



Differences in the pharmacological properties of rat and chicken brain fatty acid amidohydrolase

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1 The pharmacological properties of fatty acid amidohydrolase (FAAH) were investigated in brains of 35-day-old chickens, since nothing is known about the enzyme in avian species.

2 FAAH activity towards both [³H]-palmitoylethanolamide (PEA) [$K_M = 1.5 \mu\text{M}$] and [³H]-anandamide (AEA) [$K_M = 5.4 \mu\text{M}$] was demonstrated in the chicken brains. The chicken FAAH was inhibited by the substrate analogues oleyl trifluoromethylketone (OTMK) and diazomethylarachidonyl ketone (DAK) with similar potencies to the rat FAAH. However, in contrast to the rat brain, phenylmethanesulphonyl fluoride (PMSF) and the enantiomers of ibuprofen had very weak effects on chicken brain FAAH.

3 Indomethacin and niflumic acid were found to inhibit rat brain AEA hydrolysis. The inhibition by indomethacin was reversible and competitive, with a K_i value of $120 \mu\text{M}$. Chicken FAAH was less sensitive to indomethacin than its rodent counterpart, but the inhibition was also competitive (K_i).

4 It is concluded that chicken FAAH activity has different pharmacological properties to its rodent counterpart.

British Journal of Pharmacology (2000) **131**, 498–504

Keywords: Anandamide; palmitoylethanolamide; fatty acid amidohydrolase; ibuprofen; indomethacin

Abbreviations: AEA, anandamide; DAK, diazomethylarachidonyl ketone; FAAH, fatty acid amidohydrolase, NSAID, non-steroidal anti-inflammatory drug; OTMK, oleyl trifluoromethylketone; PEA, palmitoylethanolamide; PMSF, phenylmethanesulphonyl fluoride.

Introduction

Anandamide (AEA, arachidonyl ethanolamide) and palmitoylethanolamide (PEA) belong to a class of biologically active endogenous fatty acid amides. AEA is an agonist at cannabinoid CB₁ and CB₂ receptors (for review, see Di Marzo *et al.*, 1998) and has recently been shown to activate vanilloid receptors (Zygmunt *et al.*, 1999). PEA has been shown to prevent mast cell activation *in vitro* (Facci *et al.*, 1995) and to reduce inflammatory pain *in vivo* (Mazzari *et al.*, 1996; Calignano *et al.*, 1998; Jaggar *et al.*, 1998). Both AEA and PEA are metabolized by fatty acid amidohydrolase (FAAH, EC 3.5.1.4, also known as anandamide amidase and anandamide amidohydrolase), which in the rat brain has a cellular localization complementary, albeit with little or no actual co-localization, to the CB₁ receptors (Hillard *et al.*, 1995; Egertová *et al.*, 1998), and is located in large neurons (Tsou *et al.*, 1998). FAAH expression by non-neuronal epithelial cells has also been reported for the choroid plexus (Egertová *et al.*, 2000). The enzyme is inhibited by substrate analogues, such as oleyl trifluoromethyl ketone (OTMK) and diazomethylarachidonyl ketone (DAK) (Cravatt *et al.*, 1996; Edgemond *et al.*, 1998; see also Koutek *et al.*, 1994; Boger *et al.*, 1999), as well as by phenylmethanesulphonyl fluoride (PMSF) (Deutsch & Chin, 1993) and by various non-steroidal anti-inflammatory agents such as ibuprofen, ketoprofen and flurbiprofen (Fowler *et al.*, 1997a,b; 1999).

Most of our present knowledge concerning the pharmacological properties of FAAH has been derived from studies in mammalian species, and nothing is known as to their equivalents in avian species. In consequence, in the present

study, we have investigated the pharmacological properties of chicken brain FAAH.

Methods

Materials

DAK (diazomethylarachidonyl ketone) was a kind gift from Dr Cecilia Hillard, Medical College of Wisconsin, U.S.A. Palmitoylethanolamide-[1-³H] ([³H]PEA) and arachidonylethanolamide-[1-³H] ([³H]AEA) (both specific activity 30 Ci mmol^{-1}), were obtained from American Radiolabelled Chemicals Inc., St. Louis, MO, U.S.A. The enantiomers of ibuprofen (α -methyl-4-(2-methylpropyl) benzeneacetic acid) were obtained from Research Biochemicals International, Natick, MA, U.S.A. Niflumic acid (2-[[3-(trifluoromethyl)phenyl]-amino]-3-pyridinecarboxylic acid) was obtained from the Alexis Corp., San Diego, CA, U.S.A. PMSF (phenylmethanesulphonyl fluoride), non-radioactive PEA, indomethacin and fatty acid free bovine serum albumin were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Non radioactive AEA was obtained from Tocris-Cookson, Bristol, U.K. Oleyl trifluoromethylketone (OTMK) was obtained from the Cayman Chemical Company, Ann Arbor, MI, U.S.A. The compounds were dissolved in ethanol.

Preparation of chicken and rat brain homogenates and membranes

Frozen skulls from 35-day-old Ross chickens were obtained from Kronfågél AB, Kristianstad, Sweden. Upon thawing, the brains were removed and weighed. Cerebral cortices obtained

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from adult Sprague-Dawley rats (Umeå University) stored at -20°C , were thawed on ice and weighed. For the experiments shown in Figures 1–3, brains were homogenized in 10 mM Tris-HCl, pH 7.6 containing 1 mM EDTA, and aliquots stored frozen at -70°C . For the experiments shown in Figures 4 and 5, the brain tissues were homogenized at 4°C in homogenization buffer (20 mM HEPES pH 7.0 + 1 mM MgCl_2) using an Ultra-Turrax or glass homogenizer. These tissue homogenates were centrifuged at $36,000 \times g$ for 20 min at 4°C . The pellets were resuspended in 20 ml homogenization buffer and recentrifuged again at $36,000 \times g$ for 20 min at 4°C . Pellets were resuspended in 10 ml homogenization buffer and incubated at 37°C for 15 min. After recentrifugation at $36,000 \times g$ for 20 min at 4°C , pellets were resuspended in homogenization buffer to a final concentration of 200 mg wet weight ml^{-1} . Membrane preparations were stored in microcentrifuge tubes at -20°C until used for assay. This protocol was used partly to allow concomitant measurement of cannabinoid receptor density in the samples (results to be presented elsewhere), but also to allow the use of a membrane preparation which was necessary for the reversibility studies shown in Figure 4.

Assay of FAAH activity in chicken and rat brain homogenates

FAAH was assayed essentially as described by Omeir *et al.* (1995) who used carbon-14 labelled AEA as substrate (for full descriptions using the present tritiated substrates, see Fowler *et al.*, 1997b; Tiger *et al.*, 2000). Briefly, assay mixtures contained homogenate (10–60 μg protein assay^{-1}), test compound (when appropriate) (25 μl), and radiolabelled AEA or PEA (25 μl , containing 10 mg ml^{-1} fatty acid-free bovine serum albumin). 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 either PMSF (final concentration 1.5 mM) or distilled water instead of homogenate, as indicated. The mixtures were incubated at 37°C for 10 min (unless otherwise stated) after which reactions were stopped by placing the tubes in ice and adding 400 μl of chloroform:methanol (1:1 v v $^{-1}$). The tubes were vortex mixed, after which the phases were separated by centrifugation in a bench centrifuge. Aliquots (200 μl) of the methanol/buffer phase were removed for analyses of radioactivity by liquid scintillation spectroscopy with quench correction.

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

Determination of K_m , V_{\max} and pI_{50} values

K_m and V_{\max} values for FAAH activities were calculated using the Direct Linear Plot analysis of Eisenthal & Cornish-Bowden (1974) using the Enzyme Kinetics v 1.4 software package, Trinity Software, Campton, NH, U.S.A. Given that this analysis is non-parametric in nature, K_M and V_{\max} values have been given as medians and ranges. pI_{50} values were calculated by plotting data (in the 9–91% contiguous per cent activity remaining range, to avoid bias at high and low levels of inhibition) as \log_{10} (inhibitor concentration) vs \log_{10} (100% activity remaining) per cent activity remaining $^{-1}$). From the regression lines, pI_{50} values were determined.

Results

Assay of FAAH activity in the chicken and rat brain homogenates

Initial experiments were undertaken to determine the optimal conditions for assay of initial velocities. In the presence of chicken brain homogenate (10 μg assay^{-1}), a linear increase in the metabolism of both PEA (Figure 1A) and AEA (Figure 1B) over 20 min was found. No such increase was seen when distilled water was used in place of the homogenates. In general, PMSF (1.5 mM) is used to define blank values, since this concentration is much higher than its reported IC_{50} value (in the range of 3–25 μM ; Hillard *et al.*, 1995; Desarnaud *et al.*, 1995; Fowler *et al.*, 1997b) for rat brain samples. However, a residual activity was seen when chicken brain homogenates were incubated with this concentration of PMSF. This can be seen most clearly in Figure 2A,B, where a residual activity over blank (defined using distilled water instead of homogenate) was found for the chicken at all substrate concentrations tested. In contrast, there was no residual activity found for the rat brain homogenates (Figure 2A,B). In consequence, in the remaining experiments, blanks were either defined as the radioactivity recovered in the absence of homogenate, or (in Figures 3 and 4), by running chicken and rat experiments concomitantly, and

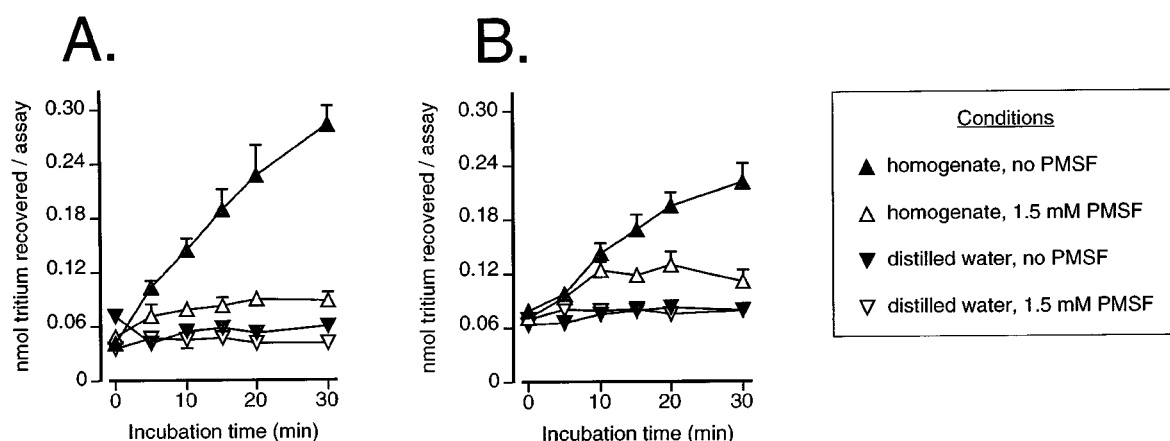


Figure 1 Time-dependent hydrolysis of 2 μM PEA (A) and 2 μM AEA (B) by chicken brain FAAH. Samples (homogenates or distilled water) were incubated in the absence or presence of 1.5 mM PMSF. Data are means \pm s.e. mean (when not enclosed by the symbols), $n = 3$.

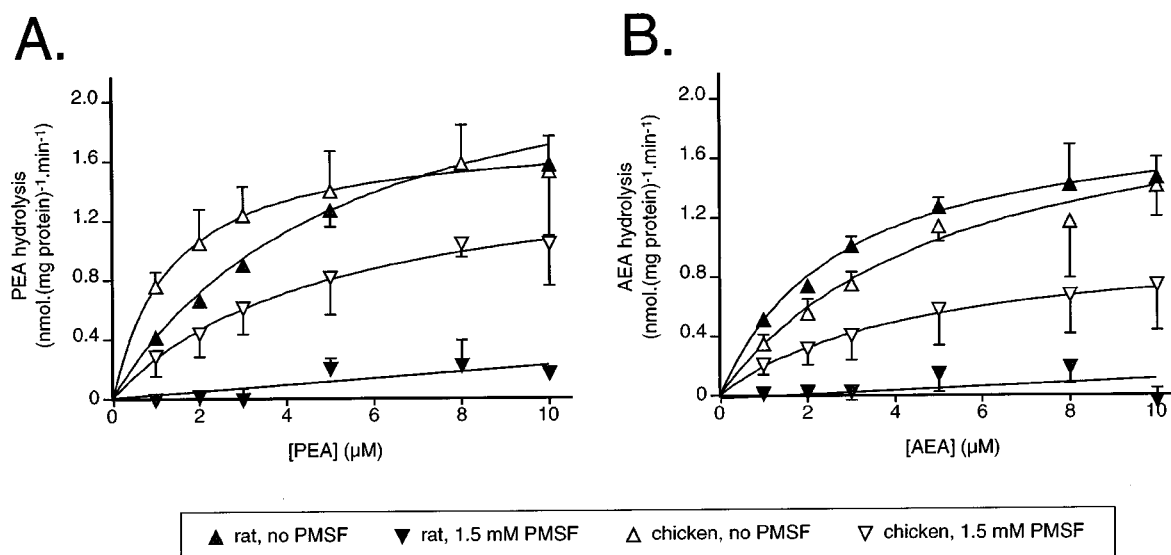


Figure 2 Concentration-dependence of the metabolism of PEA (A) and AEA (B) by rat and chicken brain FAAH in the absence or presence of 1.5 mM PMSF. Blank values were obtained for distilled water in place of the homogenates. Data are means \pm s.e.mean (when not enclosed by the symbols), $n=3$.

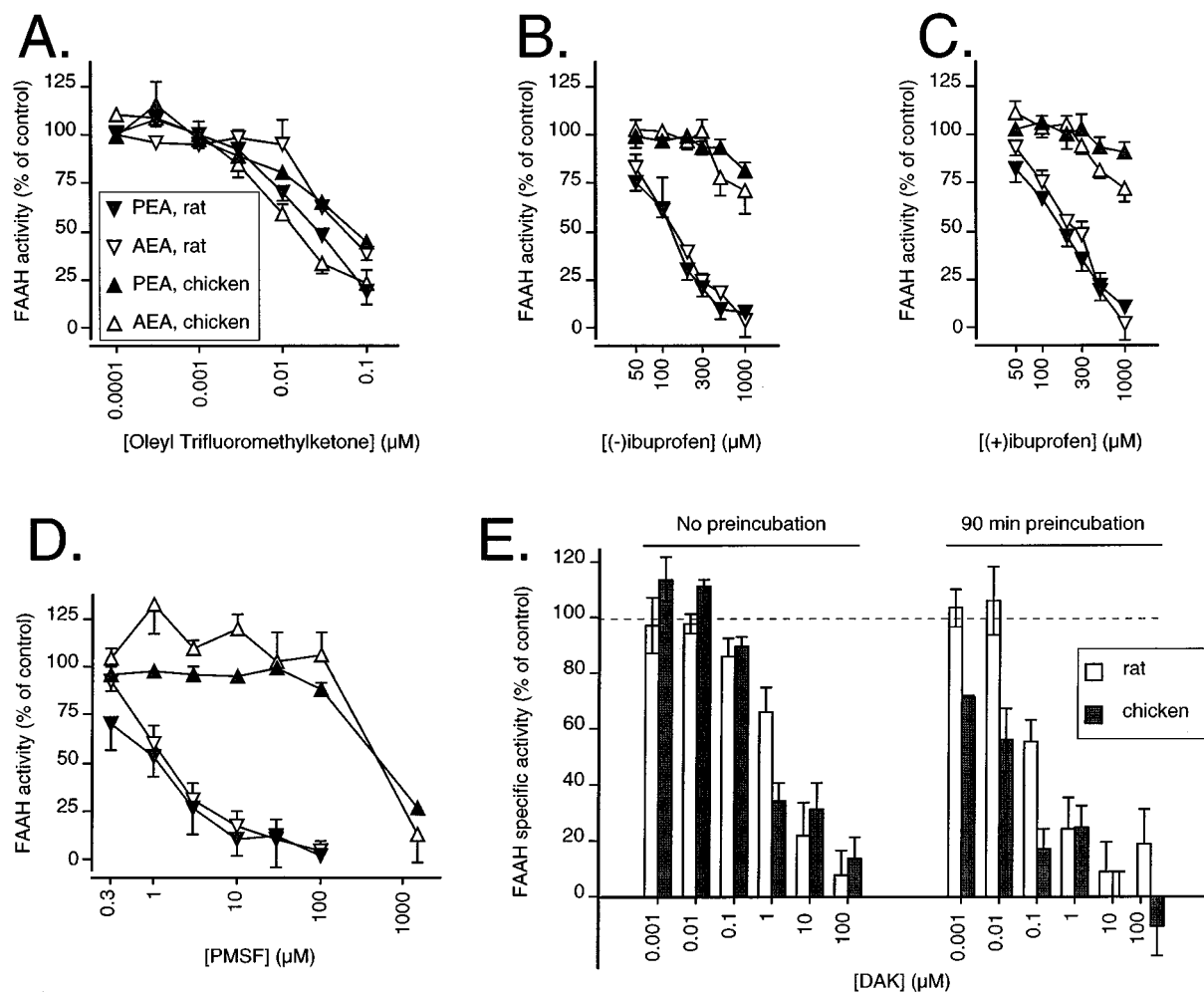


Figure 3 Inhibition of FAAH in the rat and chicken brains by OTMK (A); (-)ibuprofen (B); (+)ibuprofen (C) and PMSF (D). The substrate concentration was 2 μM. The explanation of the symbols is enclosed within A. In E, the inhibition of 2 μM PEA by DAK is shown. Blank values were those for the rat brain samples in the presence of 1.5 mM PMSF. Data are means \pm s.e.mean (when not enclosed by the symbols), $n=3$, or means and ranges, $n=2$, towards 2 μM AEA and PEA.

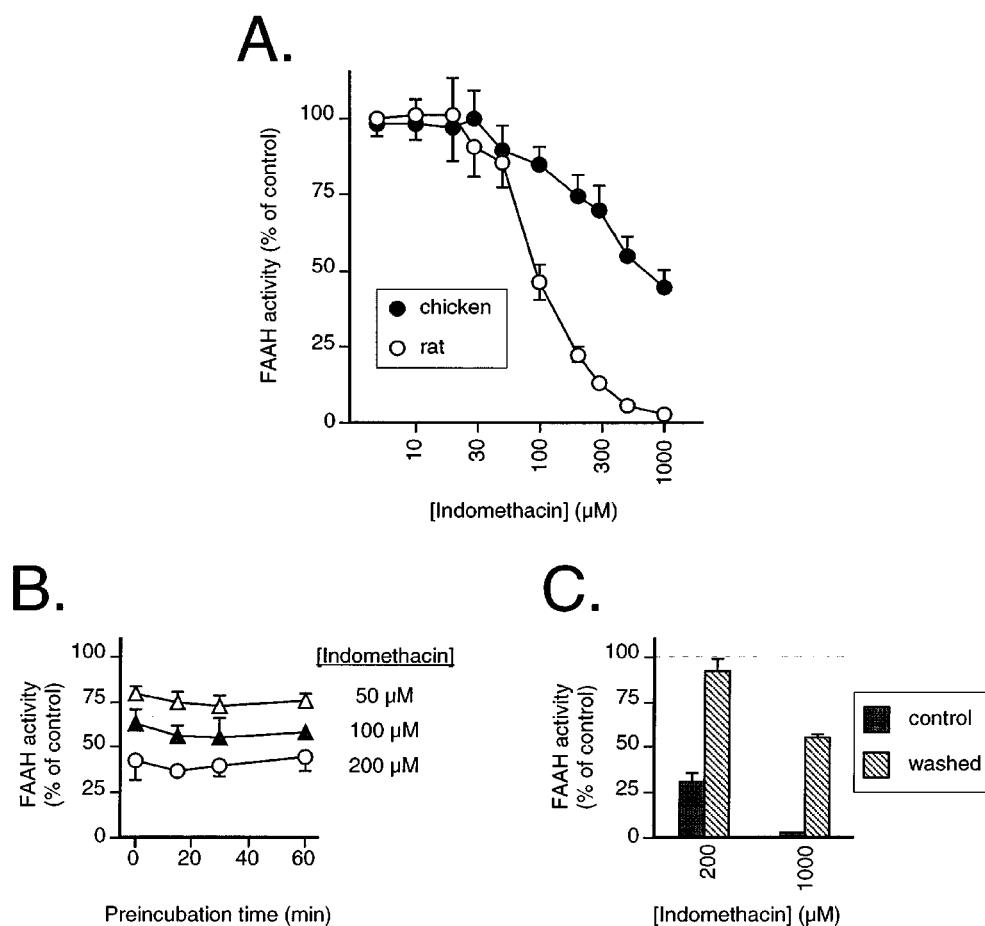


Figure 4 (A) Inhibition of FAAH in rat and chicken brain membranes by indomethacin. (B) effect of preincubation upon the inhibition FAAH in rat brains by indomethacin. (C) reversibility of the inhibition of FAAH in rat brain membranes by indomethacin. The membranes were preincubated for 60 min with either 0, 200 or 1000 μM indomethacin and then either left on ice ('control') or washed (by centrifugation followed by resuspension in the same volume) prior to addition of substrate and assay for FAAH activity. The values are given as per cent of the corresponding values for samples treated in the same way (i.e. \pm washing as appropriate) in the absence of indomethacin. All panels show results as means \pm s.e.mean (when not enclosed by the symbols), $n = 3$, using 2 μM AEA as substrate. In all cases, blank values were those for the rat brain samples in the presence of 1.5 mM PMSF.

using the rat PMSF blank, since this is equivalent to the distilled water blank. In the remaining experiments, an incubation time of 10 min was used. Using these conditions, the rates of substrate hydrolysis were saturable, with median K_M values of 1.5 (chicken) and 5.0 (rat) μM being found for PEA as substrate (Figure 2A). For AEA, the median K_M values were 5.4 (chicken) and 2.6 (rat) μM (Figure 2B).

Inhibition of chicken and rat brain FAAH activity by OTMK, PMSF, ibuprofen and DAK

The inhibition of rat and chicken brain FAAH activity by OTMK, (–)ibuprofen, (+)ibuprofen, PMSF and DAK is shown in Figure 3A–E and the pI_{50} values (with the exception of DAK) are given in Table 1. The substrate analogues OTMK and DAK inhibited chicken FAAH with roughly the same potency as for rat FAAH (Figure 3A,E). DAK, which is an irreversible inhibitor of FAAH (Edgemond *et al.*, 1998) showed the expected time-dependency (Figure 3E). In contrast to the substrate analogues, PMSF and the enantiomers of ibuprofen were very much less potent in the chicken than in the rat (Figure 3B–D). These results were seen regardless of whether PEA or AEA was used as substrate (data for DAK and AEA not shown).

Inhibition of chicken and rat brain FAAH activity by indomethacin

In order to determine whether the species difference in sensitivity to inhibition by the ibuprofen enantiomers was a property common to all non-steroidal anti-inflammatory drugs (NSAIDs), we investigated whether indomethacin could inhibit FAAH. In these experiments, membrane preparations at a pH of 7.0 were used. However, initial experiments in the rat indicated that the sensitivity of FAAH to ibuprofen was retained, concentrations of 50, 100, 150, 200, 300, 500 and 1000 μM (\pm)ibuprofen giving rates of FAAH activity (towards 2 μM [^3H]-AEA) of 82 ± 3 , 76 ± 3 , 61 ± 2 , 57 ± 3 , 44 ± 0.3 , 36 ± 1 and $23 \pm 4\%$ of control, respectively (means \pm s.e.mean, $n = 3$). The rat membranes were also sensitive to inhibition by niflumic acid, with concentrations of 50, 100, 200, 300, 500 and 1000 μM giving values of 75 ± 8 , 64 ± 12 , 61 ± 6 , 37 ± 4 , 34 ± 5 and $20 \pm 6\%$ of control, respectively.

Using 2 μM AEA as substrate, indomethacin was found to be a more potent inhibitor of rat FAAH activity than (\pm)ibuprofen, with a mean pI_{50} value of 4.03 being found (Figure 4A, Table 1). The degree of inhibition produced by indomethacin was not increased by preincubation for up to 60 min (Figure 4B) and could be reduced by washing of the membranes following a 60 min preincubation (Figure 4C) indicative of a reversible mode of action. Chicken FAAH was

less sensitive than rat FAAH to indomethacin, a mean pI_{50} value of 3.13 being found (Figure 4A, Table 1). The mode of inhibition of AEA hydrolysis was competitive for both rat (Figure 5A) and chicken (Figure 5B), with K_i values of 120 and 330 μM , respectively, being found for secondary replots of

K_{Mapp} (calculated by direct linear plot analysis of the mean data for 0 and 80 μM indomethacin (rat) and for 0, 500 and 1000 μM indomethacin (chicken)) vs the indomethacin concentration. The 160 μM indomethacin concentration was not used in these calculations, since at that concentration the $K_{Mapp} \gg$ the highest AEA concentration used, making its accurate determination impossible. For the control membranes, the K_M values (medians, $n=3$, with ranges in parentheses) towards AEA were 5.2 (4.3–8.6) μM and 3.4 (3.2–3.4) for rat and chicken, respectively.

Discussion

In a relatively short time, much has been learnt about FAAH in mammals. Rat, human and mouse FAAH have a high degree of homology and a similar (but not identical) substrate selectivity (Giang & Cravatt, 1997). Rat and human FAAH have been the subject of detailed investigations into their substrate (e.g. Giang & Cravatt, 1997; Lang *et al.*, 1999) and inhibitor (e.g. Koutek *et al.*, 1994; Maccarrone *et al.*, 1998) specificities. Recent mutagenesis studies have in addition identified a number of amino acids that are essential for mammalian FAAH activity (Goparaju *et al.*, 1999; Omeir *et al.*, 1999; Patricelli *et al.*, 1999). In contrast, little is known about FAAH in non-mammalian species, although an activity sensitive to PMSF (~50% inhibition at 250 μM) capable of metabolizing both PEA and AEA has been reported in sea urchin ovaries (Bisogno *et al.*, 1997).

In the present study, the properties of chicken brain FAAH have been compared with those of rat brain. In the rat brain, the K_M and V_{max} values, and the potencies of the inhibitors (OTMK \approx DAK [90 min preincubation] $>$ PMSF \approx DAK [0 min preincubation] $>$ (–)ibuprofen $>$ (+)ibuprofen are consistent with the literature (Hillard *et al.*, 1995; Desarnaud *et al.*, 1995; Fowler *et al.*, 1997b; 1999; Edgemond *et al.*, 1998; Tiger *et al.*, 2000). The finding that indomethacin and niflumic acid are also capable of inhibiting FAAH extends the list of NSAIDs that share this property. In theory, inhibition of FAAH might contribute to the anti-inflammatory properties of these drugs, given that both PEA and AEA have positive effects in inflammatory pain (Mazzari *et al.*, 1996; Calignano *et al.*, 1998; Jaggar *et al.*, 1998), and that blockade of FAAH potentiates the behavioural effects of AEA *in vivo* (Compton & Martin, 1997). However, significant inhibition of FAAH is unlikely to be found *in vivo* after administration of a therapeutic dose of indomethacin, given that the peak plasma concentration (e.g. ~12 μM after administration of a 75 mg controlled release preparation to elderly volunteers, Bruguero *et al.*, 1986) is much lower than the K_i value found here.

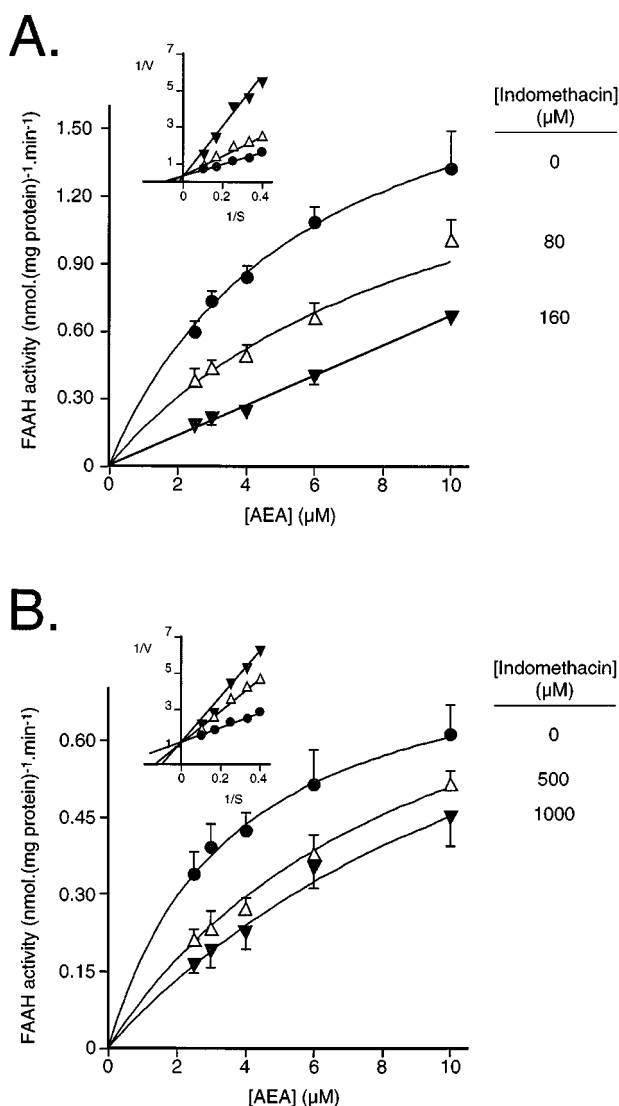


Figure 5 Mode of inhibition of FAAH by indomethacin. (A) rat brain membranes; (B) chicken brain membranes. Blank values were obtained for distilled water in place of the homogenates. Data are means \pm s.e.mean, $n=3$. Secondary Lineweaver-Burk replots of the mean data are shown as insets.

Table 1 Pharmacological properties of rat and chicken brain FAAH

Compound	rat, PEA (pI_{50})	rat, AEA (pI_{50})	chicken, PEA (pI_{50})	chicken, AEA (pI_{50})
OTMK ^a	7.58 \pm 0.079	7.24 \pm 0.077	7.14 \pm 0.045	7.82 \pm 0.014
PMSF ^a	6.11 \pm 0.33	5.74 \pm 0.15	~3.3	~3.3
(–)Ibuprofen ^a	4.02 \pm 0.061	3.85 \pm 0.038	<3	<3
(+)Ibuprofen ^a	3.75 \pm 0.074	3.64 \pm 0.037	<3	<3
(±)Ibuprofen ^b	N.D.	3.57 \pm 0.033	N.D.	N.D.
Indomethacin ^b	N.D.	4.03 \pm 0.061	N.D.	3.13 \pm 0.14
Niflumic acid ^b	N.D.	3.65 \pm 0.012	N.D.	N.D.

Values were calculated from the individual ($n=3$) experiments summarized in Figures 3 and 4 and for data summarized in the text. pI_{50} values are given as means \pm s.e.mean. pI_{50} values were not calculated for DAK since the data points were too widely spaced to allow accurate determination. ^aCrude homogenates, ^bmembrane preparations (see Materials and methods section for details). N.D., not determined.

In the case of the chicken FAAH, the enzyme shows similar K_M values towards AEA and PEA as is seen in the rat, whereas PMSF, (–)ibuprofen and (+)ibuprofen are very much less potent as inhibitors of PEA and AEA metabolism. One possible explanation for this difference is simply that when using brain homogenates, the activity of more than one FAAH isoform may be measured, and that the difference between species observed may reflect differences in the relative abundances of these enzyme species. Detailed pharmacological investigations in different subcellular fractions and in different regions of the rat brain, however, have failed to show obvious differences in the sensitivity of the FAAH to inhibition by ibuprofen (Fowler, *et al.*, 1999; Tiger *et al.*, 2000), which would argue against the notion of expression to any great extent of a 'chicken-like' FAAH activity in the rat brain. Recently, Ueda *et al.* (1999) described an enzymic activity in human CMK megakaryoblastic cells that could hydrolyze both AEA and PEA, and that was rather insensitive to PMSF. However, this enzyme was not inhibited by the substrate analogue methyl arachidonyl fluorophosphonate (Ueda *et al.*, 1999), which would suggest that it is a different enzyme to that described here in the chicken, which is highly sensitive to the related

substrate analogue OTMK. Ibuprofen inhibits rat brain FAAH in a mixed manner (Fowler *et al.*, 1997b; 1999), and a suggestion could be made that whilst the active site of chicken brain FAAH is rather similar to its mammalian equivalent, there may be species differences in parts of the molecule mediating the inhibitory effects of PMSF and ibuprofen. Indomethacin, however, was a competitive inhibitor of both rat and chicken FAAH, but was more potent towards the rodent enzyme. It is to be hoped that the molecular characterization of avian FAAH will shed light upon the mechanism behind this species difference.

We thank Dr Cecilia Hillard for her kind gift of DAK, and Kronfågel AB, Kristianstad for their help in providing the adult chicken brains. This work was supported by grants from the Swedish Medical Research Foundation (Grants nos. 12158 and 12548), Strokeförbundet, Loo and Hans Ostermans Fund, Stiftelsen Sigurd and Elsa Goljes Minne, Magnus Bergvalls Stiftelse, Stiftelse Åke Wiberg and the Research Fund of the Medical Faculty, Umeå University. The excellent technical assistance of Britt Jacobsson is much appreciated.

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(Received April 13, 2000

Revised June 23, 2000

Accepted June 29, 2000)