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Anandamide Amidohydrolase Activity in Rat Brain Microsomes

IDENTIFICATION AND PARTIAL CHARACTERIZATION*

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An amidohydrolase activity present in rat brain microsomes catalyzes the hydrolysis of *N*-arachidonoyl-[^{3}H]ethanolamine ([^{3}H]anandamide), an endogenous cannabimimetic substance, forming [^{3}H]ethanolamine and arachidonic acid. Amidohydrolase activity is maximal at pH 6 and 8, is independent of divalent cations, has an apparent K_m for [^{3}H]anandamide of 12.7 ± 1.8 μM, and has a V_{max} of 5630 ± 200 pmol/min/mg of protein. Phenylmethylsulfonyl fluoride, a serine protease inhibitor, and *p*-bromophenacyl bromide, a histidine-alkylating reagent, inhibit the activity, whereas *N*-ethylmaleimide and various nonselective peptidase inhibitors (EDTA, *o*-phenanthroline, bacitracin) have no effect. Brain amidohydrolase activity exhibits high substrate specificity for [^{3}H]anandamide; *N*-γ-linolenoyl-, *N*-homo-γ-linolenoyl-, and *N*-11,14-eicosadienoyl- are hydrolyzed at markedly slower rates. Moreover, *N*-11-eicosaenoyl- and *N*-palmitoyl-[^{3}H]ethanolamine are not hydrolyzed. [^{3}H]Anandamide hydrolysis is inhibited competitively by nonradioactive anandamide and by other *N*-acylethanolamines with the following rank order of potency: anandamide > *N*-linoleoyl- = *N*-*cis*-linolenoyl- = *N*-γ-linolenoyl- = *N*-homo-γ-linolenoyl- > *N*-11,14-eicosadienoyl- > *N*-oleoyl- > *N*-docosahexaenoyl- > *N*-docosatetraenoyl > *N*-linoelaidoyl- > *N*-eicosaenoyl- > *N*-palmitoyl ≥ *N*-elaidoyl- = *N*-eicosanoyl-ethanolamine = 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 Δ^9-tetrahydrocannabinol, the major psychoactive principle of *Cannabis sativa*, has prompted the search for an endogenous substance with cannabimimetic properties (for review, see Howlett *et al.* (1990)). This search has recently led to the isolation from porcine brain of a cannabimimetic lipid derivative, identified as *N*-arachidonoylethanolamine and named anandamide (Devane *et al.*, 1992). Like Δ^9-tetrahydrocannabinol, anandamide binds with high affinity to brain cannabinoid receptors, reduces electrically evoked contractions in mouse vas deferens, and modulates the activities of adenylyl cyclase and voltage-dependent Ca^{2+} channels in neuroblastoma cell lines (Devane *et al.*, 1992; Vogel *et al.*, 1993; Felder *et al.*, 1993; Mackie *et al.*, 1993). Moreover, anandamide produces, *in vivo*, a series of behavioral responses typical of cannabinoid drug administration, including catalepsy, hypothermia, analgesia, and activation of the hypothalamo-pituitary axis (Smith *et al.*, 1994; Weidenfeld *et al.*, 1994).

Recently, two other cannabimimetic *N*-acylethanolamines (*N*-homo-γ-linolenoyl- and *N*-docosatetraenoyl ethanolamine) have been identified in brain tissue (Hanuš *et al.*, 1993; Pertwee *et al.*, 1994), suggesting that anandamide may belong to a family of lipid mediators serving as endogenous cannabimimetic messengers in the central nervous system. Biochemical studies lend further support to this hypothesis. Rat brain preparations were shown to catalyze the synthesis of anandamide via energy-independent condensation of arachidonic acid with ethanolamine (Kruszka and Gross, 1994; Devane and Axelrod, 1994). Furthermore, studies in our laboratory have shown that rat brain neurons in primary culture produce and release anandamide and other *N*-acylethanolamines when they are stimulated with membrane-depolarizing agents or Ca^{2+} ionophores. This reaction, which is both Ca^{2+}-dependent and neuron-specific, involves the phosphodiesterase-mediated cleavage of a membrane phospholipid precursor, *N*-acylphosphatidylethanolamine (Di Marzo *et al.*, 1994). Together, these results suggest that multiple pathways of anandamide formation may coexist in nervous tissue.

Despite these important advances, the fate of endogenous anandamide in the central nervous system is still poorly documented. Before the discovery of anandamide, the pioneering studies of Schmid and co-workers have demonstrated that ethanolamides of saturated fatty acids are hydrolyzed in tissues by an amidohydrolase (amidase) activity with broad substrate specificity (Natarajan *et al.*, 1984; Schmid *et al.*, 1985, 1990). That anandamide may be a substrate for amidohydrolase activity was suggested by recent reports showing hydrolytic cleavage of exogenous [^{3}H]anandamide by rat brain homogenates (Deutsch and Chin, 1993) or by intact brain neurons in primary culture (Di Marzo *et al.*, 1994).

We report now that rat brain microsomes contain an amidohydrolase activity that acts with high selectivity on [^{3}H]anandamide and other polyunsaturated *N*-acylethanolamines. This "anandamide amidohydrolase" activity is discretely distributed in rat central nervous system and is abundant in regions where cannabinoid receptors are densely expressed. Our results suggest therefore that anandamide amidohydrolase activity may participate in the degradation of endogenous anandamide at synaptic sites.

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EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Ethanolamine (28 Ci/mol) and [^{3}H]arachidonic acid (214 Ci/mmol) were from Amersham Corp., and fatty acid chlorides were from Nu-Chek Prep (Elysian, MN). Ethanolamine, phenylmethylsulfonyl fluoride, *p*-bromophenacyl bromide, *N*-ethylmaleimide, thimerosal, bacitracin, and *o*-phenanthroline were from Sigma.

Syntheses and Purification of Substrates—Syntheses of both radioactive and nonradioactive *N*-acylethanolamines 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*-acylethanolamines, [^{3}H]ethanolamine hydrochloride (50 μCi) was dried under vacuum, resuspended in the appropriate volume of nonradioactive ethanolamine, and added to the reaction mixtures. *N*-[^{3}H]Arachidonoylethanolamine was synthesized by allowing [^{3}H]arachidonic acid (28 μmol, 100 μCi) to react with ethanolamine (164 μmol) in the presence of *N*,*N*′-diisopropylcarbodiimide (8.4 mg) in 1 ml of dichloromethane for 40 min at 0–4 °C. *N*-Acylethanolamines were purified by semipreparative reversed-phase HPLC,[1] by using a Nova-Pak HR C_{18} column (6 μm, 7.8 × 300 mm) eluted at 3 ml/min with a gradient of methanol in water (from 70 to 100% over 40 min). Fractions containing *N*-acylethanolamines were dried, resuspended in methanol to a final concentration of ≈10 mM, and stored at −80 °C. Specific radioactivities of the *N*-acyl-[^{3}H]ethanolamines used in the present study were as follows: *N*-arachidonoyl-[^{3}H]ethanolamine, 0.31 mCi/mmol; *N*-dihomo-γ-linolenoyl-[^{3}H]ethanolamine, 0.52 mCi/mmol; *N*-11,14-eicosadienoyl-[^{3}H]ethanolamine, 0.51 mCi/mmol; *N*-11-eicosamonoenoyl-[^{3}H]ethanolamine, 0.62 mCi/mmol; *N*-palmitoyl-[^{3}H]ethanolamine, 0.31 mCi/mmol; *N*-γ-linolenoyl-[^{3}H]ethanolamine, 0.69 mCi/mmol.

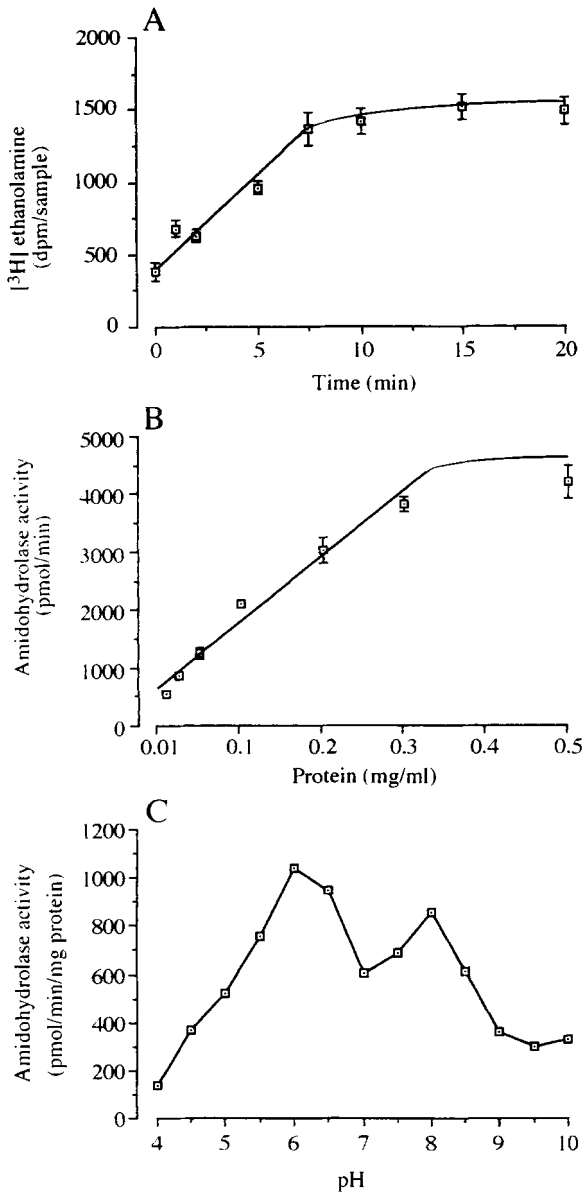


FIG. 1. **Hydrolysis of [^{3}H]anandamide by rat brain microsomes.** *A*, time dependence. Microsomes (0.2 mg of protein/ml) were incubated at 37 °C in 5 ml of Tris buffer (50 mM, pH 7.5) containing [^{3}H]anandamide (14 μM, 10,000 dpm). At the indicated times, samples of the incubation mixture were collected and diluted with methanol, and [^{3}H]ethanolamine was measured as described under "Experimental Procedures." *B*, protein dependence. Amidohydrolase activity was determined by measuring the [^{3}H]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 I

Subcellular distribution of rat brain amidohydrolase activity

Standard amidohydrolase assays and subcellular fractionations were carried out as described under "Experimental Procedures." The results are from one fractionation, representative of four, in which activity was measured in quintuplicate assays. To eliminate cellular debris, activity in the homogenate was measured after a 1-min centrifugation at 1,000 × *g*.

Fraction	Specific activity
	pmol/min/mg protein
Homogenate	675 ± 64
Crude particulate fraction	1,127 ± 215
Microsomes	2,113 ± 468
Supernatant	386 ± 89

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 EGTA. Tissue extracts were centrifuged 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 microsomes (0.2 mg of protein) and [^{3}H]anandamide (11–14 μM, 10,000 dpm/ml). [^{3}H]Anandamide and other *N*-acyl-[^{3}H]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 [^{3}H]anandamide (11 μM, 10,000 dpm). Under these conditions, [^{3}H]anandamide 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 ml) 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 (Porapak type Q, Waters) in methanol. [^{3}H]Ethanolamine was eluted in the void volume, and measured by liquid scintillation counting. Under these elution conditions, unreacted [^{3}H]anandamide and [^{3}H]ethanolamine-labeled phospholipids were quantitatively retained on the columns, which could be regenerated with ethanol (≈20 ml).

Additional Analytical Procedures—To determine whether [^{3}H]anandamide 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

[1] The abbreviation used is: HPLC, high performance liquid chromatography.

analysis was performed using a Nova-Pak C_{18} column (3.9 × 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 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 R_F 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.

Data Presentation—Each experiment was carried out in quintuplicate and repeated at least three times with identical results. Data are expressed as mean ± S.E.

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, phenylmethylsulfonylfluoride. 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.

Reagent	Concentration	Amidohydrolase activity
	mM	*% of control ± S.E.*
No addition		100
EDTA	10	116 ± 2
EGTA	10	105 ± 3
$MgCl_2$	10	106 ± 2
$CaCl_2$	10	102 ± 2
PMSF	0.05	21 ± 2
	0.025	52 ± 3
pBPB	0.05	5 ± 3
	0.025	9 ± 2
NEM	0.05	74 ± 4
	0.025	77 ± 4
o-Phenanthroline	0.1	129 ± 7
Bacitracin	1 mg/ml	114 ± 5

RESULTS AND DISCUSSION

Hydrolysis of [^{3}H]Anandamide—Exogenous [^{3}H]anandamide, labeled radioactively on the ethanolamine moiety, was rapidly degraded by rat brain microsomes forming a product which, on polydivinylbenzene columns, had the chromatographic behavior of [^{3}H]ethanolamine (Fig. 1*A*). When [^{3}H]anandamide was labeled on the arachidonate moiety, similar incubations resulted in the formation of a product which comigrated with [^{3}H]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

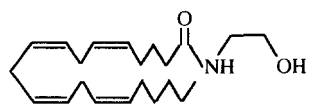
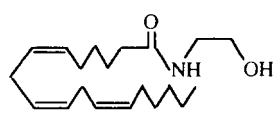
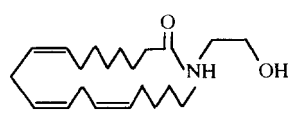
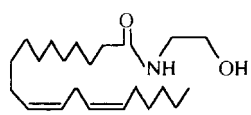
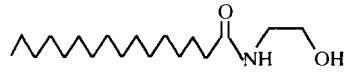
SUBSTRATE	RATE OF HYDROLYSIS (pmol/min/mg) mean ± SEM	(%)
 Anandamide (20:4 $\Delta^{5,8,11,14}$)	2926±185	100
 N-γ-linolenoyl-ethanolamine (18:3 $\Delta^{6,9,12}$)	1321±95	45
 N-homo-γ-linolenoyl-ethanolamine (20:3 $\Delta^{8,11,14}$)	1027±82	35
 N-11,14 eicosadienoyl-ethanolamine (20:2 $\Delta^{11,14}$)	1020±79	35
 N-11-eicosenoyl-ethanolamine (20:1 Δ^{11})	67±54	2
 N-palmitoyl-ethanolamine (16:0)	37±33	1

FIG. 2. **Substrate specificity of brain microsome amidohydrolase activity.** Relative rates of hydrolysis of various *N*-acyl-[^{3}H]ethanolamines are shown. The *N*-acyl-[^{3}H]ethanolamines depicted in the figure, synthesized as described under "Experimental Procedures," were incubated (28 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.

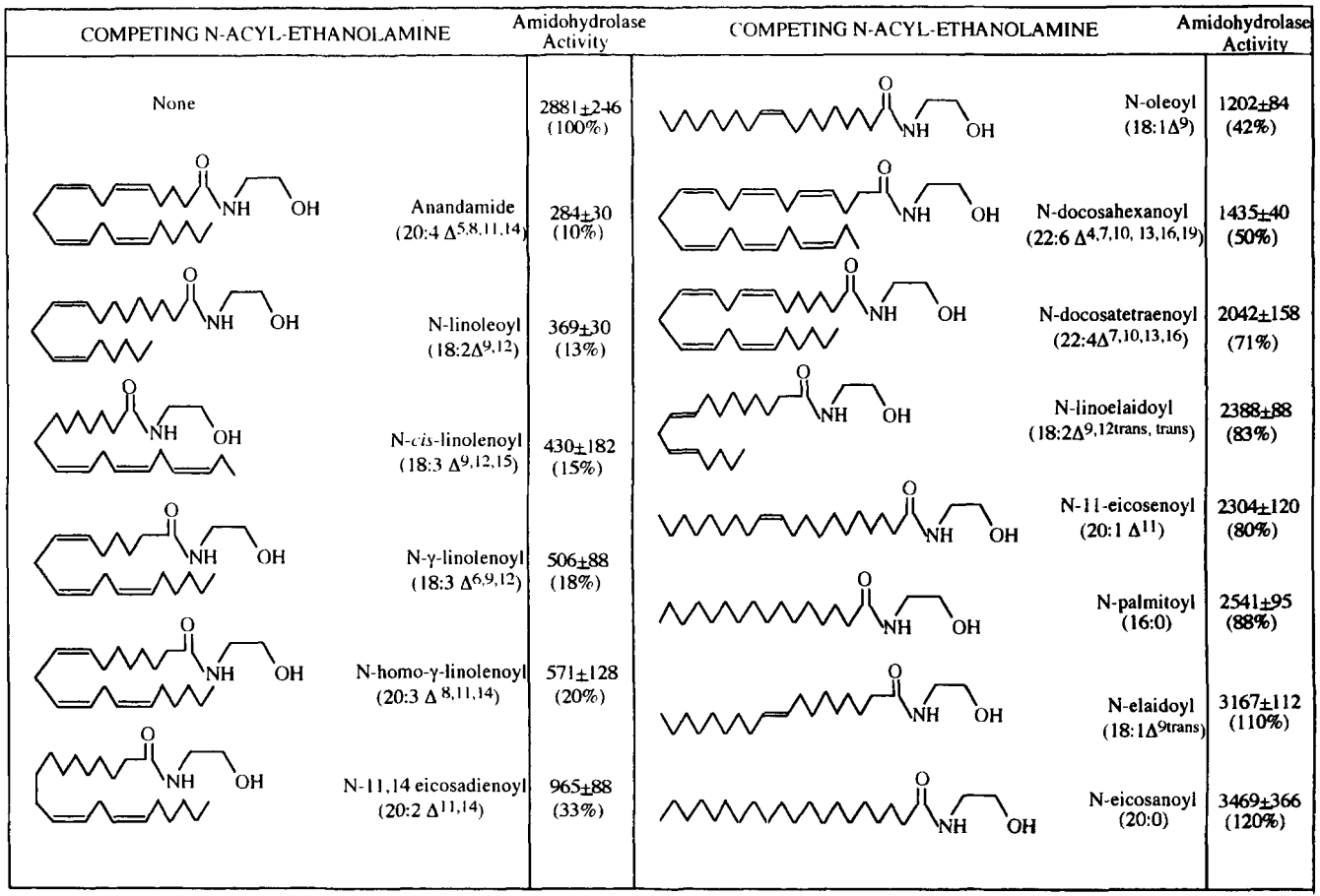


FIG. 3. **Substrate specificity of brain microsome amidohydrolase activity.** Effects of various *N*-acylethanolamines on [^{3}H]anandamide hydrolysis are shown. Standard assays were in the presence of [^{3}H]anandamide (14 μM, 10,000 dpm) and the *N*-acylethanolamines shown in the figure (200 μM). Results 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. 1*B*) and was optimal at pH 6 and 8 (Fig. 1*C*). Moreover, the activity was dependent on the concentration of [^{3}H]anandamide, with an apparent K_m of 12.7 ± 1.8 μM and a V_{max} of 5630 ± 200 pmol/min/mg of protein (n = 3). Divalent cations were neither necessary for nor stimulatory on 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, *o*-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 microsome 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 $\Delta^{5,8,11,14}$) with those of five congeners, whose structures are depicted in Fig. 2 (*N*-homo-γ-linolenoyl-[^{3}H]ethanolamine (20:3 $\Delta^{8,11,14}$), *N*-11,14-eicosadienoyl-[^{3}H]ethanolamine (20:2 $\Delta^{11,14}$), *N*-11-eicosaenoyl-[^{3}H]ethanolamine (20:1 Δ^{11}), *N*-γ-linolenoyl-[^{3}H]ethanolamine (18:3 $\Delta^{6,9,12}$), 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*-acylethanolamines are also hydrolyzed by brain amidohydrolase activity, whereas monounsaturated and saturated *N*-acylethanolamines 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 μM *N*-palmitoyl-[^{3}H]ethanolamine (39 ± 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*-acylethanolamines (Fig. 3). The concentration of [^{3}H]anandamide in these assays was 14 μM, and that of the competing products was 200 μM. 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

TABLE III
Substrate specificity of brain particulate fraction 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 1,000 ± 120 pmol/min/mg of protein. The chemical structures of the various substrates and competitors used in these experiments are depicted in Figs. 2 and 3.

	Amidohydrolase activity
	% of control ± S.E.
Substrate	
[^{3}H]Anandamide	100
N-homo-γ-linoleoyl-[^{3}H]ethanolamine	50 ± 2
N-11,14-Eicosadienoyl-[^{3}H]ethanolamine	19 ± 2
N-11eicosenoyl-[^{3}H]ethanolamine	7 ± 1
N-palmitoyl-[^{3}H]ethanolamine	20 ± 3
Competitor	
No addition	100
Anandamide	22 ± 1
N-homo-γ-linoleoylethanolamine	37 ± 1
N-11,14-Eicosadienoylethanolamine	58 ± 4
N-11-Eicosenoylethanolamine	93 ± 7
N-palmitoylethanolamine	92 ± 3

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*-acylethanolamines 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 (Hanuš *et al.*, 1993; 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-[^{3}H]ethanolamines and the inhibition of [^{3}H]anandamide hydrolysis by various nonradioactive *N*-acylethanolamines are similar in brain particulate fractions and microsomes. One notable exception is *N*-palmitoyl-[^{3}H]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*-acylethanolamines (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. 4*A*). 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. 4*B*). 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.

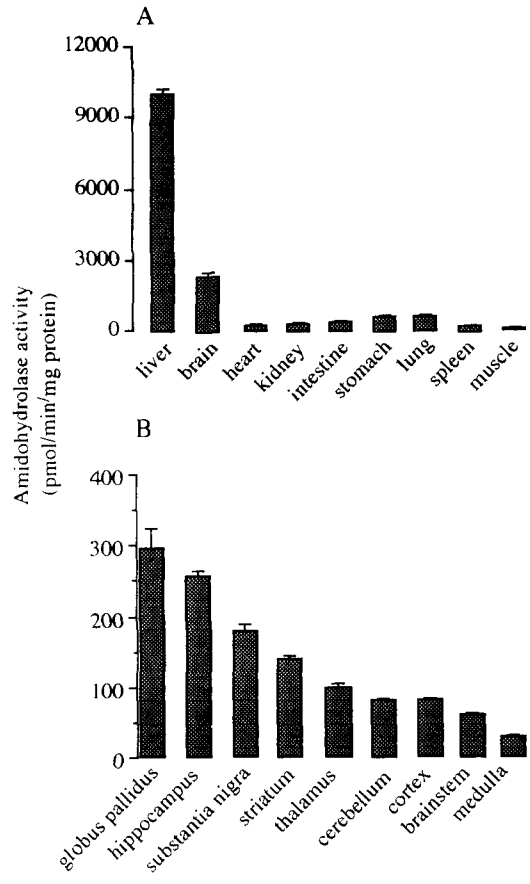


FIG. 4. **Distribution of amidohydrolase activity (*A*) in microsomes from various tissues and (*B*) in homogenates of various brain regions of the rat.** Assays were carried out on microsomes under standard conditions (*A*), or on freshly-prepared homogenates of various brain regions (1 mg/ml of protein, 10 min), with 22 μM [^{3}H]anandamide (20,000 dpm/ml) (*B*).

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 activity, unlike those described in previous studies (Natarajan *et al.*, 1984; Schmid *et al.*, 1985, 1990), appears to be highly selective for [^{3}H]anandamide. Criteria of selectivity include 1) the rate of hydrolysis, 2–100 fold faster with [^{3}H]anandamide than with a series of closely related congeners and 2) the rank potency of nonradioactive *N*-acylethanolamines to compete for [^{3}H]anandamide hydrolysis, greatest with anandamide and with its close structural congeners. Second, anandamide 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.

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REFERENCES

Abadji, V., Lin, S., Taha, G., Griffin, G., Stevenson, L. A., Pertwee, R. G., and Makriyannis, A. (1994) *J. Med. Chem.* **37,** 1889–1893

Bornheim, L. M., Kim, K. Y., Chen, B., and Correia, M. A. (1993) *Biochim. Biophys. Res. Commun.* **197,** 740–746

Butcher, L. L., and Wolfe, N. J. (1984) in *Handbook of Chemical Neuroanatomy* (Björklund, A., Hökfelt, T., and Kuhar, M. J., eds) Vol 3, part 2, p. 1

Childers, S. R., Sexton, T., and Roy, M. B. (1994) *Biochem. Pharmacol.* **47,** 711–715

Deutsch, D. G., and Chin, S. A. (1993) *Biochem. Pharmacol.* **46,** 791–796

Devane, W. A., and Axelrod, J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6698–6701

Devane, W. A., Hanuš, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) *Science* **258,** 1946–1949

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C., and Piomelli, D. (1994) *Nature* **372,** 686–691.

Felder, C. C., Briley, E. M., Axelrod, J., Simpson, J. T., Mackie, K., and Devane, W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7656–7660

Hanuš, L., Gopher, A., Almog, S., and Mechoulam, R. (1993) *J. Med. Chem.* **36,** 3032–3034

Herkenham, M., Lynn, A. B., Little, M. D., Johnson, M. R., Melvine, L. S., de Costa, B. R., and Rice, K. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1932–1936

Herkenham, M., Lynn, A. B., Johnson, M. R., Melvine, L. S., de Costa, B. R., and Rice, K. C. (1991) *J. Neurosci.* **11,** 563–582

Howlett, A. C., Bidaut-Russell, M., Devane, W. A., Melvin, L. S., Johnson, M. R., and Herkenham, M. (1990) *Trends Neurosci.* **13,** 420–423

Kruszka, K. K., and Gross, R. W. (1994) *J. Biol. Chem.* **269,** 14345–14348

Mackie, K., Devane, W. A., and Hille, B. (1993) *Mol. Pharmacol.* **44,** 498–503

Matsuda, L. A., Bonner, T. J., and Lolait, S. J. (1993) *J. Comp. Neurol.* **327,** 535–550

Natarajan, V., Schmid, P. C., Reddy, P. V., and Schmid, H. H. O. (1984) *J. Neurochem.* **42,** 1613–1619

Pertwee, R., Griffin, G., Hanuš, L., and Mechoulam, R. (1994) *Eur. J. Pharmacol.* **259,** 115–120

Schmid, P. C., Zuzarte-Augustin, M. L., and Schmid, H. H. O. (1985) *J. Biol. Chem.* **260,** 14145–14149

Schmid, H. H. O., Schmid, P. C., and Natarajan, V. (1990) *Prog. Lipid Res.* **29,** 1–43

Smith, P. B., Compton, D. R., Welsh, S. P., Razdan, R. K., Mechoulam, R., and Martin, B. R. (1994) *J. Pharmacol. Exp. Ther.* **270,** 219–227

Vogel, Z., Barg, J., Levy, R., Saya, D., Heldman, E., and Mechoulam, R. (1993) *J. Neurochem.* **61,** 352–355

Weidenfeld, J., Feldman, S., and Mechoulam, R. (1994) *Neuroendocrinology* **59,** 110–112