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Acidic Nonsteroidal Anti-inflammatory Drugs Inhibit Rat Brain Fatty Acid Amide Hydrolase in a pH-dependent Manner

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Previous studies have demonstrated that fatty acid amide hydrolase, the enzyme responsible for the metabolism of anandamide, is inhibited by the acidic non-steroidal anti-inflammatory drug (NSAID) ibuprofen with a potency that increases as the assay pH is reduced. Here we show that (*R*)-, (*S*)- and (*R*,*S*)-flurbiprofen, indomethacin and niflumic acid show similar pH-dependent shifts in potency to that seen with ibuprofen. Thus, (*S*)-flurbiprofen inhibited 2 μM [^{3}H]anandamide metabolism with IC_{50} values of 13 and 50 μM at assay pH values of 6 and 8, respectively. In contrast, the neutral compound celecoxib was a weak fatty acid amide hydrolase inhibitor and showed no pH dependency (IC_{50} values ~300 μM at both assay pH). The cyclooxygenase-2-selective inhibitors nimesulide and SC-58125 did not inhibit fatty acid amide hydrolase activity at either pH. The data are consistent with the conclusion that the non-ionised forms of the acidic NSAIDs are responsible for the inhibition of fatty acid amide hydrolase.

Keywords: Fatty acid amide hydrolase; Anandamide; Non-steroidal anti-inflammatory agents; Flurbiprofen; Indomethacin; Niflumic acid; Ibuprofen; Celecoxib

INTRODUCTION

Fatty acid amidohydrolase is an enzyme responsible for the hydrolysis of a number of biologically important endogenous *N*-acyl ethanolamines and *N*-acylamines including the endocannabinoid/endovanilloid anandamide,[1] the anti-inflammatory agent palmitoylethanolamide,[2] and the sleep-inducing agent oleamide.[3] The enzyme is predominantly localised to endoplasmic reticular and mitochondrial membranes, and can be inhibited by a variety of substrate analogues, such as, for example, oleyl trifluoromethylketone.[4,5] Brain homogenates from fatty acid amide hydrolase knockout mice lose the ability to hydrolyse anandamide, indicating that in this tissue fatty acid amide hydrolase is the predominant metabolising enzyme for this substrate.[6] The finding that these knockout mice display an increased nociceptive threshold has led to the suggestion that this enzyme may be a legitimate pharmacological target in the treatment of pain conditions.[6]

In 1996, it was reported that the non-steroidal anti-inflammatory (NSAI) agent indomethacin reduced the activity of mouse uterine fatty acid amide hydrolase measured both *ex vivo* (after a dose of 100 μg/mouse) and *in vitro* (at a concentration of 10 μM).[7] Independent studies from this laboratory have demonstrated that the ability to inhibit fatty acid amide hydrolase *in vitro* is shared by a number of NSAIDs, including ibuprofen and flurbiprofen,[8–10]although there is a large variation in potency between NSAIDs. Thus, for example, at pH 7.6, flurbiprofen, ibuprofen, ketoprofen and sulindac inhibited brain fatty acid amide hydrolase with IC_{50} values of 80, 270, 650 and >1000 μM, respectively, whereas acetylsalicylic acid and the non-NSAID compounds isobutyric acid and hydrocinnamic acid (which comprise part of the ibuprofen structure) were totally inactive even at a concentration of 1000 μM.[8,9]

More recently, Holt *et al.*[11] found that the potency of ibuprofen as an inhibitor of fatty acid amide hydrolase in rat brain homogenates increased as

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the assay pH was reduced, whereas the reverse was true for the substrate analogue oleyl trifluoromethylketone. The (*R*)-form of ibuprofen was found further to act as a mixed-type inhibitor at both acid and neutral pH, with $K_{i(slope)}$ values of 11 and 185 μM being found at pH 5.28 and 8.37, respectively. The corresponding values for $K_{i(intercept)}$ were 143 and 3950 μM, respectively. It was suggested that the increased pH sensitivity was a consequence of a preferential interaction of the non-ionised form of ibuprofen with the fatty acid amide hydrolase.[11] If this is true, then a similar pattern would be expected for other acidic NSAIDs but not for a neutral NSAID. This hypothesis has been tested in the present study.

MATERIALS AND METHODS

Compounds

Arachidonyl-ethanolamide-[1-^{3}H] (specific activity 20 Ci mmol^{-1}) was obtained from American Radiolabelled Chemicals Inc., St. Louis, MO, USA. (*R*)-Flurbiprofen and celecoxib were kind gifts from Myriad Pharmaceuticals Inc., Salt Lake City, UT, USA. (*S*)-Flurbiprofen was a kind gift from Encore Pharmaceuticals Inc., Riverside, CA. (*R*,*S*)-Flurbiprofen, nimesulide, SC-58125 (5-(4-fluorophenyl)-1-[4-(methylsulfonyl)phenyl]-3-(trifluoromethyl)-1H-pyrazole) and non-radioactive anandamide were obtained from the Cayman Chemical Company, Ann Arbor, MI, U.S.A. Niflumic acid was obtained from the Alexis Corp., San Diego, CA, U.S.A. (R)-ibuprofen was obtained from Research Biochemicals International, Natick, MA, U.S.A. Indomethacin and fatty acid free bovine serum albumin were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Assay of FAAH Activity

Rat brains were chosen as the source of fatty acid amide hydrolase and anandamide as the substrate to eliminate the risk that the recently described acidic amidase[12] will contribute to the observed activity and thereby obfuscate the results. The assay of Omeir *et al.*[13] adapted to the tritiated substrate,[8] was used. Frozen (−70°C) brains (minus cerebellum) from adult female Sprague–Dawley rats were thawed and homogenized at 4°C in 20 mM HEPES buffer, pH 7.0, with 1 mM $MgCl_2$. The homogenates were centrifuged twice (36,000*g*, 20 min at 4°C). Tissue pellets were washed by resuspension in homogenisation buffer, incubation at 37°C for 15 min, and recentrifugation as before. Membranes were then resuspended in 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA and 3 mM $MgCl_2$, and stored at −70°C until used for assay. Upon assay, membranes [165 μl, 12.5 μg/protein assay, diluted in buffer at the appropriate pH], NSAID or ethanol carrier (10 μl), [^{3}H]anandamide and assay buffer (25 μl, 10 mM Tris–HCl, 1 mM EDTA, 1% (w/v) fatty acid-free bovine serum albumin, pH 7.6) (final assay volume of 200 μl) were incubated for 10 min at 37°C. The ethanol concentration was kept constant throughout. Reactions were stopped by placing the tubes in ice. Following addition of chloroform: methanol (1:1 v/v, 400 μl) and thorough mixing, phases were separated by centrifugation. Aliquots (200 μl) of the methanol/buffer phase were analyzed for radioactivity by liquid scintillation spectroscopy with quench correction. Blanks contained distilled water instead of the membranes.

Calculation of pI_{50} Values

Data were expressed as % of control values and entered into the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, USA). The data were analysed using the built-in equation "sigmoid dose-response (variable slope)" with the "top" (i.e. uninhibited) value set to 100% and the "bottom" (i.e. minimum activity remaining) value set to zero.

RESULTS

A series of compounds were tested for their ability to inhibit rat brain [^{3}H]anandamide metabolism by fatty acid amide hydrolase. On each experimental day, the compounds were tested at both assay pH 6 and 8. The observed fatty acid amide hydrolase activity at pH 6 was 56 ± 2.5% (mean ± S.E.M., n = 16) of that seen at pH 8, consistent with the known pH activity profile of this enzyme.[14–16]

TABLE I Potencies of some NSAIDs towards inhibition of rat brain fatty acid amide hydrolase at pH 6.0 and 8.0

	pI_{50} value (IC_{50} value, μM)*	
NSAID	pH 6.0	pH 8.0
(*R*)-Ibuprofen	4.26 ± 0.07 (55)	3.69 ± 0.04 (210)
(*R*,*S*)-Flurbiprofen	4.63 ± 0.04 (23)	4.07 ± 0.03 (85)
(*R*)-Flurbiprofen	4.50 ± 0.05 (31)	4.01 ± 0.03 (97)
(*S*)-Flurbiprofen	4.90 ± 0.04 (13)	4.30 ± 0.04 (50)
Indomethacin	4.78 ± 0.06 (17)	4.27 ± 0.03 (54)
Celecoxib	3.55 ± 0.06 (280)	3.49 ± 0.027 (320)
Niflumic acid	4.17 ± 0.05 (67)	3.43 ± 0.03 (370)
Nimesulide	<3.3†	<3.3†
SC-58125	<3‡	<3‡

*Values were determined in each case using data from three experiments. † < 20% inhibition at highest concentration tested (500 μM). ‡ < 15% inhibition at 500 μM, < 30% inhibition at 1000 μM.

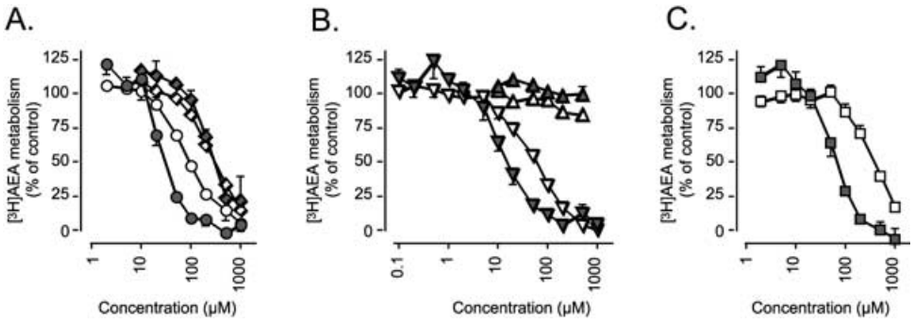

FIGURE 1 Inhibition of the metabolism of 2 μM [^{3}H]anandamide in rat brain homogenates by NSAIDs. ● ○ (*R*)-Flurbiprofen; ◆◇ Celecoxib; ▼▽ Indomethacin; ▲△ Nimesulide; ■ □ Niflumic acid. Shown are means ± S.E.M. (when not enclosed by the symbols), $n = 3$ for determinations at an assay pH of 6.0 (filled symbols) and 8.0 (unfilled symbols).

The inhibitory potencies of the NSAIDs are summarised in Table I, with examples for five of the compounds shown in Fig. 1. Consistent with our previous study,[11] (*R*)-ibuprofen was approximately four fold more potent at an assay pH of 6 than at pH 8. Similar pH dependencies in potency were seen for (*R*)-, (*S*)- and (*R*,*S*)-flurbiprofen, indomethacin and niflumic acid. In contrast, celecoxib was roughly equipotent to (*R*)-ibuprofen at pH 8, but did not show an increased potency when the assay pH was reduced to 6. Nimesulide and SC-58125 were essentially inactive at either assay pH.

DISCUSSION

It has been known since 1996 that certain NSAIDs, such as indomethacin, ibuprofen and flurbiprofen, are capable of inhibiting fatty acid amide hydrolase activity.[7–11] This effect appears to follow a very different structure activity pattern than is seen for inhibition of cyclooxygenase by these compounds, as exemplified by the flurbiprofen enantiomers, which have rather similar potencies towards fatty acid amide hydrolase, but differ profoundly with respect to their ability to inhibit cyclooxygenase.[17]

In our previous study, we found that (*R*)-ibuprofen inhibited fatty acid amide hydrolase in a mixed-type and pH-sensitive manner[11] and it was suggested that the non-ionised form of ibuprofen was responsible for the inhibition of the enzyme. Such a mechanism would predict that other acidic NSAIDs, but not neutral NSAIDs should behave in a similar manner. The present study has indicated that this indeed is the case.

The biological significance of the present results is at present unclear. An attractive hypothesis, given the role of anandamide in pain processing,[18] is that inhibition of fatty acid amide hydrolase may be a valid approach for the design of new pharmacological agents for the treatment of pain conditions. It may even be possible that some, but clearly not all, NSAIDs (such as ibuprofen and flurbiprofren) may be able to inhibit fatty acid amide hydrolase following normal dosaging in man. Whether this contributes to the efficacy of these compounds, however, remains unanswered.

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In a recent study, Gühring *et al.*[19] demonstrated that antinociceptive effects of spinally administered indomethacin could be blocked by cannabinoid receptor antagonism or deletion. Whether fatty acid amide hydrolase contributes to this effect awaits elucidation.

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