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Pharmacological Properties of Rat Brain Fatty Acid Amidohydrolase in Different Subcellular Fractions Using Palmitoylethanolamide as Substrate

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ABSTRACT. In the present study, the pharmacological properties of fatty acid amide hydrolase (FAAH) in subcellular fractions of rat brain were investigated using palmitoylethanolamide (PEA) and arachidonyl ethanolamide (anandamide, AEA) as substrates. FAAH hydrolysed [^{3}H]PEA in crude homogenates with median K_m and V_{max} values of 2.9 μM and 2.14 nmol.(mg protein)$^{-1}$.min^{-1}, respectively. [^{3}H]PEA hydrolysis was inhibited both by non-radioactive AEA (with a K_i value very similar to the K_m value for [^{3}H]AEA as substrate using the same assay) and by $R(-)$ibuprofen (mixed-type inhibition K_i and K_i' values 88 and 720 μM, respectively). FAAH activity towards both [^{3}H]PEA and [^{3}H]AEA was in the order microsomal > synaptosomal = mitochondrial > crude nuclear > myelin = cytosol, but there were no differences between the relative activities towards the two substrates in any of the fractions. [^{3}H]PEA hydrolysis in mitochondrial, myelin, microsomal, and synaptosomal fractions was inhibited by oleyl trifluoromethylketone, phenylmethylsulphonyl fluoride, and the $R(-)$- and $S(+)$-enantiomers of the nonsteroidal anti-inflammatory drug ibuprofen, with mean IC_{50} values in the ranges 0.028–0.041, 0.37–0.52, 67–110, and 130–260 μM, respectively. It is concluded that the pharmacological properties of FAAH in the different subcellular fractions are very similar. BIOCHEM PHARMACOL **59**;6:647–653, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. palmitoylethanolamide; anandamide; fatty acid amide hydrolase; ibuprofen; rat brain; subcellular fractions

In 1993, Deutsch and Chin [1] described the enzymatic degradation of the endogenous cannabinoid receptor agonist AEA† by an amidase activity in glioma and neuroblastoma cells. The amidase responsible, FAAH (EC 3.5.1.4, also known as anandamide amidase and anandamide amidohydrolase), has a deduced 579-amino-acid sequence [2] and is able to metabolise a wide range of fatty acid amides including the sleep-inducing amide oleamide and the anti-inflammatory agent PEA [2–6]. In the brain, FAAH is preferentially located in large neurons in the CNS [7], and the level of activity across the brain correlates reasonably well with the density of CB1 receptors [8; see also Reference 9]. Within brain cells, FAAH activity is found associated with synaptosomal, mitochondrial, myelin, and microsomal fractions, but not in the cytosol [6, 8, 10–12]. Although dramatic differences in relative substrate specificities are not seen in microsomal and 10,000 × *g* pellet fractions from mouse N_{18} neuroblastoma cells [3], relatively little is known about the pharmacological properties of FAAH in the different subcellular fractions. It is possible, for example, that isoforms of FAAH with different subcellular localisations and different pharmacological properties exist. In addition, most of the known pharmacology of FAAH has been worked out using AEA as substrate. In consequence, the pharmacological properties of FAAH have been investigated in subcellular fractions of rat brain, using PEA as substrate.

MATERIALS AND METHODS

Materials

[^{3}H]PEA and [^{3}H]AEA (both specific activity 30 Ci/mmol) were custom synthesised by American Radiolabelled Chemicals Inc. [^{14}C]Tryptamine bisuccinate was obtained from the same company. Non-radioactive anandamide and the enantiomers of ibuprofen (α-methyl-4-(2-methylpropyl)benzeneacetic acid) were obtained from Research Biochemicals International. OTMK was obtained from Cayman Chemical Co. Non-radioactive PEA, PMSF, and fatty acid-free BSA were obtained from the Sigma Chemical Co. OTMK, PMSF, and ibuprofen were dissolved in ethanol prior to use. The compounds were diluted with ethanol and compared with controls

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† *Abbreviations:* PEA, palmitoylethanolamide; AEA, arachidonyl ethanolamide or anandamide; FAAH, fatty acid amide hydrolase; OTMK, oleyl trifluoromethylketone; and PMSF, phenylmethylsulphonyl fluoride.

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containing the same concentration of ethanol (25 μL in a 200-μL assay volume, unless otherwise stated).

Subcellular Fractionation

A standard subcellular fractionation procedure was used [13]. Briefly, brains (minus cerebellum) from adult rats were homogenised in 0.32 M sucrose using a Braun Melsungen homogeniser, and the homogenate ("crude homogenate") was centrifuged at 1000 *g* for 10 min at 4° to give a pellet (P_1, "crude nuclear fraction") and a supernatant, S_1. S_1 was centrifuged at 13,000 *g* for 10 min at 4° to give a pellet, P_2, and a supernatant, S_2. The latter was further centrifuged at 100,000 *g* for 30 min at 4° to give a supernatant (S_3, "cytosol"), and a pellet (P_3, "microsomes"), which was resuspended in 10 mM Tris–HCl (pH 7.6) containing 1 mM EDTA. The P_2 fraction was resuspended in 0.32 M sucrose, layered on top of a discontinuous sucrose density gradient (0.8, 1.0, and 1.2 M), and centrifuged at 53,500 *g* for 60 min at 4°. The myelin, synaptosomal, and mitochondrial fractions were collected. These fractions were diluted to a 0.32 M sucrose concentration with distilled water, centrifuged at 37,000 *g* for 20 min at 4°, and resuspended in 10 mM Tris–HCl (pH 7.6) containing 1 mM EDTA. All fractions were stored in aliquots at −70° until used for assay.

Assay of [^{3}H]PEA and [^{3}H]AEA Hydrolysis

A method based upon that of Omeir *et al.* [14] was used to assay the hydrolysis of [^{3}H]PEA and [^{3}H]AEA in the homogenates [15]. Briefly, the samples were incubated in the absence or presence, as appropriate, of either OTMK, PMSF, (+)ibuprofen, (−)ibuprofen, and [^{3}H]PEA or [^{3}H]AEA. The assay volume was 200 μL (25 μL of the inhibitors dissolved in ethanol, 25 μL radioactive substrate, 150 μL homogenate). The substrate solutions contained 10 mg/mL fatty acid-free BSA. Unless otherwise stated, substrate assay concentrations were 2 μM. Reactions were stopped by the addition of chloroform:methanol (1:1 v/v). The samples were vortex-mixed, and the aqueous layer containing the [^{3}H]ethanolamine product was collected and assayed for tritium content by liquid scintillation spectroscopy with quench correction. Blanks were determined in the presence of 1.5 mM PMSF.

Marker Enzyme Assays

Monoamine oxidase activity was assayed essentially according to Otsuka and Kobayashi [16] with [^{14}C]tryptamine bisuccinate as substrate. Acetylcholinesterase was assayed by the method of Augustinsson *et al.* [17], as modified for use in a microtitre plate format [18]. 5′-Nucleotidase was assayed as described by Cammer *et al.* [19], with measurement of phosphate by a modification of the method of Fiske and SubbaRow [20]. Lactate dehydrogenase activity was measured using a cytotoxicity detection kit (Roche Molecular Biochemicals).

Determination of IC_{50}, K_m, and V_{max} Values

IC_{50} values from mean inhibition data (in the 9–91% contiguous % activity remaining range, to avoid bias at high and low levels of inhibition) were plotted as $\log_{10}$(inhibitor concentration) versus $\log_{10}$((100-% activity remaining)/% activity remaining). From the regression lines, pI_{50} and hence IC_{50} values were determined. K_m and V_{max} values were calculated using the direct linear plot analysis of Eisenthal and Cornish-Browden [21] using the Enzyme Kinetics v 1.4 software package, Trinity Software.

RESULTS

Kinetic Properties of [^{3}H]PEA Hydrolysis

Initial experiments using a mitochondrial fraction demonstrated that hydrolysis of 2 μM [^{3}H]PEA was time- and protein concentration-dependent, and was inhibited by 1.5 mM PMSF. Data for a mitochondrial fraction showed that the product recovery is a linear function of the enzyme sample concentration at least up to a concentration of 20 μg/assay following an incubation time of 10 min (Fig. 1A). In the presence of PMSF, there was no increase in product recovery with time, indicating that accurate blank values were measured. At a protein concentration of 20 μg/assay, an initial linear relationship between incubation time and product formation was clearly seen for all subcellular fractions (Fig. 1B). Using an incubation time of 10 min and at a protein concentration of 10 μg/assay, the dpm. recovered in crude homogenates (after subtraction of blank and compensation for the fact that only 50% of the total product was counted for radioactivity) at this substrate concentration was 20 ± 2% of the total radioactivity added (means ± SEM, N = 9). This value is 2.9 ± 0.3-fold higher than the corresponding blank values.

Direct linear plot analyses of 9 individual experiments with crude homogenates using [^{3}H]PEA concentrations in the range 1–10 μM gave K_m and V_{max} values of 2.9 (1.3–8.5) μM and 2.14 (1.41–4.09) nmol.(mg protein)$^{-1}$.min^{-1}, respectively (medians, with ranges in parentheses, N = 9). In a subsequent series, it was found that the V_{max} values towards [^{3}H]PEA and [^{3}H]AEA were 2.49 (1.77–2.68) and 1.73 (1.68–2.15) nmol.(mg protein)$^{-1}$.min^{-1}, respectively (medians, with ranges in parentheses, N = 3). The corresponding K_m values were 5.0 (3.9–5.5) and 2.6 (2.0–3.3) μM, respectively (data not shown). At a substrate concentration of 2 μM, the hydrolysis of [^{3}H]PEA was inhibited by non-radioactive AEA. Thus, mean (± SEM, N = 4–6) [^{3}H]PEA hydrolysis as % of control was 49 ± 7, 31 ± 6, 22 ± 5, and 17 ± 4 for 1, 2, 5, and 10 μM non-radioactive AEA, respectively. The inhibition of [^{3}H]PEA hydrolysis by 1 μM AEA was competitive in nature, with a K_i value of 0.6 μM being calculated from a secondary replot of the mean data (Fig. 2A). This value is very similar to the K_m value for [^{3}H]AEA as a substrate for rat brain FAAH

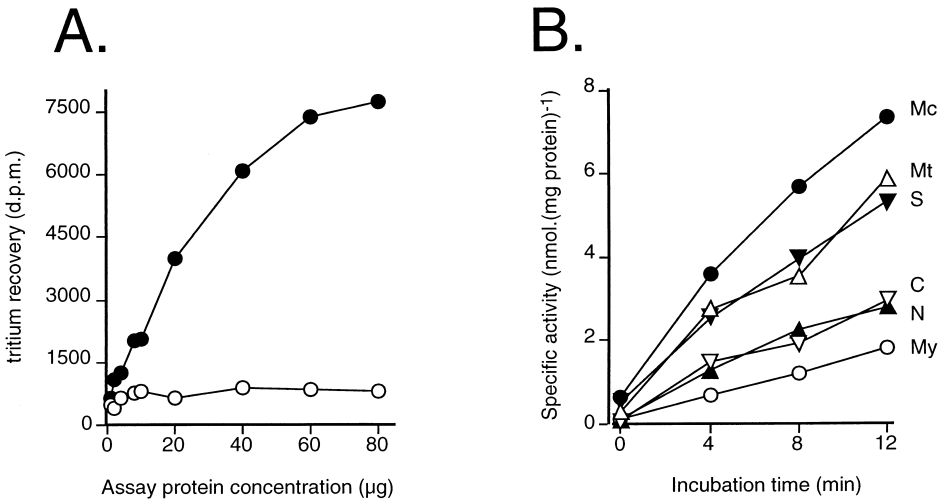

FIG. 1. (Panel A) Dependence of [^{3}H]PEA hydrolysis upon assay protein concentration. Samples were incubated for 10 min at 37° with 2 μM [^{3}H]PEA in the presence of either ethanol carrier (●, 25 μL of a 1:1 v:v ethanol/buffer mix was added) or 1.5 mM PMSF (○). Shown are means of triplicate determinations of the recovered tritium in a single mitochondrial preparation. A linear regression of the activities for the ethanol carrier minus the corresponding PMSF values for the six lowest protein concentrations (i.e. ≤20 μg) gave a slope of 154 dpm/μg protein and an r^2 value of 0.96. This slope corresponds to a specific activity of 0.69 nmol.(mg protein)$^{-1}$.min^{-1}. (Panel B) Dependence of [^{3}H]PEA hydrolysis upon incubation time. Mean specific activities of three separate determinations of single preparations of microsomes (●, Mc), mitochondria (△, Mt), synaptosomes (▼, S), cytosol (▽, C), crude nuclear (▲, N), and myelin (○, My) are shown. The assay protein concentration was in all cases 20 μg.

using the same assay conditions [9, 15, see above]. At a higher concentration (2 μM) of AEA, the inhibition of [^{3}H]PEA hydrolysis appeared to be of mixed type, with K_i and K_i' values of 0.8 and 1.6 μM, respectively, being found (Fig. 2B). *R*(−)Ibuprofen inhibited [^{3}H]PEA hydrolysis in a mixed fashion, with K_i and K_i' values of 88 and 720 μM, respectively (Fig. 2C).

Activity of FAAH towards PEA and AEA in Brain Subcellular Fractions

The activity of FAAH towards 2 μM [^{3}H]PEA and [^{3}H]AEA in the subcellular fractions was in the order microsomal > synaptosomal = mitochondrial > crude nuclear > myelin = cytosol (Fig. 3A). The mitochondrial

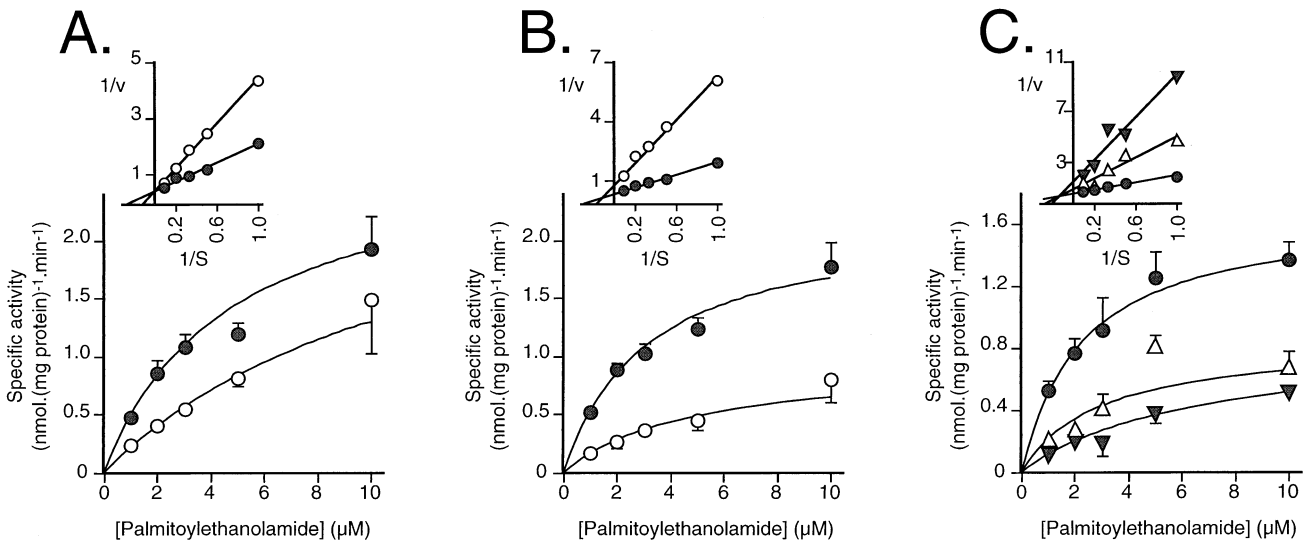

FIG. 2. Hydrolysis of [^{3}H]PEA in rat brain (minus cerebellum) homogenates. Activities were measured: (A) in the absence (●) and presence (○) of 1 μM AEA; (B) in the absence (●) and presence (○) of 2 μM AEA; and (C) in the absence (●) and presence of 200 μM (△) or 500 μM (▼) *R*(−)ibuprofen. Data are means ± SEM (unless enclosed by the symbols), N = 3–6. Secondary replots of the mean values as 1/v versus 1/S are shown in the insets.

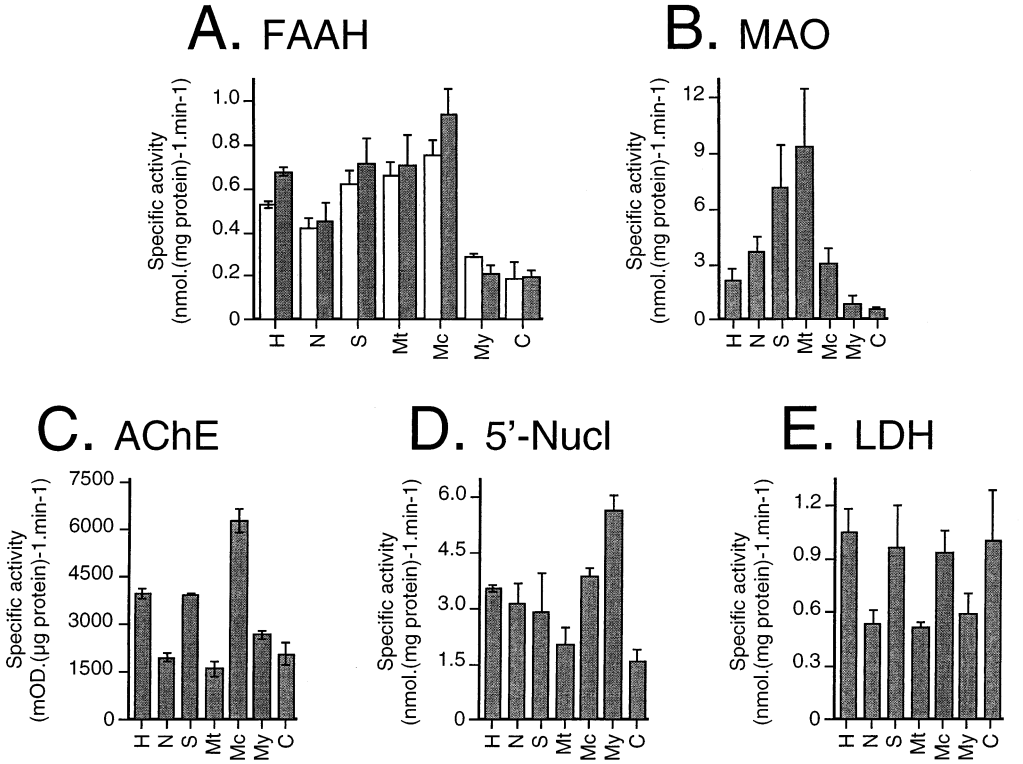

FIG. 3. The activities of (A) FAAH towards [^{3}H]AEA (unfilled columns) and [^{3}H]PEA (filled columns); (B) monoamine oxidase (MAO); (C) acetylcholinesterase (AChE); (D) 5′-nucleotidase (5′-Nucl); and (E) lactate dehydrogenase (LDH) in crude homogenate (H), crude nuclear (N), synaptosomal (S), mitochondrial (Mt), microsomal (Mc), myelin (My), and cytosolic (C) fractions derived from rat brain (minus cerebellum). Data are means ± SEM, N = 3; or means + data spread, N = 2 (where two bars are shown).

fractions showed the highest monoamine oxidase activity, the microsomal fractions the highest acetylcholinesterase activity, and the myelin fractions the highest 5′-nucleotidase activity (Fig. 3, B–E). Spearman rank correlations for the FAAH activity versus marker enzyme activity using the means of the two sets of fractions (excluding crude homogenates) where all marker enzymes were assayed gave *P* values for [^{3}H]PEA of 0.18, 0.57, 0.57, and 0.75 versus monoamine oxidase, acetylcholinesterase, 5′-nucleotidase, and lactate dehydrogenase, respectively. Similar *P* values were found for [^{3}H]AEA. The *P* value for PEA versus AEA was 0.025 (the highest significance obtainable for this sample size).

In order to rule out the possibility that the differences in FAAH activity between microsomes, synaptosomes, and mitochondria towards [^{3}H]PEA as substrate were due to differences in the relative amounts of endogenous PEA (or other competing fatty acid amides) in the subcellular fractions, K_m and V_{max} values were determined from activity measurements at six substrate concentrations (1, 2, 3, 5, 10, and 20 µM). There was no obvious difference in the K_m values between the regions, which would have been

TABLE 1. Pharmacological properties of FAAH assayed with [^{3}H]PEA in four subcellular fractions of the rat brain

	Synaptosomes	Mitochondria	Microsomes	Myelin
K_m (µM)	3.2 (0.7–4.6)	2.0 (1.7–3.4)	3.0 (2.2–4.1)	ND
V_{max}*	2.92 (0.70–3.08)	2.26 (0.48–3.18)	2.60 (1.72–3.48)	ND
IC_{50} (µM) for:				
OTMK	0.028 ± 0.004	0.030 ± 0.005	0.041 ± 0.003	0.034 ± 0.019
PMSF	0.46 ± 0.039	0.52 ± 0.066	0.52 ± 0.12	0.37 ± 0.089
R(−)ibuprofen	110 ± 3.7	110 ± 8.1	97 ± 3.9	67 ± 8.0
S(+)ibuprofen	200 ± 10	250 ± 22	260 ± 66	130 ± 21

For K_m and B_{max} values, data are means (with ranges in parentheses), N = 3. IC_{50} values were calculated from individual inhibition curves from determinations for 3–4 separate experiments carried out on 2 preparations. In a few cases, accurate IC_{50} values could not be determined due to <2 data points within the 9–91% inhibition range; these data have been excluded. ND, K_m and V_{max} values could not accurately be determined due to unfavourable ratios of specific activities: blank values at high substrate concentrations. The high blank values at these concentrations relative to the specific activities mean that small variations in blank cause great variations in the observed specific activity.

*nmol.(mg protein)$^{-1}$.min^{-1}.

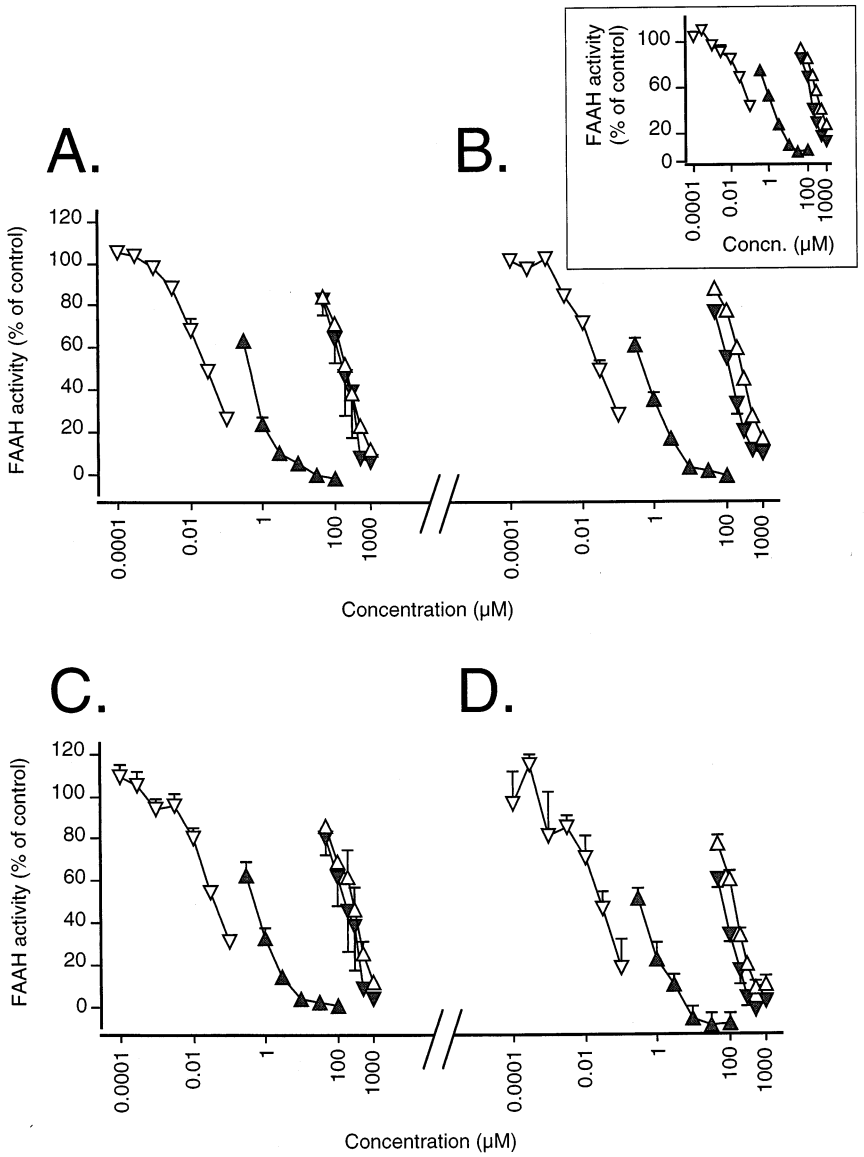

FIG. 4. Inhibition of 2 μM [^{3}H]PEA hydrolysis by OTMK (▽), PMSF (▲), *R*(−)ibuprofen (▼), and S(+)ibuprofen(△) in (A) synaptosomal, (B) mitochondrial, (C) microsomal, and (D) myelin fractions. Shown are means + SEM (unless enclosed by the symbol) for 3–4 separate experiments carried out on 2 preparations. In the inset, a single experiment (except for OTMK, where N = 3) showing inhibition of 2 μM [^{3}H]AEA hydrolysis in a mitochondrial fraction is presented.

expected if different amounts of competing PEA had been present (Table 1).

Inhibition of PEA Hydrolysis in Brain Subcellular Fractions

OTMK, PMSF, and *R*(−)- and *S*(+)ibuprofen inhibition of [^{3}H]PEA hydrolysis was studied in microsomal, synaptosomal, mitochondrial, and myelin fractions (Fig. 4). The order of potency of the compounds was OTMK ≫ PMSF ≫ *R*(−)ibuprofen > *S*(+)ibuprofen. No striking differences in potencies of the inhibitors between the fractions were seen (Table 1).

DISCUSSION

Most of the studies into the properties of FAAH have utilised AEA as substrate, and relatively little is known concerning the metabolism of PEA, despite the fact that an amidase capable of metabolising this substrate was suggested as early as 1984 [5]. In addition, the only reports of inhibition of PEA hydrolysis are those of Bisogno *et al.* [22, 23] (ovaries from *Paracentrotus lividus* and cultured RBL-2H3 basophilic leukemia cells), where single concentrations of inhibitors such as PMSF and arachidonyl trifluoromethylketone were used. Even with respect to AEA, where the inhibitory properties of the compounds used in the present study have been well documented [1, 8, 9, 15]

and shown not to vary to any great degree from brain region to brain region [9], data on inhibitor effects in subcellular fractions have been restricted to investigations either on the total particulate fraction or upon the microsomal activity [see e.g. 3, 11, 12, 22, 23]. There is thus a need to characterise the pharmacological properties of FAAH in different subcellular fractions.

There is some confusion in the literature as to the efficacy of PEA as a substrate for FAAH in rat microsomes: Desarnaud *et al.* [11] found that the rate of hydrolysis of PEA was only 1% of that for anandamide, whereas Qin *et al.* [6] found a corresponding relative rate of 82%. In all subfractions, we found PEA to be metabolised at a similar rate to AEA. In addition, the finding in crude homogenates that the hydrolysis of [^{3}H]PEA was inhibited competitively by 1 μM anandamide is in agreement with the literature [23] and is consistent with the hypothesis that [^{3}H]PEA is hydrolysed by the same FAAH as metabolises [^{3}H]AEA. This conclusion is strengthened by the finding that [^{3}H]PEA hydrolysis was inhibited in a mixed fashion by (−)ibuprofen, as a similar mode of inhibition and a similar potency were reported previously with [^{3}H]AEA as substrate [9]. The apparent mixed-type inhibition found with 2 μM AEA is unlikely to reflect a build-up of the small amount of arachidonic acid produced by metabolism of the competing AEA during the incubation period since, although arachidonic acid is capable of inhibition of FAAH activity [6], the potency is very week. It should be pointed out, however, that for the inhibition of [^{3}H]PEA by AEA, the difference between mixed and competitive was rather small and may reflect experimental error, particularly since the blank values were relatively high with respect to the specific activities at high substrate concentrations.

The metabolism of [^{3}H]PEA and [^{3}H]AEA was studied in brain subcellular fractions. Consistent with the literature [6, 8, 10–12], the bulk of the activity was membrane-bound. The relative rates of [^{3}H]AEA (and [^{3}H]PEA) metabolism (microsomes > synaptosomes = mitochondria > crude nuclear fraction > myelin = cytosol) are rather consistent with the study of Hillard *et al.* [8], who used the same fractionation procedure and a TLC methodology, although the specific activity in the myelin fraction was lower in the present study. Since myelin and microsomal fractions are sedimented at higher speeds (or sediment to a lesser extent at a given speed) than mitochondria, it is possible in theory that the FAAH activity in these fractions may be at least partly due to contamination by FAAH from mitochondrial fragments. However, the activity of the mitochondrial outer membrane marker enzyme monoamine oxidase was rather low in these fractions, suggesting this not to be a problem here. The lack of significant correlation with the marker enzymes would suggest that, although the highest activity was found in the microsomal fraction, FAAH has more than one primary subcellular localisation. Interestingly, a better correlation was found with the mitochondrial enzyme monoamine oxidase than with acetylcholinesterase, which shows the highest specific activity in the microsomes [24, 25].

The main aim of the study was to determine the properties of FAAH recovered in the different subcellular fractions. The data clearly indicate that the relative rates of AEA hydrolysis in the fractions are similar to those for PEA. Furthermore, there is no subcellular variation in the sensitivity of FAAH to inhibition by ibuprofen enantiomers, OTMK, and PMSF. The Hill slopes of the inhibitions were, in addition, reasonably near unity. This would suggest that either the same FAAH isoform is found in the different subfractions, or, alternatively, that FAAH isoforms have similar properties with respect to the substrates and inhibitors tested.

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