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DETECTION OF THE DEGRADATION PRODUCTS OF BRADYKININ BY ENZYME IMMUNOASSAYS AS MARKERS FOR THE RELEASE OF KININ *IN VIVO*

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Abstract—We developed an enzyme immunoassay (EIA) specific for Arg^1-Pro^2-Pro^3-Gly^4-Phe^5 ([1-5]-BK) for determination of the levels of this peptide in biological fluids. Previously developed EIAs for bradykinin (BK) and for des-Phe^8-Arg^9-BK ([1-7]BK) were also used. Incubation of rat plasma with glass powder resulted in the transient appearance of BK. A degradation product, [1-7]BK, could be detected in the incubation mixture for a longer period of time. When compared with BK and [1-7]BK, a larger amount of [1-5]BK was detectable even longer. In carrageenan-induced pleurisy in rats, which was associated with a peak rate of plasma exudation 5 hr after administration of carrageenan, BK was undetectable (<160 pg/rat) in the pleural exudates. By contrast, [1-7]BK was detectable over the entire course of the inflammatory response. A larger amount of [1-5]BK was detectable. The peak level of [1-5]BK was 6050 ± 1050 pg/rat, 5 hr after administration of carrageenan. Inhibition of the generation of BK by intrapleural administration of soy bean trypsin inhibitor (0.3 mg/rat) 30 min before collection of pleural fluid resulted in significant reductions in the levels of both [1-7]BK (by 51–65%) and [1-5]BK (by 63–79%) in the exudates 3, 7 and 19 hr after administration of carrageenan. Intraperitoneal administration of captopril (10 mg/kg) caused a marked reduction (by 98%) in levels of [1-5]BK in exudates 3 hr after administration of carrageenan. The reduction was accompanied by an increase in the level of BK up to 1250% of that in untreated rats. These results indicate that the newly developed EIA for [1-5]BK might be a useful tool for verifying the release of kinin *in vivo*.

Bradykinin (BK‡) is a very potent, biologically active peptide that induces vasodilatation, increased vascular permeability, increased pain sensation, increases in the glomerular filtration rate and increased excretion of sodium from kidneys. BK is considered to be involved in certain pathological states, which include inflammation [1–3], shocks [4, 5], hypertension [6, 7] and airway disease [8]. Detection of free kinin provides evidence for the involvement of kinin in such pathological states. An enzyme immunoassay (EIA) for BK has been reported from our laboratory [9] and has been used successfully in studies of endotoxin shock [5] and in *in vitro* experiments [10]. This active peptide is, however, degraded very quickly by peptidases in the plasma and other biological fluids [10–12]. The detection of free kinin itself is generally difficult even at sites where it is generated.

We have previously reported the pathways of degradation of BK in biological fluids from humans and rats. The major peptidases in the plasma and inflammatory exudates from humans and rats are kininase I and kininase II [10, 13, 14], which are equivalent to carboxypeptidase N [15] and a dipeptidyl peptidase analogous to angiotensin-converting enzyme [15], respectively. BK is degraded in plasma and inflammatory exudates to des-Arg^9-BK ([1-8]BK) by kininase I and to des-Phe^8-Arg^9-BK ([1-7]BK) by kininase II. Both [1-8]BK and [1-7]BK are degraded by kininase II to Arg^1-Pro^2-Pro^3-Gly^4-Phe^5 ([1-5]BK), which was further degraded to smaller fragments without detection of Arg^1-Pro^2-Pro^3 ([1-3]BK) [13, 14]. The contribution of kininase I to the degradation of BK is predominant in human plasma, whereas in the plasma and inflammatory exudate of rats, the major kinin-degrading enzyme is kininase II [10, 14]. Thus, in rats the degradation of BK occurs predominantly via [1-7]BK to [1-5]BK.

An EIA for [1-7]BK was developed some time ago and was used successfully in the characterization of a model of inflammation [10] and shocks [5]. We now report an EIA specific for [1-5]BK which was used to clarify the involvement of BK in carrageenan-induced pleurisy in rats.

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‡ Abbreviations: EIA, enzyme immunoassay; BK, bradykinin; [1-3]BK, Arg^1-Pro^2-Pro^3; [1-5]BK, Arg^1-Pro^2-Pro^3-Gly^4-Phe^5; [1-7]BK, des-Phe^8-Arg^9-BK; [1-8]BK, des-Arg^9-BK; BSA, bovine serum albumin; MBS, *N*-(*m*-maleimido-benzoyloxy)succinimide; PBS, sodium phosphate buffer; HMW kininogen, high-molecular-weight kininogen; LMW kininogen, low-molecular-weight kininogen; SBTI, soy bean trypsin inhibitor.

MATERIALS AND METHODS

Development of an EIA for a product of the degradation of bradykinin

Antibodies. Antibodies were raised against [1-5]-BK by conjugating its amino terminus to bovine serum albumin (BSA, Fraction V) and an EIA for

this metabolite was developed using these antibodies [9]. A solution of [1-5]BK (9 mg) and BSA (30 mg) in 30 mL of sodium phosphate buffer (pH 7.0, 0.2 M) was incubated at 25° for 4 hr with 3 mL of a 0.25% (w/v) solution of glutaraldehyde (in distilled water). One milliliter of a 1 M solution of lysine–HCl was added to the mixture which was then incubated at 25° for 1 hr. The reaction mixture was dialysed at 4° for 48 hr against 10 L of a 0.9% (w/v) solution of sodium chloride to remove the free peptide. The dialysed solution (7.4 mL, in terms of BSA 4 mg/mL) was used as the conjugated immunogen.

The solution of conjugated immunogen was emulsified with an equal volume of complete Freund's adjuvant, and then it was injected (in terms of BSA 4 mg/mL, 1 mL per animal) subcutaneously into male Japanese white rabbits (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) at 10 different sites (0.1 mL × 10 sites). As a booster, a half volume of the solution of immunogen was injected into five different sites (0.1 mL × 5 sites) every 2 weeks for 4 months. Three weeks after the last booster, rabbit blood was collected from the central artery of an ear and allowed to clot completely, and then serum was obtained by centrifugation.

β-Galactosidase-labeled peptides. For preparation of MBS [*N*-(*m*-maleimido-benzoyloxy)succinimide]-acylated peptides [17], 1 mg of [1-5]BK was dissolved in 0.2 mL of sodium phosphate buffer (PBS, 0.2 M, pH 7.0) and was incubated with 0.025 mL of 0.9% (w/v) MBS in dioxane at room temperature for 30 min. A solution (0.2 mL) of β-D-galactosidase (2.5 mg/mL, in 0.2 M PBS, pH 7.0) was mixed with 0.4 mL of a saturated solution of ammonium sulfate and allowed to settle at room temperature for 30 min. This solution was mixed with a solution of the MBS-acylated peptide prepared above and incubated at room temperature for 1 hr. This reaction mixture was dialysed at 4° for 48 hr against 0.02 M PBS (pH 7.0). This dialysed solution was applied to a column (2.7 × 40 cm, Pharmacia Fine Chemicals, Uppsala, Sweden) of Sepharose 6B and the fractions which showed a high enzyme activity were eluted with Buffer A [0.04 M PBS–0.2% (w/v) gelatin that contained 0.9% (w/v) NaCl and 0.1% (w/v) NaN_3, pH 7.4] and were pooled as labeled antigens.

Insolubilized second antibodies. Immunoglobulin raised in goat against rabbit immunoglobulin G (IgG) was coupled chemically with cell walls of *Lactobacillus plantarum* as reported previously [18].

Substrate solution. A solution of 2-nitrophenyl-β-D-galactopyranoside (27 mM), containing 1 mM $MgCl_2$, 40% (w/v) ethyleneglycol and 0.1% (w/v) NaN_3 was used [18].

Termination of the reaction. Reactions were terminated with 100 mM K_2HPO_4-NaOH (pH 11) [18].

Assays. One hundred microliters of a solution of standard peptide (400 pg–100 ng/mL) or of sample and 100 μL of diluted (1:8000) antiserum were pipetted into glass tubes (1.2 × 10 cm) and incubated at 4° for 24 hr. Two hundred microliters of the solution of enzyme-linked peptide was added into reaction tubes and the mixtures were incubated at 4° for 1 hr. Two hundred microliters of the insolubilized second antibody in suspension (continuously stirred on a magnetic stirrer) were added to the reaction tubes and the mixtures incubated at 4° for 30 min. Then, after 4 mL of a 0.9% (w/v) solution of NaCl had been added to the incubation tubes, the tubes were centrifuged (1500 *g*, 10 min) at 4° and the supernatants were discarded. Five hundred microliters of Buffer B [0.04 M PBS–0.1% (w/v) BSA containing 0.9% (w/v) NaCl and 0.1% (w/v) NaN_3, pH 7.0] were added while stirring, in order to disperse the precipitate completely. After preincubation at 37° for 5 min, 100 μL of the substrate solution were added and each mixture was incubated at 37° for 60 min. Finally 1.5 mL of the reaction terminator was added and the mixture was centrifuged again (1500 *g*, 10 min). The absorbance of the supernatant at 410 nm was measured and the concentrations of each peptide in the samples were determined by reference to the standard calibration curves for each peptide (Fig. 1).

Previously developed EIAs for BK [9, 19] and for [1-7]BK [5, 10] were also used in the present study to determine levels of BK and [1-7]BK.

Sensitivity and reproducibility of EIAs. Figure 1A shows standard curves that were obtained with 40 pg to 10 ng of authentic peptide per assay tube for each EIA. As seen in this figure, 40–80 pg/tube (100 μL) of unlabeled BK, [1-7]BK and [1-5]BK reduced absorbance at 410 nm significantly. The coefficient of variation (C.V.) was calculated for authentic BK and products of degradation of BK (500 pg/assay tube) from 15 measurements. The C.V. of EIAs for BK, [1-7]BK and [1-5]BK were 6.2%, 8.8% and 9.8%, respectively.

Specificity. Table 1 shows the cross-reactivity (%) of individual antibodies against the BK-related peptides and other biologically active peptides. The antibodies against BK showed 100% cross-reactivity with Lys-BK, Met-Lys-BK and T-kinin, whereas those against [1-5]BK showed only 20% cross-reactivity against [1-7]BK. Antibodies against [1-7]BK showed good selectivity.

Validity of EIA. Solutions of authentic [1-5]BK (1–5 μg/mL in physiological saline) were assayed both by each EIA and by reversed-phase HPLC. The HPLC analysis was performed under the conditions reported previously [10, 13, 14]. Figure 1B shows that the values determined by EIA were well correlated with those determined by HPLC.

Validity of the EIA for measurement of [1-5]BK in biological samples was tested. When the exudates from carrageenan-induced pleurisy in rats, collected at 5 hr, were diluted 2-, 4- and 8-fold with buffer A and the measured levels were plotted against the dilution on the abscissa in a logarithmic scale, the curves for the diluted samples were parallel to the standard curves of EIA for [1-5]BK. Thus, effects of any contamination that might affect the EIA for [1-5]BK can be excluded.

Glass activation of rat plasma

Blood was collected into plastic tubes which contained 1/10 of the blood volume of 3.8% (w/v) sodium citrate, from the carotid artery of male Sprague–Dawley rats (8 weeks old, specific pathogen

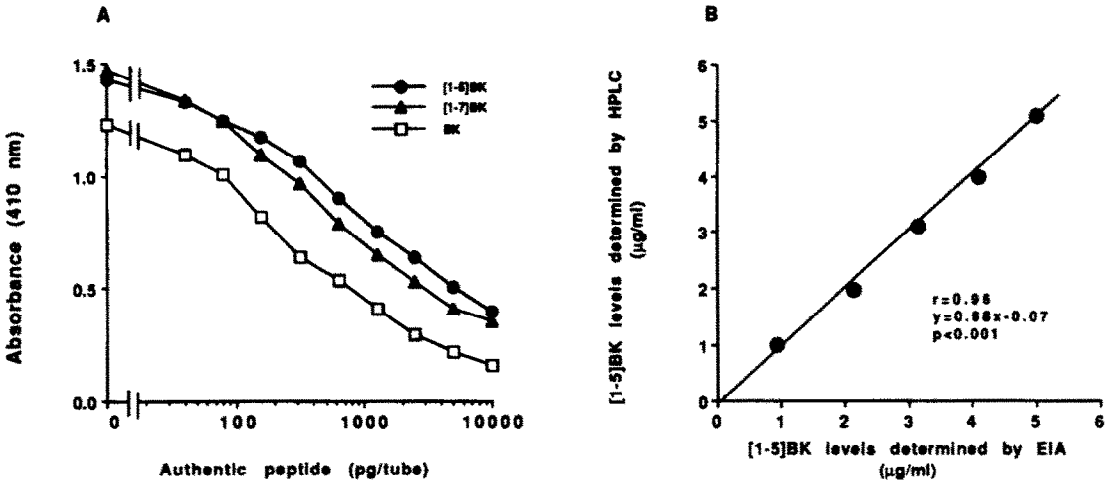

Fig. 1. Standard calibration curves for the determination of BK, [1-7]BK and [1-5]BK (panel A) and relationship between the levels of [1-5]BK determined by HPLC and EIA (panel B). In panel A, the ordinate represents absorbance at 410 nm and the abscissa represents the amounts of authentic peptides on a logarithmic scale. In panel B, the concentration, of solutions of [1-5]BK measured by HPLC (ordinate) and by EIA (abscissa) are plotted.

free, from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan) under light ether anesthesia. Citrated blood was immediately centrifuged at 1500 *g* for 15 min (25°), and plasma was obtained. Blood was collected and pooled from 20 animals. One milliliter of citrated rat plasma was placed in a plastic tube, which contained 4 mL of Tris–HCl buffer (60 mM, pH 7.8) and glass powder (100 mg/mL, Ballotini, Jencons Scientific Ltd, Hemel Hempstead, U.K.). At a given time, an aliquot (0.5 mL) was transferred to a siliconized glass tube that contained 2 mL of hot absolute ethanol. After heating at 70° for 10 min, the tube was centrifuged for 15 min at 1500 *g* at 4°. The supernatant fluid was evaporated to dryness under reduced pressure and the residue was dissolved in 2 mL of distilled water, acidified with 0.1 mL of 0.01 N HCl, and washed twice with 10 mL of diethylether to remove lipids [20]. The aqueous phase was evaporated to dryness under reduced pressure and the dried sample was dissolved in Buffer A for measurements by the EIAs described above.

Carrageenan-induced pleurisy in rats

Male Sprague–Dawley rats (8 weeks old, specific pathogen free) were anesthetized with ether lightly and 0.1 mL of a solution of λ-carrageenan [2% (w/v) in sterile physiological saline, Picnin-A] was injected into the right pleural cavity as previously described [1]. After exsanguination of rats at fixed times, 5 mL of absolute ethanol were injected into the pleural cavity for prevention of further degradation of metabolites of BK. The fluid in the pleural cavity was collected in a siliconized glass tube and centrifuged for 15 min at 1500 *g* at 4° after heating at 70° for 10 min. For the determination of BK and [1-7]BK, supernatants from four rats were pooled; for [1-5]BK, supernatants from one or two

Table 1. Specificity of EIAs of BK, [1-7]BK and [1-5]BK

Compounds	Cross-reactivity (%) BK EIA	[1-7]BK EIA	[1-5]BK EIA
Bradykinin (BK)	100	<0.1	2
Lys-BK	100	<0.1	<0.1
Met-Lys-BK	100	0.1	<0.1
T-kinin	100	<0.1	<0.1
[1-8]BK	<0.1	<0.1	2
[1-7]BK	<0.1	100	20
[1-5]BK	<0.1	<0.1	100
[1-3]BK	<0.1	<0.1	<0.1
Angiotensin II	<0.1	<0.1	<0.1
Substance P	<0.1	<0.1	<0.1
LMW kininogen	<0.1	<0.1	<0.1

rats were used. The ethanol extracts (supernatants) were evaporated to dryness and washed with diethylether as mentioned above. The washed samples were dissolved in 4 mL of distilled water that had been acidified with 0.2 mL of 0.01 N HCl and were applied to a Sep-Pak C_{18} cartridge column. After washing with 12 mL of distilled water and 4 mL of 0.1 M acetic acid, BK and products of its degradation were eluted with 6 mL of 80% (v/v) acetonitrile contained 0.1 M acetic acid [21]. The kinin fraction was evaporated under reduced pressure and the residue was dissolved in 800 μL of Buffer A, and the levels of BK and products of its degradation were determined by EIAs.

For experiments to examine recovery during the extraction, the inflammatory exudates collected 3 hr after injection of carrageenan were kept at 37° for 3 hr for complete degradation of the endogenous BK and its metabolites and then authentic BK, [1-7]BK and [1-5]BK (100 ng) were added. Immediate extraction of the kinin fraction as described above revealed that the total recovery after the extraction process was more than 85% for the individual peptides.

To test whether metabolites of BK could be attributed to generation of BK *in vivo*, the generation of BK from high-molecular-weight (HMW) kininogen by plasma kallikrein was inhibited by soy bean trypsin inhibitor (SBTI) [22]. Three hundred microliters of solution of SBTI (1 mg/mL in physiological saline) were injected into the pleural cavity of rats 30 min before the collection of pleural exudates. In control rats, only saline (0.3 mL) was injected into the pleural cavity.

To inhibit the production *in vivo* of [1-5]BK from BK via both [1-7]BK and [1-8]BK [10, 13, 14], captopril (10 mg/kg/mL in physiological saline) was administered intraperitoneally 30 min before the collection of samples. In control rats, only saline was administered (1 mL/kg, i.p.).

For the determination of the rate of exudation of plasma into the pleural cavity, pontamine sky blue (60 mg/kg/mL saline, Tokyo Kasei, Tokyo, Japan) was injected intravenously under ether anesthesia 20 min before the exsanguination. Blood was collected during exsanguination for measurements of the concentration of the dye in the serum. The concentrations of dye in the exudate and in the serum were determined spectrophotometrically from absorbance at 630 nm. The rate of exudation of plasma was expressed by the amount of dye exuded during the course of 20 min, which was corrected by reference to the concentration of dye in serum, since the concentration of dye in the serum differed between individual rats.

Agents

BK, [1-8]BK, [1-7]BK, [1-5]BK, [1-3]BK, angiotensin II and substance P were purchased from Peptide Institute (Minoh, Osaka, Japan). Low-molecular-weight (LMW) kininogen was obtained from the Biochemical Corp. (Tokyo, Japan). Captopril was supplied from the Sankyo Pharmaceutical Co. (Tokyo, Japan). SBTI was purchased from Worthington Biochemical Co. (Cleveland, OH, U.S.A.). BSA, Fraction V was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Glutaraldehyde was purchased from Nakarai Chemicals Corp. (Kyoto, Japan). Complete Freund's adjuvant from Difco Laboratories (Detroit, MI, U.S.A.) was used. λ-Carrageenan (Picnin-A) was purchased from Zushi Chemical Institute (Zushi, Kanagawa, Japan) and pontamine sky blue was obtained from Tokyo Kasei (Tokyo, Japan). Other reagents were all of analytical grade and were obtained from commercial sources.

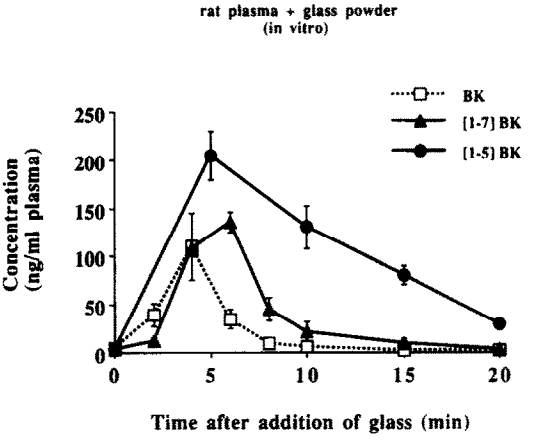

Fig. 2. Time course of the generation of BK and products of degradation of BK in diluted plasma of rats after activation with glass powder. The ordinate indicates the amounts of BK (□) and products of its degradation ([1-7]-BK, ▲; [1-5]BK, ●) per mL plasma. Each value represents mean ± SEM from four experiments.

Statistical analysis

Values were expressed as mean ± SEM, and Student's *t*-test was used to evaluate the significance of differences. When variances were heterogeneous, statistical analyses were performed by the Aspin–Welch method or by Wilcoxon's rank sum test.

RESULTS

Changes in the level of BK and products of degradation of BK after glass activation of plasma

Figure 2 shows the time course of the generation of BK, [1-7]BK and [1-5]BK after addition of glass powder to diluted plasma from rats. Immediately after addition of glass, BK was generated, but it was very rapidly degraded within 8 min under our experimental conditions. However, [1-7]BK could be detected for 10 min, although its generation was also transient. Unlike BK and [1-7]BK, [1-5]BK was detectable during the entire course of the incubation, and the maximum detectable levels of [1-5]BK (205 ± 24 ng/mL plasma) were much higher than those of BK and [1-7]BK.

Time course of exudation of plasma and accumulation of pleural fluid in rats with carrageenan-induced pleurisy

Figure 3 shows the time course of changes of the

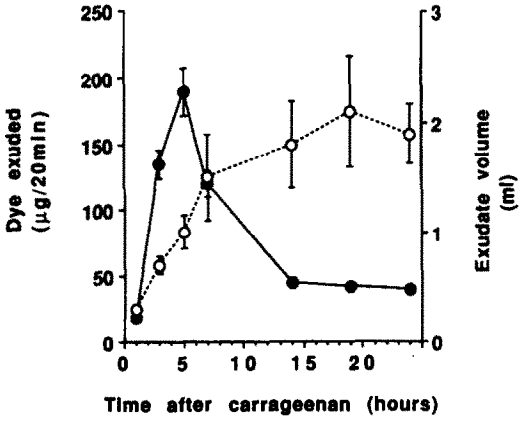

Fig. 3. Temporal changes in dye exudation and pleural fluid volume in carrageenan-induced pleurisy in rats. The left ordinate shows the dye amounts in the pleural cavity over 20 min periods (●) and right one indicates the exudate volume (○). Each value represents the mean ± SEM from four to eight animals.

rate of exudation of plasma and the volume of exudate in rats with carrageen-induced pleurisy. The accumulation of pleural exudate continued for up to 19 hr (2.1 ± 0.5 mL, N = 4) and then gradually decreased thereafter. The closed circles in the figure indicate the rate of exudation of plasma determined by leakage of dye from the circulation over 20 min periods. This rate increased rapidly up to 5 hr (190 ± 20 μg/20 min, N = 6) and then decreased gradually.

Changes in the levels of BK and products of degradation of BK in carrageenan-induced pleurisy

Figure 4 depicts temporal changes in the levels of BK, [1-7]BK and [1-5]BK in the exudates from rats with carrageenan-induced pleurisy. The limit of detection for BK and [1-7]BK in the pleural fluid was 160 pg per rat and, therefore, exudates were pooled from four rats and subjected to each EIA. Even when the exudates were pooled, the levels of BK were below the limits of detection. The degradation product, [1-7]BK, could be detected throughout the entire course of this inflammatory response at levels above 400 pg per rat. The levels of [1-7]BK were higher in the early phase of the inflammatory response. By contrast, levels of [1-5]-BK in the pleural fluid increased markedly up to 5 hr (6050 ± 1050 pg per rat, N = 6) and then decreased gradually. The amount of [1-5]BK was much greater than that of BK and [1-7]BK. The levels of BK, [1-7]BK and [1-5]BK, in the washings of the pleural cavity of normal rats, were negligible (less than 160 pg per rat).

Furthermore, the stability of [1-5]BK was tested in the pleural cavity after intrapleural injection of [1-5]BK (100 ng) or BK (100 ng). Collection of absolute ethanol injected into the pleural cavity 30 min after [1-5]BK injection revealed that nearly 20% of [1-5]BK (18.6 ± 4.9 ng per rat, N = 3) was detected. Intrapleural injection of the same amount of BK resulted in failure of detection of BK (below 0.6 ng per rat), but still 7.6 ± 0.7 ng per rat (N = 3) of [1-5]BK was detectable in the pleural cavity.

Effects of SBTI on the levels of products of degradation of BK in carrageenan-induced pleurisy

Figure 5 shows the intrapleural levels of [1-5]BK and [1-7]BK during treatment with SBTI or saline. The levels of [1-5]BK (3, 7 and 19 hr after administration of carrageenan) were significantly reduced by the treatment with SBTI by 63–79%, and those of [1-7]BK were reduced significantly by 51–65%, indicating that BK was generated in the

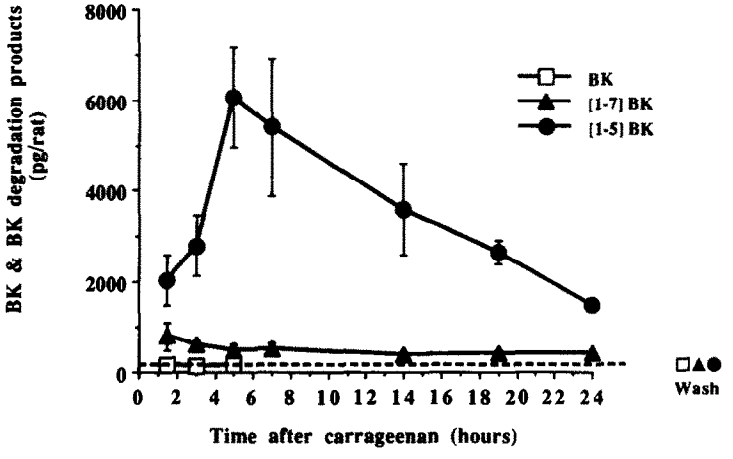

Fig. 4. Changes in intrapleural levels of BK and products of degradation of BK in rats with carrageenan-induced pleurisy. The ordinate indicates the amounts of BK (□) and products of its degradation ([1-7]BK, ▲; [1-5]BK, ●) per one rat. Wash represents the levels of BK and products of degradation of BK in the washings of the pleural cavity of normal rats. Each value represents mean ± SEM from four to eight experiments.

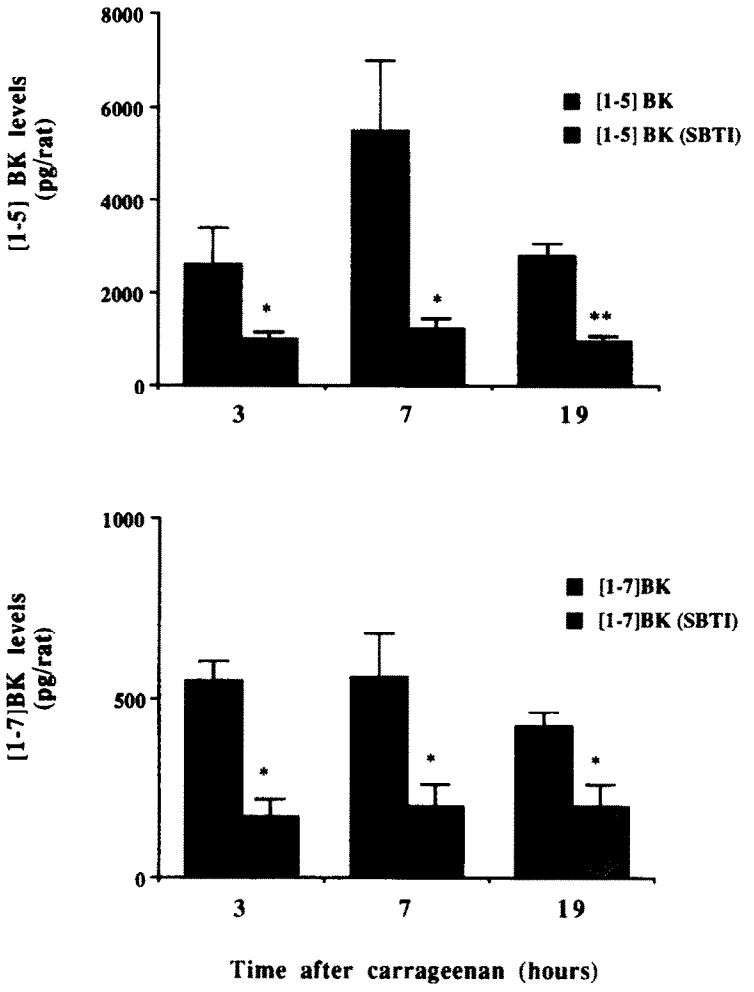

Fig. 5. Effects of SBTI on the levels of [1-5]BK (upper panel) and [1-7]BK (lower panel) in carrageenan-induced pleurisy in rats. SBTI (0.3 mg/rat) was injected intrapleurally (hatched column); control rats received only saline intrapleurally (closed column). The ordinate indicates the amounts of degradation products per rat. Each value represents mean ± SEM from four to six experiments. * $P < 0.05$; ** $P < 0.01$.

pleural cavity during the entire course of carrageen-induced pleurisy in rats.

Effects of captopril on the levels of BK and [1-5]*BK in carrageenan-induced pleurisy*

As shown in Fig. 6, the levels of BK and [1-5]BK were determined during treatments with captopril and with saline. The levels of [1-5]BK in the pleural exudate 3 hr after administration of carrageenan were markedly reduced by captopril by 98%. By contrast, the levels of BK in the exudates significantly increased during treatment with captopril to 1250% of levels in saline-treated control rats.

DISCUSSION

To verify the involvement of BK in various pathological conditions, it is necessary to detect BK at the sites where it is released, but difficulties associated with the detection of free BK have hampered such experiments. In previous reports, we proposed measuring the residual levels of precursor proteins of the kinin-forming system, e.g., plasma prekallikrein [22], HMW and LMW kininogens [19] in the biological fluids, instead of measuring free kinins, since these precursor proteins turn over slowly *in vivo*. Once plasma prekallikrein and HMW kininogen have been activated and degraded, 72 hr are required before the levels of these precursors are restored in rats [23]. In fact, residual levels of plasma prekallikrein and HMW kininogen are reduced in the pleural exudates of rats with carrageenan-induced pleurisy [1] and in plasma of rats after intravenous injection of endotoxin [5]. However, this approach to the verification of the involvement of kinin has the drawback that the precursors of kinin may be degraded by proteases other than kallikrein without formation of kinin [24–26]. Therefore, we developed assay systems to determine the stable products of the degradation of

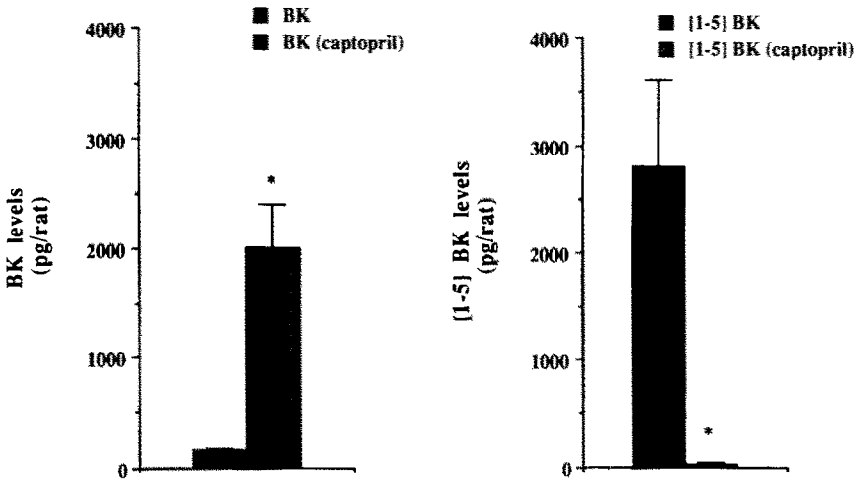

Fig. 6. Effects of captopril on the levels of BK (left panel) and [1-5]BK (right panel) in carrageenan-induced pleurisy in rats. Captopril (10 mg/kg) was injected intraperitoneally (hatched column) and as controls only saline was injected intraperitoneally (closed column). The ordinate indicates the amounts of degradation products per rat. Each value represents mean ± SEM from four to six experiments. * $P < 0.05$.

kinins in biological fluids to prove the formation of kinins.

We previously reported the pathways of degradation of bradykinin in human and rat plasma [10, 13, 14]. In human plasma, BK is degraded to [1-8]BK by kininase I and to [1-7]BK by kininase II, and both degradation products are further degraded to [1-5]BK by kininase II. The same occurs in the plasma and pleural exudates of rats, with the exception that the activity of kininase II is predominant in rats [10]. We described previously the development of EIAs for BK and for [1-7]BK [9, 10]. In the present experiments, an EIA for [1-5]BK was developed and was shown to be useful in demonstrating the activation of plasma kallikrein and release of kinin *in vitro* (Fig. 2) and *in vivo* (Fig. 4). As shown in Fig. 2, contact of rat plasma with glass powder results in activation of coagulation factors and, hence, conversion of plasma prekallikrein to plasma kallikrein, which releases bradykinin from HMW kininogen. In this *in vitro* experiment with diluted plasma, BK was certainly detectable, but its presence was fleeting. The peak level of its metabolite, [1-7]BK, was observed a little later but the level of [1-7]BK soon decreased. By contrast, the amounts of [1-5]BK detectable in the incubation mixtures were much higher. [1-8]BK was not assayed in the present experiments.

In our previous reports [10, 13, 14], incubation of [1-5]BK with rat or human plasma revealed that this peptide was degraded slowly to the smaller fragments without detection of [1-3]BK. Thus, the detection of [1-5]BK in the biological fluid was reasonable. The present assay system for [1-5]BK was sensitive and sufficiently specific (Fig. 1, Table 1), and the minimum amounts detectable were approx. 160 pg per rat. Assay systems with higher sensitivity may be possible using fluorogenic substrates and monoclonal antibodies [18]. However, the present method did allow detection of the products of degradation of BK *in vivo*. In the case of rats with carrageenan-induced pleurisy, BK was not detectable in pleural exudates even when samples from four rats were pooled. By contrast, [1-7]BK was detectable throughout the entire course of the inflammatory response induced by carrageenan. The levels of [1-5]BK in the pleural fluids increased to 6050 pg per rat at 5 hr and then decreased gradually, and levels remained higher than those of [1-7]BK throughout the entire course of this inflammatory response, indicating that [1-5]BK may be a superior marker of the release of kinin. In this carrageenan-induced pleurisy model, BK may be generated continuously in the pleural cavity, but the persistent detection of [1-5]BK in the pleural cavity after the intrapleural injection of this peptide or BK in the present experiment insured the reliability of the present method.

The successful detection of the products of degradation of BK in the pleural exudates did not identify the origin of BK, for example, plasma kallikrein–HMW kininogen system or tissue kallikrein–LMW kininogen system. Simultaneous measurements of the residual levels of precursor proteins may reveal the origin of BK, since the two kallikrein–kinin systems are activated independently *in vivo* [27]. In a previous paper [27], we reported that the residual levels of plasma prekallikrein and HMW kininogen in the pleural exudate were reduced to a negligible level, but LMW kininogen system was not activated in the pleural cavity. It is known that carrageenan shows a negative charge and activates factor XII and plasma prekallikrein successively, with resultant generation of kinin from HMW kininogen in the exudate [28]. The absence of a reduction in levels of LMW kininogen suggests that no kinin is released from LMW kininogen. It seems likely that HMW kininogen, supplied from

the blood stream, is fully converted to kinin in the pleural cavity upon contact with carrageenan [1]. The temporal changes of the amounts of kinin generated in the pleural cavity appear to parallel those in the rate of exudation of plasma from the blood stream. As shown in Figs 3 and 4, the peak rate of exudation of plasma coincided with peak levels of [1-5]BK 5 hr after administration of carrageenan.

To confirm that the detection of metabolites of BK in the pleural cavity reflected the generation of BK, SBTI was injected into the pleural cavity, since the activity of plasma kallikrein is blocked by SBTI [22]. This treatment markedly suppressed the exudation of plasma in our system [28]. The levels of both [1-7]BK and [1-5]BK were significantly reduced by SBTI (Fig. 5).

We reported that captopril, an inhibitor of kininase II, blocks the formation of [1-5]BK from BK [10, 13, 14]. Treatment with captopril in the present experiment allowed the detection of BK in the pleural fluid. Detectable levels of BK were accompanied by a marked reduction in levels of [1-5]BK. This observation suggests that BK was formed and was degraded to [1-5]BK in the pleural cavity. An inhibitor of kininase I [29] had less effect on detection of BK in the pleural cavity (data not shown) because of the lower contribution of kininase I to the degradation of BK in the pleural exudates [10]. It is convenient for the detection of BK to treat animals with an appropriate inhibitor of kininase. However, during such treatment, the inflammatory process itself is enhanced, because of the accumulation of the active peptide, BK. Furthermore, during such treatment, the effects of other chemical mediators, prostaglandins, which play a role in the inflammation process, become prominent. In kaolin-induced models of pleurisy in rats, the leakage of plasma within 20 min of administration of kaolin can be inhibited by indomethacin if rats are treated with captopril [30], indicating enhancement of the generation of prostaglandins by captopril.

In rats, a third kininogen, in addition to HMW and LMW kininogens, has been designated T-kininogen [31]. If T-kinin is generated in our model, after degradation of T-kininogen by kininase II, fragments of T-kinin might be detected by the EIA for [1-5]BK. However, this possibility is slight, because the level of T-kinin in fractions, after HPLC [10, 13, 14], of exudates from rats treated with captopril, was negligible, when it was measured by EIA for BK (data not shown).

In conclusion, it appears that the detection of products of degradation of BK is a useful strategy for verifying release of kinin *in vivo*. Among the products of degradation of BK, [1-5]BK is a superior marker in rat models of inflammation. [1-5]BK has been reported to be a stable and long-lived metabolite in human plasma [10, 13]. Thus, for human samples, the EIA for [1-5]BK will be a useful method to detect the release of kinin. In a preliminary experiment, we were able to detect [1-5]BK in the nasal cavities of patients with nasal allergy after antigen challenge (data not shown).

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