Ibuprofen Inhibits Rat Brain Deamidation of Anandamide at Pharmacologically Relevant Concentrations. Mode of Inhibition and Structure-Activity Relationship

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ABSTRACT

The ability of rat brain (minus cerebellum) homogenates to deamidate arachidonyl ethanolamide (anandamide) was determined with a custom-synthesized substrate, arachidonyl ethanalamide-[1-3H] ([3H]anandamide). Conditions whereby initial velocities were measured were established. The homogenates deamidated anandamide with a $K_m$ value of 0.8 $\mu$M and a $V_{max}$ value of 1.73 nmol · (mg protein)$^{-1}$ · min$^{-1}$. The deamidation of 2 $\mu$M [3H]anandamide was inhibited by phenylmethylsulfonyl fluoride and arachidonyl trifluoromethyl ketone with $IC_{50}$ values of 3.7 and 0.23 $\mu$M, respectively. Ibuprofen inhibited anandamide deamidation in a mixed fashion, with $K_i$ and $K'_i$ values of 82 and 1420 $\mu$M. At an anandamide concentration of 2 $\mu$M, the $IC_{50}$ values (in $\mu$M) of a series of compounds related in structure to ibuprofen were as follows: suprofen, 170; ibuprofen, 270; fenoprofen, 480; naproxen, 550; ketoprofen, 650; diclofenac, ~1000. Sulindac produced 27% inhibition at a concentration of 1000 $\mu$M, whereas isobutyric acid, hydrocinnamic acid, acetylsalicylic acid and acetaminophen were essentially inactive at concentrations $\leq$ 1 mM. We conclude that ibuprofen inhibits anandamide deamidation at pharmacologically relevant concentrations and that there is some specificity to the inhibition produced by ibuprofen and suprofen.

Since its discovery as an endogenous cannabinimetic agent (Devane et al., 1992), there has been considerable interest devoted to the pharmacological properties of anandamide. In addition to producing many of the standard pharmacological and biochemical effects of cannabinoids (Mackie et al., 1993; Vogel et al., 1993; Smith et al., 1994), anandamide has been proposed to exert neurotrophic actions in the hippocampus and to play a role in synaptic plasticity (Derkinderen et al., 1996) as well as in the modulation of long-term potentiation (Terrymanova et al., 1995) and glutamatergic transmission (Shen et al., 1996) as a result of cannabinoid receptor activation.

Anandamide is metabolized by a membrane-bound amidohydrolase activity to produce arachidonic acid (Deutsch and Chin, 1993). In view of the anti-inflammatory, antinociceptive and immunosuppressive properties of cannabinoids (Dewey, 1986), inhibitors of anandamide deamidation may be of therapeutic value. Most of our knowledge concerning such inhibitors has been gleaned from the use of structural analogs of anandamide (Koutek et al., 1994). Recently, however, we found that the nonsteroidal anti-inflammatory drug ibuprofen inhibited the metabolism of anandamide (Fowler et al., 1997). The assay, which used the PMSF-sensitive reduction in the potency of anandamide to inhibit radioligand binding to cannabinoid receptors (Childers et al., 1994), was indirect in nature and did not permit quantitative data to be obtained. Preliminary data using a direct assay of anandamide deamidation (Omer et al., 1995) and a substrate concentration of 27.7 $\mu$M (Omer et al., 1995) confirmed the enzyme inhibition by ibuprofen and gave an $IC_{50}$ value of ~400 $\mu$M (Fowler et al., 1997). Between this value is similar to the value for inhibition by ibuprofen of COX-2 in cell-free systems (Mitchell et al., 1994), it is important to investigate in more detail the inhibition of anandamide deamidation by ibuprofen. Consequently, we used the assay of Omer et al. (1995) to investigate the nature of the inhibition of anandamide deamidation by ibuprofen and to explore the relationship between the structure of this compound and its inhibitory activity.

Materials and Methods

Compounds. Arachidonyl ethanolamide [1-3H] ([3H]anandamide, specific activity 30 Ci/mmol) was custom-synthesized by American Radiolabeled Chemicals Inc., St. Louis, MO. The chemical purity of

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ABBREVIATIONS: Anandamide, arachidonyl ethanolamide; PMSF, phenylmethylsulfonyl fluoride; NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase.
the compound was found to be 97.3% using HPLC on a Zorbax SB C-18 column with acetonitrile/water/acetic acid (85:15:0.05) as the mobile phase. Arachidonyl ethanolamide-[1,2-14C] ([14C]anandamide, specific activity 120 mCi/mmol) was a kind gift from Dr. D. G. Ahern, E.I. DuPont de Nemours Co., Biomedical Product Division, Boston, MA. Anandamide (in ethanol) and arachidonyl trifluoromethyl ketone were purchased from Research Biochemicals International, Natick, MA. Ibuprofen (sodium salt), naproxen, suprofen, ketoprofen, fenoprofen, diclofenac, sulindac, isobutyric acid, hydrocinnamic acid, acetyl salicylic acid, acetaminophen and PMSF were obtained from the Sigma Chemical Co., St. Louis, MO. The NSAI compounds were dissolved in ethanol before dilution with buffer and were then further diluted with the ethanol/buffer mix to keep the ethanol concentration constant through the experiments. PMSF was dissolved in either ethanol or butanol (for assay blanks) and diluted with buffer to give a stock solution of 12 mM. Subsequent experiments demonstrated that the assay butanol concentration itself produced a 30% to 50% inhibition of [3H]anandamide amidation. However, a series of experiments using a [3H]anandamide concentration range of 1 to 27.7 μM demonstrated that the assay blank value was the same when PMSF was dissolved in ethanol (which did not itself affect the anandamide amidation rate) as when PMSF was dissolved in butanol.

**Assay of [3H]anandamide deamidation.** The assay was used that of Omeir et al. (1995). Briefly, rat brains minus cerebella (for [3H]anandamide) or cerebella (for [14C]anandamide) were homogenized in 10 mM Tris-HCl, pH 7.6, containing 1 mM EDTA, and aliquots were stored frozen at −70°C until used for assay. Assay mixtures contained homogenate, the appropriate concentration of test compound, or carrier (ethanol diluted with buffer) (25 μl), and radiolabeled anandamide (25 μl) containing 10 mg/ml fatty acid-free bovine serum albumin (Final assay volume 200 μl). Blanks contained PMSF (final concentration 1.5 mM) in place of the NSAI. The mixtures were incubated at 37°C, after which reactions were stopped by placing the tubes in ice and adding 400 μl of chloroform/methanol (1:1, v/v). 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 analysis of radioactivity by liquid scintillation spectroscopy with quench correction.

**Determination of Km and Vmax values.** Km and Vmax values of the mean data, compiled from three separate experimental series, were analyzed using the direct linear plot of Eisenthal and Cornish-Bowden (1974). Analyses were conducted using the Enzyme Kinetics computer program (Trinity Software, Campton, NH). Km and Vmax values were calculated from replots of K app/V max app vs. [ibuprofen] and 1/V max app vs. [ibuprofen], respectively (see Cornish-Bowden, 1976).

**Results**

**Assay conditions.** Because the substrate used in the present study is the result of a custom synthesis, initial experiments were undertaken to establish the validity of the assay. In the absence of enzyme, there was no time-dependent accumulation of radioactivity in the aqueous/methanol phase, which suggests that the substrate is stable under the conditions used. At a concentration of 1 μM, product formation was dependent on incubation time and protein concentration (fig. 1), a result consistent with other studies of anandamide deamidation (e.g., Desarnaud et al., 1995; Maurelli et al., 1995; Ueda et al., 1995; Omeir et al., 1995; Lang et al., 1996). Complete metabolism of [3H]anandamide, blocked by the presence of PMSF, was seen after incubation for 20 min at a protein concentration of 20 μg/assay (fig. 1). It is noteworthy that linearity was preserved until surprisingly high rates of substrate utilization were reached. A similar pattern was found when either [3H]-anandamide (whole brain minus cerebellum) or [14C]-anandamide (cerebellum) were used at a concentration of 27.7 μM (data not shown). In the remaining experiments, incubation times of 5 to 10 min were used, and protein concentrations were chosen to ensure that initial velocities were measured.

The effects of PMSF and arachidonyl trifluoromethyl ketone on the deamidation of 2 μM [3H]anandamide are shown...
in figure 2. Both compounds inhibited the deamidation of the substrate, with IC$_{50}$ values (calculated as described in the legend to table 1) of 3.7 and 0.23 µM, respectively.

**Inhibition of anandamide deamidation by ibuprofen.** Ibuprofen produced a concentration-dependent inhibition of the deamidation of 2 µM [$^{3}$H]anandamide (fig. 2). The inhibition was not changed upon preincubation of ibuprofen with homogenate for up to 90 min at 37°C before the addition of [$^{3}$H]anandamide. The data for a 90-min preincubation period are presented in figure 2; similar concentration-response curves were found after preincubations of 30 and 60 min. This lack of time-dependence was also seen for cerebellar homogenates with 27.7 µM [$^{14}$C]anandamide (data not shown), although ibuprofen was a less potent inhibitor at this high substrate concentration.

Data from three experimental series, all using the same four homogenate preparations, are shown in figure 3A. Direct linear plot analyses of the combined mean data gave a $K_{m}$ value of 0.8 µM and a $V_{max}$ value of 1.73 nmol (mg protein)$^{-1}$ min$^{-1}$. Ibuprofen was found to produce inhibition of

### TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>% of Control Anandamide Deamidation at Concentration (µM) of:</th>
<th>IC$_{50}$ (µM)$^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibuprofen</td>
<td>![Ibuprofen Structure]</td>
<td>94 ± 4*</td>
<td>86 ± 8</td>
</tr>
<tr>
<td>Naproxen</td>
<td>![Naproxen Structure]</td>
<td>99 ± 4</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>Suprofen</td>
<td>![Suprofen Structure]</td>
<td>97 ± 3</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>![Ketoprofen Structure]</td>
<td>98 ± 3</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>Fenoprofen</td>
<td>![Fenoprofen Structure]</td>
<td>103 ± 9</td>
<td>88 ± 2</td>
</tr>
<tr>
<td>Isobutyric acid</td>
<td>![Isobutyric acid Structure]</td>
<td>98 ± 1</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>Hydrocinnamic acid</td>
<td>![Hydrocinnamic acid Structure]</td>
<td>103 ± 1</td>
<td>101 ± 4</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>![Diclofenac Structure]</td>
<td>103 ± 2</td>
<td>98 ± 5</td>
</tr>
<tr>
<td>Sulindac</td>
<td>![Sulindac Structure]</td>
<td>81 ± 2</td>
<td>85 ± 3</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>![Acetylsalicylic acid Structure]</td>
<td>101 ± 1</td>
<td>103 ± 2</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>![Acetaminophen Structure]</td>
<td>99 ± 2</td>
<td>103 ± 4</td>
</tr>
</tbody>
</table>

* Shown are means ± S.E., n = 3 using a [$^{3}$H]anandamide concentration of 2 µM. The ethanol carrier concentration was kept constant for each experimental series. For compounds where two values are given at the concentration of 500 µM, two sets of experiments were undertaken; one over a range of 30 to 500 µM and one with 500 and 1000 µM, the second series using a higher ethanol concentration to permit solubilization of the compounds.

$^{b}$ IC$_{50}$ values were calculated by Hill analysis of the mean data at each concentration. Only concentrations giving an inhibition in the range of 10% to 90% were included to prevent data bias at the ends of the scales. In all cases, the regression lines gave $r^2$ values >0.91.
enzyme activity at all concentrations tested, the inhibition appearing to be mixed in nature (fig. 3B). When the low (1–5 μM) and high (20–40 μM) experiments were combined (data shown in fig. 3A), the $K_m$ and $V_{max}$ values calculated by direct linear plot from the mean data were as follows: [μM and nmol · (mg protein)$^{-1}$ · min$^{-1}$, respectively] control, 0.7 and 1.70; 200 μM ibuprofen, 1.3 and 1.46; 500 μM ibuprofen, 2.7 and 1.25. One-way ANOVA for repeated measures, calculated using the individual values, showed a significant effect of ibuprofen on $V_{max}$ ($F_{2,6} = 50$, $P > 0.0005$), and a nearly significant effect on $K_m$ ($F_{2,6} = 4.3$, $P = 0.069$). From the mean $K_m$ and $V_{max}$ data, $K_i$ and $K'_{i}$ values of 82 and 1420 μM can be calculated.

**Structure-activity relationships.** The potencies of a series of compounds related in structure to ibuprofen are shown in table 1. Suprofen was the most potent, with an IC$_{50}$ value of 170 μM. This was followed by ibuprofen (270 μM), fenoprofen (480 μM), naproxen (550 μM), ketoprofen (650 μM) and diclofenac (1000 μM). Sulindac produced 27% inhibition at a concentration of 1000 μM, whereas isobutyric acid, hydrocinnamic acid, acetylsalicylic acid and acetaminophen were essentially inactive at concentrations ≤ 1 mM.

**Discussion**

In the present study, anandamide deamidation in homogenates of whole brain minus cerebellum has been measured using a custom-synthesized substrate, arachidonyl ethanolamide-[1-3H]. Metabolism of this substrate gives arachidonic acid and [3H]ethanolamine, which can easily be separated from [3H]anandamide. This method is based on the assay of Omeir et al. (1995), who used arachidonyl ethanolamide-[1,2-14C] as substrate. The validity of the assay was confirmed with respect to 1) the sensitivity to PMSF and arachidonyl trifluoromethyl ketone, 2) the linearity of product formation with time up to relatively high rates of substrate utilization and 3) the saturability of the activity at high substrate concentrations. The sensitivities to PMSF and arachidonyl trifluoromethyl ketone found in the present study are consistent with the literature. Thus IC$_{50}$ values for PMSF on the order of 12 and 25 μM have been reported for rat brain homogenates and microsomes, respectively (Hillard et al., 1995; Desarnaud et al., 1995). With respect to arachidonyl trifluoromethyl ketone, a concentration of 7.5 μM was found to inhibit the deamidation of 27.7 μM anandamide by ~90% in rat brain homogenates (Koutek et al., 1994).

The $K_m$ and $V_{max}$ values found in the present study (0.8 μM and 1.73 nmol · (mg protein)$^{-1}$ · min$^{-1}$, respectively) are in reasonable agreement with the values of 3.4 μM and 2.2 nmol · (mg protein)$^{-1}$ · min$^{-1}$ found at 30°C via an assay where [14C]arachidonic acid was separated from [14C]anandamide by thin-layer chromatography (Hillard et al., 1995). On the other hand, higher values ($K_m = 12.7 μM$ and $V_{max} = 5.6 nmol · (mg protein)^{-1} · min^{-1}$) were found when rat brain microsomes were used (Desarnaud et al., 1995). This raises the possibility of subcellular heterogeneity, especially given that anandamide amidohydrolase activity is found in all subcellular fractions of rat brain except the cytosol (Hillard et al., 1995).

In their study, Hillard et al. (1995) found that arachidonic acid inhibited 3 μM anandamide deamidation, with an inhibition of ~30% over the concentration range 1 to 10 μM arachidonic acid and increasing to ~40% and ~95% at 30 and 100 μM arachidonic acid, respectively, being found. The linearity with time found in the present study, however, would suggest that there is little functional product inhibition by arachidonic acid under the conditions used. Whether this is due to insufficient build-up of arachidonic acid (in the case of 1 μM anandamide) or to a mode of product inhibition that...
decreases with increasing substrate concentration (in the case of 27.7 μM anandamide) awaits investigation.

It can thus be concluded, in agreement with the study of Omer et al. (1995), that the use of anandamide labeled in the ethanalamide position provides a simple and robust assay of anandamide deamidation. The assay has been used to establish the nature of inhibition of anandamide deamidation by the NSAID ibuprofen and to explore the relationship between the structure of this compound and its inhibitory activity. Ibuprofen was found to be a mixed-type inhibitor of anandamide deamidation, with K_i and K' values of 82 and 1420 μM being found. Whether the mixed-type nature of the inhibition reflects the absolute mode of inhibition or a mixture of actions on a heterogeneous enzyme population awaits elucidation. Be that as it may, the K_i value (and the IC_50 value of 270 μM found at 2 μM [3H]anandamide) is lower than the IC_50 value of ~800 μM for inhibition by ibuprofen of COX-2 activity in broken cells (Mitchell et al., 1994). With respect to clinical data, peak plasma ibuprofen concentrations of 110 and 150 μM have been reported after 2 × 200-mg single doses of two over-the-counter ibuprofen preparations (Kartunen et al., 1990). Thus the data presented here suggest that ibuprofen is a sufficiently potent inhibitor of anandamide deamidation to affect anandamide metabolism in vivo, particularly in the case of individuals taking higher doses for rheumatoid arthritis.

In order further to explore the effect of ibuprofen on anandamide deamidation, we evaluated the potencies of a series of related compounds. All of the compounds tested with an isobutyric acid side-chain coupled to an aromatic moiety inhibited anandamide deamidation with IC_50 values ranging from 170 to 650 μM. The aromatic moiety was necessary for inhibition, because isobutyric acid itself was inactive. For the compounds with an isobutyric acid side-chain, some structure-activity elements can be seen. Thus replacement of the isobutylphenyl group of ibuprofen with the more bulky 6-methoxynaphthyl group of naproxen reduced the inhibitory potency by a factor of 2. On the other hand, replacement of the isobutyryl group of ibuprofen with a thiencarboxylic group (suprofen) produced a slight increase in potency and resulted in the most potent compound tested, despite the more bulky nature of this compound. The potency of suprofen is presumably a consequence of the polarity of the thiophene, because its replacement with a phenyl group (ketoprofen) reduced the inhibitory activity in rat brain microsomes. Identification and partial characterization. J. Biol. Chem. 270: 6030–6035, 1995.

Ibuprofen and Anandamide Deamidation

In conclusion, the present findings demonstrate that ibuprofen is able to inhibit anandamide deamidation in a mixed manner at pharmacologically relevant concentrations. The clinical relevance of this finding is unclear: although ibuprofen (and suprofen) have been shown to be superior to aspirin with respect to analgesic efficacy in studies of oral surgical pain after removal of impacted third molars (Cooper et al., 1986; Forbes et al., 1992), there is no obvious clinical correlate for the differences in inhibitory potencies with respect to anandamide deamidation of the various proipionic acid NSAIDs (Insel, 1996). Nevertheless, the finding that pradavoline, which has agonist efficacy at cannabinoid receptors in addition to its COX-inhibitory properties, has greater antinociceptive efficacy than other NSAIDs (see D’Ambra et al., 1992) raises the possibility that a combined anandamide amidohydrolase/COX-2 inhibitor may be a therapeutically interesting prospect. This possibility is underlined by the recent finding that intrathecal administration of the cannabinoid receptor antagonist SR 141716A (N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) produces hyperalgesia in mice as assessed by the hot-plate test (Richardson et al., 1997), which suggests a tonic cannabinoid receptor activity and, by extension, a role of endogenous cannabimimetics in some aspects of nociception.

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