CENTRAL ACTION OF BRADYKININ (I)
ELECTROENCEPHALOGRAM OF BRADYKININ AND
ITS DEGRADATION SYSTEM IN RAT BRAIN

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Abstract—Both in vivo and in vitro experiments were performed to elucidate
the relationship between the action of bradykinin (BK) and the possibility
of release of pharmacologically active fragments from BK in the rat brain.
In in vivo experiments, the activities of electroencephalogram (EEG)
increased immediately after the intracerebral administration of 5 nmole BK.
The effect was prolonged by intracerebral pretreatment of o-phenanthroline
which inhibits plasma kininases. In vitro experiments, o-phenanthroline
inhibited partially purified enzyme of rat brain which released fragments
of BK possessing phenylalanine, serine, proline and/or arginine as N-
terminal amino acids. Only arginine and minute amounts of phenylalanine
were observed after the incubation with o-phenanthroline. The evidence
suggests that the inhibition of the enzyme by o-phenanthroline resulted
in a prolongation of the excited phase produced by BK on the EEG and on
behavior. Supporting evidence indicates that Ser-Pro containing frag-
ments derived from BK are concerned with the sedative phase of BK which
was observed after the excitative stage.

Behavioral changes after intracerebral administration of bradykinin (Arg-Pro-Pro-
Gly-Phe-Ser-Pro-Phe-Arg) (BK) have been reported by several investigators (1-3). BK
caused a short-term excitation and sedation followed. There is some evidence that
degraded fragments of BK also have pharmacological activities. Iwata et al. suggested
that the excited state was induced by the native BK molecule whereas its metabolites
causd a depression (2). Our colleagues synthesized a number of BK fragments, and
suggested that the structure of Ser-Pro at the C-terminal position was required to
prolong pentobarbital-induced sleeping time in the mouse (4).

Regarding enzymatic degradation of BK in the brain, Shikimi and Iwata purified the
enzyme that released Arg and Phe from the C-terminal position of BK (5). Marks and
Pirotta suggested that the primary point of cleavage occurred at the Phe-Ser bond (6).
Camargo and Graeff (7) and Oliveira et al. (8) separated kininase A which cleaved the
peptide bond of Phe-Ser and kininase B which released Phe-Arg from the rabbit
cerebral cortex.

We now report the EEG in conscious rats after the intracerebral administration of BK
and the possibility that pharmacological
active peptides are released from BK by partially purified BK degrading enzyme of the rat brain is discussed.

MATERIALS AND METHODS

EEG recording: Male Sprague-Dawley strain rats (JCL), weighing 250–300 g were anesthetized with pentobarbital (30 mg/kg, i.p.) and fixed on a stereotaxic instrument (Narishige SN-1). After lidocaine was sprayed on the head skin, the skull was exposed and 1.0 mm diameter holes bored for the implantation of electrodes and cannula. According to the stereotaxic coordinates of Groot (9) bipolar electrodes were inserted into the nucleus caudatus (NC; A. 6.6, L. 3.5, d. 4.5), hippocampus (HPC; A. 4.2, R. 2.0, d. 3.5) and corpus callosum (CC; A. 1.0, R. 1.5, d. 2.5) to record the EEG. A 10 mm stainless steel needle (23G) was also implanted (P. 1.5, L. 2.0, d. 0.05) as the cannula for the injection of drugs, and the upper end was connected to a polyethylene tube. These electrodes were connected to a small socket and fixed to the skull with dental cement. All wound edges were sutured and dermatol solution was applied. After one or two weeks, the EEG was recorded in a small box (30 x 30 x 50 cm) which had been placed in a shield box (40x80x105 cm). After the unrestricted rats had adapted to the environment, spontaneous EEG’s were recorded on the pen recorder under the monitoring polygraph (Nihon Kohden RM-85). The drugs were given through a cannula; recording and observations were carried out for 60 min. Five rats with chronically implanted electrodes and a cannula were used in each experiment.

Enzyme preparation: Partially purified BK degrading enzymes were prepared according to the modified procedures of Oliveira et al. (8). Both carotid arteries were cannulated with polyethylene tubes to wash out blood with cold saline, when the rats were under pentobarbital anesthesia (50 mg/kg, i.p.). The whole brain was homogenized with a Teflon pestle in five volumes of 0.25 M sucrose. After the homogenate was centrifuged at 25,000× g for 60 min, the supernatant was adjusted to pH 5.0 by the addition of 0.5 M acetic acid and then left 4°C for 2 hr. The suspension was centrifuged at 900× g for 15 min, and this supernatant was adjusted to pH 7.5 by adding 0.5 N NaOH, after which the preparation was dialyzed against a large volume of 50 mM sodium phosphate buffer (pH 7.5). The activity of the enzyme was stable for several months in the lyophilized powder. Protein was determined by the method of Lowry et al. (10), using bovine serum albumin as a standard.

Bioassay system: The rat uterus was used to measure the amounts of BK, using a Magnus apparatus. Female Sprague-Dawley strain rats were given 100 μg estradiol benzoate s.c. about 36 hr before sacrifice. The uterus was isolated and bathed in 10 ml of De Jalon’s solution containing 1.7×10⁻⁶ M atropine at 25°C. The kinin degrading enzyme activity was calculated from the concentration of residual BK in an incubation mixture which initially contained 0.5 nmole BK, and 2.3–2.5 μg enzyme protein in a total of 500 μl of 50 mM sodium phosphate buffer (pH 7.5). The incubations were carried out for 15 min at 37°C. All glasswares including the Magnus bath were coated with silicon to avoid adsorption of peptides to the surface.

Determination of the peptides released from BK: Thin-layer chromatography was applied in this experiments. Two hundred μl of 50 mM sodium phosphate buffer (pH 7.5) containing 25 nmole of BK and 4.6–5.0 μg protein of the partially purified enzyme was incubated at 37°C for 30 to 60 min. Before the chromatography, dansylation of amino acids and peptides were carried out according
to the method of Gray (11). After drying the dansylated mixture in vacuo, 50 μl of 6 N HCl was added. The tubes were then sealed and hydrolysis was carried out for 16 hr at 105 °C. After the hydrolysis, the tubes were opened and HCl was removed in vacuo. The residue was dissolved in ethanol and 5 μl of the solution was spotted on polyamide thin-layer (Cheng Chin Trading Co., LTD. Taiwan R.O.C.) for the separation of dansylated amino acids. As described by Woods and Wang with the rerun in two dimension (12), applied samples were developed with water : 90% formic acid (200 : 3, v/v) as the first solvent system, and then with benzene : acetic acid (9 : 1, v/v) as the second. Dansylated amino acids on the chromatogram were observed using a UV lamp (360 nm), as a strong yellow fluorescence, dansyl-OH a blue-green and dansyl-NH₂ a dark orange fluorescence.

Reagents: Synthetic BK was purchased from Protein Research Foundation (Osaka). Other chemicals used were of special grade. BK was dissolved in artificial cerebrospinal fluid of the following composition; NaCl 8.1 g, KCl 0.25 g, CaCl₂ 0.14 g, MgCl₂ 0.11 g and NaHCO₃ 1 g/l (13) and 25 μl was given to each rat.

RESULTS

Effect of some compounds on BK degradation by partially purified rat brain kininase: The enzyme activity was measured by the bioassay system using rat uterus. As BK was degraded linearly by the enzyme for 40 min (Fig. 1), the incubation was carried out for 15 min in further studies. Figure 2 shows the effect of some compounds related to the inactivating enzyme of BK in rat brain. α-Phenanthroline inhibited the BK degrading enzyme at concentrations from 10⁻² to 10⁻³ M. The other compounds used did not inhibit the enzyme activity in this system.

Fig. 1. Time as related to the activity of the BK degrading enzyme partially purified from rat brain. Enzyme and BK were incubated and the residual BK was bioassayed as described in MATERIALS AND METHODS. Vertical bars represent ± S.D. from five observations.

Fig. 2. Effect of various compounds on the BK degrading activities of enzymes partially purified from rat brain. Enzyme and BK were incubated for 15 min under the presence of □—□, α-phenanthroline (O-Phe); △—△, 8-hydroxyquinoline (8-OHQ) and ×—×, diethyldithiocarbamate (DDC) in doses shown along abscissa. The results show mean percent inhibition ± S.D. from five different observations.

Relationship between EEG and behavior on rats treated with BK: Figure 3 illustrates the EEG of rat treated with 5 n mole BK intracerebrally. The artificial cerebrospinal fluid did not effect either the EEG or the behavior of unrestricted rats (data not shown). The activities of NC and CC
showed low voltage of less than 100 μV, and irregular high amplitude activity (100–300 μV) was predominant in HPC before the injection of BK. Intracerebral administration of 5 nmole BK caused a brief irregular change of amplitude in NC and CC. The activity in HPC produced higher voltages and more spiked waves than were produced before BK treatment. These changes were observed for 1–2 min after the injection. However, spiked waves in HPC continued for 10 min or more. Some behavior was characterized by signs of excitation such as restlessness and trembling which were observed in the early period, followed by sedation and crouching. The latter signs were observed

**Fig. 3.** Effect of intracerebrally injected BK (5 nmole/25 μl) on the EEG of a conscious rat. A typical EEG pattern obtained from the results of five rats is illustrated in this figure. NC: nucleus caudatus; HPC: hippocampus; CC: corpus callosum.

**Fig. 4.** Effect of BK (5 nmole/25 μl) on the EEG in a rat pretreated with o-phenanthroline (25 pmole/50 μl). o-Phenanthroline was intracerebrally injected 15 min before the injection of BK and the effect of BK on EEG was observed as shown in Fig. 1. The figure shows a typical EEG.
for about 30 min after excitation, but the animal recovered normally. The EEGs in the sedative phase showed a light, slow frequency and high amplitude in NC and HPC. These changes of EEG and behavior induced by intracerebral administration of BK were obtained in a dose dependent manner from 2.5 to 7.5 n mole of BK. No changes were seen in five rats with doses of less than 1.0 n mole, whereas a dose of 2.5 n mole did produce changes, albeit unclear. The changes in the EEG and behavior were seen following a 5 n mole BK injection. With doses over 7.5 n moles, strong excitation appeared and recording of the EEG was not feasible.

O-Phenanthroline produced no effect on the EEG (data not shown). However, in contrast to the EEG observed when BK was injected alone, pretreatment of o-phenanthroline (25 pmole/50 μl, i.c.) produced a prolongation of a high amplitude period on the EEG, particularly in the NC and CC (Fig. 4). EEG in the HPC which was characterized by irregular high amplitude and spike activity also continued for about 20 min. Behavioral signs such as sedation and crouching were observed for about 1 hr.

Identification of degraded products of BK by partially purified enzyme: To determine the position of hydrolysis on the BK molecule, the N-terminal amino acids of the products formed during incubation of BK with the enzyme were investigated. In this experiment, the enzymatic reaction was carried out for 30 and 60 min. Figure 5 illustrates the chromatographic properties of the N-terminal amino acids of peptides cleaved from BK by the rat brain enzyme. After the incubation for 30 min, dansylated arginine, phenylalanine, serine and proline were found on the thin-layer. The intensities of fluorescence of arginine and phenylalanine were strong, while those of serine medium and those of proline faint. The same four spots were observed after an incubation of 60 min and here the fluorescence was even more intense.

As shown in Fig. 6, under the presence of o-phenanthroline (5×10⁻⁴ M) in the incubation mixture, intense dansylated arginine

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Fig. 5. Typical thin-layer chromatogram of N-terminal amino acids of the hydrolyzed products of BK. After incubation for the indicated times, the mixture was dansylated according to the method of Gray (11) and the hydrolysis and development were carried out as described in MATERIALS AND METHODS.

Fig. 6. Effects of various compounds on the hydrolyzed patterns of BK. Incubations were carried out in the presence of compounds (5×10⁻⁴ M) for 60 min and N-terminal amino acids of the products were determined as described in MATERIALS AND METHODS.
and faint phenylalanine were observed but serine and proline were not detected after an incubation of 60 min. Fluorescent spots of arginine and phenylalanine were seen in the incubation with 8-hydroxyquinoline, but the intense fluorescence of arginine, phenylalanine, and faint proline and serine were observed in the case of the incubation with diethylidithiocarbamate.

**DISCUSSION**

Biphasic behavioral changes of animals after intracerebral administration of BK have been reported by several authors (1-3). Graeff et al. found in conscious rabbits that behavioral excitation was associated with desynchronization and the subsequent phase of sedation was associated with synchronization in the electrocorticogram (1). We also observed the same biphasic behavior. Further, results of the EEG showed that the excited phase was accompanied by an irregular activity in all regions, and high voltage and spike activity were predominant in the HPC. Light slow frequency and amplitude in NC and HPC were obtained during the sedation phase in unrestricted rats. Related to this evidence, it was reported that the excited state was elicited by the authentic BK whereas the metabolites tended to cause depression (2). Previous reports showed that some fragments of BK containing Ser-Pro prolonged the pentobarbital-induced sleeping time (4). This evidence indicates that these fragments may be released from BK in the brain. This postulation was supported by our present results. As shown in Fig. 5, dansylated serine appeared on the chromatogram and this evidence suggested that serine or peptides containing serine at N-terminal position such as Ser-Pro, Ser-Pro-Phe or Ser-Pro-Phe-Arg were cleaved from BK by the partially purified degrading enzyme system in rat brain. Further, phenylalanine and/or peptides like Phe-Ser-Pro-Phe-Arg, Phe-Ser-Pro, Phe-Ser and Phe-Arg may be identified by the appearance of dansylated phenylalanine on chromatogram. However, it is unclear whether arginine was released from the C- or N-terminal of BK. On the other hand, we have reported that Phe-Ser-Pro was one of the most potent peptides which prolonged pentobarbital-induced sleeping time in the mouse (4). From these results, it is reasonable that BK may be hydrolyzed and may release some pharmacological active peptides in rat brain.

With regard to detection of amino acids cleaved from BK by brain kininase, Shikimi and Iwata (5) using the electrophoresis after incubation of BK with rat brain kininase, showed that arginine and phenylalanine are released from the C-terminal of BK. Marks and Pirotta (6) suggested that the primary point of cleavage was at Phe-Ser bond by peptidase purified from rat brain. Camargo et al. (14) and Oliveira et al. (8) separated two kininases from rabbit cerebral cortex which they termed kininase A, which cleaved the peptide bond of Phe-Ser, and kininase B which released Phe-Arg from BK. Our results obtained from the thin-layer chromatogram show that peptides released from BK by the action of partially purified enzyme from rat brain have phenylalanine, serine and proline as N-terminal amino acids. This evidence indicates that this enzyme preparation contains several kinds of enzyme. In regard to this evidence, it has been well demonstrated that o-phenanthroline inhibited plasma kininases (15), and also these experiments have shown that the high voltage effects of BK on the EEG were prolonged by intracerebral pretreatment with o-phenanthroline. Furthermore, inactivation of BK by the enzyme is effectively inhibited by o-phenanthroline in the bioassay system. Arginine and small amounts of phenylalanine were detected in the thin-layer chromatogram.
after incubation for 60 min. These results suggest that one of the enzymes which is inhibited by o-phenanthroline in the rat brain cleaved a BK molecule at the peptide bond of Pro-Phe. However, inhibition of the cleavage of a C-terminal arginine from BK was not determined. 8-Hydroxyquinoline and diethyldithiocarbamate may have no effect on this bond. Namely, these data indicate that the prolongation of the effect of BK on the EEG, induced by intracerebral pretreatment of o-phenanthroline, may be due to the protection of BK degradation in rat brain by the BK degrading enzyme system.

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