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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 ^{14}C-labeled substrates, the apparent K_m 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 arachidonyl 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.

An endogenous agonist for cannabinoid receptor was isolated from porcine brain, and this compound referred to as anandamide was identified to be arachidonylethanolamide (1). It inhibited the specific binding of radiolabeled ligands to cannabinoid receptors, reduced cAMP production, and caused the inhibition of N-type calcium currents and calcium channel antagonist binding (1–5). Anandamide also inhibited electrically evoked contraction of vas deferens isolated from mice (1) and mimicked *in vivo* effects of cannabinoids such as antinociception, hypothermia, hypoactivity, and catalepsy in mice (6–8).

It was shown that anandamide was rapidly degraded by an amidase (amidohydrolase) activity which was found in the membrane fraction of cultured neuroblastoma and glioma cells and homogenates of rat tissues (9). In fact, the addition of PMSF,[1] a serine protease inhibitor (9), increased an apparent 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-^{14}C]Arachidonic acid and [1-^{14}C]linoleic acid were purchased from Amersham International (Amersham), [1-^{14}C]oleic acid and [1-^{14}C]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), peptidyl 4-methylcoumaryl-7-amide (MCA) substrates from Peptide Institute (Osaka), and precoated Silica Gel 60 F_{254} glass plates for TLC (20 cm × 20 cm, 0.25-mm thickness) from Merck (Darmstadt). Anandamide and [1-^{14}C]anandamide were chemically prepared from ethanolamine and nonradioactive or [1-^{14}C]arachidonic acid, respectively, as described previously (15). Ethanolamides of other ^{14}C-labeled fatty acids were also synthesized according to the previous method described for [1-^{14}C]anandamide (15). [^{14}C]Ceramide (*N*-oleoylsphingosine) was chemically prepared from sphingosine and [1-^{14}C]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 (v/w) 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 12 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-ml 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 nmol/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-

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[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; PCMB, *p*-chloromercuribenzoic acid; MCA, 4-methylcoumaryl-7-amide.

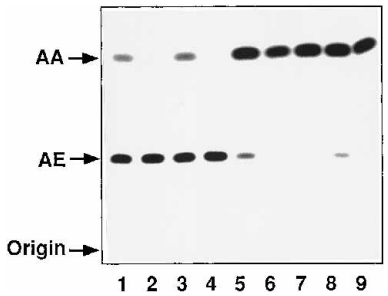

FIG. 1. **Anandamide amidohydrolase and synthase activities as examined by TLC.** The solubilized protein (31 μg of protein, *lanes 1*, *5*, and *6*), the heat-treated solubilized protein (31 μg of protein, *lanes 2* and *7*), the partially purified enzyme (0.94 μg of protein, *lanes 3* and *8*), or the protein-free buffer (*lanes 4* and *9*) was incubated with [1-^{14}C]anandamide (*lanes 1–4*) or with [1-^{14}C]arachidonic acid in the presence (*lanes 5* and *7–9*) or absence (*lane 6*) of ethanolamine under the standard conditions. The enzyme was denatured at 90 °C for 10 min before the enzyme assay. *AA*, arachidonic acid; *AE*, anandamide.

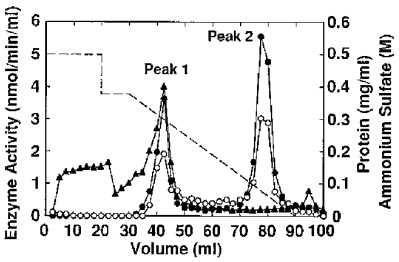

FIG. 2. **Hydrophobic chromatography of anandamide amidohydrolase and synthase.** The solubilized protein of porcine brain microsome (8.8 mg of protein) was applied onto a Phenyl-5PW column, and 2.5-ml fractions were collected as described under "Experimental Procedures." *Closed circles*, amidohydrolase activity; *open circles*, synthase activity; *closed triangles*, protein concentration; *broken line*, ammonium sulfate concentration.

lows. The partially purified enzyme was concentrated 7 times by ultrafiltration using an Amicon XM-50 membrane. During this procedure, the concentration of ammonium sulfate was reduced to about 20 mM. This material (1.3 mg of protein in 5 ml) was loaded onto a Tosoh DEAE-5PW column (7.5 mm inside diameter × 7.5 cm) which had been equilibrated with 20 mM citrate-sodium phosphate buffer (pH 6.0) containing 0.05% Triton X-100 (solution B). The flow rate was 1.0 ml/min during the entire procedure. After loading the sample, the column was washed with 15 ml of solution B, and adsorbed proteins were eluted in 2.5-ml fractions with a 50-ml linear gradient of NaCl (0–1 M) and then with 20 ml of solution B containing 1 M NaCl.

Enzyme Assay—For the amidohydrolase activity, the enzyme was incubated with 100 μM [1-^{14}C]anandamide (10,000 cpm in 5 μl of ethanol) at 37 °C for 20 min in 200 μl of 50 mM Tris-HCl (pH 9.0). For the anandamide synthase activity, the enzyme was incubated with 250 μM [1-^{14}C]arachidonic acid (50,000 cpm in 5 μl of ethanol) in 200 μl of 250 mM ethanolamine-HCl (pH 9.0) at 37 °C for 20 min. The reaction was terminated by the addition of 0.3 ml of a mixture of ethyl ether/methanol/1 M citric acid (30:4:1) and 10 μl of 2 N HCl. The ethereal extract was spotted on a Silica Gel 60 F_{254} glass plate, which was developed in the organic phase of a mixture of isooctane/ethyl acetate/water/acetic acid (50:110:100:20, v/v) for 15 min (10-cm plate) or for 75 min (20-cm plate) at room temperature. Distribution of radioactivity on the plate was detected by a Fujix BAS2000 imaging analyzer. Assays were performed in duplicate. For the ceramidase assay (18), the enzyme was incubated with 50 μM [^{14}C]ceramide (10,000 cpm) at 37 °C for 60 min in 200 μl of 100 mM buffer (Tris-HCl buffer at pH 9 or at pH 7.4 or citrate-Na_2HPO_4 buffer at pH 5) including 0.1% sodium cholate and 0.05% Triton X-100. The produced free [^{14}C]oleic acid was separated from the remaining [^{14}C]ceramide by TLC using a mixture of chloroform/methanol/acetic acid (94:1:5, v/v). The protease assay was performed with various peptidyl-MCA substrates as described in Ref. 19.

RESULTS

A homogenate of porcine brain was subjected to differential centrifugation. Each fraction was allowed to react with [1-^{14}C]anandamide, and free [^{14}C]arachidonic acid as a hydrolytic product was separated from the remaining [^{14}C]anandamide by TLC. Approximately 50%, 35%, 13%, and 7% of the total activity of the whole homogenate were recovered in the 2,000 × *g* pellet, 20,000 × *g* pellet, 105,000 × *g* pellet (microsomal fraction), and 105,000 × *g* supernatant (cytosol), respectively. The specific enzyme activities in these fractions were 5.4, 5.5, 7.5, and 1.7 nmol/min/mg of protein. The yield of the activity in the 105,000 × *g* pellet was low, but its specific enzyme activity was the highest. Therefore, the pellet referred to as the microsomal fraction was chosen as a starting material for purification of the anandamide amidohydrolase.

The microsomal fraction was treated with 1% Triton X-100. As shown in Fig. 1, the solubilized protein hydrolyzed [^{14}C]anandamide to free [^{14}C]arachidonic acid (*lane 1*). The same preparation also synthesized [^{14}C]anandamide from free [^{14}C]arachidonic acid in the presence of a high concentration of ethanolamine (*lane 5*), but not in its absence (*lane 6*). For identification of the product as anandamide, it was purified by TLC and reverse-phase high performance liquid chromatography with a solvent mixture of methanol/water/acetic acid (85:15:0.01) and analyzed by gas chromatography/mass spectrometry. Significant ion peaks were observed at *m/z* 329 (M − 18, dehydration of the parent ion), 315, 301, 287, 273, 259, 245, 232, 218, 204, 192, 178, 164, 139, 125, 124, 98 (product of a γ-cleavage fragment), and 85 (base peak, McLafferty rearrangement ion). Essentially the same spectra were obtained with synthetic anandamide. Either the amidohydrolase or synthase activity was not observed with heat-treated solubilized protein (*lanes 2* and *7* of Fig. 1).

For enzyme purification, the solubilized protein was directly loaded on a Tosoh Phenyl-5PW column. As shown in Fig. 2, when the proteins were separated by decreasing the concentration of ammonium sulfate, two peaks with the amidohydrolase activity were observed. The amidohydrolase in the peak 2 fractions was purified about 22-fold in a yield of 31% and showed an average specific enzyme activity of 368 (240–540) nmol/min/mg of protein at 37 °C. When the peak 2 fractions were subjected to 8.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, staining with silver showed several major bands. The intensity of a protein band corresponding to about 60 kDa changed in parallel with the amidohydrolase activity from fraction to fraction. Peak 1 also contained anandamide amidohydrolase in a bulk of protein. When the peak 1 fractions were pooled and loaded again on the Phenyl-5PW, the enzyme 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 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-5PW 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 amidohydrolase and synthase activities of the partially purified enzyme increased depending on the amount of protein almost in a linear fashion (Fig. 4*A*). The amidohydrolase reaction proceeded linearly up to 30 min while the rate of the anandamide synthase decreased gradually (Fig. 4*B*). The amidohydrolase activity increased depending on the concentrations of anandamide with an apparent K_m value of about 60 μM

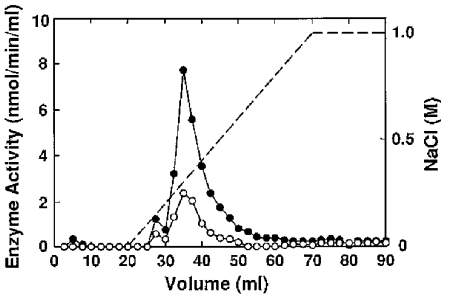

FIG. 3. **Cochromatography of the anandamide amidohydrolase and synthase activities on a DEAE-5PW column.** The partially purified enzyme (1.3 mg) was applied onto a DEAE-5PW column as described under "Experimental Procedures." *Closed circles*, amidohydrolase activity; *open circles*, synthase activity;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

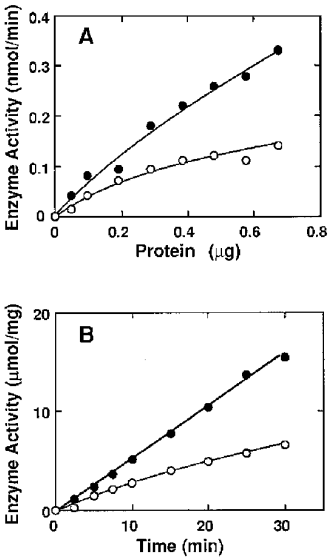

FIG. 4. **Dependence on protein amount and time course of the anandamide amidohydrolase and synthase reactions.** *A*, different amounts of the partially purified enzyme were assayed for amidohydrolase activity (*closed circles*) and synthase activity (*open circles*) under the standard conditions. *B*, the partially purified enzyme (0.48 μg of protein) was allowed to react for the indicated time periods for amidohydrolase activity (*closed circles*) and synthase activity (*open circles*).

(Fig. 5*A*). The synthase activity depended on the concentrations of arachidonic acid (Fig. 5*B*) and ethanolamine (Fig. 5*C*). Their apparent K_m values were approximately 100 μM and 50 mM, respectively. V_{max} values of the hydrolase and synthase reactions were 0.48 μmol/min/mg of protein and 0.11 μmol/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. 5*A*). At 300 μM concentration, the relative amidohydrolase activity was 44% with linoleylethanolamide, 27% with oleoylethanolamide, and 19% with palmitoylethanolamide as compared with arachidonylethanolamide (anandamide). On the other hand, the rate of ethanolamide synthesis was not very different with palmitic, oleic, linoleic, and arachidonic acids (Fig. 5*B*).

We tested whether the partially purified enzyme had a certain protease activity with hydrophobic peptidyl-MCA sub-

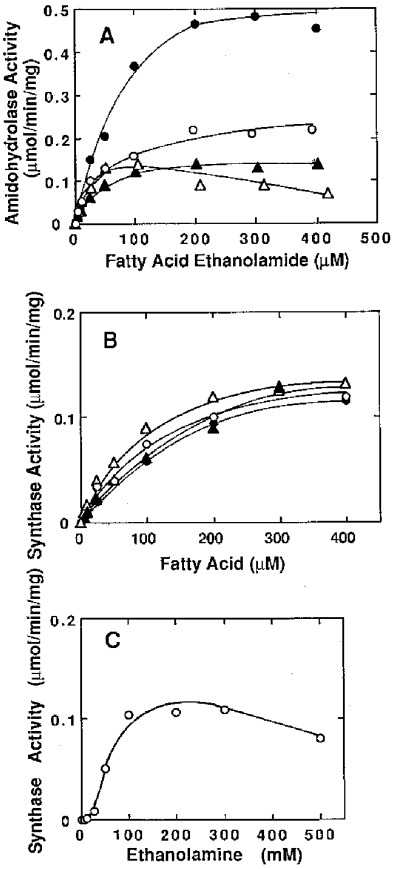

FIG. 5. **Substrate specificity of the enzyme reactions.** The partially purified enzyme (0.94 μg of protein) was allowed to react under the standard conditions with various concentrations of substrates as follows. *A*, ethanolamides of various ^{14}C-labeled fatty acids (*closed circles*, arachidonylethanolamide; *open circles*, linoleylethanolamide; *closed triangles*, oleoylethanolamide; *open triangles*, palmitoylethanolamide); *B*, various ^{14}C-labeled free fatty acids in the presence of 250 mM ethanolamine (*closed circles*, arachidonic acid; *open circles*, linoleic acid; *closed triangles*, oleic acid; *open triangles*, palmitic acid); and *C*, ethanolamine in the presence of 250 μM arachidonic acid.

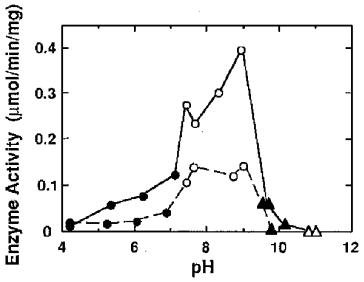

FIG. 6. **pH dependence of the anandamide amidohydrolase and synthase reactions.** The partially purified enzyme (0.94 μg of protein) was allowed to react at various pH values for amidohydrolase activity (*solid line*) and synthase activity (*broken line*) under the standard conditions. pH was adjusted with the following buffers: *closed circles*, citrate-Na_2HPO_4; *open circles*, Tris-HCl; *closed triangles*, Na_2CO_3-$NaHCO_3$; *open triangles*, $NaHCO_3$-NaOH.

strates; *t*-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 peptidyl-MCA substrates were inactive with our enzyme preparation. For the assay of ceramidase activity, [^{14}C]ceramide (*N*-oleoylsphingosine) was incubated with the Triton X-100-

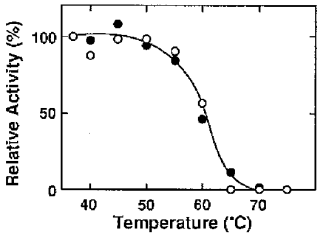

FIG. 7. **Heat inactivation of anandamide amidohydrolase and synthase.** The partially purified enzyme was kept at various temperatures for 5 min. An aliquot (0.94 μg of protein) was removed for the standard assays of amidohydrolase (*closed circles*) and synthase (*open circles*). The activity of untreated enzyme was expressed as 100%; amidohydrolase, 0.29 μmol/min/mg of protein; and synthase, 0.13 μmol/min/mg of protein.

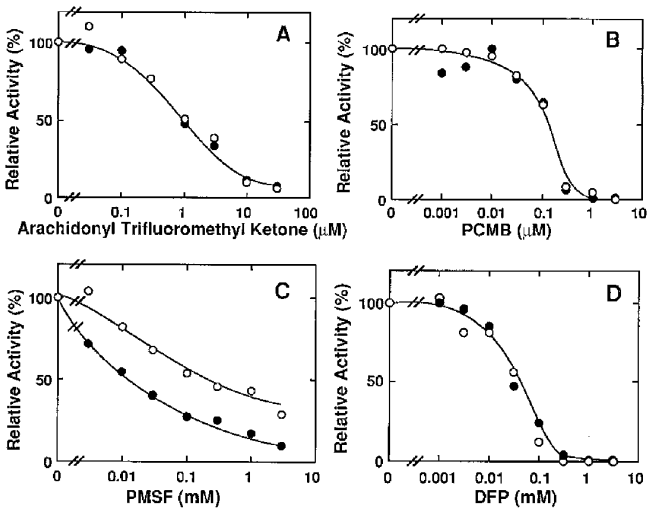

FIG. 8. **Various inhibitors for anandamide amidohydrolase and synthase reactions.** The partially purified enzyme (0.94 μg of protein) was assayed for amidohydrolase activity (*closed circles*) and synthase activity (*open circles*) under the standard conditions in the presence of different concentrations of arachidonyl trifluoromethyl ketone (*A*), PCMB (*B*), PMSF (*C*), and diisopropyl fluorophosphate (*D*). The activity in the control run was expressed as 100%; amidohydrolase, 0.27 μmol/min/mg of protein; and synthase, 0.10 μmol/min/mg of protein.

solubilized enzyme and the partially purified enzyme, and the produced [^{14}C]oleic acid was separated from the remaining [^{14}C]ceramide by TLC. The cholate extract of the 27,000 $\times$ *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 A_2 inhibitor) (20) have been reported to inhibit the anandamide hydrolysis with crude enzyme preparation. 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. 8*A*) and PCMB (Fig. 8*B*) inhibited both the amidohydrolase and synthase activities almost in parallel. PMSF (Fig. 8*C*) 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. 8*D*).

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 radiolabeled 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 the present paper, we attempted to isolate and purify the anandamide amidohydrolase from porcine brain, from which anandamide was first isolated (1). The enzyme was solubilized from the microsomal fraction with 1% Triton X-100, and the solubilized enzyme was partially purified by hydrophobic chromatography of high performance (Fig. 2). We tried several conventional column chromatographies such as ion exchange chromatography, hydrophobic chromatography, and gel filtration. However, probably due to its hydrophobicity, we have so far been unsuccessful in high purification of the enzyme protein by these methods giving only a low yield of the enzyme activity and a low purification of the enzyme. By the method presented here we could prepare the enzyme constantly with a specific activity of 240–540 nmol/min/mg of protein. This is the first report of the preparation of partially purified enzyme which could be used for enzymological studies.

When the partially purified enzyme was allowed to react with ethanolamides of arachidonic, linoleic, oleic, and palmitic 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*-acylsphingosine) to sphingosine and a fatty acid, was distinguished from our enzyme which was inactive with *N*-oleoylsphingosine.

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.

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. 5*B*). 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

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. 8*C*) 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. 5*C*, 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 K_m 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 K_m for ethanolamine by either covalent modification of 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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