Kinin-Forming Enzyme in Rat Brain Mitochondria Fraction and Biological Activity of a Kinin Released from Rat Plasma Kininogen by This Enzyme

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Accepted October 4, 1983

Abstract—The kinin-forming enzyme of rat brain was studied by bioassaying kinin using a rat uterus. This enzyme released a kinin from the partially purified kininogen of rat plasma. The activity is exclusively distributed in the mitochondrial fraction and was detected in the pH range of 2.5–4.0 (optimally at pH 3.0). The enzyme was potently inhibited by pepstatin, but not by aprotinin. Released kinin was extracted by *n*-butanol and it was purified using Amberlite CG-50 absorption and CM-cellulose column chromatography. The elution profile of kinin from the CM-cellulose column did not coincide with that of bradykinin, Lys-bradykinin or Met-Lys-bradykinin. Isolated kinin was inactivated by treatment with chymotrypsin, but not with trypsin. In addition to the contractile activity on rat uterus, the kinin caused contraction of guinea pig ileum, with the response being potentiated by the presence of bradykinin-potentiator B. It also relaxed a rat duodenum, decreased rat blood pressure, and increased the vascular permeability in guinea pigs. Relative potencies of kinin on these pharmacological activities did not coincide with those of bradykinin. From these results, it is concluded that a kinin-forming enzyme is present in the rat brain. It is a cathepsin D-like enzyme, and furthermore, the enzyme releases a kinin-like peptide from the plasma kininogen fraction.

Many physiologically active peptides located in the peripheral tissues such as substance P, neurotensin, cholecystokinin and vasoactive intestinal polypeptide have been demonstrated in the brain or nervous tissues (1–4). However, evidence regarding the presence of kinin has never been reported, until a recent paper demonstrated the presence of a bradykinin-like immunoreactive substance in the rat brain by Corra et al. (5). Extensive work has been done on the kallikrein-kinin system in peripheral tissues; however, very little is known about its role in the brain. Among components reported are a kallikrein-like enzyme (6, 7), a kininogen-like substance (7, 8) and kininase (9, 10) in the brain; kallikrein-like activity has been reported in the microsomal fraction of the rabbit brain, but its activity was detected only by the pretreatment with acetone or

acid (6). In the rat brain, the formation of activity corresponding to a kinin-like substance was observed in the acidic pH range using bioassay (7). The evidence showing the possibility for the presence of a kinin-forming enzyme in the brain, however, was uncertain as to whether the substance causing contraction of the isolated smooth muscle was kinin under the experimental conditions employed.

In the present paper, we studied the kinin-forming activity in rat brain by using partially purified kininogen from rat plasma as the substrate.

Materials and Methods

Materials

DEAE-Sephadex A-50 was from Pharmacia Fine Chemicals (Uppsala, Sweden), CM-cellulose from Nakarai Chemicals

(Kyoto, Japan), and Amberlite CG-50 resin from Organo Company (Tokyo, Japan). Bradykinin, Lys- and Met-Lys-bradykinin, bradykinin potentiator B and pepstatin were purchased from the Protein Research Foundation (Osaka, Japan). Trypsin (bovine pancreas, type III), chymotrypsin (bovine pancreas, Type II), soybean trypsin inhibitor, diisopropylfluorophosphate (DFP) and phenylmethyl sulfonylfluoride (PMSF) were obtained from Sigma Chemical Company (U.S.A.). Aprotinin was a gift from Hoechst Japan, and highly purified porcine pancreatic kallikrein was kindly supplied by Bayer Yakuin Ltd. (Japan).

Bioassay of kinin

An isolated rat uterus preparation was used for assays of kininogen concentration or kinin-forming activity. Virgin rats weighing 180–250 g were injected s.c. with 0.2 mg/kg of estradiol benzoate (Teikoku Zoki, Tokyo, Japan) 16 hr before assay. The isolated uterus was suspended at 28°C in a 10 ml siliconized organ bath filled with de Jalon's solution containing 2 μ M atropine and 4 μ M di-phenhydramine gassed with air. Contractile responses were measured isotonicly for 90 sec at 2 min intervals. Synthetic bradykinin was used as the standard.

Preparation of kininogen fraction from rat plasma

Partially purified kininogen was obtained from rat plasma according to the procedure of Li et al. (11). The preparation was dialyzed against 0.15 M NaCl, and the content was 0.84 nmole bradykinin equiv./mg protein.

Enzyme preparation from rat brain

Male Sprague-Dawley rats, weighing 200–350 g, were anesthetized with pentobarbital Na (30 mg/kg, i.p.), and the infusion of cold saline, through a cannula inserted in each carotid artery, was completed for removal of blood from the brain. After exsanguinating from the jugular veins, the whole brain was removed. The tissue was homogenized with 9 vol. of 0.32 M sucrose (buffered with 10 mM Tris-HCl, pH 7.0), with subcellular fractionation being carried out according to the procedure of Koenig et al. (12). The homogenate was centrifuged at 800 g for 10 min to separate the fraction containing nuclei and unbroken cells. The supernatant

fluid was centrifuged at 15,700 g for 20 min, and the pellet, the crude mitochondrial fraction, was washed once with the sucrose solution. The combined supernatant was centrifuged at 100,000 g for 60 min. The pellet was designated as the microsomal fraction. The pellets were suspended in an appropriate volume of 0.15 M NaCl, and the soluble supernatant was dialyzed against 0.15 M NaCl.

Assay of kinin-forming activity

Kinin-formation was detected by bioassay using a rat uterus. Incubation was carried out in 250 μ l of 50 mM glycine-HCl buffer (pH 3.0) containing 0.5 mM EDTA, 0.24 mg protein of the kininogen preparation, and 0.05–0.4 mg protein from the enzyme preparations, using siliconized tubes at 37°C. After incubating for 1 hr, the mixture was chilled in ice, and the formed kinin was assayed using the rat uterus.

For comparison, the kinin-forming activity of purified porcine pancreatic kallikrein was determined, using partially purified beef plasma kininogen as a substrate, according to the method of Moriwaki et al. (13).

Extraction and purification of kinin from the incubation mixture

Isolation of kinin from the reaction mixture was performed using the procedure of bradykinin extraction (14).

1. Reaction and n-butanol extraction: The reaction mixture contained 60 mg protein of the crude mitochondria of rat brain, 36 mg protein of partially purified plasma kininogen, 0.5 mM EDTA and 50 mM glycine-HCl buffer (pH 3.0) in a total volume of 37.5 ml. When the amount of kinin attained a plateau after incubation for 6 hr at 37°C, the reaction was terminated by adjusting the pH to 2.0 with 1.0 N HCl.

This mixture was saturated with NaCl, and the solution was shaken twice with 60 ml of n-butanol. The butanol phases were combined, and 30 g of anhydrous sodium sulfate was added to eliminate the water. The suspension was agitated for 15 min and stored overnight at –20°C. After the organic phase was separated by centrifugation, 12 ml of ethylether was added. The solution was agitated for 5 min with 12 ml distilled water, and extraction was repeated. The combined

aqueous phase was lyophilized.

2. Amberlite CG-50 absorption: The lyophilizate was dissolved in 10 ml distilled water, and 3 g of Amberlite CG-50 resin (100–200 mesh, H^+ -form) was added. The pH was adjusted to 4.0 with 0.5 N NaOH, and gentle stirring was continued for 10 min. The mixture was centrifuged, and the resin was washed three times with 20 ml distilled water. Kinin was eluted by shaking twice with 10 ml of 50% acetic acid. The elute was lyophilized.

3. CM-cellulose chromatography: The lyophilizate was dissolved in 5 ml of 0.01 M ammonium formate (pH 5.0), and the solution was applied to a column of CM-cellulose (0.6×20 cm) previously equilibrated with 0.01 M ammonium formate (pH 5.0) containing 1.0 mM EDTA. The column was washed with 30 ml of the buffer, and then the elution was performed with a linear-gradient of the buffer (100 ml) against 0.5 M ammonium formate (pH 7.5) containing 1.0 mM EDTA (100 ml) (flow rate 20 ml/hr, 3 ml/fraction). Kinin activity in the elute was measured using the rat uterus.

Protein determination

Protein was determined by the modified Lowry procedure (15) with bovine serum albumin as the standard.

Fig. 1. Influence of pH on the kinin-forming activity of the crude mitochondrial fraction in rat brains. Points and vertical bars represent means $\pm$ S.D. for four experiments, expressed as pmoles bradykinin equiv./mg protein/min. Each 50 mM buffer was adjusted to yield the following pH in the presence of 0.5 mM EDTA: glycine-HCl (2.0–4.0), acetate (4.0–6.0), sodium phosphate (6.0–8.0), Tris-HCl (8.0–9.0).

Results

Influences of pH on the kinin-forming activity: Figure 1 shows the influence of pH on the kinin-forming activity of the crude mitochondria of the rat brain. Kinin liberation from the plasma kininogen fraction was only observed in the acidic pH range, optimally at pH 3.0. At pH 3.0, the kinin formation increased linearly up to 2 hr of incubation and to a concentration of 3.0 mg protein/ml of the enzyme preparation (Fig. 2a and 2b).

Subcellular distribution of the kinin-forming activity at pH 3.0: Table 1 shows the subcellular distribution of the kinin-forming activity of the rat brain at pH 3.0. Specific activity was higher in the crude mitochondrial fraction than in the other fractions. More

Fig. 2. Kinin-forming activities as a function of incubation time and as a function of the amount of crude mitochondrial fraction from the rat brain. a) the time course of activity; b), effect of the amount of crude mitochondrial fraction on kinin-formation. Points and vertical bars represent means $\pm$ S.D. for four experiments, expressed as a) nmoles/mg protein and b) pmoles/min.

than 70% of the total activity was observed in this fraction.

Effects of inhibitors: Table 2 shows the effects of various proteinase inhibitors on the kinin-forming activity in the rat brain and the purified porcine pancreatic kallikrein. The rat brain enzyme was potently inhibited by pepstatine, but porcine kallikrein was not inhibited at all. Aprotinin, which is a potent inhibitor of pancreatic kallikrein, did not affect the brain enzyme. The brain enzyme was inhibited by PMSF and DFP, but not by SBIT.

Purification of kinin from the incubation

mixture and its identification: As summarized in Table 3, kinin liberated from the plasma kininogen fraction by incubating with the crude mitochondrial fraction was extracted and purified by the procedures described in Materials and Methods. The column chromatogram obtained using CM-cellulose is illustrated in Fig. 3, in comparison to authentic bradykinin by assay with rat uterus. Kinin extracted from the incubation mixture was detected as one peak, and this eluted with a elution volume different from those of authentic bradykinin, Lys-bradykinin and Met-Lys-bradykinin (Fig. 3a and 3b). When the

Table 1. Subcellular distribution of kinin-forming enzyme in rat brain

Fraction	Specific activity ^a (pmoles/mg protein/min)	Total activity (%)
Whole homogenate	1.89±0.18	100
Nuclei & unbroken cells	0.69±0.14	8.9
Crude mitochondria	2.51±0.10	71.4
Microsomes	0.27±0.04	2.4
Supernatant	1.04±0.80	8.4

^aValues represent means±S.D. as bradykinin equivalent for four experiments.

Table 2. Effects of some proteinase inhibitors on the kinin-formation by the crude mitochondrial fraction of rat brain and porcine pancreatic kallikrein

Inhibitor	Concentration	% inhibition	
		Brain crude mitochondria ^d	Pancreatic kallikrein ^e
Pepstatin	2 nM	21	— ^g
	20	32	—
	200	95	0
PMSF ^a	0.2 mM	9	—
	2.0	37	0
DFP ^b	0.1 mM	21	100
	1.0	58	—
	10.0	100	—
Aprotinin	50 KIU/ml ^f	—	100
	500	0	—
SBTI ^c	0.1 mg/ml	—	—
	1.0	0	0

Values represent means of % inhibition for three experiments. ^aPhenylmethyl sulfonyl fluoride, ^bDiisopropyl fluorophosphate, ^cSoybean trypsin inhibitor. Specific activity of control was ^d2.27 pmoles/mg protein/min or ^e680 nmoles/mg protein/min, expressed as bradykinin equivalent assayed by rat uterus. ^fKallikrein inhibiting units, ^gNot determined. Purified porcine pancreatic kallikrein was incubated at the concentration of 1 µg/ml with partially purified beef plasma kininogen in 50 mM Tris-HCl buffer, pH 8.0, at 30 °C for 1 min.

Table 3. Purification of kinin from the incubation mixture

Step	Volume (ml)	Concentration (nmoles/ml)	Total (nmoles)	Yield (%)
Incubation mixture	37.5	0.68	25.5	100
Butanol extract	10.0	2.53	25.3	99.2
Amberlite adsorption	5.0	2.82	14.1	55.3
CM-cellulose chromatography	5.0	2.10	10.5	41.2

The amounts of kinin are expressed as bradykinin equivalent assayed by rat uterus.

Fig. 3. CM-cellulose chromatography of extracted kinin and standard kinins. The solutions of kinins in 10 mM ammonium formate buffer, pH 5.0, were applied to CM-cellulose columns (0.6×20 cm). Columns were washed and eluted as described in Materials and Methods. Fractions of 3 ml were collected. a), standard kinins (each 1 nmole) were separately applied: bradykinin (—○—), Lys-bradykinin (—△—) and Met-Lys-bradykinin (—●—). b), extracted kinin (3.6 nmole bradykinin equivalent using a rat uterus) of Amberlite CG-50 elute. c), mixture of bradykinin (1 nmole) and extracted kinin (3.6 nmole bradykinin equivalent using a rat uterus). Fractions were assayed by using a rat uterus, expressed as nmoles bradykinin equiv./fraction.

mixture of extract and the authentic bradykinin was applied, two peaks were detected (Fig. 3c).

Effects of trypsin and chymotrypsin

Fig. 4. Effects of trypsin and chymotrypsin digestion on the contractile activity of isolated kinin on a rat uterus. Eluted kinin from CM-cellulose column and bradykinin were incubated with trypsin (10 μ g/ml) or chymotrypsin (10 μ g/ml) in 0.12 M Tris-HCl buffer, at pH 7.8, each time. Reactions were stopped by the addition of SBTI (50 μ g/ml) for trypsin or DFP (2 mM) for chymotrypsin. Residual kinins were assayed by use of a rat uterus and expressed as % of the control. Isolated kinin incubated with trypsin (---○---) and chymotrypsin (---△---). Bradykinin with trypsin (—●—) and chymotrypsin (—▲—). Points and vertical bars represent means $\pm$ S.D. for three experiments.

digestion on the activity of purified kinin:

Figure 4 shows the contractile activities of purified kinin eluted from the CM-cellulose column and authentic bradykinin incubated with trypsin and chymotrypsin as a function of incubation time. The contractile activity of the kinin on the rat uterus was not affected with trypsin treatment, similar to what was observed for bradykinin. By chymotrypsin treatment, the kinin was inactivated in a manner similar to bradykinin, but the inactivation rate was slower than that for bradykinin.

Biological activities of purified kinin: As shown in Fig. 5, the purified kinin con-

Fig. 5. Pharmacological activities of isolated kinin. The preparations of A), an isolated guinea pig ileum, and B), an isolated rat duodenum, were suspended at 28°C in a 10 ml organ bath filled with Tyrode's solution containing 2 μ M atropine and 4 μ M diphenhydramine gassed with air. The response of contraction or relaxation was recorded isotonicly for 2 min. The doses of kinin were in A): a, bradykinin (5 nM); b, bradykinin (10 nM); c, bradykinin-potentiator B (0.25 μ M) followed by bradykinin (5 nM); d, isolated kinin (10 nM); e, bradykinin-potentiator B (0.25 μ M) followed by isolated kinin (10 nM); and in B): a, bradykinin (0.05 nM); b, bradykinin (0.075 nM); c, isolated kinin (0.04 nM); d, isolated kinin (0.08 nM). C), blood pressure of rat: a male Sprague-Dawley rat, weighing 450 g, was anesthetized with urethane (1.5 g/kg, i.p.), and the blood pressure was recorded at the carotid artery. Kinins were dissolved in saline and injected into the carotid vein at a volume of 50 μ l. The doses of kinins were: bradykinin, 1.25 (a) and 2.5 (b) nmole; isolated kinin, 0.05 (c) and 0.10 (d) nmole. The amounts of isolated kinin were expressed as bradykinin equivalent assayed by a rat uterus.

tracted the guinea pig ileum preparation, relaxed the rat duodenum and depressed rat blood pressure, actions similar to those of bradykinin. Pretreatment of the guinea pig ileum with bradykinin potentiator B peptide, which is known to be a decapeptide (16), increased the potential for contractile activity

Fig. 6. Effects of isolated kinin on vascular permeability increasing activity in guinea pig. Abdominal hair of the animals (350 g, male) was removed using an electric clipper before experiments. One ml of 0.5% Evans blue in saline was injected into the basilic vein, and then saline, isolated kinin and authentic bradykinin were administered intracutaneously into the animals at 0.1 ml volume. Ten minutes later, the animal was decapitated and skinned, and the blueing of the injection areas was observed visually.

by the kinin.

The permeability increasing activity of isolated kinin is presented in the photograph of Fig. 6. No blueing area was observed at the top of the center of the skin injected with saline. On the other hand, injection of isolated kinin caused visible blueing in dose-dependence. The authentic bradykinin showed a little lower activity than that of purified kinin. However, when the amounts of isolated kinin were converted to the bradykinin equivalent assayed by the rat uterus, their effects seemed similar.

Table 4 summarizes the potencies of the kinin as compared to those of bradykinin. Assuming that the kinin was equipotent with bradykinin on the rat uterus, the potencies on guinea pig ileum and rat duodenum were about 3 and 1.5 times less than those of bradykinin, respectively. The potency on rat blood pressure was about 20-fold that of bradykinin.

Discussion

In order to clarify the presence of a kinin-forming enzyme in the brain, the kinin formed from the kininogen fraction of rat

Table 4. Comparison of potencies of the isolated kinin and bradykinin

	Relative amounts of bradykinin required for the same response as isolated kinin
Rat uterus contraction	1
Guinea pig ileum contraction	0.32
Rat duodenum relaxation	0.77
Blood pressure fall in rat	20
Vascular permeability in guinea pig	1

Values are expressed as the isolated kinin to be equipotent with bradykinin on rat uterus.

Values are means of four experiments.

plasma by a rat brain preparation was determined using bioassay. Hori provided primary evidence for the presence of a kinin-forming enzyme in rabbit brain (6). This enzyme was only activated by treatment with acid or acetone, acts at neutral pH on plasma kininogen, and is distributed in the microsomal fraction of the brain. In the present results, however, the kinin-formation was detected only in the acidic pH ranges, optimally at pH 3.0, and was exclusively distributed in the crude mitochondrial fraction of the rat brain. Furthermore, in our preliminary experiment, no kinin-forming activity in the present enzyme preparation was detected at neutral pH even by acid treatment (pH 5.0, 2 hr) as reported by Hori (6). The reason for these distinctions regarding the presence of the neutral kinin-forming enzyme in the brain might depend on different procedures used in the treatments of brains from different species. However, another reason may be the presence of potent kininase activity at the neutral pH range. In the present preparation, kininase activity measured by bradykinin inactivation was detected over broad pH ranges, maximally at pH 6.5–8.5 (data not shown). Therefore, the kinin-forming activity in the neutral range may be masked by the degrading enzyme. In this respect, the kinin-forming activity detected in the acidic pH range seems to be unaffected by kininases because in the pH range below 5.0, kininase activity was hardly detected.

The kinin-forming enzyme of the brain found in this experiment appears to be a cathepsin-D like acid protease because 1) the activity was located exclusively in the crude mitochondrial fraction, which is rich in lysosomes (12), and 2) the activity was

potently inhibited by pepstatin, a potent inhibitor of cathepsin-D (17). The CM-cellulose column elution profile of the extracted kinin from the reaction mixture was not consistent with those of the authentic bradykinin, Lys-bradykinin, and Met-Lys-bradykinin. Moreover, the kinin is about 20-fold more potent in decreasing rat blood pressure and about 3-fold less potent in contracting the guinea pig ileum than bradykinin, in contrast with its action on the rat uterus. Those pharmacological potencies show that the formed kinin in this experiment is not bradykinin. In addition to this evidence, the treatment of kinin with trypsin caused neither increased potential nor decreased the action of kinin on rat uterus. The above results support the idea that the kinin may not contain the peptide sequence of bradykinin, which is unlikely to be Lys-, or Met-Lys-bradykinin as shown in Fig. 3. In these points, the enzyme observed in the present experiment seems to be different from splenic acid kininogenases, which are catheptic enzymes and form Met-Lys-bradykinin from plasma kininogen (18).

If the kinin is formed in the brain under pathological conditions such as an injury and inflammation, this may play a role in various processes, as in the peripheral tissues (19). For example, in multiple sclerosis, considered one of the most serious neurological diseases, the cathepsin-D-like acid proteases activity is elevated at the edge of active plaques (20), and it has been suggested that the elevated enzyme activity may play an important role in the mechanism of myelin breakdown in this allergic inflammatory disease (21). The acid protease appears to be identical with the kinin-

forming enzyme because of its pepstatin susceptibility (22). Therefore, it is considered that the kinin may be produced in lesions and may participate in inflammatory processes.

From the above evidences, it is impressive that the kinin-forming enzyme in the nervous tissue releases the kinin-like peptide from the plasma kininogen fraction. Further experiments are being carried out for elucidating the physiological or pathological significance of the kinin-forming enzyme in the brain.

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