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Distribution of anandamide amidohydrolase in rat tissues with special reference to small intestine

Kazuhisa Katayama [b], Natsuo Ueda [a], Yuko Kurahashi [a], Hiroshi Suzuki [a], Shozo Yamamoto [a,*], Itsuo Kato [b]

[a] *Department of Biochemistry, Tokushima University, School of Medicine, Kuramoto-cho, Tokushima 770, Japan*
[b] *Department of Cardiovascular Surgery, Tokushima University, School of Medicine, Kuramoto-cho, Tokushima 770, Japan*

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Abstract

Anandamide (arachidonylethanolamide), an endogenous ligand for cannabinoid receptors, is hydrolyzed by an amidohydrolase and its biological activity is lost. Previously, we partially purified the enzyme from porcine brain and anandamide synthesis by its reverse reaction was proposed (Ueda et al., (1995) J. Biol. Chem. 270, 23823–23827). The anandamide hydrolase and synthase activities were examined with various rat tissues. Rat liver showed the highest specific activities (4.4 ± 0.3 and 4.5 ± 0.5 nmol/min/mg protein) for the hydrolase and synthase, respectively. In most other tissues such as brain, testis and parotid gland, the ratio of synthase/hydrolase activity was 0.7–1.6. However, small intestine showed a relatively high synthase/hydrolase ratio of about 5.0 (1.0 ± 0.1 and 0.2 ± 0.1 nmol/min/mg protein). When a homogenate of small intestine was subjected to acetone extraction to remove lipids, a higher hydrolase activity was found (2.0 ± 0.2 nmol/min/mg protein). Furthermore, Northern blotting detected an intense mRNA band of anandamide hydrolase in small intestine as well as liver and brain. These results demonstrated for the first time a high content of anandamide hydrolase in small intestine.

Keywords: Anandamide; Cannabinoid; Arachidonic acid; Amidohydrolase; Small intestine; Rat

1. Introduction

Anandamide (arachidonylethanolamide) was found as an endogenous ligand for cannabinoid receptors [1] of either the brain type (CB1) or the peripheral type (CB2) with a wide tissue distribution [2]. Hydrolysis of anandamide to arachidonic acid and ethanolamine results in the loss of its biological activity. This reaction is catalyzed by an amidohydrolase the activity of which was detected in brain, liver, eye and several other tissues [3–8]. On the other hand, anandamide can be formed by the condensation of arachidonic acid with ethanolamine by 'anandamide synthase' which is found in brain, eye, uterus and oviduct [3,5,8–11]. Previously we partially purified the hydrolase from the microsome of porcine brain, and suggested that the anandamide synthase activity was due to the reverse reaction of the hydrolase itself [5]. Since the K_m value for ethanolamine in the synthase reaction was so high, 27–50 mM [5,9], the enzyme seemed to act as the hydrolase rather than the synthase under physiological conditions. On the basis of our enzymological findings on the porcine hydrolase,

Abbreviations: TLC, thin-layer chromatography.

* Corresponding author. Fax: +81 886 33 6409.

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we examined extensively tissue distribution of the enzyme in rats by assaying both anandamide hydrolase and synthase activities. We also performed Northern analysis by using the 'fatty-acid amide hydrolase' cDNA which was recently cloned by Cravatt et al. [12], and appears to be identical to the anandamide hydrolase.

2. Materials and methods

2.1. Materials

[1-^{14}C]Arachidonic acid (2.04×10^6 GBq/nmol) was purchased from Amersham International (Amersham, UK), arachidonic acid from Nu-Chek-Prep (Elysian, MN), anandamide from Cayman Chemical Company (Ann Arbor, MI), protein assay dye reagent concentrate from Bio-Rad (Hercules, CA), and precoated silica gel 60 F_{254} glass plates for TLC (20 cm × 20 cm, 0.25 mm thickness) from Merck (Darmstadt, Germany). [1-^{14}C]Anandamide was chemically prepared from [1-^{14}C]arachidonic acid and ethanolamine as described previously [13]. Reagents used for Northern blotting were purchased as described previously [14].

2.2. Enzyme preparation

Wistar rats (280–320 g weight) were anesthetized by diethyl ether, and sacrificed by cervical dislocation. Various organs were removed and homogenized in 5 times the volume (v/w) of ice-cold 20 mM Tris-HCl buffer (pH 8.0) containing 0.32 M sucrose with a Potter-Elvehjem homogenizer or a Polytron homogenizer. Each homogenate (2 ml) was mixed with 18 ml of cold acetone, and kept at 0°C for 20 min. The mixture was then centrifuged at $10\,000 \times g$ for 15 min. The resultant precipitate was dried under nitrogen gas, resuspended in 1 ml of 20 mM Tris-HCl buffer (pH 8.0), and used as an acetone-treated homogenate. The liver microsome fraction ($105\,000 \times g$ pellet) was prepared from homogenate by sequential centrifugation at $1000 \times g$ for 10 min, at $10\,000 \times g$ for 20 min and at $105\,000 \times g$ for 40 min. The microsomes were then suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 1% Triton X-100, kept at 4°C for 12 h, and centrifuged at $105\,000 \times g$ for 40 min. The supernatant thus prepared was used as a solubilized enzyme. All the enzyme preparations were stored at −80°C until use. Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard [15].

2.3. Enzyme assay

For the anandamide hydrolase assay, the enzyme was incubated with 100 μM [1-^{14}C]anandamide (10 000 cpm in 5 μl ethanol) at 37°C for 20 min in 200 μl of 50 mM Tris-HCl (pH 9.0). The assay for the anandamide synthase activity was carried out by incubation of the enzyme with 250 μM [1-^{14}C]arachidonic acid (50 000 cpm in 5 μl ethanol) in 200 μl of 250 mM ethanolamine-HCl (pH 9.0) at 37°C for 20 min. The reaction was terminated by addition of 0.4 ml of a mixture of diethyl ether/methanol/1 M citric acid (30:4:1, v/v) and 20 μl of 1 *N* HCl. The ethereal extract was spotted on a silica gel 60 F_{254} glass plate (10 cm length), and subjected to TLC with chloroform/methanol/ammonium hydroxide (80:20:2, v/v) for 18 min at room temperature. Radioactivity on the plate was quantified by a BAS 2000 imaging analyzer (Fujix, Tokyo, Japan).

2.4. Northern blotting

A cDNA fragment for rat fatty-acid amide hydrolase [12] was prepared by reverse transcriptase-polymerase chain reaction using rat liver poly(A)$^+$ RNA as a template. The primers used were: an upstream primer 5′-GCCTGAAAGCTCTACTGTGTGAGC-3′ and a downstream primer 5′-GCTCTAGATTAC-GATGGCTGCTTTTGAGG-3′. The prepared DNA fragment was then digested with *Xba*I resulting in the formation of a 781-bp fragment containing 1014-1787 (the numbers are due to reference 12), and labeled with [α-^{32}P]dCTP by the random primer DNA labeling system. Total RNA was extracted from rat tissues by the use of ISOGEN (a mixture of guanidium isothiocyanate and phenol) according to the manufacturer's instruction. The RNA (25 μg) was denatured, subjected to electrophoresis on a 1% agarose formaldehyde gel, transferred to a Hybond-N$^+$ membrane, and hybridized with the ^{32}P-labeled cDNA probe. Distribution of the radioactivity on the

membrane was visualized by a BAS 2000 imaging analyzer. Staining of 28S and 18S rRNA bands with ethidium bromide confirmed that essentially the same amount of RNA was applied on each lane.

3. Results

The anandamide hydrolase and synthase activities were screened in various tissues of rat. As shown in Fig. 1, when the homogenate of rat liver was allowed to react with radioactive anandamide or arachidonic acid, [arachidonyl-1-^{14}C]anandamide was converted to arachidonic acid (lane 1), but not with a protein-free buffer control (lane 3). On the other hand, [1-^{14}C]arachidonic acid was converted to anandamide in the presence of ethanolamine (lane 4), but not in its absence (lane 5). When similar experiments were carried out with a homogenate of rat small intestine, anandamide hydrolysis occurred to a much lesser extent (lane 2), whereas the small intestine was as active as the liver in the production of anandamide (lane 6).

Table 1 summarizes the enzyme activities tested with various rat tissues. By far the highest hydrolase activity was found in liver with a specific enzyme activity of 4.4 ± 0.3 nmol/min/mg protein. Brain, testis, parotid gland, kidney and submaxillary gland also showed considerable hydrolase activities. The anandamide synthase activity was also the highest in liver with a specific enzyme activity of 4.5 ± 0.5 nmol/min/mg protein. In most tissues, the synthase activity was comparable to the hydrolase activity under our assay conditions. However, small intestine showed a much higher synthase activity (1.0 ± 0.1

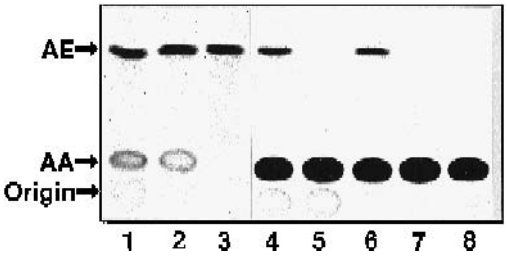

Fig. 1. Anandamide hydrolase and synthase activities in homogenates of rat liver and small intestine as examined by TLC. A homogenate (0.1 mg protein) of rat liver (lanes 1, 4 and 5) or small intestine (lanes 2, 6 and 7) or the protein-free buffer (lanes 3 and 8) was incubated either with [arachidonyl-1-^{14}C]anandamide (lanes 1–3) or with [1-^{14}C]arachidonic acid (lanes 4–8) under the standard conditions. Ethanolamine was present (lanes 4, 6 and 8) or absent (lanes 5 and 7). AA, arachidonic acid; AE, anandamide.

Table 1
Distribution of the anandamide hydrolase and synthase activities in native and acetone-treated homogenates of various rat tissues

Tissues	Enzyme activities (nmol/min/mg protein) [a]			
	Hydrolase		Synthase	
	Native	Acetone-treated	Native	Acetone-treated
Liver	4.36 ± 0.28	5.00 ± 0.06	4.49 ± 0.51	4.71 ± 0.10
Cerebrum	0.86 ± 0.04	0.79 ± 0.11	0.58 ± 0.06	0.83 ± 0.11
Cerebellum	0.56 ± 0.05	0.40 ± 0.02	0.40 ± 0.30	0.34 ± 0.06
Testis	0.55 ± 0.02	0.82 ± 0.08	0.61 ± 0.13	0.63 ± 0.04
Parotid gland	0.42 ± 0.09	0.32 ± 0.04	0.34 ± 0.03	0.25 ± 0.07
Kidney	0.30 ± 0.10	0.23 ± 0.03	0.30 ± 0.07	0.33 ± 0.01
Submaxillary gland	0.28 ± 0.02	0.32 ± 0.02	0.30 ± 0.06	0.30 ± 0.02
Small intestine	0.22 ± 0.09	2.02 ± 0.20	1.00 ± 0.11	1.88 ± 0.17
Stomach	0.14 ± 0.10	0.67 ± 0.04	0.48 ± 0.11	0.62 ± 0.02
Lung	0.14 ± 0.01	0.26 ± 0.03	0.22 ± 0.01	0.27 ± 0.03
Spleen	0.13 ± 0.06	0.13 ± 0.06	0.19 ± 0.07	0.14 ± 0.01
Colon	0.07 ± 0.06	0.48 ± 0.04	0.42 ± 0.36	0.54 ± 0.02
Esophagus	N.D.	N.D.	N.D.	N.D.
Heart	N.D.	N.D.	N.D.	N.D.
Skeletal muscle	N.D.	N.D.	N.D.	N.D.

[a] Assays were carried out with 0.1 mg of native and acetone-treated homogenates for the hydrolase and synthase under standard conditions. Values are shown as mean $\pm$ S.D. ($n = 4$). N.D., not detectable.

nmol/min/mg protein) than hydrolase activity (0.2 ± 0.1 nmol/min/mg protein). The synthase activity was also higher than the hydrolase activity in stomach and colon.

The rat liver homogenate showed the anandamide hydrolase and synthase activities increasing as the enzyme amount was raised (Fig. 2A). However, in small intestine the synthase activity did not increase depending on the enzyme amount, and only a low hydrolase activity was detected although the protein amount was raised (Fig. 2B). This finding suggested the presence of endogenous inhibitors of the two enzyme activities, especially of the hydrolase. The homogenate from small intestine (0.1 mg protein) inhibited the hydrolase activity of rat liver microsome by 50%, and these inhibitory factors were heat-stable (data not shown).

When we extracted the homogenate of small intestine with acetone, and tested the acetone extract on the hydrolase and synthase activities of the solubilized liver enzyme, it was found that the hydrolase was inhibited dose-dependently and the synthase was also inhibited, but to a lesser degree (Fig. 3). We questioned if lipids in the acetone extract were inhibitory to the enzyme activity. Upon TLC the inhibitory activities were mainly detected in the bands corresponding to free fatty acids, polar lipids and monoacylglycerols (Fig. 4). In agreement with this finding, when 500 μM of pure oleic acid, 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine or 2-arachidonoylglycerol was included in the reaction mixture, the hydrolase activity was reduced to 21%, 51% and 28%, and the synthase activity was reduced to 52%, 73% and 63%, respectively ($n = 2$). For removal of the lipid inhibitors, proteins in the homogenate of small intestine were precipitated with 90% cold acetone, and resuspended in a buffer. This acetone-treated homogenate was then subjected to the enzyme assays. As shown in Fig. 2C, the hydrolase activity was now clearly detected, and the acetone treatment increased the specific activities of hydrolase and synthase by 4- to 5-fold and 1- to 2-fold, respectively.

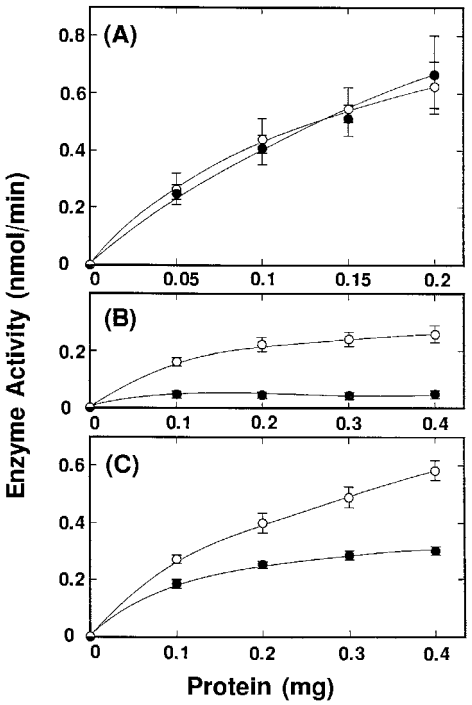

Fig. 2. Dependence of the anandamide hydrolase and synthase reactions on protein amount. Different amounts of the native homogenate of liver (A), the native homogenate of small intestine (B) and the acetone-treated homogenate of small intestine (C) were assayed for anandamide hydrolase (closed circles) and synthase (open circles) under the standard conditions. Values are shown as mean ± S.D. ($n = 4$).

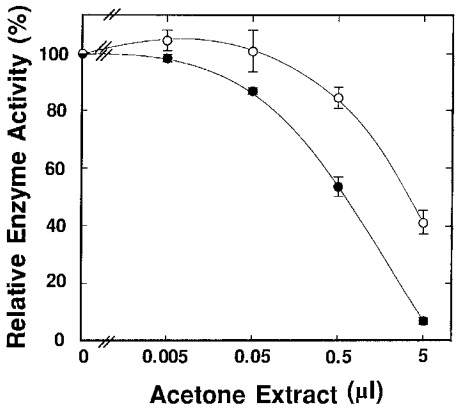

Fig. 3. Inhibition of the anandamide hydrolase and synthase reactions by an acetone extract of small intestine. The acetone extract was prepared from the homogenate (17 mg protein) of rat small intestine, and dissolved in 0.3 ml of ethanol. The solubilized enzyme of rat liver microsome (30 μg of protein) was assayed for hydrolase (closed circles) and synthase (open circles) in the presence of different amounts of the acetone extract dissolved in 5 μl ethanol. Values are shown as mean ± S.D. ($n = 4$). Enzyme activities in the absence of acetone extract were indicated as 100% (16 nmol/min/mg protein for the hydrolase and 19 nmol/min/mg protein for the synthase).

On the basis of these results, we re-examined the tissue distribution of the hydrolase and synthase activities with the acetone-treated homogenates. As shown in Table 1, the hydrolase activity was comparable to the synthase activity in all the tissues tested. The highest hydrolase activity was found in liver (a specific activity of 5.0 ± 0.1 nmol/min/mg protein), followed by small intestine (a specific activity of 2.0 ± 0.2 nmol/min/mg protein). Stomach and colon also showed considerable hydrolase activities.

Northern blotting for the hydrolase mRNA was performed with various rat tissues using a ^{32}P-labelled probe (Fig. 5). A major radioactive band around 2.5 kb was detected in the RNA preparations from small intestine and stomach as well as liver and brain. Only faint bands around 2.5 kb were observed from the RNA of testis, parotid gland, kidney, submaxillary gland and spleen. Another slightly bigger band was observed in small intestine and other organs in our study and also in the work of Cravatt et al. [12]. Its identification awaits further investigations.

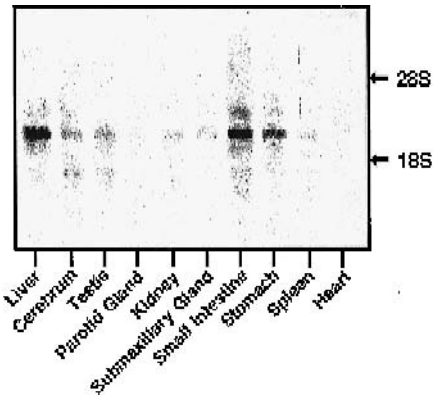

Fig. 5. Northern blot analysis of rat anandamide hydrolase. Total RNAs (25 μg) isolated from various rat tissues were subjected to Northern blotting with a probe as described in Section 2. 28S and 18S show the positions of 28S and 18S rRNA bands.

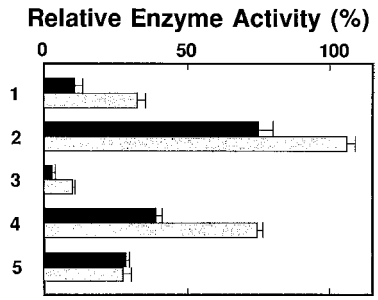

Fig. 4. Inhibition of the anandamide hydrolase and synthase activities by endogenous lipids of small intestine. An acetone extract obtained from 52.8 mg protein of rat small intestine homogenate was applied to TLC and developed with the organic phase of a solvent mixture of ethyl acetate/isooctane/acetic acid/H_2O (110:50:20:100, v/v) up to the height of 20 cm. The bands corresponding to non-polar lipids (3.0–5.0 cm from the top), free fatty acids (5.5–8.5 cm), monoacylglycerols (9.5–11.5 cm) and polar lipids (16.5–19.0 cm) were scraped separately. The lipids were then eluted with methanol, which was evaporated under nitrogen gas, and the residue was dissolved in 200 μl of ethanol. Assays for the hydrolase (solid bar) and the synthase (stippled bar) were performed with the solubilized protein of rat liver microsome (30 μg protein) in the presence of the ethanol solution (5 μl) including each fraction of total lipids (lane 1), non-polar lipids (lane 2), free fatty acids (lane 3), monoacylglycerols (lane 4) or polar lipids (lane 5). Values are shown as mean $\pm$ S.D. ($n = 4$). Enzyme activities in the absence of lipids were expressed as 100% (13 nmol/min/mg protein for the hydrolase and 17 nmol/min/mg protein for the synthase).

4. Discussion

Since we suggested previously that 'anandamide amidohydrolase' from porcine brain could also catalyze the reverse reaction, namely, the formation of anandamide from arachidonic acid and ethanolamine [5], we attempted to expand this observation to the enzyme of other tissues, and carried out simultaneous determinations of both the hydrolase and synthase activities in various tissues of rat which is an easily available experimental animal. Previously, Desarnaud et al. screened only the hydrolase (but not the synthase) in various rat tissues [4].

In most tissues examined the synthase activity was comparable to the hydrolase activity as reported in our previous work with porcine brain enzyme [5]. However, rat small intestine showed a very high ratio of synthase/hydrolase activity. A similar tendency was observed with stomach and colon. In view of these observations, we presumed the occurrence of tissue-specific isozymes with different catalytic properties, but our attempts to separate the two possible isozymes have so far been unsuccessful. Furthermore, we predicted that these tissues may have endogenous factors affecting the enzyme activities. We extracted the inhibitory factors with acetone from the ho-

mogenate of small intestine, and the substances were tentatively identified as free fatty acids, polar lipids and monoacylglycerols. Various free fatty acids were shown to be substrates for the synthase and products by the hydrolase [5]. Thus, endogenous fatty acids and their related compounds may bind to the enzyme as inhibitors. An earlier study by Schmid et al. showed that rat liver *N*-acylethanolamine amidohydrolase, presumably identical to anandamide amidohydrolase, was inhibited by free oleic acid [16]. Since the enzyme activities were stable in cold acetone, the removal of lipids by the acetone treatment increased the specific enzyme activities. Thus, the enzyme assay with the native homogenates was misleading, and the acetone-treated homogenates showed that small intestine had a high hydrolase activity. When a highly purified preparation of the enzyme is available, the mechanism of how the endogenous lipid factors inhibited the hydrolase more potently than the synthase and the structure-activity relationship of these lipids would be interesting subjects of enzymological investigation.

Fatty-acid amide hydrolase, which hydrolyzes oleamide as a putative endogenous sleep inducer, was recently cloned [12]. The recombinant enzyme, overexpressed in COS-7 cells, preferred anandamide as the substrate, and the fatty-acid amide hydrolase is presumed to be identical to the anandamide hydrolase, with which we worked in the present study. Its mRNA was abundant in liver, brain, and testis where the anandamide hydrolase activity was high [12]. Although digestive organs were not examined by Cravatt and co-workers, we found that the small intestine was rich in this mRNA.

Although we found a considerable anandamide hydrolase activity in alimentary tract, its physiological role is still unclear. The enzyme may play a role in digestion and detoxification of various exogenous fatty acid amides since the enzyme hydrolyzes not only anandamide but also ethanolamides of other fatty acids and oleamide [5,12,17]. Chocolate was shown to contain anandamide and other fatty acid ethanolamides [18], which may be hydrolyzed by the enzyme in gastrointestinal organs. Cannabinoids and anandamide inhibit electrically evoked contraction of myenteric plexus of guinea pig intestine [19]. Since cannabinoids probably exert immunosuppressive and anti-inflammatory effects through CB2 receptor expressed in immune cells [2], the immune system in the alimentary tract may be a target for anandamide. There are intestinal cells of various structures and functions. It is important to identify in which type of cell the anandamide hydrolase is localized. Preparation of a specific antibody and its application may give a clue to elucidate physiological functions of the enzyme in alimentary tract.

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