A BRADYKININ-LIKE SUBSTANCE IN RAT STOMACH

Motoki KOBAYASHI, Tadahiro SHIKIMI*, Sadao MIYATA**
and Katsuya OHATA

Department of Pharmacology, Kyoto College of Pharmacy,
Yamashina, Kyoto 607, Japan

Accepted May 27, 1980

Abstract—A bradykinin (BK)-like substance (P1) in the rat stomach was extracted with acetic acid, n-butanol, distilled water and methanol. The gradient and equilibrium chromatography were carried out on SP-Sephadex C-25 columns with the extract containing P1. P1 had a different retention time from BK, kallidin and methionyl-lysyl-bradykinin (MLBK), on the equilibrium chromatography. The apparent molecular weight of P1 estimated by gel chromatography was over 1,300. P1 was classified as a biologically BK-like peptide of mammalian origin which is distinct from BK, kallidin and MLBK. Another kind of biologically active substance (P2) which contracts the isolated rat uterus and duodenum was detected during the course of the extraction and purification of P1. The contractile activity of P2 was abolished by the presence of dibenamine or methysergide, but was not influenced by chymotrypsin, trypsin or papain digestion. The hypotensive effect of P2 on rabbit blood pressure was similar to that of serotonin (5-HT). The retention times of P2 on the equilibrium chromatography on the SP-Sephadex C-25 column, and on the gel chromatography were the same as those of 5-HT. P2 proved to be 5-HT.

In a previous paper, we reported the existence of a kinin-like substance in the rat stomach and classified it as a bradykinin (BK)-like substance from its biological activities (1). During the course of the extraction and purification of this substance, a different type of biologically active substance was detected. This report describes the extraction and purification procedures and properties of these biologically active substances.

MATERIALS AND METHODS

Extraction procedures: Male Wistar rats weighing about 200 g were fasted for 18 hr and exsanguinated from the carotid artery. The stomach was extirpated and washed with cold Ringer's solution. After removal of the serosal membrane, the tissue was separated into fore- and glandular stomach. The BK-like substance was extracted by a modification of the method of Diniz and Carvalho (2) and the method of Abe et al. (3). The glandular stomach was homogenized with 10 vol. of cold 0.2% acetic acid. The homogenate was heated in a boiling water bath for 30 min. After centrifugation at 700 g for 10 min, the supernatant fraction was adjusted to pH 2.0 with 1 N HCl, and then the solution was saturated with NaCl. After addition of the same vol. of n-butanol, the mixture was shaken.
for 10 min, and centrifuged at 700 g for 10 min. The n-butanol phase was pipetted to another tube. The extraction with n-butanol was then repeated. After addition of 10 g of anhydrous sodium sulfate per 40 ml of the combined n-butanol phase, the solution was stored overnight at -20°C. After centrifugation at 700 g for 10 min, the supernatant fraction was transferred to another tube and 2 volume of petroleum ether were added. The mixture was extracted with a one tenth volume of distilled water under shaking for 10 min, centrifuged at 700 g for 10 min and the aqueous phase was pipetted to another tube. The extraction with distilled water was repeated. The combined aqueous phase was adjusted to pH 7.0 with 1 N NaOH, and evaporated to dryness at below 35°C. The dried residue was extracted twice with the same volume of methanol as 0.2% acetic acid, under shaking for 10 min. After centrifugation at 700 g for 10 min, the supernatant fraction was evaporated to dryness at below 35°C, a part of the dried residue was dissolved in 0.9% saline, and the resultant solution was used as the stomach extract to be tested with the isolated rat uterus and duodenum.

**Gradient chromatography on SP-Sephadex C-25:** The dried residue derived from 15 g of the wet tissue, obtained from the extraction procedure, was dissolved in 100 ml of distilled water and chromatographed at room temperature on a SP-Sephadex C-25 column, 0.9×8 cm, by the method of Nakajima (4). The column resin was suspended in 0.5 M ammonium formate for 24 hr and washed with distilled water before use. The dried residue dissolved in distilled water described above was applied to the column, the column was washed with 30 ml of distilled water, then developed with a linear gradient of ammonium formate solution, pH 5.4 (0-0.6 M). The fractions of 1.5 ml each were collected, lyophilized, and a part of each preparation was dissolved in 0.9% saline. The resultant solution was tested with the isolated rat uterus and duodenum.

**Equilibrium chromatography on SP-Sephadex C-25:** The lyophilized preparation containing uterine contractile activity, obtained from gradient chromatography, and standard materials were dissolved in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.08 M NaCl. The resultant solutions were chromatographed at room temperature on a SP-Sephadex C-25 column, 0.6×9 cm, by the method of Hial et al. (5). The fractions of 1.5 ml each were collected, and assayed with the isolated rat uterus.

**Desalting:** The fractions containing uterine contractile activity, separated by equilibrium chromatography, were pooled and chromatographed on a SP-Sephadex C-25 column by the method of Nakajima (4), for desalting.

**Gel chromatography on Sephadex G-25:** The desalted preparations and standard materials were dissolved in 3% acetic acid and chromatographed on a Sephadex G-25 column, 1.7×56 cm. The flow rate was 32 ml/hr. The fractions of 5 ml each were collected, and the optical density was determined at 280 nm. As for the materials with uterine contractile activity, these fractions were lyophilized, and a part of each preparation was dissolved in 0.9% saline. The resultant solutions were tested with the isolated rat uterus. The following substances were used for estimation of the molecular weight of the test materials: serotonin (5-HT), leu²-enkephalin, BK, methionyl-lysyl-bradykinin (MLBK), angiotensin I
(human), neurotensin and \( \alpha \)-endorphin.

**Enzymatic treatment:** Digestions with trypsin and chymotrypsin were carried out by incubating 1 ml of the eluate containing uterine contractile activity, obtained from the equilibrium chromatography, with 0.2 ml of enzyme solution (1 mg/ml in distilled water) at 37°C for 20 min. The reactions were terminated by boiling for 5 min. After centrifugation at 700 g for 10 min, the supernatant fractions were assayed with the isolated rat uterus.

The digestion with papain was done by incubating 1 ml of the preparation containing uterine contractile activity, obtained from the gel chromatography, with 0.2 ml of papain solution (1 mg/ml in 0.2 M ammonium acetate containing 0.03 M mercaptoethanol, pH 5.6) at 37°C for 20 min. The reaction was terminated by boiling for 5 min. After centrifugation at 700 g for 10 min, the supernatant fraction was lyophilized, dissolved in 0.9% saline, and the resultant solution was assayed with the isolated rat uterus.

**Biological procedures:** Bioassays were carried out by the same method as in our previous paper (6). The isolated rat uterus or duodenum was suspended in a 10 ml bath filled with aerated De Jalons' solution at 30°C. In addition, De Jalons' solution containing atropine sulfate (10^{-6} g/ml) with or without dibenamine HCl (10^{-7} g/ml) was used to suspend the isolated rat uterus, and the solution containing additional diphenhydramine HCl (10^{-6} g/ml) and propranolol HCl (10^{-8} g/ml) was used to suspend the isolated rat duodenum. A few experiments were performed using methysergide (10^{-8} g/ml) instead of dibenamine (10^{-7} g/ml). The uterine contractile activity in the test preparation was expressed in terms of \( \mu \)g BK or 5-HT equivalent.

The blood pressure was recorded from the carotid artery of urethanized rabbits. The test preparations were administered into a cannula fixed to the vena marginalis.

**Recovery:** The recovery of added standard BK was about 75% in the extraction step, and that in the SP-Sephadex C-25 and Sephadex G-25 steps was about 90%.

**Materials:** The following reagents were used: SP-Sephadex C-25 Fine and Sephadex G-25 Fine (Pharmacia, Sweden); BK, kallidin, MLBK, leu\textsuperscript{5}-enkephalin, angiotensin I (human), neurotensin and \( \alpha \)-endorphin (Protein Research Foundation, Osaka); trypsin from bovine pancreas Type III (2 \times crystallized), chymotrypsin from bovine pancreas (3 \times crystallized), papain (2 \times crystallized) and Tris-aminomethane (Sigma Chemical Co., U.S.A.); atropine sulfate (E. Merck AG, Germany); dibenamine HCl and 5-HT (Nakarai Chemicals LTD., Kyoto); propranolol HCl (ICI Pharmaceuticals Division, England); diphenhydramine HCl (Tanabe Seiyaku Co., LTD., Osaka) and methysergide (Sandoz, Switzerland).

**RESULTS**

**Effects of the stomach extract on isolated rat uterus and duodenum:** Ten \( \mu \)l of the stomach extract/ml contracted the rat uterus, and this contractile activity was equivalent to 1.0 ng of BK/ml (Fig. 1-A). The contractile activity of the stomach extract was not affected by the presence of atropine (Fig. 1-B), but diminished to the equivalent of 0.3 ng of BK/ml with the additional presence of dibenamine (Fig. 1-C). The stomach extract
contracted the rat duodenum (Fig. 1-D), and this contractile activity was not affected by
the presence of atropine, diphenhydramine and propranolol (Fig. 1-E), whereas the stomach
extract relaxated the duodenum in the presence of dibenamine (Fig. 1-F). The same result
was observed in the presence of methysergide.

**Gradient chromatography of the stomach extract on SP-Sephadex C-25:** Linear gradient
chromatography was carried out with the stomach extract corresponding to 14.5 g wet
tissue of glandular stomach (4.32 μg BK equivalent on assay with the isolated rat uterus in
the presence of atropine and dibenamine). The uterine contractile activity was detected in
fractions no. 150 to 200 in the presence of atropine and dibenamine (Fig. 2). These fractions
contracted the rat duodenum in the presence of atropine, diphenhydramine and propranolol,

![Fig. 1](image_url)

**Fig. 1.** Effects of the stomach extract (SE) on isolated rat uterus and duodenum.
The tissue was suspended in De Jalon's solution containing the following
antagonists. A and D: no antagonists. B: atropine sulfate (10^{-6} g/ml). C:
additional dibenamine HCl (10^{-7} g/ml) to B. E: atropine sulfate (10^{-6} g/ml),
diphenhydramine HCl (10^{-8} g/ml) and propranolol HCl (10^{-8} g/ml). F: ad-
ditional dibenamine HCl (10^{-7} g/ml) to E.
and relaxed it in the presence of dibenamine.

Separation and identification of the active substances: The panel A of Fig. 3 shows the elution pattern of standard kinins, such as BK, kallidin and MLBK, on the equilibrium chromatography on the SP-Sephadex C-25 column, detected by assay with the rat uterus. The lyophilized preparation containing uterine contractile activity, obtained from the gradient chromatography, was applied to the equilibrium chromatography. Two peaks were detected by assay with the rat uterus (Fig. 3, Panel B). The uterine contractile active substances (P1 and P2) contained in the first and second peaks were eluted with the different retention times from that of the standard BK (Fig. 3, Panel C). The standard 5-HT was eluted with the same retention time as that of P2 (Fig. 3, Panel D).

Effects of P1 and P2 on the isolated rat uterus and duodenum: P1 and P2 contracted the rat uterus (Fig. 4-A), and these contractile potencies were not affected by the presence of atropine (Fig. 4-B). The contractile activity of P1 on the rat uterus was observed even in the presence of dibenamine, while that of P2 was abolished (Fig. 4-C). P1 relaxed the rat duodenum (Fig. 4-D), and this relaxant activity was not affected by the presence of atropine, diphenhydramine, propranolol and dibenamine (Fig. 4-F). P2 contracted the rat duodenum (Fig. 4-D), and this contractile activity was not affected by the presence of atropine, diphenhydramine and propranolol (Fig. 4-E), but was abolished by the presence of dibenamine (Fig. 4-F). The same result was observed in the presence of methysergide.

Enzymatic treatment: The contractile activity of P1 on the rat uterus was abolished by the chymotrypsin and papain treatments but was not affected by trypsin. Upon digestion of P1 (0.02 μg BK equivalent on assay with the isolated rat uterus) and standard MLBK (100 μg) with trypsin, the uterine contractile activity of P1 and the retention time of P1 on the equilibrium chromatography were not affected, whereas the uterine contractile activity of MLBK was increased to about 3-fold, and the retention time of MLBK on the equilibrium...
chromatography was completely transferred to that of standard BK. The contractile activity of P2 on the rat uterus was not influenced by the enzymes.

*Gel chromatography of P1 and P2 on Sephadex G-25:* Each region containing P1 or P2, obtained from the equilibrium chromatography, was desalted and applied to the gel

![Diagram](image)

Fig. 3. Equilibrium chromatography of lyophilized preparation, containing uterine contractile activity, obtained from the gradient chromatography. The elution pattern of the following materials is shown in each panel. A: standard kinins, BK, kallidin and MLBK. B: the preparation obtained from the gradient chromatography. C: the preparation obtained from the gradient chromatography plus standard BK. D: standard 5-HT. Column size: 0.6 x 9 cm. Elution buffer: 0.05 M Tris-HCl buffer (pH 8.0) containing 0.08 M NaCl. Flow rate: 7.7-9.5 ml/hr. Fraction vol.: 1.5 ml. Fractions were assayed with the isolated rat uterus suspended in De Jalon's solution and without addition of antagonists.
chromatography on Sephadex G-25. The protein concentration of the eluates was monitored at 280 nm, and the uterine contractile activities of P1 and P2 were determined. Figure 5 shows the elution pattern of the region containing P1. Three peaks were observed by monitoring at 280 nm. The uterine contractile activity of P1 was located at the first peak.

Figure 6 shows the elution positions of P1, P2 and peptidic substances of known molecular weight. The apparent molecular weight of P1 obtained from this plot was over 1,300. P2 was eluted with the same retention time as that of standard 5-HT.

In the separate experiment using the rat tissues, the lyophilized preparations containing P1 or P2, obtained from the gel chromatography, showed the same biological properties as shown in Fig. 4.

**Effects of P1 and P2 on rabbit blood pressure:** The lyophilized preparation containing

![Fig. 4. Effects of P1 and P2 on isolated rat uterus and duodenum. The aliquots of eluates containing P1 and P2 (1 and 2 respectively) obtained from the equilibrium chromatography were tested with the isolated tissue suspended in De Jalon's solution containing the following antagonists. A and D: no antagonists. B: atropine sulfate (10^{-6} g/ml). C: additional dibenamine HCl (10^{-7} g/ml) to B. E: atropine sulfate (10^{-6} g/ml), diphenhydramine HCl (10^{-5} g/ml) and propranolol HCl (10^{-8} g/ml). F: additional dibenamine HCl (10^{-7} g/ml) to E.]
P1 or P2, obtained from the gel chromatography, was dissolved in 0.9% saline, and was tested on rabbit blood pressure. P1 (0.01 μg BK equivalent on assay with the isolated rat uterus/kg), given i.v., produced a fall on blood pressure, whereas P2 which was equivalent to P1 had no effects on the blood pressure (Fig. 7-A). The intravenous administration of

![Fig. 5. Gel chromatography of P1 on Sephadex G-25. The eluate containing P1, obtained from the equilibrium chromatography, was collected, desalted and applied. The optical density of the fractions was determined at 280 nm, and those fractions were lyophilized. The lyophilized preparations were dissolved in 0.9% saline, and the resultant solutions were tested with the isolated rat uterus suspended in De Jalon's solution and without addition of antagonists. Column size: 1.7 x 56 cm, Fraction vol.: 5.0 ml, Eluent: 3% acetic acid, Flow rate: 32 ml/hr.](image1)

![Fig. 6. Estimation of molecular weight of P1 and P2. The condition was the same as shown in Fig. 5. The elution position of P2 was determined in the isolated rat uterus, similar to that of P1 in Fig. 5. The void vol. (Vo) was determined with blue dextran 2000. The elution vol. (Ve) was chosen at the peak of the eluted material. Each point indicates the elution positions of the following substances: (1) 5-HT, (2) leu5-enkephalin, (3) BK, (4) angiotensin I (human), (5) MLBK, (6) neurotensin and (7) α-endorphin.](image2)
P2 (36 μg 5-HT equivalent on assay with the isolated rat uterus/kg) produced a fall in blood pressure (Fig. 7-B).

DISCUSSION

We have already reported the existence of a BK-like substance in the rat stomach (1).

In the present extraction and purification procedure of this BK-like substance, two different biologically active substances, P1 and P2, were isolated. P1 contracted the isolated rat uterus in the presence of atropine and dibenamine, and relaxed the isolated rat duodenum in the presence of atropine, diphenhydramine, propranolol and dibenamine, thereby indicating that the effects are not mediated by acetylcholine, histamine or 5-HT nor do they involve α- or β-adrenergic receptors. After the chymotrypsin and papain treatments, P1 lost its uterine contractile activity but not so after the trypsin treatment. P1 produced a fall in rabbit blood pressure. The apparent molecular weight of P1 was over 1,300 as estimated by the gel chromatography. P1 had a different retention time from BK, kallidin and MLBK as shown in Fig. 3. P1 may not contain the sequence of BK in the molecule because the trypsin digestion did not convert P1 to BK. From these results, P1 is classified as a biologically BK-like peptide of mammalian origin which is distinct from BK, kallidin and MLBK, according to the definition by Bertaccini (7). P2 contracted the isolated rat uterus and duodenum in the presence of atropine or in the presence of atropine, diphenhydramine and propranolol, respectively. The contractile activity of P2 was abolished by the presence of dibenamine or methysergide. In the previous work (1), P2 was not detected because the activity of P2 had been abolished by the presence of dibenamine. The uterine contractile
activity of P2 was not influenced by the chymotrypsin, trypsin or papain treatments. The hypotensive effect of P2 on rabbit blood pressure was similar to that of 5-HT, as shown in Fig. 7. The retention time of P2 as seen by the equilibrium and gel chromatography was the same as that of 5-HT as shown in Fig. 3 and Fig. 6, respectively. From these results, P2 was shown to be 5-HT, and the content of 5-HT in the rat glandular stomach was 0.95 μg/g of wet tissue.

In the purification procedure, P1 and P2 could not be separated by the gradient chromatography on SP-Sephadex C-25, but were separated completely by the equilibrium chromatography on SP-Sephadex C-25 (Fig. 3-B). In Fig. 1, the stomach extract contracted the rat uterus, and its uterine contractile activity was diminished to approx. 30% in the presence of dibenamine. The stomach extract contracted the rat duodenum in the absence of dibenamine and relaxed it in the presence of dibenamine. These pharmacological effects of the stomach extract can probably be ascribed to the sum total of the activities of P1 and P2.

Acknowledgement: We thank Messrs. H. Okugawa and Y. Tou for technical assistance.

REFERENCES
1) Kobayashi, M., Shikimi, T., Miyata, S. and Ohata, K.: Existence of a bradykinin-like substance, and influence of carbachol and atropine on kinin-forming and destroying activities, and kinin and kininogen contents in rat stomach. Japan. J. Pharmacol. 30, 755-758 (1980)
2) Diniz, C.R. and Carvalho, I.F.: A micromethod for determination of bradykininogen under several conditions. Am. N.Y. Acad. Sci. 104, 77-89 (1963)
3) Abe, K., Watanabe, N., Kumagai, N., Mouri, T., Seki, T. and Yoshinaga, K.: Estimation of kinin in peripheral blood in man. Tohoku J. exp. Med. 89, 103-112 (1966)
4) Nakajima, T.: Occurrence of a new active peptide in smooth muscle and bradykinin in the skin of Rana nigromaculata Hallowell. Chem. Pharm. Bull. 16, 769-770 (1968)
5) Hial, V., Keiser, H.R. and Pisano, J.J.: Origin and content of methionyl-lysyl-bradykinin, lysyl-bradykinin and bradykinin in human urine. Biochem. Pharmacol. 25, 2499-2503 (1976)
6) Kobayashi, M., Shikimi, T., Miyata, S. and Ohata, K.: Studies on kinin-forming enzyme in rat stomach. Japan. J. Pharmacol. 29, 947-950 (1979)
7) Bertaccini, G.: Active polypeptides of nonmammalian origin. Pharmacol. Rev. 28, 127-177 (1976)