Title
Anandamide amidohydrolase activity in rat brain microsomes: Identification and partial characterization

Permalink
https://escholarship.org/uc/item/7dc5p4vr

Journal
Journal of Biological Chemistry, 270(11)

ISSN
0021-9258

Authors
Desarnaud, F
Cadas, H
Piomelli, D

Publication Date
1995-03-17

License
CC BY 4.0

Peer reviewed
An amidohydrolase activity present in rat brain microsomes catalyzes the hydrolysis of N-arachidonoyl-[3H]ethanalamine ([3H]anandamide), an endogenous cannabinimetic substance, forming [3H]ethanolamine and arachidonic acid. Amidohydrolase activity is maximal at pH 6 and 8, is independent of divalent cations, has an apparent \(K_a\) for [3H]anandamide of 12.7 ± 1.8 \(\mu\)M, and has a \(V_{\text{max}}\) of 5630 ± 200 pmol/min/mg of protein. Phenylmethylsulfonyl fluoride, a serine protease inhibitor, and \(\beta\)-bromopenacyl bromide, a histidine-alkylating reagent, inhibit the activity, whereas N-ethylmaleimide and various nonselective peptide inhibitors (EDTA, \(\alpha\)-phenanthroline, bacitracin) have no effect. Brain amidohydrolase activity exhibits high substrate specificity for [3H]anandamide; \(N\)-\(\gamma\)-linolenoyl-, \(N\)-homo-\(\gamma\)-linolenoyl-, and \(N\)-11,14-ecosadienoyl- are hydrolyzed at markedly slower rates. Moreover, \(N\)-11-ecosanoyl- and \(N\)-palmitoyl-[3H]ethanalamone are not hydrolyzed. [3H]Anandamide hydrolysis is inhibited competitively by nonradioactive anandamide and by other \(N\)-acylthanolamines with the following rank order of potency: anandamide > \(N\)-linoleoyl > \(N\)-cis-linolenoyl = \(N\)-\(\gamma\)-linolenoyl = \(N\)-homo-\(\gamma\)-linolenoyl > \(N\)-11,14-ecosadienoyl > \(N\)-oleoyl > \(N\)-docosahexaenoyl > \(N\)-docosatetraenoyl > \(N\)-linoleialoyl > \(N\)-ecosanoyl > \(N\)-palmitoyl. Thus, amidohydrolase for \(N\)-ecosanoyl-ethanalamone = amidohydrolase for \(N\)-ethanalamone = \(N\)-oleoyl-ethanalamone = \(N\)-linoleoyl-ethanalamone = \(N\)-linoleialoyl-ethanalamone = \(N\)-linolenoyl-ethanalamone = \(N\)-\(\gamma\)-linolenoyl-ethanalamone = \(N\)-homo-\(\gamma\)-linolenoyl-ethanalamone = \(N\)-11,14-ecosadienoyl-ethanalamone = \(N\)-docosahexaenoyl-ethanalamone = \(N\)-docosatetraenoyl-ethanalamone = \(N\)-linoleialoyl-ethanalamone = \(N\)-ecosanoyl-ethanalamone = \(N\)-palmitoyl-ethanalamone = no effect. Amidohydrolase activity is high in liver and brain and low in heart, kidney, intestine, stomach, lung, spleen, and skeletal muscle. Within the central nervous system, highest activity is found in globus pallidus and hippocampus, two regions rich in cannabinoid receptors, and lowest activity is found in brainstem and medulla, where cannabinoid receptors are sparse. The results, showing that brain amidohydrolase activity is selective for anandamide and enriched in areas of the central nervous system with high density of cannabinoid receptors, suggest that this activity may participate in the inactivation of anandamide at its sites of action.

The discovery of a G protein-coupled membrane receptor that recognizes \(\Delta^8\)-tetrahydrocannabinol, the major psychoactive principle of *Cannabis sativa*, has prompted the search for an endogenous substance with cannabinimetic properties (for review, see Howlett et al. (1990)). This search has recently led to the isolation from porcine brain of a cannabinimetic lipid derivative, identified as \(N\)-arachidonoylthanolamine and named anandamide (Devane et al., 1992). Like \(\Delta^9\)-tetrahydrocannabi-

---

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Contributed equally to this work.

§ To whom correspondence should be addressed: The Neurosciences Inst., 10630 John Jay Hopkins Dr., San Diego, CA 92121. Tel.: 619-626-2170; Fax: 619-626-2199.
EXPERIMENTAL PROCEDURES

Materials—[3H]Ethanolamine (28 Ci/mmol) and [3H]arachidonic acid (214 Ci/mmol) were from Amer sham Corp., and fatty acid chlorides were from Nu-Check Prep (Elysian, MN). Ethanolamine, phenylmethylsulfonyl fluoride, p-bromophenacyl bromide, N-ethylmaleimide, thimerosal, bacitracin, and α-phenan throlne were from Sigma.

Syntheses and Purification of Substrates—Syntheses of both radioactive and nonradioactive N-acyl ethanolamines were performed essentially as described previously (Devane et al., 1992). Briefly, ethanolamine (300 μmol) was allowed to react with the appropriate fatty acyl chloride (30 μmol) in dichloromethane (4 ml) at 0–4 °C, and the reaction was stopped after 15 min by extracting excess ethanolamine with water (15 ml). To prepare radioactive N-acyl ethanolamines, [3H]ethanolamine hydrochloride (50 μCi) was dried under vacuum, resuspended in the appropriate volume of nonradioactive ethanolamine, and added to the reaction mixtures. N-[3H]Arachidonoyl ethanolamine was synthesized by allowing [3H]arachidonic acid (28 μmol, 100 μCi) to react with ethanolamine (164 μmol) in the presence of N,N'-dipropylcarbodimide (8.4 mg) in 1 ml of dichloromethane for 40 min at 0–4 °C. N-Acylethanolamines were purified by semipreparative reversed-phase HPLC, by using a Nova-Pak HR C18 column (6 μm, 7.8 × 300 mm) eluted at 3 ml/min with a gradient of methanol (30% at 0 min, 70 to 100% over 40 min). Fractions containing N-acyl ethanolamines were dried, resuspended in methanol to a final concentration of ~10 mm, and stored at ~80 °C. Specific radioactivities of the N-acyl-[3H]ethanolamines used in the present study were as follows: N-arachidonoyl-[3H]ethanolamine, 0.51 mCi/mmol; N-dihomo-γ-linolenoyl-[3H]ethanol amine, 0.52 mCi/mmol; N-11,14-eicosadienoyl-[3H]ethanolamine, 0.51 mCi/mmol; N-11-eicosanoenooyl-[3H]ethanolamine, 0.62 mCi/mmol; N-palmityl-[3H]ethanolamine, 0.31 mCi/mmol; N-γ-linolenoyl-[3H]ethanol amine, 0.69 mCi/mmol.

Preparation of Rat Tissue Homogenates and Microsome Fraction—Wistar rats were sacrificed by cervical dislocation, and brain and other tissues were homogenized in 20 mM Tris, pH 7.5, containing 0.32 M sucrose and 1 mM EDTA. Tissue extracts were fractionated sequentially at 1,000 × g (1 min), 22,000 × g (30 min), and 105,000 × g (60 min). The soluble fraction obtained in the last centrifugation step (microsome fraction) was stored at ~80 °C until use. In some experiments, the brains were cut manually into ~4-mm slices with a razor blade, and individual brain structures were dissected and homogenized in 1 ml of Tris buffer (50 mM, pH 7.5).

Amidohydrolase Assay—Standard amidohydrolase assays were carried out for 5 min at 37 °C in 1 ml of Tris buffer (50 mM, pH 7.5) containing 1 mg of microsome (0.5 mg of protein) and [3H]andamide (14 μM, 10,000 dpm/ml). [3H]Andamide and other N-acyl-[3H]ethanolamines were added in methanol to yield a final concentration of 0.2% (v/v). Identical incubations were carried out in the absence of tissue: these “blank” samples contained ~40 dpm/sample, which were subtracted from values obtained with tissue samples. Amidohydrolase activity in brain regions was measured in freshly dissected tissue homogenates (1 mg/ml), which were incubated for 10 min at 37 °C in the presence of [3H]andamide (11 μM, 10,000 dpm). Under these conditions, [3H]Andamide hydrolysis was found in preliminary experiments to be linear with respect to time and protein concentration (data not shown). The reaction mixtures were diluted with methanol (4 μl) and centrifuged to eliminate precipitated protein, and samples (1 ml) were applied onto glass wool-plugged Pasteur pipettes, which had been previously packed with a suspension (100 mg/ml, 1 ml) of polydivinylbenzene (Perapak type Q. Waters) in methanol. [3H]Ethanolamine was eluted in the void volume, and measured by liquid scintillation counting. Under these elution conditions, unreacted [3H]andamide and [3H]ethanolamine-labeled phospholipids were quantitatively retained on the columns, which could be regenerated with ethanol (~20 ml).

Additional Analytical Procedures—To determine whether [3H]Andamide undergoes oxidative metabolism in brain microsomes, samples of amidohydrolase assays carried out for 10 min were diluted with an equal volume of methanol and extracted with chloroform (2 ml). The organic phases were applied onto Pasteur pipettes packed with a slurry of silica gel G in chloroform to yield a final column volume of ~0.5 ml. Radioactive products were eluted from the columns with 1 ml of ethyl acetate and brought to dryness under vacuum. Reversed-phase HPLC

FIG. 1. Hydrolysis of [3H]Andamide by rat brain microsomes. A, time dependence. Microsomes (0.2 mg of protein/ml) were incubated at 37 °C in 5 ml of Tris buffer (50 mM, pH 7.5) containing [3H]andamide (14 μM, 10,000 dpm). At the indicated times, samples of the incubation mixtures were collected and diluted with methanol, and [3H]ethanolamine was measured as described under “Experimental Procedures.” B, protein dependence. Amidohydrolase activity was determined by measuring the [3H]ethanolamine formed in assay mixtures containing varying quantities of microsome protein and incubated under standard conditions (see “Experimental Procedures”). C, pH dependence. Standard amidohydrolase assays were carried out in the following buffer solutions: sodium acetate (100 mM), pH 4–6, Tris (100 mM), pH 6.5–9; sodium borate (100 mM), pH 9–10.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>675 ± 64</td>
</tr>
<tr>
<td>Crude particulate</td>
<td>1,127 ± 215</td>
</tr>
<tr>
<td>Microsomes</td>
<td>2,113 ± 468</td>
</tr>
<tr>
<td>Supernatant</td>
<td>386 ± 89</td>
</tr>
</tbody>
</table>

1 The abbreviation used is: HPLC, high performance liquid chromatography.
analysis was performed using a Nova-Pak C18 column (3.9 x 150 mm, Waters) eluted at 1 ml/min with a gradient of methanol in water (from 60 to 100% over 20 min). Elution of products was followed by monitoring

### Table II

**Effects of divalent cations and enzyme inhibitors on rat brain microsome amidohydrolase activity**

Illustrated are the results from one experiment, carried out in quintuplicate and repeated twice with identical results, in which control amidohydrolase activity was 2289 ± 46 pmol/min/mg of protein. Abbreviations used: NEM, N-ethylmaleimide; pBPB, p-bromophenacyl bromide; PMSF, phenylmethylsulfonyl fluoride. Most drugs were diluted in standard assay buffer from dimethyl sulfoxide or methanol stock solutions, yielding concentrations of 0.05-0.1%, which had no effect on amidohydrolase activity. Bacitracin was dissolved in assay buffer.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amidohydrolase activity (pmol/min/mg)</th>
<th>% of control ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td></td>
<td>160 ± 2</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>105 ± 3</td>
<td>67 ± 2</td>
</tr>
<tr>
<td>EGTA</td>
<td>10</td>
<td>106 ± 2</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>MgCl2</td>
<td>10</td>
<td>102 ± 2</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>CaCl2</td>
<td>0.05</td>
<td>21 ± 2</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.025</td>
<td>52 ± 3</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>pBPB</td>
<td>0.05</td>
<td>5 ± 3</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>NEM</td>
<td>0.05</td>
<td>74 ± 4</td>
<td>120 ± 7</td>
</tr>
<tr>
<td>α-Phenanthroline</td>
<td>0.1</td>
<td>114 ± 5</td>
<td></td>
</tr>
<tr>
<td>Bacitracin</td>
<td>1 mg/ml</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

UV absorbance at 214 nm. 1-min fractions were collected for liquid scintillation counting. Thin layer chromatography analysis was carried out on silica gel G-coated plastic plates (Merck) eluted with a solvent system of chloroform/methanol (9:1). The Rf of anandamide in this solvent system was ~0.5. After having visualized the lipids with iodine vapors, 0.5-cm bands were cut, and radioactivity in the bands was determined by liquid scintillation counting.

**RESULTS AND DISCUSSION**

**Hydrolysis of [3H]Anandamide**—Exogenous [3H]anandamide, labeled radioactively on the ethanolamine moiety, was rapidly degraded by rat brain microsomes forming a product which, on polyvinylbenzene columns, had the chromatographic behavior of [3H]ethanolamine (Fig. 1A). When [3H]anandamide was labeled on the arachidonate moiety, similar incubations resulted in the formation of a product which comigrated with [3H]arachidonic acid on thin layer chromatography. In contrast, no degradation occurred in boiled tissue samples (data not shown). The results suggest that rat brain microsomes contain an amidohydrolase activity that catalyzes the cleavage of exogenous anandamide to ethanolamine and arachidonic acid. The subcellular fractionation illustrated in Table I shows that this activity was present in both particulate and microsomal fractions and was enriched in the latter.

Mouse liver microsomes incubated in the presence of

---

**Fig. 2. Substrate specificity of brain microsome amidohydrolase activity.** Relative rates of hydrolysis of various N-acetyl-[3H]ethanolamines are shown. The N-acetyl-[3H]ethanolamines depicted in the figure, synthesized as described under “Experimental Procedures,” were incubated (29 μM, 20,000 dpm/ml) for 5 min at 37°C with 0.2 mg/ml of microsome protein (specific radioactivities are listed under “Experimental Procedures”). Results are from one experiment, representative of three, carried out in quintuplicate.
NADPH convert anandamide into several oxygenated products, possibly formed by cytochrome P-450-dependent monooxygenation (Bornheim et al., 1993). Under our experimental conditions, oxidative metabolism does not appear, however, to participate in \( ^{3}H \)anandamide degradation. When samples from amidohydrolase assays (10-min incubation) were analyzed by reversed-phase HPLC, no evidence was found for the formation of polar \( ^{3}H \)anandamide metabolites (data not shown).

Amidohydrolase activity in microsomes was dependent on the concentration of protein (Fig. 1B) and was optimal at pH 6 and 8 (Fig. 1C). Moreover, the activity was dependent on the concentration of \( ^{3}H \)anandamide, with an apparent \( K_m \) of 12.7 \( \pm 1.8 \mu M \) and a \( V_{max} \) of 5630 \( \pm 200 \) pmol/min/mg of protein (n = 3). Divalent cations were neither necessary nor stimulatory for amidohydrolase activity; adding EGTA, EDTA, \( CaCl_2 \), or \( MgCl_2 \) (each at 10 mM) had little or no effect (Table II).

The serine protease inhibitor, phenylmethylsulfonyl fluoride, was shown to prevent the degradation of anandamide in brain homogenates (Deutsch and Chin, 1993) and to improve its metabolic stability in binding assays carried out on rat brain membranes (Abadji et al., 1994; Childers et al., 1994). In our experiments, phenylmethylsulfonyl fluoride inhibited amidohydrolase activity in a concentration-dependent manner (Table II). The effects of other protein-alkylating reagents (N-ethylmaleimide and \( p \)-bromophenacyl bromide), as well as of nonselective peptidase inhibitors (EDTA, \( \alpha \)-phenanthroline, bacitracin) are also shown in Table II. Among these, only \( p \)-bromophenacyl bromide, which alkylates histidine residues on proteins, produced a significant inhibition of the activity.

Substrate Specificity in Brain Microsomes—To study the substrate specificity of rat brain microsomal amidohydrolase, in a first series of experiments, we compared the rate of hydrolysis of \( ^{3}H \)anandamide (short-hand designation of the N-acyl chain \( 20:4 \Delta^2,5,8,11,14 \)) with those of five congeners, whose structures are depicted in Fig. 2 (N-homo-\( \gamma \)-linolenoyl-\( ^{3}H \)ethanolamine \( 20:3 \Delta^2,4,8,11,14 \), N-11,14-eicosadienoyl-\( ^{3}H \)ethanolamine \( 20:2 \Delta^2,11,14 \), N-11-eicosanoyl-\( ^{3}H \)ethanolamine \( 20:1 \Delta^1 \), N-\( \gamma \)-linolenoyl-\( ^{3}H \)ethanolamine \( 18:3 \Delta^2,5,9,12 \), and N-palmitoyl-\( ^{3}H \)ethanolamine \( 16:0 \)). The results indicate that, among the substrates tested, \( ^{3}H \)anandamide is hydrolyzed at the highest rate (Fig. 2). Although more slowly than anandamide, other polyunsaturated N-acyl ethanolamides are also hydrolyzed by brain amidohydrolase activity, whereas mono- and saturated N-acyl ethanolamides are not. Indeed, very little hydrolysis of N-palmitoyl-\( ^{3}H \)ethanolamine was observed even when the microsomes were incubated in the presence of 30 \( \mu M \) N-palmitoyl-\( ^{3}H \)ethanolamine (39 \( \pm 2 \) pmol/min/mg of protein).

In a second series of experiments, \( ^{3}H \)anandamide hydrolysis was measured in the presence of various nonradioactive N-acyl ethanolamides (Fig. 3). The concentration of \( ^{3}H \)anandamide in these assays was 14 \( \mu M \), and that of the competing products was 200 \( \mu M \). The results show that \( ^{3}H \)anandamide hydrolysis was best inhibited by unsaturated ethanolamides with a fatty acyl chain containing 18–20 carbon atoms and a number of cis double bonds comprised between two and four (Fig. 3). Structural modifications that resulted in reduced inhibitory efficacy included the following: 1) elongating the fatty acyl chain above 20 carbon atoms; 2) replacing cis double bonds...
Anandamide Amidohydrolase in Rat Brain Microsomes

### Table III

**Substrate specificity of brain particulate fraction amidohydrolase activity**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amidohydrolase activity % of control ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Anandamide</td>
<td>100</td>
</tr>
<tr>
<td>N-homo-γ-linoleoyl-[3H]ethanolamine</td>
<td>50 ± 2</td>
</tr>
<tr>
<td>N-11,14-Eicosadienoxy-[3H]ethanolamine</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>N-11-eicosenoxy-[3H]ethanolamine</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>N-palmitoyl-[3H]ethanolamine</td>
<td>29 ± 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Amidohydrolase activity % of control ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>100</td>
</tr>
<tr>
<td>Anandamide</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>N-homo-γ-linoleoylethanolamine</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>N-11,14-Eicosadienoylethanolamine</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>N-11-Eicosenoylethanolamine</td>
<td>93 ± 7</td>
</tr>
<tr>
<td>N-palmitoylethanolamine</td>
<td>92 ± 3</td>
</tr>
</tbody>
</table>

with *trans* double bonds; 3) decreasing the number of double bonds to one or eliminating them (Fig. 3).

Although our experiments do not provide a complete characterization of the structural requirements of brain microsome amidohydrolase activity, which may be best accomplished on a purified enzyme preparation, they do suggest that this activity is highly selective for anandamide. That other unsaturated N-acylthanolamines may be also substrates for this activity is in agreement with studies showing that more than one anandamide may be produced and released by stimulated neurons (Hanus et al., 1995; Pertwee et al., 1994; Di Marzo et al., 1994).

**Substrate Specificity in Brain Particulate Fractions**—The results shown in Table III demonstrate that the relative rates of hydrolysis of various N-acyl-[3H]ethanolamines and the inhibition of [3H]anandamide hydrolysis by various nonradioactive N-acylthanolamines are similar in brain particulate fractions and microsomes. One notable exception is N-palmitoyl-[3H]ethanolamine, which is hydrolyzed by particulate fractions at a rate that greatly exceeds that observed in microsomes. This result suggests that component(s) of particulate fraction may contain a nonspecific short chain amidohydrolase, which may participate in the hydrolysis of endogenous saturated N-acylthanolamines (Di Marzo et al., 1994).

**Tissue Distribution**—High levels of amidohydrolase activity were found in microsomes prepared from liver and brain tissues, whereas activity was low in kidney, intestine, stomach, lung, and spleen and barely detectable in skeletal muscle (Fig. 4A). Preliminary experiments showed that the substrate specificity of liver microsome amidohydrolase activity was similar to that observed in brain microsomes (data not shown).

**Regional Distribution in Brain**—Within the central nervous system, highest levels of amidohydrolase activity were measured in homogenates of globus pallidus, hippocampus, and substantia nigra; intermediate levels were measured in striatum, thalamus, cerebellum, and cortex; and lowest levels were measured in brainstem and medulla (Fig. 4B). Quantitative autoradiographic studies have shown that cannabinoid receptor binding is dense in globus pallidus, substantia nigra, hippocampus, entopeduncular nucleus, and cerebellum; moderate in cerebral cortex and caudate-putamen; and sparse in brainstem and spinal cord (Howlett et al., 1990; Herkenham et al., 1990, 1991). Thus, the levels of amidohydrolase activity in brain were in good correlation with the distribution of cannabinoid receptors. An important exception was constituted by the cerebellum, where to high levels of receptor density corresponded intermediate levels of amidohydrolase activity. This discrepancy may be accounted for by the fact that, within the cerebellum, cannabinoid receptors are densely concentrated in the molecular layer and only sparsely present elsewhere (Herkenham et al., 1991). Because our measurements were carried out in homogenates of whole cerebella, they likely reflect average levels of amidohydrolase activity in cerebellar tissue.

**Conclusions**—Two lines of evidence suggest that the amidohydrolase activity identified and partially characterized in the present study participates in the physiological degradation of anandamide in the central nervous system. First, this amidohydrolase activity, unlike those described in previous studies (Natarajan et al., 1984; Schmid et al., 1985, 1990), appears to be highly selective for [3H]anandamide. Criteria of selectivity include 1) the rate of hydrolysis, 2–100 fold faster with [3H]anandamide than with a series of closely related congeners and 2) the rank potency of nonradioactive N-acylthanolamines to compete for [3H]anandamide hydrolysis, greatest with anandamide and with its close structural congeners. Second, ananandamide amidohydrolase activity is discretely distributed in the rat central nervous system where its localization parallels, by and large, that of cannabinoid receptors (Herkenham et al., 1990, 1991; Matsuda et al., 1993). As for other neurotransmitter-metabolizing enzymes (e.g. acetylcholinesterase, Butcher and Wolfe (1984)), such parallel distribution supports the possibility that anandamide amidohydrolase activity participates in terminating the actions of anandamide at its sites of action.
Acknowledgments—We thank Dr. V. Di Marzo for participating in initial experiments and Prof. J.-C. Schwartz for helpful suggestions.

REFERENCES