Partial Purification and Characterization of the Porcine Brain Enzyme Hydrolyzing and Synthesizing Anandamide

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Anandamide (arachidonylethanolamide) is known as an endogenous agonist for cannabinoid receptors. An amidohydrolase, which hydrolyzed anandamide, was solubilized from the microsomal fraction of porcine brain with 1% Triton X-100. The enzyme was partially purified by Phenyl-5PW hydrophobic chromatography to a specific activity of approximately 0.37 µmol/min/mg of protein at 37 °C. As assayed with 14C-labeled substrates, the apparent Km value for anandamide was 60 µM, and anandamide was more active than ethanolamides of linoleic, oleic, and palmitic acids. Ceramidase and protease activities were not detected in our enzyme preparation. The purified enzyme also synthesized anandamide from free arachidonic acid in the presence of a high concentration of ethanolamine with a specific activity of about 0.16 µmol/min/mg of protein at 37 °C. On the basis of cochromatographies, pH dependence, heat inactivation, and effects of inhibitors such as arachidonoyl trifluoromethyl ketone, p-chloromercuribenzoic acid, diisopropyl fluorophosphate, and phenylmethylsulfonyl fluoride, it was suggested that the anandamide amidohydrolase and synthase activities were attributable to a single enzyme protein.

Affinity of anandamide for cannabinoid receptors, probably due to protecting the compound from hydrolysis (10, 11). Recently, some properties of the enzyme were reported with a microsomal preparation of rat brain (12). On the other hand, the synthesis of anandamide from free arachidonic acid and ethanolamine was shown with rat (9), bovine (13), and rabbit (14) brain and was reported to be independent of ATP and coenzyme A (14). However, the enzyme(s) hydrolyzing and synthesizing anandamide has not yet been purified and well characterized, and it is still unknown whether the two enzyme activities are attributed to a single enzyme protein or two enzymes.

EXPERIMENTAL PROCEDURES

Materials—[1-14C]Arachidonic acid and [1-14C]linoleic acid were purchased from Amersham International (Amersham), [1-14C]oleic acid and [1-14C]palmitic acid from DuPont NEN, various fatty acids from Nu-Chek-Prep (Elysian), sphingosine from BIOMOL Research Laboratories (Plymouth Meeting, PA), PMSF from Sigma, p-chloromercuribenzoic acid (PCMB) and diisopropyl fluorophosphate from Wako Pure Chemical Industries (Osaka), arachidonyl trifluoromethyl ketone from Cayman Chemical Co. (Ann Arbor, MI), peptideyl 4-methylcoumaryl-7-amide (MCA) substrates from Peptide Institute (Osaka), and precoated Silica Gel 60 F254 glass plates for TLC (20 cm × 20 cm, 0.25-mm thickness) from Merck (Darmstadt). Anandamide and [1-14C]anandamide were chemically prepared from ethanolamine and nonradioactive or [1-14C]arachidonic acid, respectively, as described previously (15). Ethanolamides of other 14C-labeled fatty acids were also synthesized according to the previous method described for [1-14C]anandamide (15). [14C]Ceramide (N-oleylsphingosine) was chemically prepared from sphingosine and [1-14C]oleic acid (16).

Enzyme Preparation—Porcine brain was obtained at a local slaughterhouse. The brain (approximately 100 g) was homogenized in 9 times the volume (w/v) of ice-cold 20 mM Tris-HCl (pH 8) containing 0.32 M sucrose with a Potter-Elvehjem homogenizer. The following procedures were performed at 4 °C. The homogenate was centrifuged at 2,000 × g for 10 min, and the supernatant was further centrifuged at 20,000 × g for 20 min and at 105,000 × g for 40 min, successively. The resultant pellet (microsomal fraction, 300 mg of protein) was suspended in 42 ml of 50 mM Tris-HCl buffer (pH 8) containing 1% Triton X-100, kept for 2 h, and centrifuged at 105,000 × g for 40 min. The supernatant was stored as the solubilized protein at −80 °C until use. The solubilized protein (6–9 mg) was diluted in 20 ml of 20 mM citrate-sodium phosphate buffer (pH 6.0) containing 0.5 M ammonium sulfate and 0.05% Triton X-100 (solution A), passed through a 0.22-µm membrane filter, and loaded onto a Tosoh Phenyl-5PW column (7.5 mm inside diameter × 7.5 cm). The column was equipped with a Pharmacia fast protein liquid chromatography (FPLC) system and had been equilibrated with solution A. The chromatography was carried out at room temperature, and flow rate was 1.0 ml/min during the entire procedure. After loading the sample, the column was washed with 10 ml of solution A in which the concentration of ammonium sulfate was changed to 0.375 M, and adsorbed proteins were eluted in 2.5-ml fractions with a 60-mM linear gradient of ammonium sulfate (0.375–0 M) and then with 10 ml of ammonium sulfate-free solution A. Fractions with anandamide amidohydrolase activity of more than 0.2 nmoI/min/100 µl were pooled and stored at −80 °C. An appreciable loss of the enzyme activity was not observed either at 4 °C for 10 h or at −80 °C for at least 2 months. Protein concentration was determined by the method of Bradford (17) with bovine serum albumin as standard.

DEAE-ion exchange column chromatography was performed as fol-
Fractionation of the partially purified enzyme by DEAE-Sephacel. Anandamide amidohydrolase and synthase activities were purified 22-fold in a yield of 31% and showed an average specific enzyme activity of 368 (240–540) nmol/min/mg of protein. The amidohydrolase activity was detected only in the original position. It is unclear whether the enzyme found in peak 1 is an isozyme or a tight aggregate of the enzyme and other proteins.

Anandamide synthase activity was also found in peaks 1 and 2 (Fig. 2). The synthase in the peak 2 fractions showed an average specific enzyme activity of 160 nmol/min/mg of protein at 37°C in a yield of 29%. Hydrolysis and synthesis of anandamide by the partially purified enzyme are shown in lanes 3 and 8 on thin layer chromatograms (Fig. 1). When the partially purified enzyme (the peak 2 fractions in Fig. 2) was applied onto a DEAE-Sephacel column and the adsorbed protein was eluted by increasing NaCl concentration, both the amidohydrolase and synthase activities cochromatographed in one major peak (Fig. 3).

The amidohydrolyse and synthase activities of the partially purified enzyme were collected as described under "Experimental Procedures." Closed circles, amidohydrolase activity; open circles, synthase activity; closed triangles, protein concentration; broken line, ammonium sulfate concentration. 

**Fig. 1.** Hydrophobic chromatography of anandamide amidohydrolase and synthase. The solubilized protein of porcine brain microsomes (8.8 mg of protein) was applied onto a Phenyl-5PW column, and 2.5-ml fractions were collected as described under "Experimental Procedures." The enzyme activity was monitored by TLC.
The synthase activity depended on the concentrations of arachidonic acid (Fig. 5B) and ethanolamine (Fig. 5C). Their apparent \(K_{\text{m}}\) values were approximately 100 \(\mu\text{M}\) and 50 \(\text{mM}\), respectively. \(V_{\text{max}}\) values of the hydrolase and synthase reactions were 0.48 \(\text{mmol/min/mg}\) of protein and 0.11 \(\text{mmol/min/mg}\) of protein. With the same amount of enzyme, the amidohydrolase activity was 3–4 times higher than the synthase activity.

The two enzymes were active between pH 7 and 9 (Fig. 6). Furthermore, when the enzyme was preincubated at various temperatures for 5 min, the amidohydrolase and synthase activities were lost almost in parallel as the temperature was raised (Fig. 7).

Substrate specificity of the amidohydrolase reaction was examined with different fatty acyl ethanolamides (Fig. 5A). At 300 \(\mu\text{M}\) concentration, the relative amidohydrolase activity was 44% with linoleylethanolamide, 27% with oleylethanolamide, and 19% with palmitoyethanolamide as compared with arachidonyethanolamide (anandamide). On the other hand, the rate of ethanolamide synthesis was not very different with palmitic, oleic, linoleic, and arachidonic acids (Fig. 5B).

We tested whether the partially purified enzyme had a certain protease activity with hydrophobic peptidyl-MCA substrates; \(\alpha\)-butoxycarbonyl-Val-Leu-Lys-MCA (a substrate for plasmin), Leu-MCA (for aminopeptidase), succinyl-Ala-Ala-Ala-MCA (for elastase), succinyl-Ala-Ala-Pro-Phe-MCA (for chymotrypsin), succinyl-Ala-Pro-Ala-MCA (for elastase), succinyl-Leu-Leu-Val-Tyr-MCA (for chymotrypsin), and Met-MCA. These peptide-MCA substrates were inactive with our enzyme preparation. For the assay of ceramidase activity, \([14\text{C}]\)ceramide (N-oleylsphingosine) was incubated with the Triton X-100-

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Fig. 3. Cochromatography of the anandamide amidohydrolase
and synthase activities on a DEAE-5PW column. The partially
purified enzyme (1.3 mg) was applied onto a DEAE-5PW column as
described under "Experimental Procedures." Closed circles, amidohy-
drolase activity; open circles, synthase activity; broken line, NaCl
ccentration.

Fig. 4. Dependence on protein amount and time course of the
anandamide amidohydrolase and synthase reactions. A, different
amounts of the partially purified enzyme were assayed for amidohydro-
lase activity (closed circles) and synthase activity (open circles) under
the standard conditions. B, the partially purified enzyme (0.48 \(\mu\text{g}\) of
protein) was allowed to react for the indicated time periods for amidohy-
drolase activity (closed circles) and synthase activity (open circles).

Fig. 5. Substrate specificity of the enzyme reactions. The par-
tially purified enzyme (0.94 \(\mu\text{g}\) of protein) was allowed to react under
the standard conditions with various concentrations of substrates as
follows. A, ethanolamides of various \(14\text{C}\)-labeled fatty acids (closed
circles, arachidonylethanolamide; open circles, linoleylethanolamide;
closed triangles, oleylethanolamide; open triangles, palmitoylethanol-
amide); B, various \(14\text{C}\)-labeled free fatty acids in the presence of 250 \(\mu\text{m}\)
ethanolamine (closed circles, arachidonic acid; open circles, linoleic acid;
closed triangles, oleic acid; open triangles, palmitic acid); and C, etha-
nolamine in the presence of 250 \(\mu\text{m}\) arachidonic acid.

Fig. 6. pH dependence of the anandamide amidohydrolase
and synthase reactions. The partially purified enzyme (0.94 \(\mu\text{g}\) of
protein) was allowed to react at various pH values for amidohydrolase
activity (solid line) and synthase activity (broken line) under the stand-
ard conditions. pH was adjusted with the following buffers: closed
circles, citrate-Na\(_2\)HPO\(_4\); open circles, Tris-HCl; closed triangles,
Na\(_2\)CO\(_3\)-NaHCO\(_3\); open triangles, NaHCO\(_3\)-NaOH.

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Solubilized enzyme and the partially purified enzyme, and the produced [14C]oleic acid was separated from the remaining [14C]ceramide by TLC. The cholate extract of the 27,000 × g pellet of 16-day-old rat brain (18) was used as a positive control. The result showed that a low ceramidase activity (approximately 16 pmol/min/mg of protein) was detected in the Triton X-100-solubilized enzyme at pH 7.4 and pH 9, but not at pH 5. However, the partially purified enzyme did not show a detectable ceramidase activity at pH 5, 7.4, or 9.

We also tested the effects of various inhibitors on the amidohydrolase and synthase activities (Fig. 8). PMSF (9) and arachidonyl trifluoromethyl ketone (a cytosolic phospholipase amidohydrolase and synthase activities (Fig. 8). PMSF also inhibited the anandamide synthase activity (13). Sulfhydryl-reactive agents such as PCMB inhibited rat liver N-acylethanolamine amidohydrolase (21). In our assays, arachidonyl trifluoromethyl ketone (Fig. 8A) and PCMB (Fig. 8B) inhibited both the amidohydrolase and synthase activities almost in parallel. PMSF (Fig. 8C) also inhibited both the activities although its higher concentration was required for the inhibition of the synthase. Diisopropyl fluorophosphate, another serine protease inhibitor, inhibited both of the activities in a similar manner (Fig. 8D).

**DISCUSSION**

In consideration of potent psychoactivity of cannabinoids, there must be an in vivo mechanism which metabolizes and inactivates anandamide as an endogenous cannabinoid receptor agonist. It was reported that radioisotopically labeled anandamide was hydrolyzed rapidly to free arachidonic acid and ethanolamine in neuroblastoma and glioma cells (9). The brain and several other tissues of rats also hydrolyzed anandamide (9, 12). In these works, however, the enzyme was not found in various mammalian tissues (22), and the enzyme of rat liver membrane was solubilized with sodium taurodeoxycholate (21). In these works, however, the enzyme was not purified, and its reactivity with anandamide was not described.

Our purified anandamide amidohydrolase catalyzed the reverse reaction and produced anandamide from arachidonic acid and ethanolamine. The synthase was also reactive with other fatty acids (palmitic acid, oleic acid, and linoleic acid) at similar reaction rates (Fig. 5B). It was reported that arachidonic acid was a better substrate for the rabbit brain anandamide synthase than palmitic, oleic, and linoleic acids, but the reactivity was assayed at a low substrate concentration (5 μM) below K_m and was not compared in terms of V_max (14). Another report showed that with bovine hippocampal P2 membrane arachidonic acid was more active than palmitic acid at 30 μM-1 mM (13). It is unknown at the present time whether or not the discrepancy of substrate specificity between these results and our finding was attributable to different animal species or different assay conditions.

Both the amidohydrolase and synthase activities were copurified as peak 2 on a Phenyl-5PW column (Fig. 2) and cochromatographed as one major peak on a DEAE-5PW column (Fig. 3). The two activities were lost essentially in parallel by heat inactivation (Fig. 7) and various inhibitors (Fig. 8). Although our enzyme preparation was not purified to homogeneity, the results suggested that a single enzyme protein catalyzed both acids as substrates, anandamide (arachidonylethanolamide) was the most active, suggesting that the physiological role of this enzyme was the metabolic inactivation of anandamide. While we were preparing this manuscript, a similar substrate specificity was reported with the rat brain microsome (12). Moreover, several hydrophobic peptidyl-MCA substrates tested were inactive with our anandamide amidohydrolase, which could not be attributed to a certain protease with wide substrate specificity. Ceramidase, an amidohydrolase to hydrolyze ceramide (N-acylphosphosine) to sphingosine and a fatty acid, was distinguished from our enzyme which was inactive with N-oleylphosphosine.

Previously, N-acylethanolamine amidohydrolase activity was found in various mammalian tissues (22), and the enzyme of rat liver membrane was solubilized with sodium taurodeoxycholate (21). In these works, however, the enzyme was not purified, and its reactivity with anandamide was not described.

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the synthesis and hydrolysis of anandamide. A previous report suggested that the anandamide synthesis and hydrolysis were catalyzed by separate enzymes since the synthase activity was not inhibited by PMSF (9). However, we found that the synthase was also inhibited by PMSF with the partially purified enzyme (Fig. 8C) and the Triton X-100-solubilized protein (data not shown), although PMSF was less effective on the synthase than on the amidohydrolase. It should be noted that other lipid-related amidohydrolases such as N-acylethanolamine amidohydrolase of rat liver mitochondria (21) and ceramidase (23) are thought to be catalytically reversible enzymes. In agreement with this view, several lines of evidence so far available with our partially purified enzyme support the attribution of the hydrolysis and synthesis of anandamide to one enzyme protein. This conclusion must be confirmed by further purification of the enzyme to homogeneity and by expression of cDNA for this enzyme.

As presented in Fig. 5C, the ethanolamine concentration which gave a half-maximum activity of the anandamide synthase was as high as 50 mM. Other investigators also reported a high Km value for ethanolamine (27 ± 4 mM) with bovine hippocampal P2 membrane (13). Since such a high concentration of ethanolamine must be supplied for the enzyme to work as anandamide synthase, it is unlikely that the anandamide synthesis is catalyzed by this enzyme under physiological conditions. However, we cannot rule out a certain mechanism to activate the enzyme and to lower the Km for ethanolamine by either covalent modification or allosteric effect on the enzyme protein. As an alternative biosynthetic pathway, it was recently proposed that anandamide was released from N-arachidonyl phosphatidylethanolamine by the catalysis of a certain phospholipase D (24, 25).

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