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Proinflammatory characteristics of a nonpeptide bradykinin mimic, FR190997, *in vivo*

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1 Proinflammatory potency of the nonpeptide bradykinin (BK) B_2 receptor agonist FR190997 (8-[2,6-dichloro-3-[N-[(E)-4-(N-methylcarbamoyl)cinnamidoacetyl]-N-methylamino]benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline) was investigated.

2 Intradermal injection of FR190997 (0.03–3 nmol site^{-1}) into dorsal skin of rats increased vascular permeability in a dose-dependent manner. The effect was less than that of BK, but it was long-acting and was inhibited by treatment with FR173657 (3 mg kg^{-1}, p.o.). Captopril (10 mg kg^{-1}, i.p.) did not enhance the plasma extravasation by FR190997 (0.3 nmol site^{-1}) in the presence of soybean trypsin inhibitor (SBTI, 30 μg site^{-1}).

3 Subcutaneous injection of FR190997 (3 nmol site^{-1}) into the hindpaw of mice markedly induced paw swelling. The oedema lasted up to 3 h after the injection. Administration of indomethacin or NS-398 (10 mg kg^{-1}, i.p.) significantly reduced it at 3 h after the injection.

4 Simultaneous i.p. injection of prostaglandin (PG) E_2 (1 nmol site^{-1}) or beraprost sodium (0.5 nmol site^{-1}) with FR190997 (5 nmol site^{-1}) greatly enhanced frequency of writhing reactions in mice.

5 FR190997 (0.3–30 nmol kg^{-1}, i.v.) showed less increase in airway opening pressure (Pao) in the guinea-pig after i.v. injection. Furthermore, FR190997 (0.03–30 nmol) resulted in a very weak contraction of tracheal ring strips and lung parenchymal sections *in vitro*.

6 In mice sponge implants, topical application of FR190997 increased angiogenesis and granulation with enhanced expressions of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) mRNAs.

7 These results indicate that FR190997 has proinflammatory long-lasting characteristics and it might be 'a stable tool' for studying the role of BK B_2 receptor *in vivo*.

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Abbreviations: bFGF, basic fibroblast growth factor; BK, bradykinin; COX, cyclo-oxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MBP, mean arterial blood pressure; Pao, airway opening pressure; PECAM-1, platelet endothelial cell adhesion molecule-1; PG, prostaglandin; RT–PCR, reverse transcription-polymerase chain reaction; SBTI, soybean trypsin inhibitor; VEGF, vascular endothelial growth factor

Introduction

Bradykinin (BK) is an important endogenous mediator involved in inflammation, since it induces plasma extravasation, smooth muscle contraction/relaxation, bronchoconstriction, nociception receptor (Bhoola *et al*., 1992; Dray & Perkins, 1993; Regoli & Barabé, 1980) and release of prostaglandins by binding the B_2 receptor (Burch & Axelrod, 1987). Therefore, BK has a potentially crucial role in inflammatory diseases, such as asthma, rhinitis, arthritis, endotoxin shock (Proud & Kaplan, 1988) and pancreatitis (Griesbacher, 2000). Recently, orally active non-peptidic B_2 receptor antagonists, FR173657 and FR167344, have been reported (Aramori *et al*., 1997b; Asano *et al*., 1997a, b). The role of BK in inflammatory diseases has demonstrated using experimental animal models and several BK receptor antagonists (Griesbacher & Legat, 1997; Griesbacher *et al*., 1997; Majima *et al*., 1997; Rizzi *et al*., 1997). FR190997 (8-[2,6-dichloro-3-[N-[(E)-4-(N-methylcarbamoyl)cinnamidoacetyl]-N-methylamino]benzyloxy]-2 -methyl-4-(2-pyridylmethoxy) quinoline), which is a nonpeptide mimetic of BK, was reported to stimulate phosphatidylinositol hydrolysis in Chinese hamster ovary cells expressing the human bradykinin B_2 receptor (Asano *et al*., 1998). BK has been administered to reproduce the actions of intrinsic BK *in vivo*. However, the dose of exogenously administered BK is usually high. Because of the susceptibility to degrading enzymes, the biological activity of BK is diminished rapidly, even after the i.v. administration *in vivo*.

The effect of FR190997 may be long-acting due to it being a nonpeptide, suggesting that it should be an effective tool for analysis of the biological function of BK *via* activation of the B_2 receptor (Aramori *et al*., 1997a; Rizzi *et al*., 1999). Taking the place of biologically unstable BK, this nonpeptide agonist

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might enable us to reproduce physiological and pathological events *via* the B_2 receptor with certain stability, leading to potential uses of the B_2 receptor antagonists towards therapeutics. In the present study, we examined the ability of FR190997 to elicit inflammatory reactions *in vivo* and the effects were compared to BK.

Methods

Vascular permeability increase

Animals were housed in the Experimental Animal Center of the School for 1 week and thereafter acclimatized in the laboratory for 1 h before the commencement of each experiment. All of the following procedures were conducted in accordance with the guideline principles for the care and use of laboratory animals of the Animal Care Committee of Kitasato University and with guiding principles for the care and use of laboratory animals approved by The Japanese Pharmacologcal Society.

Male 8-week-old SD rats (SLC Japan, Shizuoka, Japan) were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.p.). Five minutes after i.v. injection of pontamine sky blue (50 mg kg^{-1}, i.p.), 0.1 ml of various concentrations of a given agent dissolved in 0.01 N HCl was injected intradermally into eight sites of the shaved dorsal skin. The rats were killed by exsanguination 20 min after i.d. injection of agents for the determination of leaked dye for 20 min, Figure 1A. Blood was taken before exsanguination to obtain serum. The exudated dye in the skin at each site was extracted by the method as reported previously (Hayashi & Majima, 1999). In brief, excised skin was incubated in 1 ml of 1 N KOH at 37°C overnight; then 2.5 ml of 0.6 N phosphoric acid was added to neutralize the base, and the exudate dye was extracted by addition of 7.5 ml of acetone. After centrifugation at $1200 \times g$ for 20 min, the optical density of each supernatant and serum were measured at 620 nm with a

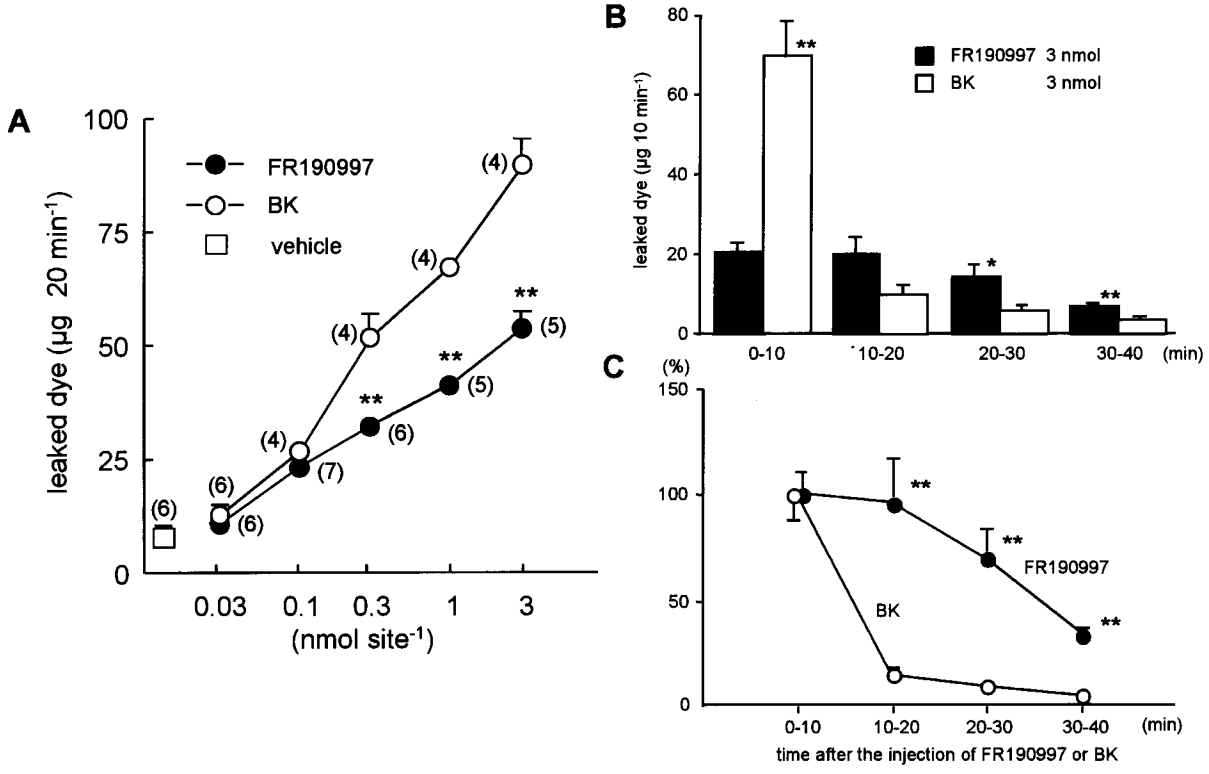

Figure 1 Vascular permeability increase by i.d. injection of FR190997 or BK in rats. (A) 5 min after i.v. injection of pontamine sky blue (50 mg kg^{-1}, i.p.) into anaesthetized rats, 0.1 ml of various concentration of a given agent in 0.01 N HCl was injected intradermally into eight sites of the shaved dorsal skin of rats. The doses of agents were expressed as mol $site^{-1}$. The rats were killed by exsanguination 20 min after i.d. injection of the agents. The exudated dye in the skin at each site was extracted and the amount of dye at each site was expressed as serum equivalents (μl serum eq.) in the same rat for uniformity. Results are mean$\pm$s.e.mean with the number of observations indicated in parentheses, $^*P<0.05$ (ANOVA plus *post-hoc* Dunnett's test, *c.f.* BK-injected animals). Where no error bars are shown error lies within the dimensions of the symbol. (B) FR190997 (3 nmol) or BK was injected intradermally as above. To determine dye leakage for 10 min-interval in (B) and (C), rats received the dye 10 min before exsanguination. In detail, for 0–10 min-determination, dye and agent were injected at 0 min and rat was killed at 10 min. For 10–20 min-determination, agent was injected at 0 min and the dye was administered at 10 min, then rat was sacrificed at 20 min after the injection of agent. For 20–30 min-determination, agent was injected at 0 min and the dye was administered at 20 min, then rat was sacrificed at 30 min after the injection of agent. Results show amounts of leaked dye in the skin (μg 10 min^{-1}) and are mean$\pm$s.e.mean, $n=5$, $^*P<0.05$, $^{**}P<0.01$ (ANOVA plus *post-hoc* Dunnett's test, *c.f.* BK-injected animals). (C) Values are calculated from the data of (B), and are expressed as a percentage when the amount of leaked dye during the initial 10 min after injection of FR190997 or BK is as 100%. Results are mean$\pm$s.e.mean, $n=5$, $^{**}P<0.01$ (ANOVA plus *post-hoc* Dunnett's test, *c.f.* BK-injected animals).

spectrophotometer. The amount of dye at each site was expressed as serum equivalents (μl serum eq.) in the same rat for uniformity.

To determine dye leakage at 10 min-intervals in Figure 1B, C, rats received the dye 10 min before exsanguination. In detail, for 0 to 10 min-determination, the dye and agent were injected at 0 min and the rats were killed at 10 min. For 10 to 20 min-determination, agent was injected at 0 min and the dye was administered at 10 min, and the rats were sacrificed 20 min after the injection of agent. For 20 to 30 min-determination, agent was injected at 0 min and the dye was administered at 20 min, and the rats were sacrificed 30 min after the injection of agent.

In the experiments to assess effects of BK B_2 receptor antagonist, FR173657, angiotensin-converting enzyme (kininase I) inhibitor, captopril, and an inhibitor for plasma kallikrein, soybean trypsin inhibitor (SBTI) in Figure 2A, B, rats received pontamine sky blue 5 min prior to the administration of BK or FR190997 as described above. Rats were killed by exsanguination 10 min after the injection of the agents. FR173657 (3, 30 or 100 mg kg^{-1}, 6 mg ml^{-1} in 5% w v^{-1} gum arabic) or captopril (10 mg kg^{-1}, 4 mg ml^{-1} in saline) was administered orally or intraperitoneally, 1 h or 30 min before the injection of FR190997, respectively. SBTI (30 μg $site^{-1}$) was injected with FR190997 or BK simultaneously.

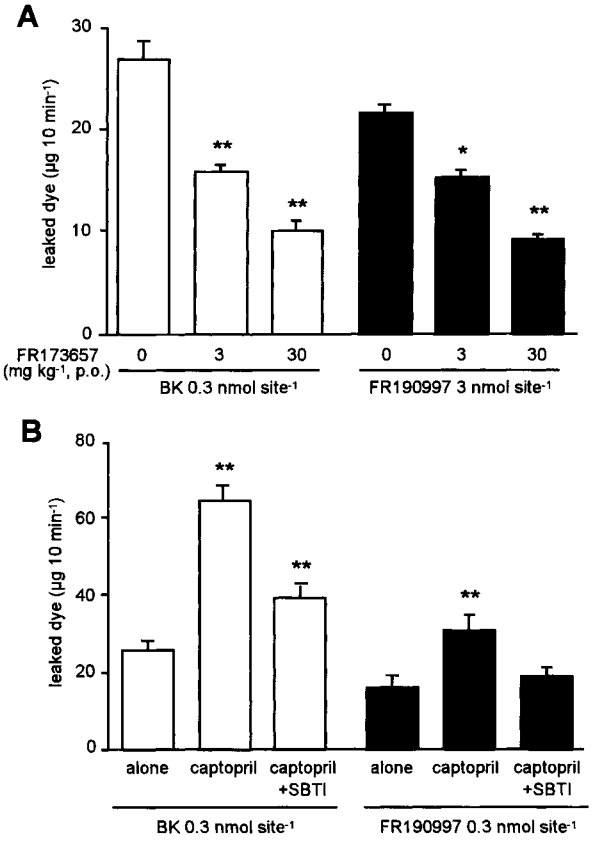

Figure 2 Effects of FR173657 (A) and captopril (B) on vascular permeability induced by FR190997 or BK. FR190997 (3 nmol in A, 0.3 nmol in B) or BK (0.3 nmol) was injected intradermally. FR173657 (indicated doses) or captopril (10 mg kg^{-1}) was administered orally or intraperitoneally, 1 h or 30 min before the injection, respectively. Rats received pontamine sky blue 5 min prior to the administration of BK or FR190997. Rats were killed by exsanguination 10 min after the injection of agents. SBTI (30 μg $site^{-1}$) was injected with FR190997 or BK simultaneously. Results show amounts of leaked dye in the skin (μg 10 min^{-1}), and are mean$\pm$s.e.mean, $n=6$, $^*P<0.05$, $^{**}P<0.01$ (ANOVA plus *post-hoc* Dunnett's test).

Paw oedema

Male 5-week-old ICR mice (SLC Japan) were used. Hindpaw volume was measured mercury plethysmography *via* a low-pressure transducer (TMI BLH, LPU-0.1-350-0-II, No.21171, Natsume Corp., Tokyo, Japan) and recorded with a transducer (1829, NEC San-ei Instruments Ltd., Tokyo, Japan), an amplifier case (7903, NEC San-ei Instruments Ltd.), and a recorder (Rectigraph-8K 1155, NEC San-ei Instruments Ltd.). Paw oedema was induced by intraplantar injection of 25 μl of FR190997 (3 nmol) dissolved in 0.01 N HCl, BK (3 nmol), or the same volume of 0.01 N HCl as the vehicle. Indomethacin (10 mg kg^{-1}, 4 mg ml^{-1} in saline), or NS-398 (10 mg kg^{-1}, 4 mg ml^{-1} in 5% w v^{-1} gum arabic) was injected intraperitoneally 30 min before injection of FR190997 or BK.

Writhing reaction

Writhing reaction was induced by i.p. injection of FR190997 (5 nmol in 50 μl of 0.05 N HCl), BK (5 nmol) or desArg^9-bradykinin (desArg^9-BK, 5 nmol) into 4-week-old male ICR mice (SLC Japan). Prostaglandin (PG) E_2 (1 nmol) or beraprost sodium (sodium ($\pm$)-(1R, 2R, 3αS,8βS)-2,3,3α, 8β-tetrahydro-2-hydroxy-1-[(E)-(3S)-3-hydroxy-4-methyl-I-octen-6-ynyl]-1H-cyclopenta[β]bensofuran-5-butyrate, 0.5 nmol) was injected intraperitoneally with FR190997, BK or desArg^9-BK simultaneously. Frequency of writhing response was counted for 30 min after the injection of stimulants.

Measurements of bronchoconstriction and systemic blood pressure

Male Hartley guinea-pigs (SLC Japan) weighing 350–450 g, and 7–8-weeks-old male SD rats (SLC Japan) were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.p.) and placed on a heated blanket, which maintained body temperature at approximately 37°C. The trachea was cannulated with a polyethylene cannula and the lungs were artificially ventilated with room air (Rodent Respirator, Harvard Apparatus, Millis, MA, U.S.A.) at 70 strokes min^{-1} with a tidal volume of 1 ml 100 g body $weight^{-1}$. Airway opening pressure (Pao) was monitored by means of a pressure transducer (TMI BLH, LPU-0.1-350-0-II, No.21171, Natsume Corp.) as an index of changes in tracheobronchial resistance to airflow at the side arm of the trachea cannula. Systemic mean arterial blood pressure (MBP) was measured *via* cannula (PE-50, Becton Dickinson and Co., Parsippany, NJ, U.S.A.) inserted into the carotid artery. Pao and MBP were recorded using a polygraph system composed of a transducer (1829, NEC San-ei Instruments Ltd.), an amplifier case (7903, NEC San-ei Instruments Ltd.), and a recorder (Rectigrapg-8K 1155, NEC San-ei Instruments Ltd.).

Agonists were administered by a single bolus injection through a cannula (PE-50, Becton Dickinson and Co.) inserted into jugular vein. FR173657 (30 mg kg^{-1}, 6 mg ml^{-1} in 5% w v^{-1} gum Arabic) was administered orally 1 h before the injection of FR190997. A BK B_1 receptor antagonist, desArg^9[Leu^8]-bradykinin (desArg^9[Leu^8]-BK) dissolved in a physiological saline at a concentration of 100 nmol ml^{-1}, was infused into the right femoral vein at a constant rate (10 nmol min^{-1}) with a syringe pump (model 11, Harvard Apparatus).

Contraction of tracheal smooth muscle and lung parenchymal section

Male Hartley guinea-pigs weighing 350–450 g and 7–8-week-old male SD rats (SLC Japan) were used. Animals were stunned and exsanguinated. The trachea was excised from guinea-pigs, cleaned carefully of adhering fat and connective tissue, cut transversely into 4–5 rings and then opened by cutting longitudinally through the cartilage rings diametrically opposite the tracheal smooth muscle. Each tracheal segment was sutured together. For preparation of lung parenchymal section of guinea-pig and rat, a lung section with 1.5 cm-length was excised. These segments were mounted under a resting tension in 5 ml-volume in siliconized glass organ bath containing 5 ml of Krebs bicarbonate solution, maintained at 37°C and continuously bubbled air. The composition of the Krebs bicarbonate solution was (in mM): NaCl 117, KCl 5.36, $NaHCO_3$ 25, KH_2PO_4 1.03, $MgSO_4 \cdot H_2O$ 0.57, $CaCl_2$ 2.5, D-glucose 11.1. Changes in tension were recorded isometrically *via* a force-displacement transducer (45196A, NEC Medical Systems Ltd., Tokyo, Japan) connected to a transducer amplifier and a recorder. Following a 30 min equilibration period, during which the tissues were washed every 5 min, tissues were exposed to FR190997 or BK.

The mice sponge model

A circular polyether polyurethane sponge disc (5 mm-thick, 12 mm in diameter) was implanted into the subcutaneous tissue of the back of male ICR mice (SLC, specific pathogen free, 5-week-old) under light ether anaesthesia, as reported previously (Majima *et al*., 2000b). FR190997, BK, and desArg^9-BK (10 mM) were dissolved in dimethyl sulphoxide. Thirty nmol of each agonist was spotted onto a 5.5 mm diameter sterile filtered paper (Advantec Toyo, Tokyo) and air dried. The disc spotted with 3 μl of dimethy sulphoxide alone was used as vehicle control. The paper disc was inserted in the middle of the sponge disc. Mice were sacrificed 7 days after the implantation.

Neovascularization was assessed by measuring the concentration of haemoglobin in the granulation tissues and the enclosed sponge implants. The granulation tissues plus the sponge were taken immediately after the sacrifice and were weighed. Distilled water, four times the weight of the sample granulation tissues, was added to each sample, which was then homogenized with a Polytrone (Kinematica GmbH, Luzern, Switzerland). The homogenate was centrifuged at 4°C, 10,000 × *g* for 15 min. Fifty μl of the supernatant was mixed with 50 μl of 0.67 mM phosphate buffer (pH 7.2) containing 3.5 mM sodium lauryl sulphate. The absorbance at 540 nm of the mixture was measured to determine using haemoglobin concentration. Mouse haemoglobin (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) was used as the standard. For the determination of hydroxyproline, 100 μl of the above homogenate was added to 400 μl of 7.5 N hydrochloric acid to hydrolyze at 110°C for 16 h. The hydrolysate was evaporated to dryness. The specific activity of hydroxyproline in the hydrolysate was measured after converting hydroxyproline into pyrrol (Kivirikko *et al*., 1967). 4-hydroxyproline was used as the standard.

Reverse transcription-polymerase chain reaction (RT–PCR)

Approximately 50 mg of the granuloma tissues were removed from the mouse, and homogenized in 1 ml of TRIZOL Reagent (GIBCO BRL, Rockville, MD, U.S.A.). Total RNA was prepared according to the instruction. Single-stranded cDNA from 250 ng of the RNA was synthesized using 0.4 μg of oligo-p(dT)$_{15}$ primer and four units of AMV reverse transcriptase (Roche Diagnostics, Basel, Switzerland). PCR was performed in 20 μl of 20 mM Tris-HCl (pH 8.7) containing 10 mM KCl, 5 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 μM of forward and reverse primers, and 0.5 unit of Taq DNA polymerase (Qiagen GmbH, Germany). Complementary DNA from 25 ng of total RNA was used as the template. Oligonucleotide primers used were: for platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), 5′-CGGGATCCAGGAAAGCCAAGGCCAAA-3′ (bases 1941–1958), 5′-CGGAATTCTTGACTGTCTTAAGTTCC-3′ (bases 2288–2274); vascular endothelial growth factor (VEGF), 5′-AACCATGAACTTTCTGCTCT-3′ (bases −4-+16) 5′-CCGAAACCCTGAGGAGCTC-3′ (bases 720–701); bFGF, 5′-ACCAGCCACTTCAAGGAC-3′ (bases 63–81), 5′-TATGGCCTTCTGTCCAGGTC-3′ (bases 435–416); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCCTTATTGACCTCAACTACATGGT-3′ (bases 100–125), 5′-GAGGGGCCATCCACAGTCTTCTG-3′ (bases 569–547). These PCR were run for 35 cycles (PECAM-1, bFGF, and GAPDH) or 40 cycles (VEGF). Cycling conditions were: 94°C, 30 s; 58°C, 45 s; 72°C, 45 s for 30 cycles, followed by a final extension for 10 min at 72°C. Products of PCR were analysed on 1.5% agarose gel electrophoresis and the products sizes were as predicted from the sequences.

Statistical analysis

Results show mean ± s.e.mean with the number of observations indicated in parentheses. Statistical significance of differences between groups was determined by ANOVA followed by Dunnett's test. In the experiment of writhing response (Figure 5), statistical analysis was conducted with Student's *t*-test. A probability (*P*) value of less than 0.05 was taken to indicate statistical significance.

Drugs and chemicals

FR190997 and FR173657 were kindly supplied from Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). BK, desArg^9-BK, and desArg^9[Leu^8]-BK were purchased from Peptide Institute (Osaka, Japan). The following drugs were used: Captopril (Wako Pure Chemicals Industries, Ltd.,

Osaka, Japan), indomethacin (Wako Pure Chemicals Industries, Ltd.), NS-398 (N-[2-(cyclohexyloxy-4-nitrophenyl)-methane-sulphonamide, Cayman Chemical Company, Ann Arbor, MI, U.S.A], and SBTI (Sigma-Aldrich Co.). Other reagents were all of analytical grade and were obtained from commercial sources.

Results

Vascular permeability increase induced by FR190997 or BK in the rat

Intradermal injection of FR190997 into the back of the rat caused an apparent increase in vascular permeability in a dose-dependent manner (Figure 1A). Doses of more than 0.3 nmol site^{-1} of FR190997 were significantly less potent than those of BK ($P<0.01$). At a dose of 3 nmol site^{-1}, the efficacy of FR190997 was approximately 60% of BK. Duration of the vascular permeability increase was compared between FR190997 and BK. At the time lapse of 0–10 min after the injection of 3 nmol site^{-1}, the effect of FR190997 on the vascular permeability was approximately one-third of that of BK. However, FR190997 was more potent than BK at the time lapses of 20–30 min and 30–40 min after the injection (Figure 1B). The activity of FR190997 did not change at the time lapse of 10–20 min and 70% of the activity during the initial 10 min still remained at the 20–30-min period after the injection. By contrast, the vascular permeability caused by BK was markedly reduced from 20 to 10 min (Figure 1C).

Effects of FR173657 and captopril on vascular permeability increase induced by FR190997 or BK

Effect of a BK B_2 receptor antagonist, FR173657, or an inhibitor of kininase II, captopril on the vascular permeability increased by FR190997 was examined. FR173657 (3 or 30 mg kg^{-1}) was orally administered at 30 min before injection of FR190997 (3 nmol site^{-1}) or BK (0.3 nmol site^{-1}), which are almost equi-effective doses. The B_2 receptor antagonist significantly suppressed the vascular permeability increased by both BK and FR190997 ($P<0.01$ at 30 mg kg^{-1}) (Figure 2A). Captopril (3 μg site^{-1}) was intradermally injected into dorsal skin simultaneously together with those agonists. The vascular permeability induced by FR190997 or BK (0.3 nmol site^{-1}) was significantly enhanced by treatment with captopril ($P<0.01$). However, the dye leakage by FR190997 was not affected by combined treatment with captopril and soybean trypsin inhibitor (SBTI, 30 μg site^{-1}), whereas that by BK was significantly enhanced (Figure 2B).

Paw oedema induced by FR190997 or BK in mice

FR190997 or BK (3 nmol) were injected into the hindpaw of mice and paw swelling measured for up to 6 h. A transient increase in paw volume was observed at 15 min after injection of BK, after which swelling rapidly reduced to the initial volume. Following injection of FR190997, paw volume was greatly increased to peak at 15 min after the injection and then gradually decreased. In comparison with BK, FR190997 caused long-acting swelling of the hindpaw up to 3 h after the injection. The increase in paw volume induced by FR190997 was much greater than that by BK during the time course (Figure 3). Paw swelling induced by FR190997 at 3 h was significantly reduced by pretreatment of mice with indomethacin (10 mg kg^{-1}, i.p., $P<0.01$) or NS-398 (10 mg kg^{-1}, i.p., $P<0.05$) (Figure 4).

Writhing response induced by FR190997 or BK in mice

Pain action was compared between FR190997, BK and desArg^9-BK by using writhing model in mice (Figure 5). Intraperitoneal injection of each 5 nmol of the agonists provoked a few times of writhing (stretching) responses. There was no significant difference between them. Effects of PGs on writhing response induced by FR190997 were tested. Writhing reaction was not apparent by i.p. injection of PGE_2 (1 nmol mouse^{-1}) or a stable derivative of PGI_2, beraprost sodium (0.5 nmol mouse^{-1}) alone. Simultaneous injection of PGE_2 or beraprost sodium with FR190997 induced a significant increase in the frequency of writhing reaction. In contrast, writhing response induced by BK was also significantly enhanced by beraprost sodium, but not by PGE_2. Both PGs did not enhance the reaction by desArg^9-BK. Enhancement of writhing responses by FR190997 and beraprost sodium or PGE_2 was significantly greater than that by BK and desArg^9-BK ($P<0.05$).

Effects of FR190997 on bronchoconstriction and systemic blood pressure in guinea-pig in vivo

Effects of FR190997 on bronchoconstriction in guinea-pig were examined. Intravenous injection of FR190997 *via*

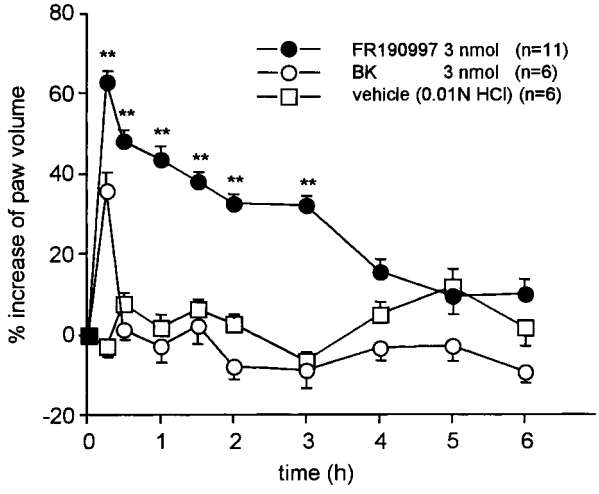

Figure 3 Induction of paw oedema by FR190997 or BK in mice. Paw oedema was induced by s.c. injection of 50 μl of 0.01 N HCl containing 5 nmol of FR190997, 5 nmol of BK or vehicle alone into the right hindpaw of ICR mice. Increase in paw volume was measured at the indicated time after injection of each stimulant. Hindpaw volumes are expressed as an increased percentage when the paw volume at 0 h is as 100%. Results are mean$\pm$s.e.mean with the number of observations indicated in parentheses, $^{**}P<0.01$ (ANOVA plus *post-hoc* Dunnett's test, *c.f.* 0.01 N HCl-injected animals).

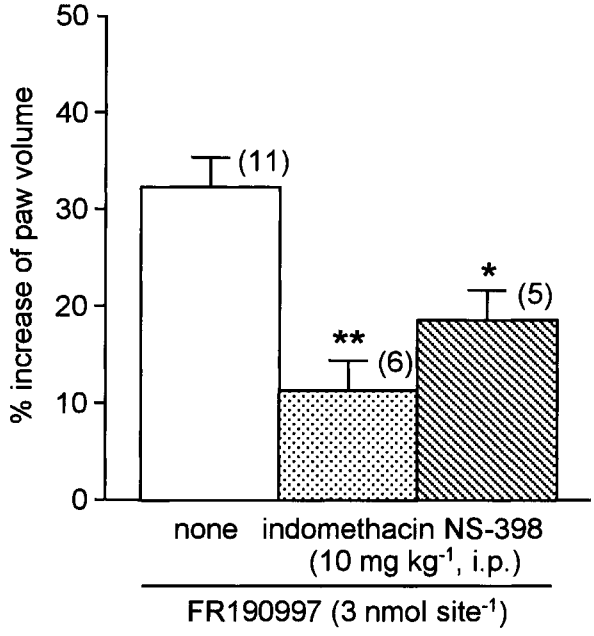

Figure 4 Effects of indomethacin and NS-398 on paw oedema induced by FR190997. Indomethacin or NS-398 (10 mg kg^{-1}, i.p.) was administered 30 min before injection of 3 nmol of FR190997 into right foot hindpaw. Hindpaw volume was measured at 3 h after the injection. Values are expressed as an increased percentage when the paw volume before the injection of FR190997 is as 100%. Results are mean ± s.e.mean with the number of observations indicated in parentheses, $*P<0.05$, $**P<0.01$ (ANOVA plus *post-hoc* Dunnett's test).

catheter cannulated into jugular vein did not show a significant increase in Pao (Figure 6A), while it decreased in MBP in a dose-dependent manner (Figure 6B). In contrast, doses more than 1 nmol kg^{-1} of BK elicited both a significant increase in Pao and a significant decrease in MBP when compared with FR190997. The efficacies of FR190997 in the increase in Pao and the decrease in MBP were less than 10 and 60%, respectively, of those of BK at a dose of 30 nmol kg^{-1}. An increase in Pao by BK (3 nmol kg^{-1}) was significantly inhibited by a pretreatment with a BK B_2 receptor antagonist, FR173657 (30 mg kg^{-1}) ($P<0.01$), but not a B_1 receptor antagonist desArg^9[Leu^8]-BK (Figure 6C). Bolus i.v. injection of desArg^9-BK (10–100 nmol kg^{-1}) did not cause a significant increase in Pao (Figure 6C).

In vitro *comparison of contractile activities of FR190997 to those of BK in isolated tracheal ring strips and lung parenchymal sections*

Contractile responses to tracheal and lung parenchymal sections by FR190997 were further evaluated *in vitro*. Up to 30 nmol of FR190997 showed less contractile activity on ring preparation of trachea isolated from guinea-pig (Figure 7A). A very weak activity was observed in lung parenchymal sections isolated from guinea-pig and rat (Figure 7A,B). BK induced concentration-dependent contraction of both tracheal ring strips isolated from guinea-pig and lung parenchymal sections from guinea-pig and rat.

Angiogenesis induced by kinin receptor agonists

When a circular sponge disc was implanted into the mice subcutaneous tissue, the granuloma tissues around the implant developed and grew. In this model, the newly formed tissues were constituted mainly of collagen fibres and the neovascularization was developed in and around the granuloma (Majima *et al*., 2000b). Filtered paper contained 30 nmol of each kinin receptor agonist was inserted into the sponge. The effects of the agonists on formation of granuloma and neovascularization were examined. Granulation and neovascularization were quantified by the determination of hydroxyproline contents and haemoglobin concentrations in the granuloma tissues, respectively. Application of 30 nmol of FR190997, BK, desArg^9-BK significantly increased mass of granuloma, contents of hydroxyproline and haemoglobin in the granuloma tissues compared to those in vehicle-treated mouse (Figure 8). FR190997 showed more potent formation of granuloma than BK and desArg^9-BK ($P<0.01$) (Figure 8A,C). Angiogenesis induced by those agonists was confirmed by expression of PECAM-1, which is a marker of vascular endothelium. Moreover expressions of VEGF and bFGF mRNAs were enhanced in the granuloma tissues of the agonists-treated mouse (Figure 9).

Discussion

Investigation of the physiological actions of BK has been greatly hampered because its effects are curtailed by rapid proteolysis in blood and tissues (Kuribayashi *et al*., 1993; Shima *et al*., 1992). FR190997 has been reported to show a high affinity binding to the human B_2 receptor, but not B_1 receptor, permanently expressed in Chinese hamster ovary cells, followed by a marked stimulation of phosphatidylinositol hydrolysis (Aramori *et al*., 1997a). *In vivo*, FR190997 induces prolonged hypotensive responses in the rat (Aramori *et al*., 1997a; Majima *et al*., 2000a). Therefore, it is a highly potent and subtype-selective nonpeptide agonist that displays high intrinsic activity. Thus this compound represents a powerful tool for exploring the physiology and pathophysiology of BK receptors.

In the present study, the proinflammatory *in vivo* effects of the nonpeptide BK B_2 receptor agonist, FR190997 was investigated. Vascular permeability increase by i.d. injection of FR190997 when assessed by the dye-leakage method in the rat was significant, but less effective than that of BK. The maximal response induced by FR190997 was approximately 60% of that induced by BK. This phenomenon agrees with the previous report, in which contractile activities of FR190997 in guinea-pig ileum (Asano *et al*., 1998), human umbilical vein and rabbit jugular vein (Rizzi *et al*., 1999) were also less effective than those of BK *in vitro*. The effect of FR190997 on vascular permeability increase was inhibited by treatment with FR173657, a nonpeptidic BK B_2 receptor antagonist in a dose-dependent manner. These results indicate that FR190997 has high selectivity to the B_2 receptor and its intrinsic activity is lower than BK *in vivo* as well as *in*

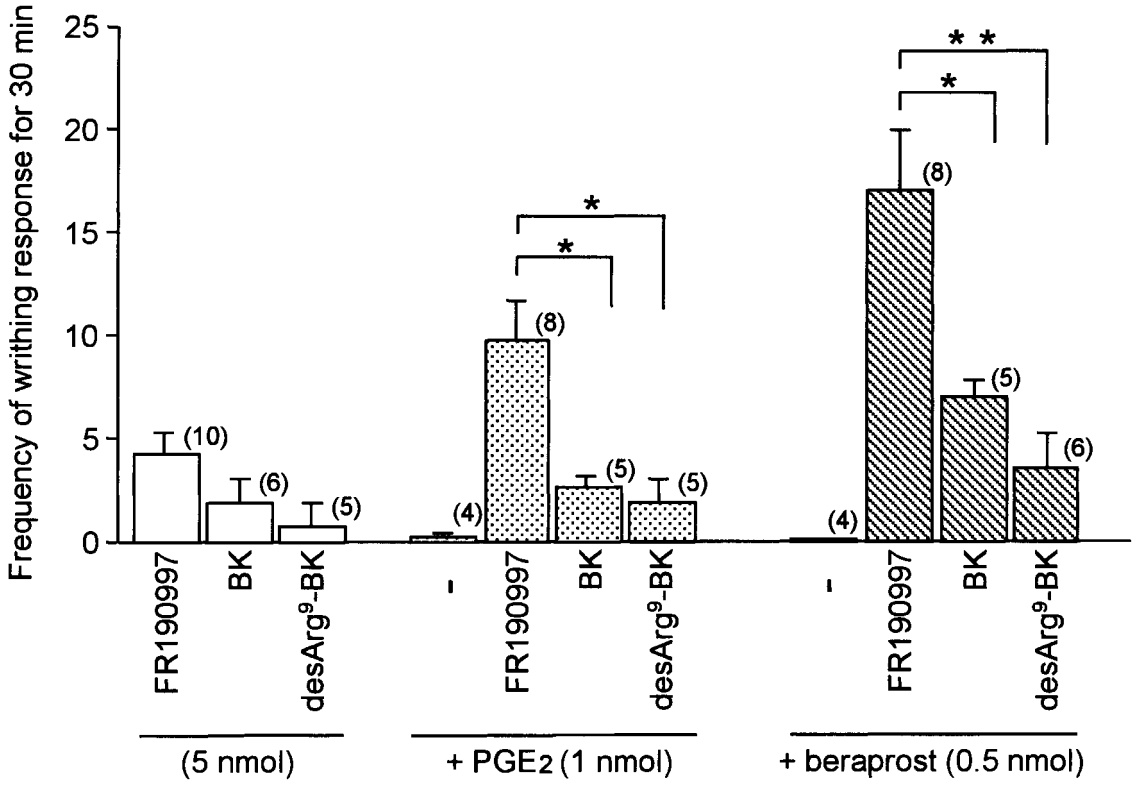

Figure 5 Writhing reaction induced by i.p. injection of FR190997 and synergistic effects between FR190997 and PGE_2 or beraprost sodium. Frequency of the stretch response (writhing) was counted for 30 min after i.p. injection of FR190997 (5 nmol mouse^{-1}), BK (5 nmol mouse^{-1}) or desArg^9 -BK (5 nmol mouse^{-1}). PGE_2 (1 nmol mouse^{-1}) or beraprost sodium (0.5 nmol mouse^{-1}) was intraperitoneally injected with or without FR190997, BK or desArg^9-BK. Results are mean$\pm$s.e.mean with the number of observations indicated in parentheses, $^*P<0.05$, $^{**}P<0.01$ (ANOVA plus *post-hoc* Dunnett's test). *·**Indicates statistically significant difference between FR190997-injected and BK- or desArg^9-BK-injected groups by Student's *t*-test at $P<0.05$.

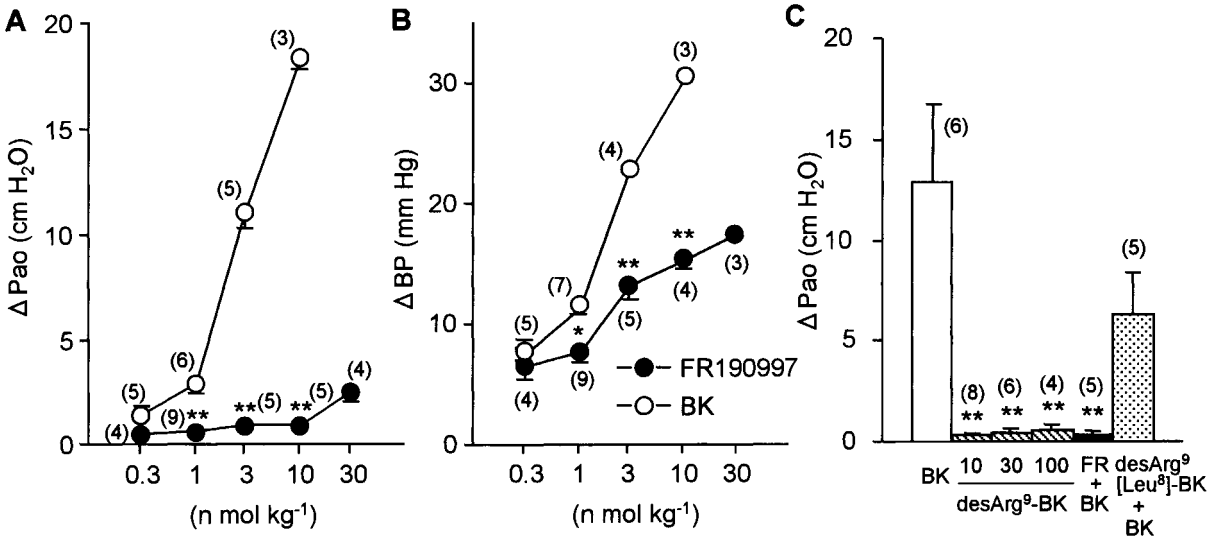

Figure 6 Effects of BK receptor agonists on airway opening pressure (A and C) and systemic mean arterial blood pressure (B) in guinea-pig. (A) ΔPao (airway opening pressure) represents the difference between the maximum response value and base-line value after the i.v. administration of FR190997 or BK at indicated doses. (B) ΔBP represents the maximum hypotensive response value of mean arterial pressure after i.v. administration of FR190997 or BK at indicated doses subtracted from the value before the injection. (C) BK (3 nmol kg^{-1}) or desArg^9-BK (10–100 nmol kg^{-1}) were given intravenously and ΔPao was measured. FR173657 (FR, 30 mg kg^{-1}) was administered orally 1 h before the injection of the agonists. DesArg^9[Leu^8]-BK was infused into the right femoral vein at a constant rate (10 nmol min^{-1}). Results are mean$\pm$s.e.mean with the number of observations indicated in parentheses, $^*P<0.05$, $^{**}P<0.01$ (ANOVA plus *post-hoc* Dunnett's test, *c.f.* BK-injected animals). Where no error bars are shown error lies within the dimensions of the symbol.

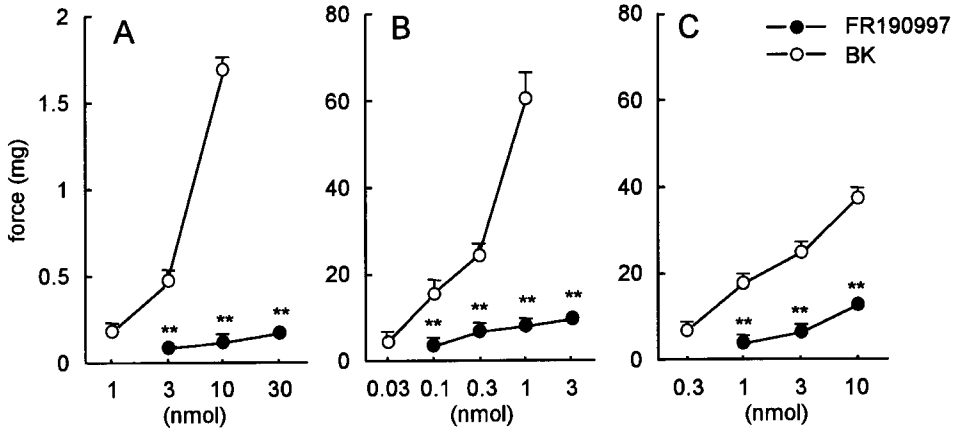

Figure 7 Effects of FR190997 and BK on the isolated tracheal strip from guinea-pig (A) and lung parenchymal sections of guinea-pig (B) and rat (C). Tracheal strip or lung parenchymal section was mounted in 5 ml-volume of siliconized glass organ bath under a resting tension. Changes in tension after the addition of indicated doses of FR190997 or BK into the organ bath were recorded isometrically *via* a force-displacement transducer. Results show contractile force (mg) and are mean ± s.e.mean, $n=5$, $^{**}P<0.01$, ANOVA plus *post-hoc* Dunnett's test, *c.f.* BK-injected animals. Where no error bars are shown error lies within the dimensions of the symbol.

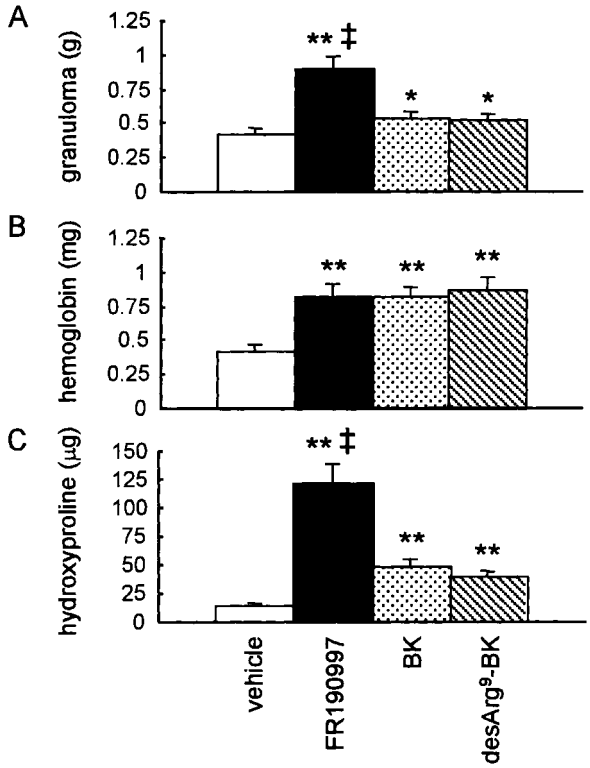

Figure 8 Granulation and angiogenesis induced by kinin receptor agonists sponge implants in mice. Seven days after implantation, the sponges with the surrounding granulation tissue were excised to measure the wet weight (A), the haemoglobin content (B) as an index of angiogenesis, and the hydroxyproline content (C) as an index of granulation. Each 30 n mol of FR190997, BK, or desArg^9-BK was spotted onto filtered paper, which was inserted into polyether sponge. Values are means ± s.e.mean of 5–6 experiments. $^{**}P<0.01$ vs vehicle group; $\ddagger P<0.01$ vs BK- or desArg^9-BK-treated group. ANOVA plus *post-hoc* Dunnett's test was used.

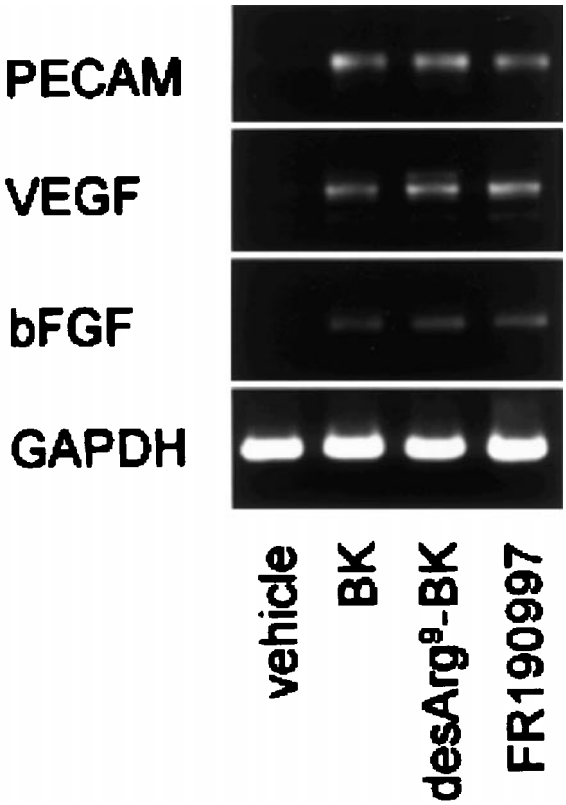

Figure 9 Expression of PECAM-1, VEGF, and bFGF mRNAs by the stimulation with kinin receptor agonists in sponge granulomas. The granulation tissue were excised 7 days after the implantation of sponge as described in Figure 8. Total RNA was extracted from the tissues. PCR was performed with reverse transcribed cDNA, the products were subjected to electrophoresis on 1.5% agarose gels and DNA subsequently visualized after staining with ethidium bromide. RT–PCR product sizes for PECAM-1, VEGF, bFGF, and GAPDH were 348, 724, 373 and 470 bp, respectively.

vitro. Another nonpeptide agonist, L-162,313, which bound to angiotensin AT_1 receptor stably expressed in COS-7 cells, has also been shown to have 50% of the maximal angiotensin II response to stimulate inositol phosphate accumulation (Perlman *et al*., 1995). Therefore it is possible that the interaction between FR190997 and the binding site of the B_2 receptor cannot completely alter the receptor into a fully active conformation leading to maximal responses. However, the vascular permeability increase by FR190997 was long-acting as was its hypotensive effect (Aramori *et al*., 1997a; Majima *et al*., 2000a). Captopril enhanced the vascular permeability increase by BK in the presence of SBTI, a plasma kallikrein inhibitor, but did not by FR190997. This difference between these agonists indicates that FR190997 is resistant to ACE (kininase II) due to its nonpeptidic structure, thus the effect *in vivo* might be long-acting. In the absence of SBTI, enhanced dye-leakage by FR190997 under treatment with captopril was seen. Initial stimulation by the vascular permeability induced by FR190997 may cause a subsequent activation of the plasma kallikrein-kinin system, resulting a further plasma extravasation.

The long-acting characteristics of FR190997 were also confirmed by investigation of paw oedema formation in mice. A significant increase in hindpaw volume lasted up to 3 h after intraplantar injection of FR190997 into foot pad. Such a long-lasting action cannot be explained by the direct effect of FR190997 alone. Since indomethacin or a cyclo-oxygenase (COX)-2 inhibitor, NS-398, significantly reduced the paw swelling induced by FR190997, a possible linkage of BK B_2 receptor to the production of COX-mediated metabolites could be also suggested in this *in vivo* model (Samadfam *et al*., 2000). Synergistic effects between FR190997 and PGs were seen in writhing responses in mice. Although i.p. injection of PGE_2 or beraprost sodium, a stable PGI_2 analogue, alone failed to cause the responses, simultaneous injection of the PGs with FR190997 greatly enhanced frequency of the reactions. This indicates a crucial interaction of the B_2 receptor with PGs in nociceptive responses to inflammatory stimuli (Poole *et al*., 1999). Potentiation of bradykinin-induced nociceptive response was reported, in that the reflex hypertensive response to BK was diminished by the treatment with indomethacin (Ferreira *et al*., 1973) and was enhanced dose dependently by PGI_2 rather than PGE_2 (Hori *et al*., 1986). Writhing response induced by acetic acid in prostacyclin receptor-deficient mice was reduced to the level of the indomethacin-pretreated wild-type of mice (Murata *et al*., 1997). The present data also shows greater potentiation of FR190997 as BK by a PGI_2 analogue than PGE_2, suggesting a predominant interaction between PGI_2 and BK B_2 receptor in pain response.

Although the experiments of oedema and writhing were carried out in a unanaesthetized mice, where affects of anaesthesia such as lowered blood pressure or analgesia are negligible, the results provided us with definitive *in vivo* information. FR190997 succeeded to show that signalling from BK B_2 receptor can truly activate COX-1 or COX-2 and can exhibit synergism with PGs *in vivo* as *in vitro*. In contrast, exogenous BK failed to reproduce the activation and the synergism because of its rapid degradation *in vivo*. This is a limitation for natural peptide. Concomitantly, this observation indicates pain induction or long-lasting oedema formation should be considerable as one of peripheral side effects of stable BK B_2 receptor agonists when they have developed therapeutic usefulness.

BK is known to be a potent bronchoconstrictor *in vivo* and to cause contraction of isolated trachea. BK receptor involved in airway microvascular leak and bronchoconstriction in guinea-pig was classified as a B_2 receptor (Ichinose & Barnes, 1990). A prominent difference of biological activity between FR190997 and BK was found in airway responses. FR190997 showed almost no increase in Pao after the i.v. injection in guinea-pig, although its hypotensive response was observed as reported previously (Aramori *et al*., 1997a). This failure to cause bronchoconstriction could be partly explained by the following results from *in vitro* experiments. In isolated trachea ring strips and lung parenchymal sections, FR190997 displayed a very weak contractile activity, suggesting less bronchoconstriction *in vivo*. Furthermore, it is unlikely that the lack of effect observed with FR190997 is due to the different animal species used, since significant contraction of those tissue preparations were not observed in both rats and guinea-pigs, and even in rats less increase in Pao at a dose of 30 nmol kg^{-1} of FR190997 was also seen (data not shown). In the present experiment, under non-inflammatory condition, the B_1 receptor was not seen to be involved, based on the fact that desArg^9-BK did not exhibit an increase in Pao and the B_1 receptor antagonist, desArg^9[Leu^8]-BK did not significantly antagonize the increase induced by BK. BK B_2 and B_1 receptor antagonists have been reported to be ineffective in trachea and lung of guinea-pig in [^{3}H]-BK binding experiments (Farmer *et al*., 1989). Taken together, this information with the present observation, trachea or pulmonary tissue in airway may contain a novel BK receptor other than B_1 and B_2, which may be involved in BK-induced bronchoconstriction. However, critical evaluation based on use of selective and stable antagonists and agonists for BK receptors has been pointed out (Regoli *et al*., 1998). Further studies for the elucidation will be required to identify a cDNA encoding a novel BK receptor in airway by molecular cloning. Assuming that both FR190997 and FR173657 specifically bind to the B_2 receptor, another mechanism or conformational state for the ligand to activate BK receptor, which will be specific in those tissues, would be also considerable.

BK has been also shown to promote cell proliferation and to induce activation of mitogen-activated protein kinase in some cell types *in vitro* (Drube & Liebmann, 2000). This information leads to a possibility in that kinin is involved in pathophysiological processes of granulation and angiogenesis *in vivo*. On the other hand, kinin B_1 receptor was suggested to mediate angiogenic effect (Hu & Fan, 1993). In the sponge implant model used here, topical applications of FR190997, BK and desArg^9-BK enhanced mass of granuloma, hydroxyproline and haemoglobin contents in the granulation tissues. This indicates that both kinin B_1 and B_2 may be implicated in formation of the granuloma and the neovascularization. Kinin has a potent vascular permeability, therefore it is easy to suppose that kinin promotes cell proliferation *via* the action by which growth factors could be supplied from leaked plasma around tissues to enhance cell migration and growth. However in the granuloma tissues, expressions of VEGF and bFGF, which have been characterized as consequential proliferative and angiogenic factors, increased by the

stimulation with kinin B_1 and B_2 agonists. Thus, one possible mechanism to promote neovascular and cell proliferative responses may be a direct induction of such growth factors by kinins *in vivo*. Since BK could be rapidly metabolized to desArg^9-BK *in vivo*, it cannot be specified whether its action is mediated by kinin B_1 or B_2 as long as BK is used to evaluate the B_2-mediated events. Therefore, this would emphasize that FR190997 can be effectively used to identify effects of the B_2 specifically without interaction with kinin B_1 receptor.

In conclusion, the nonpeptide mimic of BK, FR190997 was demonstrated to exert proinflammatory effects, such as plasma extravasation through the B_2 receptor, oedema formation, pain production, granulation and angiogenesis *in vivo*, although less effective on airway response. Thus, it can be expected that FR190997 would be a major advance for the elucidation of pathological and physiological roles of the kinin system, especially the B_2 receptor-mediated events by using as 'a stable tool' *in vivo*. Furthermore, exploration with FR190997 could support a potential use of BK B_2 receptor antagonist in pathophysiological condition.

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