptive at low doses *via* effects on cannabinoid receptors, activation of peripheral nociceptors at high doses *via* effects on vanilloid receptors), cell proliferation and lung function (Calignano *et al.*, 1998; 2000; Jaggar *et al.*, 1998; Richardson *et al.*, 1998; De Petrocellis *et al.*, 1998; Sarker *et al.*, 2000; Maccarrone *et al.*, 2000; Gauldie *et al.*, 2001). Palmitoylethanolamide is inactive at cannabinoid receptors *in vitro* (Lambert *et al.*, 1999), but has been shown to prevent substance P-induced mast cell degranulation and to have beneficial effects upon inflammatory pain *in vivo* (Aloe *et al.*, 1993; Mazzari *et al.*, 1996; Calignano *et al.*, 1998; Jaggar *et al.*, 1998). Given that

the synthesis of fatty acid amides is increased under some conditions of cellular stress and inflammation (for review, see De Petrocellis *et al.*, 2000), the enzyme responsible for their metabolism may be a useful therapeutic target for the treatment of certain inflammatory states.

Fatty acid amide hydrolase, FAAH, is a membrane-associated enzyme capable of the metabolism of a large number of fatty acid amides (see Boger *et al.*, 2000) and is expressed in a number of tissues, in particular the liver, brain, and testis (Deutsch & Chin, 1993). In the case of AEA, FAAH-catalyzed hydrolysis produces arachidonic acid and ethanolamine (Deutsch & Chin, 1993). A number of inhibitors of FAAH have been described, including substrate analogues such as oleyl trifluoromethylketone (OTMK) (Patterson *et al.*, 1996) and arachidonyl serotonin (AA-5HT) (Bisogno *et al.*, 1998), non-steroidal anti-inflammatory drugs such as ibuprofen (Fowler *et al.*, 1997), and other substances such as the serine protease inhibitor phenyl-methylsulphonyl fluoride (PMSF) (Deutsch & Chin, 1993). In 1996, the cDNA encoding the enzyme was cloned and shown to be a member of the amidase signature sequence group of enzymes (Cravatt *et al.*, 1996). Subsequent mutation studies have identified some of the residues both

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within and outside the amidase signature sequence that are of importance for catalytic activity (Goparaju *et al.*, 1999; Omeir *et al.*, 1999; Patricelli *et al.*, 1999; Patricelli & Cravatt, 2000), although information is lacking as to whether mutations retaining FAAH activity have altered pharmacological properties.

FAAH from a variety of sources, including both the naturally occurring enzyme (from the rat brain, for example) and the recombinant enzyme expressed in COS-7 cells, has been reported in several studies to have a pH optimum around pH 9 (Ueda *et al.*, 1995; 1999; Maurelli *et al.*, 1995; Hillard *et al.*, 1995; Omeir *et al.*, 1995; Bisogno *et al.*, 1997; Patricelli *et al.*, 1998); although one group found an apparently biphasic pH dependency for the metabolism of AEA by rat brain microsomes (Desarnaud *et al.*, 1995). More recently, Ueda *et al.* (1999) reported the expression in a human megakaryoblastic cell line (CMK) of an AEA hydrolysing activity with a pH optimum of 5.0 and rather different pharmacological properties to FAAH. It was, however, not established whether the lack of sensitivity of this acid amidase to PMSF and the AEA substrate analogue methyl arachidonyl fluorophosphonate (MAFP) was a property of the enzyme *per se* or a reflection of the pH used. If the latter was the case, then similar anomalous sensitivities of inhibition of FAAH would be expected at acidic pH. In the present study, we have: (a) reinvestigated the pH dependency of the ability of rat brain homogenates to metabolise AEA, using two different buffer systems and (b) determined whether different assay pH values affect the pharmacological properties of FAAH. In the latter case, we have used both rat brain homogenates and COS-7 cells transfected with either wild type (wt) or FAAH with mutations within the amidase signature sequence that retain catalytic activity as enzyme sources.

Methods

Materials

Arachidonyl-ethanolamide-[1-³H] ([³H]-AEA) (specific activity 60 Ci mmol⁻¹) was obtained from American Radiolabelled Chemicals Inc., St. Louis, MO, U.S.A. Non-radioactive AEA and the enantiomers of ibuprofen (α-methyl-4-(2-methylpropyl) benzeneacetic acid) were obtained from Research Biochemicals International, Natick, MA, U.S.A. Oleyl trifluoromethylketone (OTMK) and arachidonoyl-serotonin (AA-5HT) were obtained from the Cayman Chemical Company, Ann Arbor, MI, U.S.A. Phenylmethylsulphonyl fluoride (PMSF) and fatty acid free bovine serum albumin were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). AEA, ibuprofen enantiomers, OTMK, AA-5HT and PMSF were dissolved in ethanol. COS-7 cells were a kind gift of Dr Göran Bucht, Swedish Defence Research Agency, NBC Defence Division, Department of Microbiology, Umeå, Sweden.

Preparation of rat brain homogenates

Adult (9 month old) male Wistar and Sprague Dawley rats were used in the study. The animals were killed by carbon dioxide exposure followed by decapitation, and whole brains (minus the cerebellum) were dissected and frozen. For preparation of homogenates, the tissue was thawed, weighed and homogenized in 10 mM Tris-HCL buffer pH 7.6 containing 1 mM EDTA, in a volume of 2.5 ml/g wet weight. After determination of protein concentration, the homogenates were stored in aliquots of 250 μl at −80°C prior to assay of FAAH activity.

Preparation of homogenates of COS-7 cells transfected with wild-type and mutant FAAH

The pcDNA3.1 vectors containing wild-type or mutated FAAH cDNAs have been described previously (Omeir *et al.*, 1999). The vectors were transported to Umeå and recovered as described by Rosman & Miller (1990) before being transformed into competent *E. coli* strain JM 109. Colonies were picked and propagated in Luria-Bertani medium. After pelleting the bacteria, the plasmids were purified using Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany). Aliquots of 1.5×10^5 COS-7 cells were seeded into 6-well plates and grown for 18–24 h prior to transfection. Transfections were performed using the Lipofectamine™ or Lipofectamine2000™ protocol (Life Technologies, Tåstrup, Denmark). A 1.5 μg aliquot of plasmid (pFAAH-wt, pFAAH-S152A, pFAAH-C249A or pFAAH-S218A) was used for each transfection. As a control, cells were treated with the Lipofectamine™ or Lipofectamine2000™ alone ('mock transfection'). After growing the transfected cells overnight, the cells were supplemented with fresh Dulbecco's minimal essential medium containing 10% FBS, and were grown for an additional 20 h. The cells were then trypsinized, pelleted by centrifugation, and stored at −70°C. Upon thawing, the samples were suspended in 10 mM Tris-HCl buffer with 1 mM EDTA, pH 8.0 and frozen in aliquots at −80°C prior to assay of FAAH activity.

Assay of FAAH activity

FAAH was assayed essentially as described by Omeir *et al.* (1995) who used carbon-14 labelled AEA as substrate (for full description using [³H]-AEA, see Fowler *et al.*, 1997). Briefly, assay mixtures contained homogenate or cell lysate, test compound (when appropriate), and radiolabelled AEA (containing 10 mg ml⁻¹ fatty acid-free bovine serum albumin) in an assay volume of 200 μl. Test compounds were diluted with ethanol and compared with controls containing the same concentration of ethanol (25 μl in a 200 μl assay volume). Blanks contained homogenization buffer instead of homogenate or cell lysate. The mixtures were incubated at 37°C for the times stated in the figure legends. The reactions were stopped by placing the samples on ice, adding 400 μl methanol-chloroform (1 : 1), and vortexing and centrifuging the samples. The unmetabolized [³H]-AEA is retained in the chloroform whereas the [³H]-ethanolamine formed by the action of FAAH upon AEA is soluble in the methanol/buffer phase. Aliquots (200 μl) of the methanol/buffer phase were removed for analyses of radioactivity by liquid scintillation spectroscopy with quench correction. To obtain the actual pH during the experiments, the assays were scaled up (albeit using homogenization buffer in place of the homogenate or cell lysate) and the pH was measured after incubation at 37°C.

Protein determination Protein contents were measured using the method of Harrington (1990) using bovine serum albumin as standard.

Determination of pI_{50}, K_M and V_{max} values

For the inhibition curves, the specific activity at each inhibitor concentration in each experiment was expressed as per cent of control and pIC_{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.), with 'top' (i.e. control) and 'bottom' (i.e. blank) values fixed at 100 and 0, respectively. This method was used to mimimize the impact of inter-experimental variations in enzyme specific activity.

However, when the inhibition curve for PMSF at pH 5.5 was analysed using the raw data at each inhibitor concentration (but excluding the control and blank values), the calculated 'top' and 'bottom' values agreed well with those found for the uninhibited and blank samples, respectively. In addition, the pIC_{50} value obtained (5.84 ± 0.18) agreed well with that found using the data as percent of control (Table 1). K_M and V_{max} values (or apparent K_M and V_{max} values for experiments determining the mode of inhibition by ($-$)ibuprofen) for FAAH activities were calculated 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. Since this analysis is non-parametric in nature, K_M and V_{max} values have been given as medians and ranges. For the experiments with ($-$)ibuprofen, the apparent K_M and V_{max} values calculated from the mean data at each inhibitor concentration were used in secondary replots to determine $K_{i(slope)}$ and $K_{i(intercept)}$ values. Significance testing was undertaken using the Statview™ computer programme (SAS Institute Inc., Cary, NC, U.S.A.).

Results

pH dependency of AEA hydrolysis by rat brain homogenates

Two different combinations of buffers were used. One consisted of sodium acetate, tris-HCl, and disodium tetraborate (Figure 1a), as was used in the study of Desarnaud *et al.* (1995) (where a biphasic pH curve for rat brain microsomal FAAH activity was seen). The other consisted of sodium citrate, tris-HCl, and sodium carbonate (Figure 1b) as was used in the study of Ueda *et al.* (1999) (where monphasic pH curves with pH optima of ~ 9 and ~ 5 were seen for AEA metabolising activity from RBL2H3 and CMK cells, respectively). An optimum around pH 8–9 was seen for both buffer systems, with no sign of any obvious top at acid pH values. The 100 mM disodium tetrabrate buffer used to give the highest pH values in Figure 1a appeared to inhibit [^{3}H]-AEA metabolism by the rat brain homogenates.

The effect of variations in the pH upon the K_M and V_{max} of FAAH was determined (Figure 2). For the three pH values tested using the same tris-HCl buffer system, the difference in activity was found to be due to differences in V_{max}, rather

Table 1 pIC_{50} values for the inhibition of rat brain FAAH at pH 5.28 and 8.37

	pIC_{50} value (mean $\pm$ s.e.mean)	
	pH 5.28	pH 8.37
Oleyltrifluorometylketone (OTMK)	8.07 ± 0.064	8.91 ± 0.12
Arachidonyl serotonin (AA-5HT)	5.58 ± 0.054	6.05 ± 0.032
Phenylmethyl sulphonyl fluoride (PMSF)	5.92 ± 0.028	4.16 ± 0.036
($-$)Ibuprofen	4.38 ± 0.040	3.14 ± 0.048
($+$)Ibuprofen	4.16 ± 0.030	$<3^*$

Data (summarized in Figure 3) were calculated using the built-in equation 'sigmoidal dose-response, variable slope' of the GraphPad Prism computer programme. The data was presented as per cent of control, with 'top' (i.e. control) and 'bottom' (i.e. blank) values set to 100 and 0, respectively. In all cases, the 95% confidence limits of the calculated Hill slopes straddled unity (e.g. 0.94–1.21 for PMSF, pH 5.28). *The calculated pIC_{50} value (2.69 ± 0.092) was outside the highest concentration tested (1000 μM).

than K_M values. Thus, K_M values (medians, $n=3$, with ranges in brackets) of 0.36 [0.35–0.37], 0.33 [0.30–0.38] and 0.41 [0.31–0.66] μM were found at assay pH values of 6.3, 7.27 and 8.2, respectively. The corresponding V_{max} values were 645 [471–885], 828 [698–1124] and 1374 [1050–1572] pmol (mg protein)$^{-1}$ min^{-1}, respectively. The Friedman nonparametric test for analysis of variance by ranks of matched samples gave a significant effect of pH upon the V_{max} values ($P<0.05$) but not on the K_M values.

Pharmacological properties of FAAH at different pH values.

To see if the sensitivity of FAAH to different known inhibitors changes with the pH, homogenates were incubated with different concentrations of the inhibitors at pH 5.28 and 8.37 (Figure 3). PMSF and the two ibuprofen enantiomers were found to be more potent inhibitors of [^{3}H]-AEA metabolism at pH 5.28 than at pH 8.37, whereas the reverse was true for OTMK and AA-5HT. The pI_{50} values calculated from these experiments are summarized in Table 1.

The mode of inhibition of FAAH by ($-$)ibuprofen was determined at pH 5.28 and 8.37 (Figure 4). The inhibition was mixed-type at both pH values, although once again the potency of ($-$)ibuprofen was greater at the acidic pH.

Pharmacological properties of wt and mutant FAAH transiently expressed in COS-7 cells

One of the drawbacks associated with the use of rat brain homogenates is that they can represent a heterogeneous population of enzyme activities, although no evidence for pharmacological differences in FAAH in different brain regions or in different subcellular fractions has been presented (Fowler *et al.*, 1999; Tiger *et al.*, 2000). In order to circumvent this drawback, and to provide data on the pharmacological properties of catalytically active FAAH mutants, we investigated the sensitivity of wt, S152A and C249A enzymes expressed in COS-7 cells. The latter mutant is of particular interest since the rodent variant (used here) is catalytically active, whereas the porcine variant is not (Omeir *et al.*, 1999; Goparaju *et al.*, 1999).
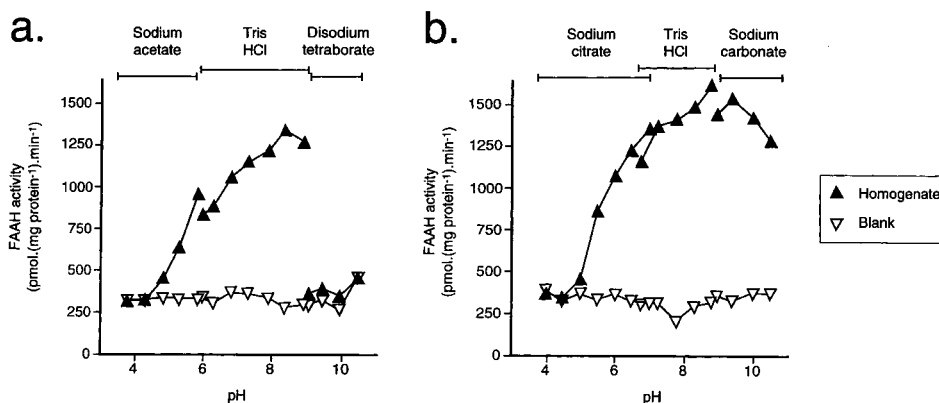
Figure 1 pH dependency of the hydrolysis of 2 μM [^{3}H]-AEA by rat brain homogenates. Two different buffer systems were used: (a) 100 mM sodium acetate buffer, 100 mM tris-HCl buffer, and 100 mM disodium tetraborate buffer (b) 50 mM sodium citrate, 50 mM tris-HCl and 50 mM sodium carbonate buffer. The pH values shown are the actual assay pH values, tested in scaled up assays, rather than the nominal pH of the buffers added to the homogenates. Data are means from two separate experiments using an incubation time of 20 min and of 4 μg protein/assay.
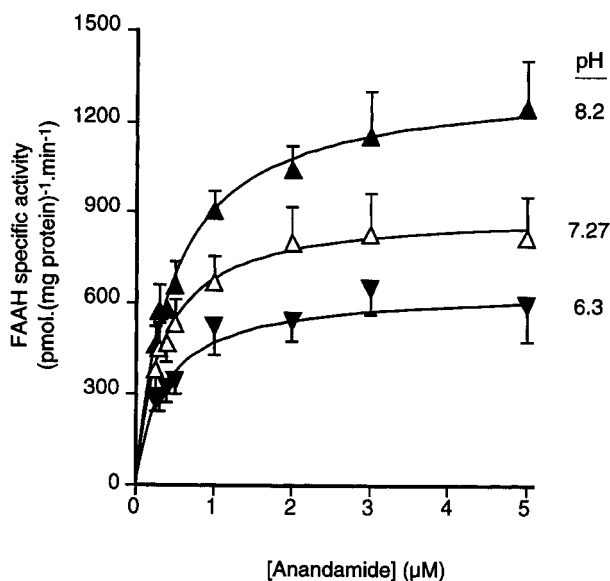
Figure 2 Hydrolysis of a range of concentrations of [^{3}H]-AEA by rat brain homogenates at different pH values. In each case, 50 mM tris-HCl was used as assay buffer. The pH values shown are the actual assay pH values, tested in scaled up assays, rather than the nominal pH of the buffers added to the homogenates. Shown are means $\pm$ s.e.mean of three experiments using an incubation time of 10 min and 2.5 μg protein/assay.

Two transfection strategies were used, one with Lipofectamine™ reagent and one with Lipofectamine2000™ reagent. Both strategies gave essentially identical data in terms of relative enzyme activities and pharmacological properties. Initial time-course experiments indicated that good expression levels of the enzymes, $\sim$20 μg lysate/assay of cells expressing wt, S152A and C249A FAAH being able completely to metabolise essentially all available substrate within 120 min of incubation (Figure 5a). Mock transfected cells showed very low, but measureable, [^{3}H]-AEA metabolizing activity. However, this potentially confounding factor was not seen when more diluted samples and shorter incubation

times were used (Figure 5b). A fourth variant, S218A, was also tested, but the activity was rather low and was therefore not used in subsequent experiments (Figure 5a,b). The relative activities of the mutants are in agreement with the original study of Omeir *et al.* (1999), although these authors required longer incubation periods to assess activity, presumably reflecting lower transfection efficiencies.

Using assay conditions where no relevant metabolism of [^{3}H]-AEA by mock transfected cells could be seen, the effects of OTMK and ($-$)ibuprofen upon wt, S152A and C249A FAAH were assessed at pH 6.1 and 8.0 (Figure 5c). The pattern seen for the rat brain was repeated, with OTMK being less potent and ($-$)ibuprofen more potent at the lower pH. There was no marked difference in the sensitivities to inhibition between the wt and mutant FAAH. Thus, the pIC_{50} values calculated from the mean data for wt, S152A and C249A FAAH, respectively, were OTMK pH 6.1 : 6.84, 6.79 and 6.84; OTMK pH 8.0 : 7.45, 7.55 and 7.50; ($-$)ibuprofen pH 6.1 : 4.19, 4.23 and 4.18; ($-$)ibuprofen, pH 8.0 : 3.15, 3.13 and 3.35.

Discussion

In the present study, the pharmacological properties of FAAH have been investigated at different pH values, since information is lacking in this respect. Consistent with previous studies using homogenates from cow brain (Omeir *et al.*, 1995) and membranes from rat brain (Hillard *et al.*, 1995), mouse N18TG2 neuroblastoma cells (Maurelli *et al.*, 1995), rat RBL2H3 and RBL-1 basophilic leukaemia cells (Bisogno *et al.*, 1997; Ueda *et al.*, 1999), purified recombinant rat FAAH (Patricelli *et al.*, 1998), and partially purified FAAH from pig brain microsomes (Ueda *et al.*, 1995), a single optimum at pH 8-9 was seen. The variation in activity, at least between pH 6.3 and 8.2, was due to a variation in V_{max} rather than K_M, indicating that in this range the variation does not merely reflect pH-dependent changes in the ionization state of the substrate but rather a change in the catalytic activity of the enzyme. Although the present study does not shed light into the molecular mechanisms giving rise
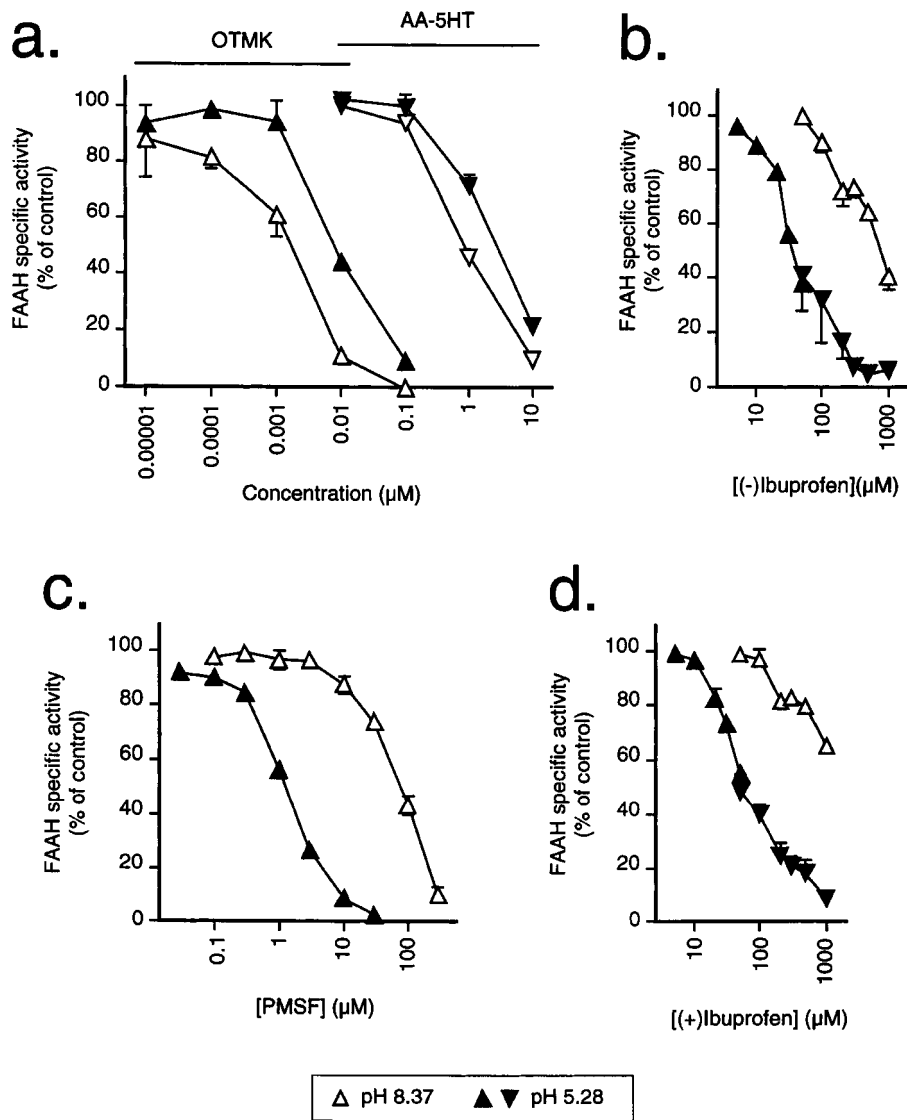
Figure 3 Inhibition of the hydrolysis of 2 μM [³H]AEA by rat brain homogenates by: (a) OTMK and AA-5HT; (b) (−)ibuprofen; (c) PMSF; and (d) (+)ibuprofen. Assays were performed either at pH 5.28 using 50 mM sodium citrate or at pH 8.37 using 50 mM tris-HCl as assay buffer. The pH values shown are the actual assay pH values, tested in scaled up assays, rather than the nominal pH of the buffers added to the homogenates. Shown are means $\pm$ s.e. mean, $n=3$ using an incubation time of 20 min and either 8 (pH 5.28) or 4 (pH 8.37) μg protein/assay. The two filled symbols shown in Panels (b) and (d) indicate data from separate experimental series at pH 5.28.

to the pH sensitivity of the enzyme, it has been reported that the pH profile found here has been seen in both wild type FAAH and mutant FAAH lacking the putative transmembrane region of the enzyme near the N-terminus (Patricelli et al., 1998). Thus, the large predicted cytoplasmic region of the molecule defines the pH sensitivity.

The lack of a peak at low pH values would suggest that in the rat brain homogenates used here, there is no sign of any significant expression of an acid amidase capable of the hydrolysis of AEA, such as that expressed in CMK cells (Ueda et al., 1999). It is of course possible that microsomal fractions contain appreciable acid amidase, and thereby give the biphasic pH curve seen by Desarnaud et al. (1995) who used a microsomal fraction from rat brain and the buffer system shown in Figure 1a. Appreciable expression of such

activity, however, would be expected to affect the observed sensitivity of microsomal [³H]-AEA metabolism to inhibition by PMSF relative to other subcellular fractions, given that the acid amidase activity found in CMK cells has a very low sensitivity to this inhibitor (Ueda et al., 1999). Such an anomalous sensitivity of microsomal fractions has not been seen (Tiger et al., 2000).

The pharmacological properties of FAAH at pH values near the optimum and at more acidic pH values is consistent for the enzyme activity in rat brain homogenates to that expressed following transfection of COS-7 cells with wt and mutant plasmids. In all cases, the potency of OTMK is lower at the lower pH, whereas the potency of (−)ibuprofen is higher. At both pH values tested, (−)ibuprofen acted as a mixed-type inhibitor, a result consistent with previous studies
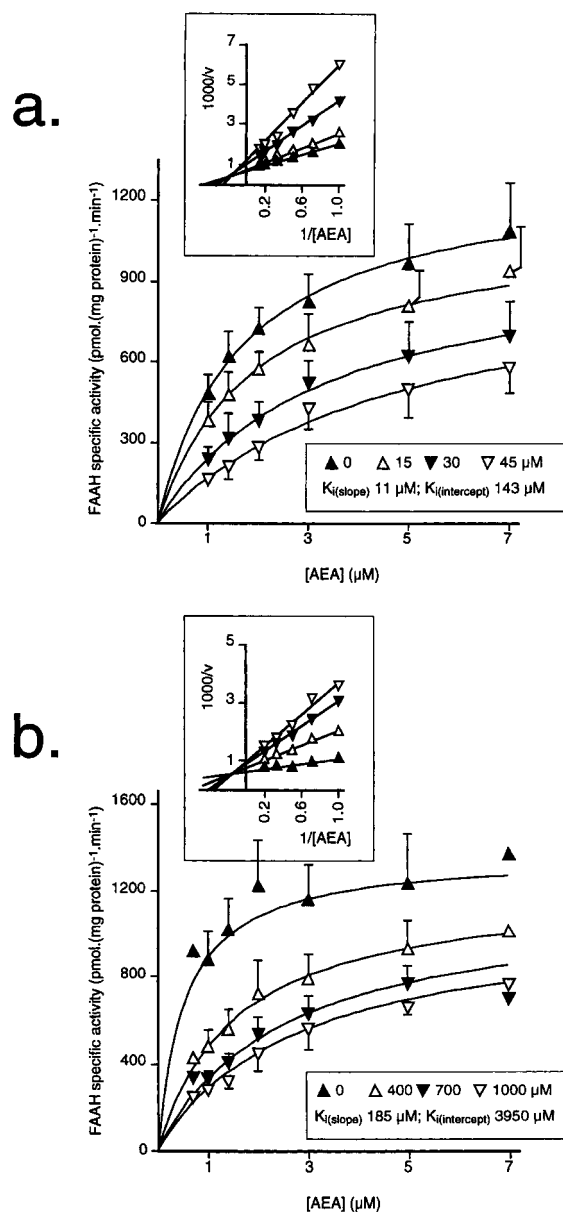
Figure 4 Mode of inhibition by (−)ibuprofen of the metabolism of [³H]-AEA by rat brain homogenates. The pH values (at assay) were: (a) pH 5.28 and (b) pH 8.37 using 50 mM sodium citrate and 50 mM tris-HCl, respectively, as assay buffers. Shown are means ± s.e.mean of three experiments using an incubation time of 10 min and 4 μg protein/assay. Inserts to the figures are secondary replots of the mean data to illustrate the mixed-type nature of the inhibition. The $K_{i(slope)}$ and $K_{i(intercept)}$ values were calculated as described in Methods.

at pH 7.6 using both this enantiomer and racemic ibuprofen (Fowler *et al.*, 1997; 1999).

In the brain samples, the pattern seen with (−)ibuprofen was also seen with its (+)-enantiomer (albeit with a slightly lower potency, consistent with previous data, Fowler *et al.*, 1999) and with PMSF, whereas the potency of AA-5HT behaved more like OTMK. The increased potency of PMSF at low pH may at least in part be a reflection of the better stability of this compound in acidic aqueous solutions than in alkaline solutions (James, 1978). In the case of ibuprofen, there is a report that basic solutions can promote the

racemization of ibuprofen enantiomers (Xie *et al.*, 2000). However, such racemization cannot account for the pH difference in potencies of the ibuprofen enantiomers, since the enantiomeric selectivity of ibuprofen for FAAH is rather small, and indeed would have been expected to result in an increase in the potency of the (+)enantiomer, since this is less potent than the racemate (Fowler *et al.*, 1999).

At first sight, the increased potency of the ibuprofen enantiomers at acid pH is at odds with the decreased potency of OTMK and AA-5HT. However, if it is argued that only the uncharged form of ibuprofen inhibits FAAH, an increased potency at acid pH would be predicted by the Henderson-Hasselbach equation. Indeed, this equation predicts a greater difference in potency than is actually found, which might reflect a general decrease in sensitivity of FAAH to all inhibitors at acid pH. This would be consistent with the data for OTMK and AA-5HT. This interpretation would predict that a plot of pIC$_{50}$ for (−)ibuprofen vs assay pH would be linear. Simple regression analysis of the mean pIC$_{50}$ values found here for the rat brain and wt FAAH inhibition by (−)ibuprofen at the four pH values tested indeed appeared linear ($r^2 = 0.979$) and predicted a pI$_{50}$ value at pH 7.6 of 3.42. This is in reasonable agreement with our previously published data at this pH using rat brain samples and 2 μM [³H]-AEA as substrate where pI$_{50}$ values in the range 3.54 – 3.80 were found (Fowler *et al.*, 1999; 2000).

In conclusion, the present data would indicate (a) that there is no significant expression in brain homogenates (relative to FAAH) of an acid amidase such as is found in CMK cells; (b) that the sensitivities to inhibition by OTMK and (−)ibuprofen for two active FAAH mutants, S152A and C249A FAAH are similar to the wild type enzyme; (c) that the uncharged form of ibuprofen is presumed to account for its inhibitory potency towards FAAH. This latter observation raises a possibility previously discussed (Fowler *et al.*, 1997; 1999) that FAAH inhibition may contribute to the antiinflammatory properties of this compound *in vivo*, given (a) that certain inflammatory stimuli produce an increased synthesis of AEA and palmitoylethanolamide, both of which have beneficial effects upon inflammatory pain (see Introduction) and (b) that the peak plasma concentration of ibuprofen following oral administration of 2 × 200 mg ibuprofen tablets is in the range 110 – 150 μM (Karttunen *et al.*, 1990). This compares with the IC$_{50}$ values for (−) and (+)ibuprofen of 42 and 70 μM (calculated from the mean pI$_{50}$ values in Table 1) at pH 5.28. Although this pH value is admittedly low (the IC$_{50}$ value for racemic ibuprofen at pH 7.6 is 270 μM (Fowler *et al.*, 1997)), there is some data suggestive of a reduced intracellular pH in certain inflammatory conditions in addition to the well-documented changes in extracellular pH. Andersson *et al.* (1999) reported a reduction in both tissue and intracellular pH in periarticular soft tissue from arthritic knee joints of rats relative to the contralateral control joints following antigen-induced arthritis. It is tempting to speculate that under such conditions, the reduced pH$_i$ may result not only in an impaired ability of FAAH to metabolise AEA *per se* (on the basis of the pH optimum curve), but also in an increased sensitivity of FAAH to inhibition by ibuprofen so that significant inhibition can be produced *in vivo* following administration of this compound at the high doses generally used by patients with rheumatoid arthritis.
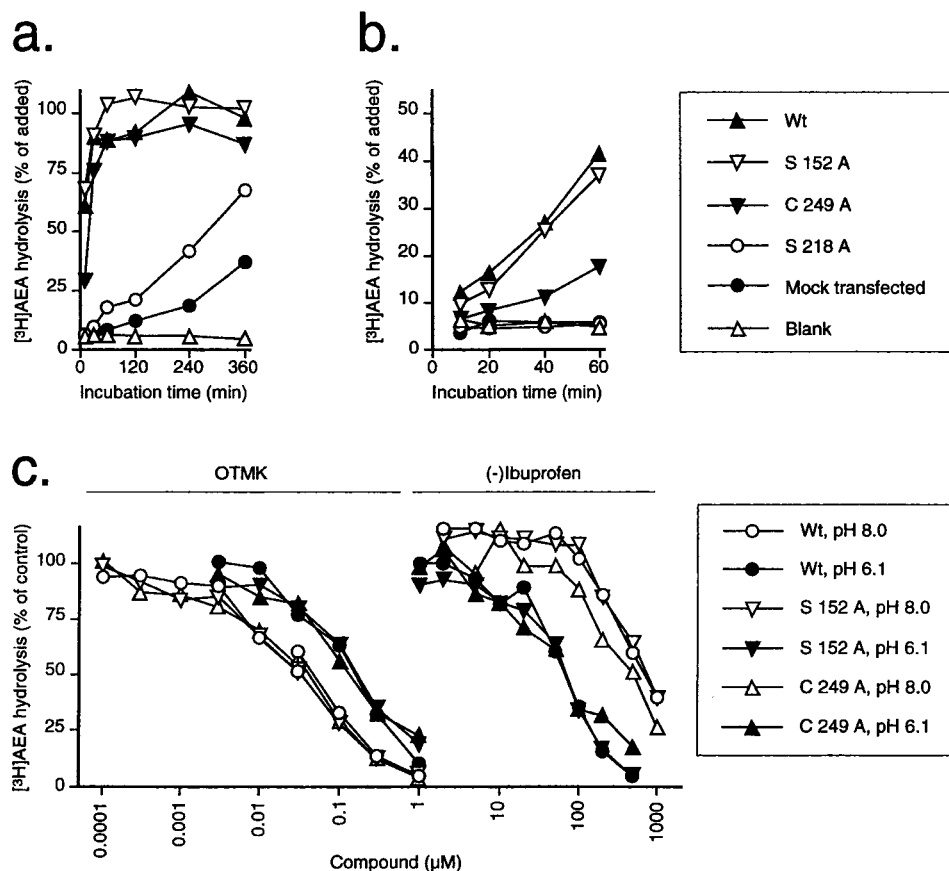
Figure 5 Properties of wt and mutant FAAH expressed in COS cells using 2 μM [^{3}H]-AEA as substrate (a) and (b) show mean data from single experiments using lysates from cells transfected using the Lipofectamine^{TM} reagent; where the assay protein concentration of the samples in (a) was set to $\sim$20 and $\sim$1.5 μg in (b). The assay buffer used was 10 mM Tris HCl, pH 8.0 containing 1 mM EDTA. In (c), the inhibition of 2 μM [^{3}H]-AEA metabolism by OTMK and ($-$)ibuprofen is shown. The pH values (at assay) were pH 6.1 and pH 8.0 using 10 mM tris-HCl containing 1 mM EDTA as assay buffer in both cases. Shown are means of two experiments using one set of samples from cells transfected using the Lipofectamine^{TM} reagent and one set using cells transfected using the Lipofectamine2000^{TM} reagent using incubation times of 30 min and the appropriate protein contents to ensure that initial velocities were measured.

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