British Journal of Pharmacology (1997) 120, 617–624

The identification of an orally active, nonpeptide bradykinin B_2 receptor antagonist, FR173657

[1]Masayuki Asano, Noriaki Inamura, Chie Hatori, Hiroe Sawai, Tatsujiro Fujiwara, Akira Katayama, *Hiroshi Kayakiri, *Shigeki Satoh, *Yoshito Abe, *Takayuki Inoue, *Yuki Sawada, Kunio Nakahara, *Teruo Oku & Masakuni Okuhara

Department of Pharmacology and *Department of Chemistry, Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-3, 5-chome, Tokodai, Tsukuba, Ibaraki 300–26, Japan.

1 An orally active, nonpeptide bradykinin (BK) B_2 receptor antagonist, FR173657 (E)-3-(6-acetamido-3-pyridyl)-N-[N-[2-4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl]phenyl]-N-methylaminocarbonylmethyl]acrylamide) has been identified.

2 This compound displaced [^{3}H]-BK binding to B_2 receptors present in guinea-pig ileum membranes with an IC_{50} of 5.6×10^{-10} M and in rat uterus with an IC_{50} of 1.5×10^{-9} M. It did not inhibit different specific radio-ligand binding to other receptor sites.

3 In human lung fibroblast IMR-90 cells, FR173657 displaced [^{3}H]-BK binding to B_2 receptors with an IC_{50} of 2.9×10^{-9} M and a K_i of 3.6×10^{-10} M, but did not reduce [^{3}H]-des-Arg^{10}-kallidin binding to B_1 receptors.

4 In guinea-pig isolated preparations, FR173657 antagonized BK-induced contractions with an IC_{50} of 7.9×10^{-9} M, but did not antagonize acetylcholine or histamine-induced contractions even at a concentration of 10^{-6} M. FR173657 caused parallel rightward shifts of the concentration-response curves to BK at concentrations of 10^{-9} M and 3.2×10^{-9} M, and a little depression of the maximal response in addition to the parallel rightward shift of the concentration-response curve at a concentration of 10^{-8} M. Analysis of the data yield a pA_2 of 9.2 ± 0.2 ($n=5$) and a slope of 1.5 ± 0.2 ($n=5$).

5 *In vivo*, the oral administration of FR173657 inhibited BK-induced bronchoconstriction dose-dependently in guinea-pigs with an ED_{50} of 0.075 mg kg^{-1}, but did not inhibit histamine-induced bronchoconstriction even at 1 mg kg^{-1}. FR173657 also inhibited carrageenin-induced paw oedema with an ED_{50} of 6.8 mg kg^{-1} 2 h after the carrageenin injection in rats.

6 These results show that FR173657 is a potent, selective, and orally active bradykinin B_2 receptor antagonist.

Keywords: Bradykinin; antagonist; B_2 receptor; nonpeptide; orally active; FR173657

Introduction

Bradykinin (BK), an endogenous nonapeptide produced by kallikrein, has various biological actions such as bronchoconstriction, plasma extravasation, release of prostaglandins/leukotrienes, smooth muscle contraction/relaxation and nociception (Burch *et al*., 1990; Bhoola *et al*., 1992). Therefore, BK has potentially important roles in inflammatory diseases such as asthma, rhinitis, arthritis and pancreatitis. The effects of BK are mediated through specific G-protein-coupled cell surface receptors (Burch & Axelrod, 1987). At least, two subtypes of BK receptor designated as B_1 and B_2 have been identified by molecular cloning and pharmacological means (Regoli & Barabé, 1980; Hess *et al*., 1992; Menke *et al*., 1994). Most biological actions of BK are thought to be mediated by the B_2 receptors.

To investigate the pathophysiological role of BK and to develop a drug for inflammatory diseases, many BK antagonists have been synthesized (Burch *et al*., 1990; Stewart, 1995). [D-Phe^7]-BK was shown to be one of the first BK antagonists (Vavrek & Stewart, 1985). Incorporation of β-(2-thienyl)-alanine residues at position 5 and 8 of [D-Phe^7]-BK converted a weak antagonist to a much more potent antagonist (Stewart, 1995). But these 'first-generation' BK antagonists had relatively low affinity for B_2 receptors compared to BK itself, and had a limited lifetime *in vivo*. Although the first-generation BK antagonists were useful for studying the involvement of BK in many pathophysiological processes, they did not have a good therapeutic potential *in vivo*.

Recently the 'second-generation' BK antagonists such as Hoe 140, CP-0127 and S 16118 have been described (Hock *et al*., 1991; Wirth *et al*., 1991; Cheronis *et al*., 1992; Félétou *et al*., 1995b). The second-generation BK antagonists have unusual amino acid residues or dimeric peptides in the structure. Therefore, they have much higher affinity for B_2 receptors and longer lifetimes *in vivo* than the first-generation BK antagonists. However, these antagonists are all peptide analogues and their therapeutic use is limited because of their poor oral bioavailability. Several nonpeptide B_2 antagonists have already been described (Salvino *et al*., 1993; Sawutz *et al*., 1994), but an orally active nonpeptide B_2 antagonist has not yet been identified.

The present study describes the identification of an orally active, nonpeptide B_2 receptor antagonist, FR173657, (E)-3-(6-acetamido-3-pyridyl)-N-[N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl]phenyl]-N-methylaminocarbonylmethyl]acrylamide (Figure 1), which was obtained by optimization of a lead compound discovered by random screening of Fujisawa's chemical library. To our knowledge, this is the first account of an orally active, nonpeptide B_2 receptor antagonist.

Methods

Receptor binding

Guinea-pig ileum The specific binding of [^{3}H]-BK (a high affinity B_2 ligand) was assayed according to a method previously described (Manning *et al*., 1986) with minor mod-

[1] Author for correspondence.

ifications. Male Hartley guinea-pigs (from Charles River Japan, Inc.) were killed by exsanguination under anaesthesia. The ilea were removed and homogenized in ice-cold buffer (50 mM sodium trimethylamino-ethanesulphonate (TES) and 1 mM 1,10-phenanthroline, pH 6.8) with Polytron. The homogenate was centrifuged to remove cellular debris ($1000 \times g$, 20 min, 4°C) and the supernatant was centrifuged ($100{,}000 \times g$, 60 min, 4°C). Then, the pellet was resuspended in ice-cold assay buffer (50 mM TES, 1 mM 1,10-phenanthroline, 140 μg ml^{-1} bacitracin, 1 mM dithiothreitol, 1 μM captopril and 0.1% bovine serum albumin (BSA), pH 6.8), and was stored at −80°C until use.

In the binding assay, membranes (0.2 mg protein ml^{-1}) were incubated with [^{3}H]-BK (final concentration 0.06 nM) and varying concentrations of test compounds (FR173657: 1×10^{-10} to 3.2×10^{-8} M, 6 concentrations; Hoe 140: 3.2×10^{-12} to 1×10^{-9} M, 6 concentrations: NPC 567: 3.2×10^{-9} to 1×10^{-6} M, 6 concentrations; des-Arg^9-[Leu^8]BK: 1×10^{-6} to 1×10^{-5} M, 2 concentrations) or unlabelled BK at room temperature for 60 min. Receptor-bound [^{3}H]-BK was harvested by filtration through Whatman GF/B glass fibre filters under reduced pressure and the filter was washed 5 times with 300 μl of ice-cold buffer (50 mM Tris-HCl). The radioactivity retained on the washed filter was measured with a liquid scintillation counter. Specific binding was calculated by subtracting the nonspecific binding (determined in the presence of 1 μM unlabelled BK) from total binding. The experiments were performed four times in duplicate.

Rat uterus Uteri from female Sprague-Dawley rats (from Japan SLC, Inc.) were removed immediately after the animals had been killed by exsanguination under anaesthesia. The uteri were homogenized and centrifuged by the same method as described above for guinea-pig ileum. Membrane preparations of rat uteri were used for binding assay with the same methods as those described above for guinea-pig ileum (FR173657: 1×10^{-11} to 1×10^{-7} M, 5 concentrations; Hoe 140: 1×10^{-12} to 1×10^{-8} M, 5 concentrations). The experiments were performed four times in duplicate.

Human fibroblast cells IMR-90, human foetal lung fibroblasts (obtained from the American Type Culture Collection) were grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing penicillin (100 μg ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% foetal bovine serum. The cells were cultured into 24-well tissue culture plates at the concentration of 10^5 cells per well before the assay. In B_1 assay, IMR-90 cells were treated with interleukin-1β (IL-1β) (1 ng ml^{-1}) for 6 h before assay to enhance B_1 receptor expression. The cells were washed twice with phosphate-buffered saline containing 0.1% BSA, then incubated with [^{3}H]-BK (final concentration 1 nM) for B_2 assay or [^{3}H]-des-Arg^{10}-kallidin (a high affinity B_1 ligand, final concentration, 1 nM) for B_1 assay and test compounds (for B_2 assay FR173657: 1×10^{-10} to 3.2×10^{-8} M, 6 concentrations; Hoe 140: 3.2×10^{-11} to 1×10^{-8} M, 6 concentrations; des-Arg^9-[Leu^8]BK: 1×10^{-6} to 1×10^{-5} M, 2 concentrations; for B_1 assay FR173657: 1×10^{-6} to 1×10^{-5} M, 2 concentrations; Hoe 140: 1×10^{-6} to 1×10^{-5} M 2 concentrations; des-Arg^9-[Leu^8]BK: 3.2×10^{-8} to 1×10^{-5} M, 6 concentrations) for 90 min at room temperature in 0.5 ml of assay buffer (20 mM HEPES, 125 mM N-methyl-D-glucamine, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM 1,10-phenanthroline, 1 mM dithiothreitol, 1 μM captopril and 0.1% BSA, pH 7.4). Non-specific binding was de-

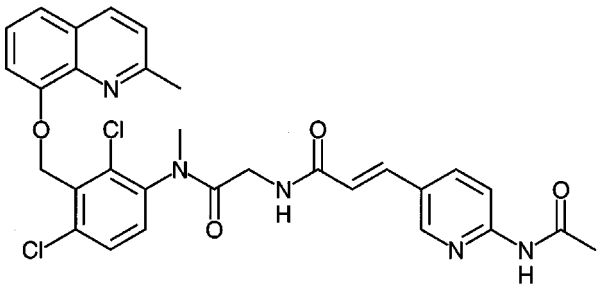

Figure 1 Structure of FR173657 ((E)-3-(6-acetamido-3-pyridyl)-N-[N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl]phenyl]-N-methylaminocarbonylmethyl]acrylamide).

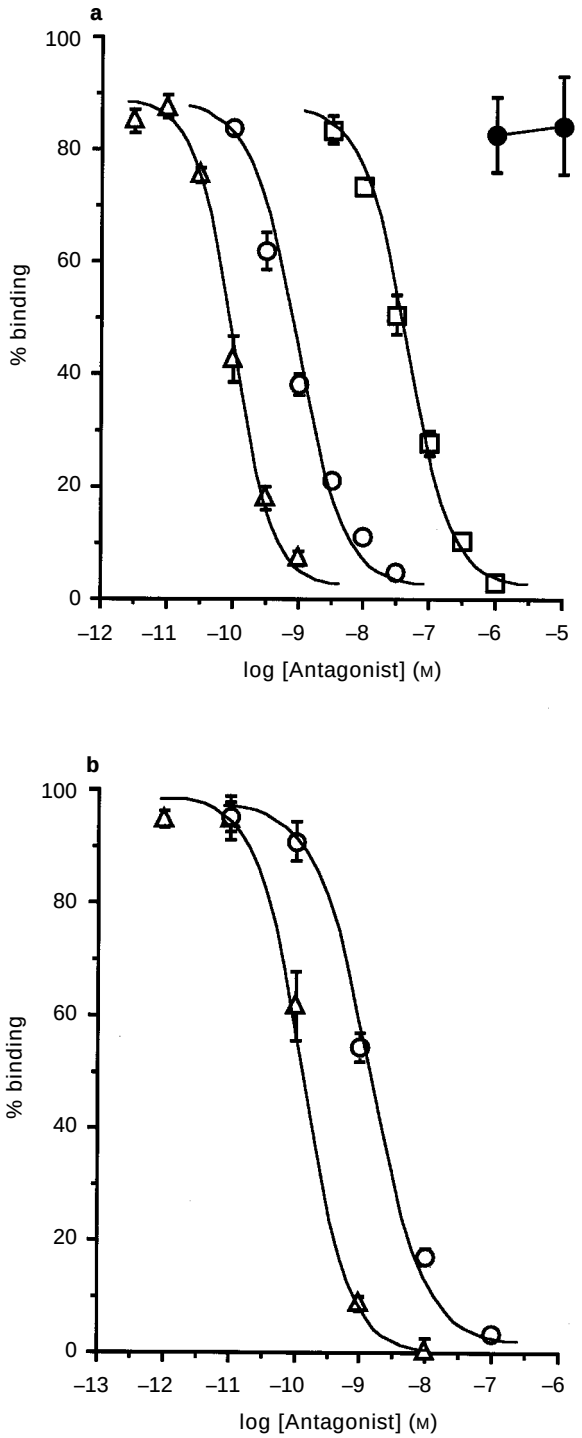

Figure 2 Effect of F173657 on [^{3}H]-BK binding to B_2 receptors in guinea-pig ileum membranes or rat uterus membrane. (a) Guinea-pig ileum membranes were incubated with [^{3}H]-BK and increasing concentrations of FR173657 (○), Hoe 140 (△), NPC 567 (□) or des-Arg^9-[Leu^8]BK (●). (b) Rat uterus membranes were incubated with [^{3}H]-BK and increasing concentrations of FR173657 (○) or Hoe 140 (△). Data are expressed as mean and vertical lines show s.e.mean ($n = 4$).

termined in the presence of 1 μM unlabelled BK or des-Arg^{10}-kallidin. At the end of the incubation, the buffer was aspirated and the cells were washed three times with phosphate-buffered saline containing 0.1% BSA. Bound radioactivity was determined by solubilizing with 1% sodium dodecyl sulphate containing 0.05 M NaOH and quantitating in a liquid scintillation counter. In Scatchard analysis, the concentration of the [^{3}H]-BK was varied from 0.03 to 1 nM. The experiments were performed four times in triplicate.

Other ligand binding assays The specific binding of [^{3}H]-5′-N-ethylcarboxamidoadenosine (adenosine, non-selective), [^{3}H]-prazosin (α_1-adrenoceptors, non-selective), [^{3}H]-quinuclidinylbenzilate (muscarinic, non-selective), [^{3}H]-pyrilamine (histamine, H_1), [^{125}I]-endothelin-1 (endothelin-1 ET_A), [^{3}H]-substance P (neurokinin, NK_1), [^{3}H]-leukotriene D_4 (leukotriene, cysLT_1) were assayed according to the methods of Bruns *et al*. (1986), Reader *et al*. (1987), Luthin & Wolfe (1984), Haaksma *et al*. (1990), Ambar *et al*. (1989), McLean *et al*. (1993) and Norman *et al*. (1990), respectively. The receptor sources of these binding assays are bovine striatal membranes, rat forebrain membranes, guinea-pig bladder membranes, bovine cerebellar membranes, A10 cells, rat submaxillary gland membranes and guinea-pig lung membranes, respectively. The final ligand concentrations were 4, 0.5, 0.2, 2, 0.06, 1.4 and 0.2 nM, respectively. Incubations were carried out at 25°C, in 50 mM Tris-HCl (pH 7.7) for 60 min, in 50 mM Tris-HCl (pH 7.7) for 60 min, in 50 mM Tris-HCl (pH 7.4) for 60 min, in 50 mM Na-KPO_4 (pH 7.5) for 30 min, in 50 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ for 90 min, in 20 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 30 mM KCl, 0.02% BSA, 0.1 mM thiorphan for 30 min and in 50 mM Tris-HCl (pH 7.7) for 60 min, respectively. Nonspecific binding was determined in the presence of 10 μM [^{3}H]-5′-N-ethylcarboxamidoadenosine, 1 μM prazosin, 1 μM atropine, 10 μM triprolidine, 100 nM endothelin-1, 1 μM substance P and 1 μM leukotriene D_4, respectively. The reactions were terminated by rapid vacuum filtration on glass fibre filters. Radioactivity trapped on the filters was determined and compared to control values. The experiments were performed twice.

Smooth muscle contraction in guinea-pig ileum

Guinea-pig ileum contraction by BK, acetylcholine (ACh) or histamine was measured by the method of Hock *et al*. (1991). Segments of ileum (1.5 cm) were isolated from male Hartley guinea-pigs (from Japan SLC, Inc.) and suspended in 25 ml organ baths containing Tyrode solution (composition in g l^{-1}. NaCl 8.0, KCl 0.2, $MgCl_2$ 0.1, $CaCl_2$ 0.2, $NaHCO_3$ 1.0, $NaHPO_4$ 0.05 and glucose 1.0), maintained at 37°C (for BK-induced contraction) or 27°C (for ACh- or histamine-induced contraction) and bubbled with 95% O_2, 5% CO_2. Tension was measured isometrically with force transducers and responses were recorded on a multi-channel polygraph recorder. Initial tension was set at 1.0 g (for BK-induced contraction) or 0.5 g (for ACh or histamine-induced) and after an equilibration period of about 30 min, a stable baseline tone was reached and two or three contractions were obtained to BK (6×10^{-8} M), ACh (1×10^{-6} M) or histamine (5×10^{-7} M). After the contraction, the isolated tissue was washed three times. Following a period of 10 min the segments had relaxed to original baseline levels and only segments exhibiting reproducible responses (100±15%) were used. The last control response was taken as 100% and subsequent responses to BK, ACh or histamine obtained in the presence of BK antagonists were expressed as a percentage of this. The segments were incubated with the BK antagonists for 10 min before BK, ACh or histamine was added.

A full Schild plot was performed in different strips from the same animal. The strips were contracted with BK in a cumulative manner, 9 concentrations between 10^{-9} M and 10^{-5} M being used. Responses to BK either in absence or presence of FR173657 were normalized towards the maximal

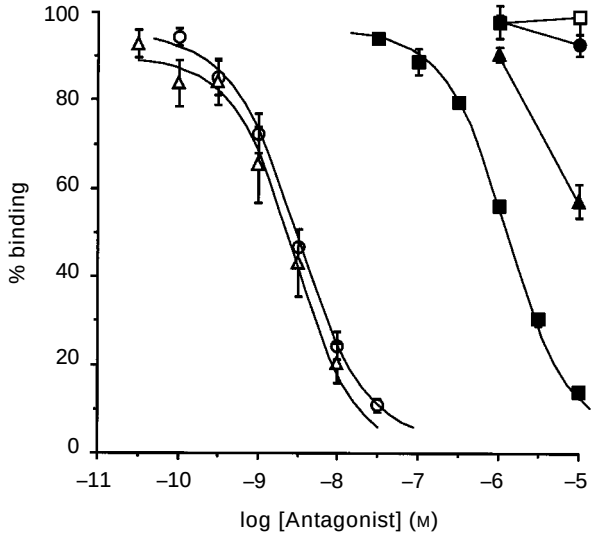

Figure 3 Effect of FR173657 on [^{3}H]-BK binding to B_2 receptors or [^{3}H]-des-Arg^{10}-kallidin binding to B_1 receptors in human IMR-90 cells. B_2: IMR-90 cells were incubated with [^{3}H]-BK and increasing concentrations of FR173657 (○), Hoe 140 (△) or des-Arg^9-[Leu^8]BK (□): B_1: IL-1β-treated IMR-90 cells were incubated with [^{3}H]-des-Arg^{10}-kallidin and increasing concentrations of FR173657 (●), Hoe 140 (▲) or des-Arg^9-[Leu^8]BK (■). Data are expressed as mean and vertical lines show s.e.mean ($n=4$).

effect of BK reached with the first curve. Different concentrations of FR173657 were applied 10 min before the BK-induced contraction was measured in its presence. A dose-ratio (DR) was calculated from the ED_{50} of the concentration-response curve in the presence of FR173657 divided by the ED_{50} for the individual concentration-response curve of bradykinin alone. The pA_2 and slope were calculated by Schild plot (Schild, 1947) and the mean values are quoted.

BK-induced bronchoconstriction in guinea-pigs

Male Hartley guinea-pigs weighing 470–750 g (from Charles River Japan, Inc.) were anaesthetized by intraperitoneal injection of sodium pentobarbitone (30 mg kg^{-1}), and the trachea, jugular vein, and oesophagus were cannulated. The animals were ventilated at a tidal volume of 10 ml kg^{-1} and at a frequency of 60 breaths min^{-1} through the tracheal cannula. To suppress spontaneous respiration, alcuronium chloride (0.5 mg kg^{-1}) was administered intravenously through the jugular vein cannula. Then, propranolol (10 mg kg^{-1}) was also administered subcutaneously and after 10 min, BK (5 μg kg^{-1}, dissolved in saline with 0.1% BSA) was administered intravenously through the cannula. Bronchoconstriction was measured by the modified Konzett and Rossler method as the peak increase of pulmonary insufflation pressure (PIP) (Asano *et al*., 1992). FR173657 suspended in 0.5% methylcellulose solution or vehicle was administered through the oesophageal cannula after the first BK-induced bronchoconstriction. After 30 min, BK was administered again and the bronchoconstriction was measured in the same manner. Zero % response was determined as PIP before the administration of BK and the 100% response was determined as the first BK-induced bronchoconstriction before drug administration. % response was calculated from following the formula: % response = $(\Delta PIP_{\text{ after drug}}/\Delta PIP_{\text{ before drug}})\times100$.

The measurement of histamine-induced bronchoconstriction was performed according to the method described above for BK-induced bronchoconstriction with minor modifications described below. Propranolol was not administered; histamine (5 μg kg^{-1}, dissolved in saline) was administered in-

travenously, and the administration of histamine was repeated several times of 30 min until reproducible bronchoconstriction (100±10%) was obtained. After the last histamine-induced bronchoconstriction, FR173657 was administered orally.

Carrageenin-induced paw oedema in rats

The carrageenin-induced paw oedema model was performed by the method previously described (Winter *et al*., 1962). Male Sprague-Dawley rats (8 weeks old, from Clea Japan, Inc.) deprived of food overnight were treated orally with FR173657, 15 min before carrageeninin was injected into the right hind paw. Paw volume was measured by water plethysmometer before and 1, 2, 3 and 4 h after injection of carrageenin. FR173657 was suspended in 0.5% methylcellulose solution and administered at a volume of 5 ml kg^{-1}. Carrageenin was made up 1% in saline. Each rat received 0.1 ml of the irritant. Plasma concentrations of FR173657 were measured by high performance liquid chromatography with uv spectroscopic detection after the extraction with ethyl acetate.

Materials

FR173657 and Hoe 140 were chemically synthesized in Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). BK, NPC 567, des-Arg^9-[Leu^8]BK, BSA, DMEM, penicillin, streptomycin, N-

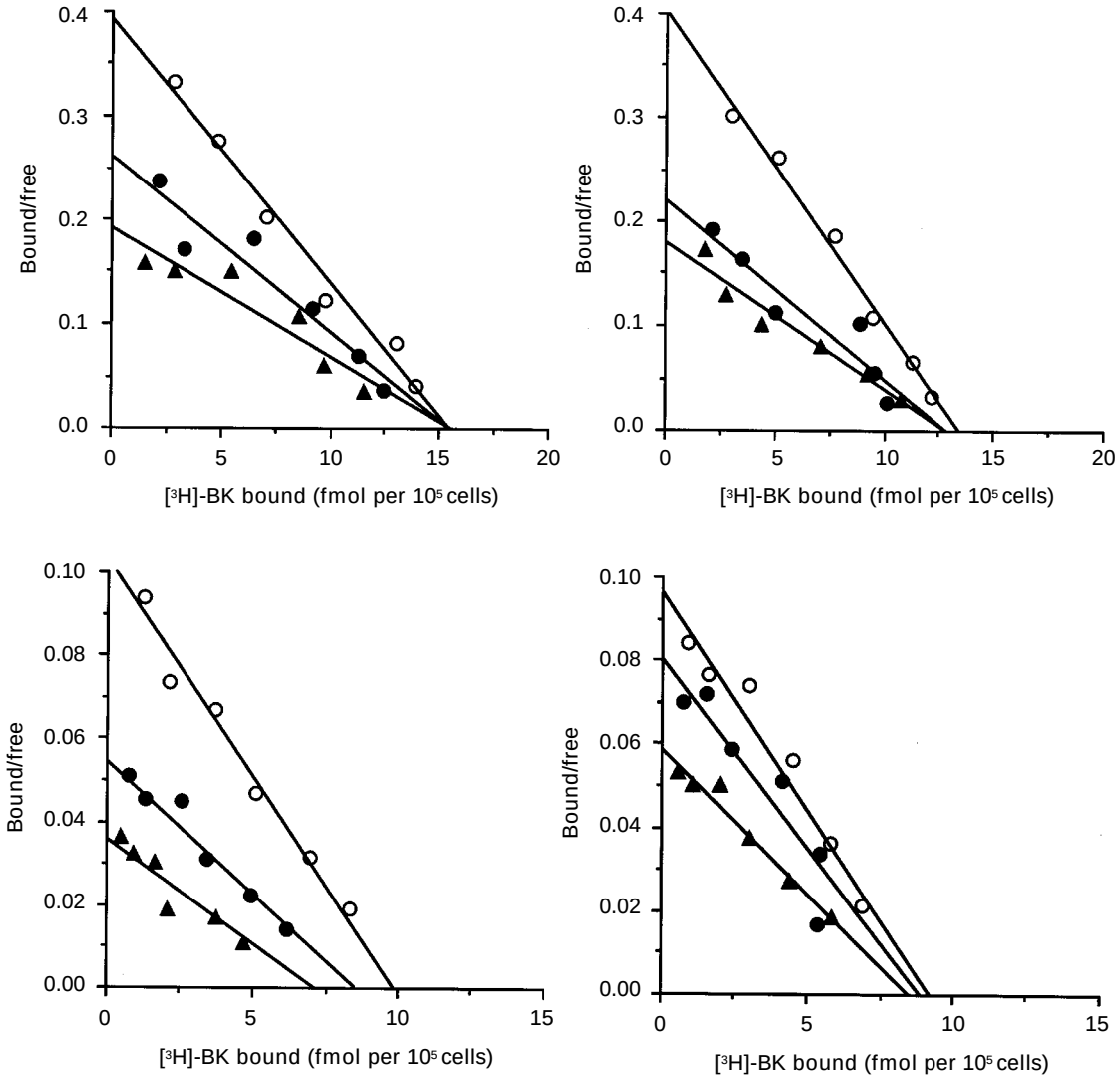

Figure 4 Effect of FR173657 on Scatchard analysis of specific [^{3}H]-BK binding to human B_2 receptors in IMR-90 cells. IMR-90 cells were incubated with varying concentrations of [^{3}H]-BK in the absence (○) or presence of 0.25 nM (●) or 0.5 nM (▲) FR173657. Each graph shows the data from each experiment.

Table 1 Effect of FR173657 on specific radio-ligand binding to B_1 receptors in man and B_2 receptors in guinea-pigs, rats and man

		IC_{50} (nM)			
Receptor binding		*FR173657*	*Hoe 140*	*NPC 567*	*Des-Arg^9-[Leu^8]BK*
Guinea-pig ileum	(B_2)	0.56±0.06	0.09±0.01	34±5	>10000
Rat uterus	(B_2)	1.5±0.3	0.16±0.03	NT	NT
Human	(B_2)	2.9±0.6	2.7±0.9	NT	>10000
Human	(B_1)	>10000	>10000	NT	1300±70

Data are expressed as mean±s.e.mean (n=4). NT: not tested.

methyl-D-glucamine, captopril, ACh, histamine, propranolol and carrageenin were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). [^{3}H]-BK and [^{3}H]-des-Arg^{10}-kallidin were purchased from Dupont/NEN Research Products (Wilmington, U.S.A.). Des-Arg^{10}-kallidin was purchased from Peninsula Laboratories, Inc. (Belmont, U.S.A.). Alcuronium chloride was purchased from Roche Japan, Inc. (Tokyo, Japan). All other compounds were purchased from Nacalai Teque, Inc. (Kyoto, Japan). *In vitro*, FR173657 was dissolved in dimethylsuphoxide and diluted with appropriate buffer.

Statistical analysis

The results are expressed as the mean ± s.e.mean, and statistical significance between groups was analysed by means of one way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. IC_{50} value was obtained by using the non-linear curve fitting methods with a specific computer programme made by our company's engineer. K_i was calculated by the method of Cheng and Prusoff.

Results

Receptor binding

FR173657, Hoe 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK, a potent second-generation B_2 antagonist) (Hock *et al*., 1991) and NPC 567 (D-Arg-[Hyp^3, D-Phe^7]BK, a first-generation B_2 antagonist) (Stewart, 1995) displaced [^{3}H]-BK binding to B_2 receptors in guinea-pig ileum membrane preparation, but des-Arg^9-[Leu^8]BK (a B_1 antagonist) (Regoli & Barabé, 1980) did not (Figure 2a). IC_{50}, K_i and Hill coefficient of FR173657 were 5.6×10^{-10} M, 1.1×10^{-10} M and 0.8, respectively. In rat uterus, FR173657 displaced [^{3}H]-BK binding to B_2 receptors with an IC_{50} of 1.5×10^{-9} M. Hoe 140 was also effective in this assay (Figure 2b).

FR173657 potently inhibited [^{3}H]-BK binding to B_2 receptors expressed in IMR-90 cells (Figure 3), but not [^{3}H]-des-Arg^{10}-kallidin binding to IL-1β induced B_1 receptors (Figure 3) in IMR-90 cells. IC_{50}, K_i and Hill coefficient of FR17367 were 2.9×10^{-9} M, 3.6×10^{-10} M and 0.9, respectively, in untreated IMR-90 cells (B_2 receptor binding). Hoe 140 inhibited B_2 binding, but not B_1 receptor binding (Figure 3). Des-Arg^9-[Leu^8]BK inhibited B_1 binding with an IC_{50} of 1.3×10^{-6} M, but not B_2 receptor binding (Figure 3). In our assay system, untreated IMR-90, human fibroblast cells expressed about 70,000 B_2 receptors per cell (Figure 4), and IMR-90 cells treated by IL-1β expressed about 40,000 B_1 receptors per cell (from Scatchard analysis). These results are consistent with data previously described (Menke *et al*., 1994). Scatchard analysis in the absence and presence of FR173657 showed a reduction of the slope, but no change in the intercept (Figure 4). FR173657 (0, 0.25, 0.5 nM) increased the K_d (140 ± 40, 190 ± 40, 240 ± 50 pM) without changing the B_{max} value

Table 2 Effect of FR173657 (10^{-6} and 10^{-5} M) on different specific radio-ligand binding assays

Receptor binding	*% inhibition* 10^{-6} M	10^{-5} M
Adenosine (non-selective)	−1.3	5.0
Adenoceptor (α_1, non-selective)	−1.8	1.8
Muscarinic (non-selective)	−3.5	17.7
Histamine (H_1)	10.4	7.9
Endothelin (ET_A)	1.5	−0.6
Neurokinin (NK_1)	−2.8	25.0
Leukotriene D_4 ($CysLT_1$)	2.4	5.6

Data are expressed as mean ($n=2$).

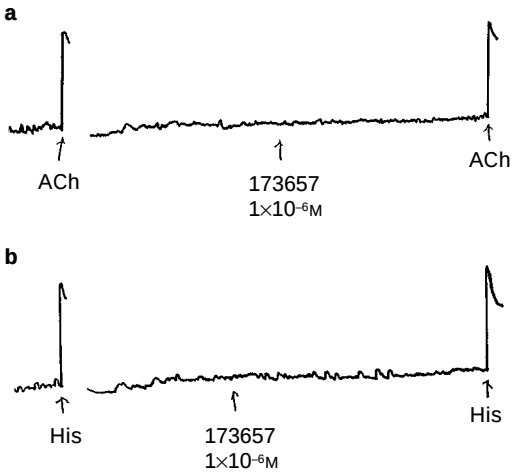

Figure 5 The typical traces of (a) acetylcholine (ACh, 1 μM) and (b) histamine (His, 0.5 μM) -induced contractions in the absence and presence of FR173657. Final concentration of FR173657 was 10^{-6}M.

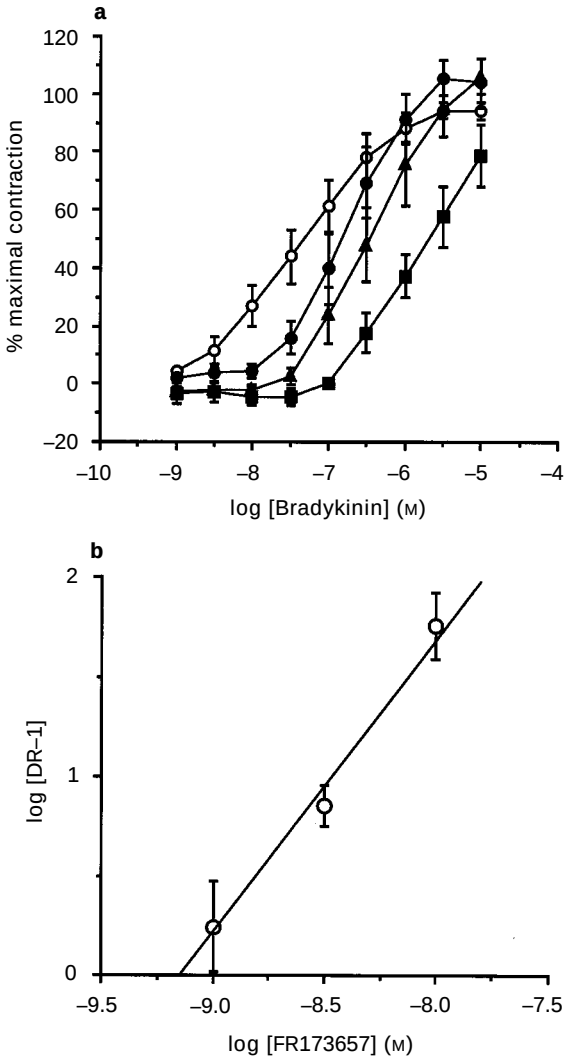

Figure 6 Effect of FR173657 (10^{-9}, 3.2×10^{-9} and 10^{-8} M) on concentration-contractile response curves to BK in guinea-pig isolated ileum and Schild analysis. (a) Dose-response curves; (○) control, (●) FR173657 1 nM, (▲) FR173657 3.2 nM, (■) FR173657 10 nM. (b) Schild analysis. Data are expressed as mean and vertical lines show s.e.mean ($n=5$). The data yielded a pA_2 value of 9.2 ± 0.2 ($n=5$) and a slope of 1.5 ± 0.2 ($n=5$).

(12.0 ± 1.5 fmol per 10^5 cells) (Figure 4). The IC_{50} values for all ligands from the B_1 and B_2 receptor binding experiments are shown in Table 1.

As shown in Table 2, FR173657 has almost no effect on different specific radio-ligand binding to other receptor sites.

Smooth muscle contraction in guinea-pig ileum

The *in vitro* functional activity of FR173657 was examined. In guinea-pig isolated ileum-preparations, it antagonized BK-induced contractions concentration-dependently with an IC_{50} of 7.9×10^{-9} M and had no agonistic effect ($n=4$). Hoe 140 also antagonized BK-induced contractions with an IC_{50} of 6.7×10^{-9} M ($n=4$). FR173657 did not inhibit ACh or histamine-induced guinea-pig ileum contractions even at a concentration of 10^{-6} M (% inhibition was -1.2 ± 3.4 or 3.0 ± 3.4, respectively, $n=3$); typical traces are shown in Figure 5.

FR173657 caused parallel rightward shifts of the concentration-response curves to BK at concentrations of 10^{-9} M and 3.2×10^{-9} M, but little depression of the maximal response in addition to the parallel rightward shift of the concentration-response curve at a concentration of 10^{-8} M (Figure 6a). There was no significant difference between control contractions to 10^{-5} M BK and those in the presence of 10^{-8} M FR173657. Analysis of the data gave a pA_2 value of 9.2 ± 0.2 ($n=5$) and a slope of 1.5 ± 0.2 ($n=5$) (Figure 6b). The slope was not significantly different from unity.

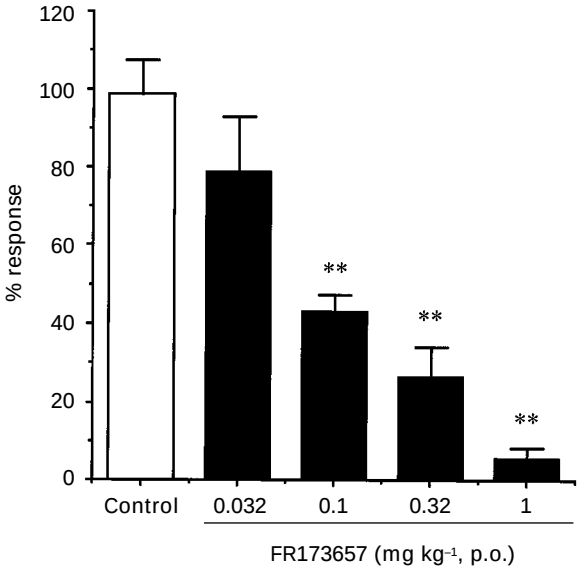

Figure 7 Inhibition of BK-induced bronchoconstriction by oral administration of FR173657 (0.032, 0.1, 0.32 and 1 mg kg^{-1}) in guinea-pigs. Data are expressed as mean and vertical lines show s.e.mean ($n=6$). Open column shows value in control animals. Solid columns show values in FR173657 treated animals. $^{**}P<0.01$ vs control (Dunnett's test).

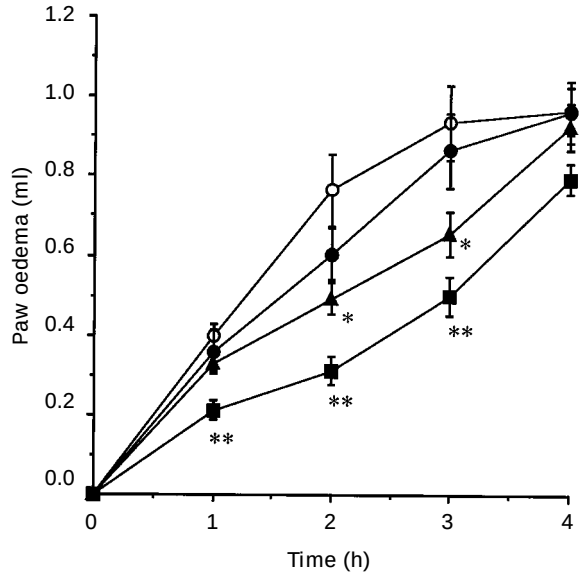

Figure 8 Inhibition of carrageenin-induced paw oedema by oral administration of FR173657 (1, 3.2 and 10 mg kg^{-1}) in rats. (○) Control, (●) FR173657 1 mg kg^{-1}, (▲) FR173657 3.2 mg kg^{-1}, (■) FR173657 10 mg kg^{-1}. Data are expressed as mean and vertical lines shown s.e.mean ($n=6$). $^{*}P<0.05$, $^{**}P<0.01$ vs control (Dunnett's test).

BK-induced bronchoconstriction

The effect of oral administration of FR173657 on BK-induced bronchoconstriction was examined *in vivo*. Exogenously administered BK (5 μg kg^{-1}, i.v.) induced an increase in PIP, indicating bronchoconstriction in guinea-pigs (29.7 ± 2.1 cmH_2O, $n=6$). At 30 min after the oral administration of vehicle, BK induced the same increase in PIP as the first BK-induced increase in PIP before the oral administration of vehicle. Oral administration of FR173657 inhibited the BK-induced increase in PIP dose-dependently with an ED_{50} of 0.075 mg kg^{-1} ($n=6$) (Figure 7), but did not inhibit histamine-induced increases in PIP even at 1 mg kg^{-1} (% inhibition was 6.2 ± 2.6, $n=5$). At 30 min after the oral administration of FR173657 (1 mg kg^{-1}), the plasma concentration was 0.6 μg ml^{-1} (10^{-6} M) and the oral bioavailability of FR173657 was 44%.

Carrageenin-induced paw oedema in rats

In the carrageenin-induced paw oedema in rats, the oral administration of FR173657 inhibited the paw swelling dose-dependently at 1, 2 and 3 h but did not at 4 h after the carrageenin injection (Figure 8). The ED_{50} of FR173657 was 6.8 mg kg^{-1} at the 2 h time point. In rats, the maximal plasma concentrations and the oral bioavailability of FR173657 were 0.12 μg ml^{-1} (2×10^{-7} M) and 7%, respectively, with a dose of 3.2 mg kg^{-1}.

Discussion

We have obtained an orally active nonpeptide B_2 antagonist FR173657 (a quinoline derivative) by optimization of a lead compound discovered by random screening. The present study demonstrates that FR173657 inhibits BK binding to B_2 receptors in guinea-pigs, rats and man, and that its oral administration inhibits not only the BK-induced bronchoconstrictive response in guinea-pigs, but also carrageenin-induced inflammatory responses in rats. Although FR173657 is less potent than Hoe 140 in inhibiting BK binding to B_2 receptors, it is potent enough to inhibit BK-induced response *in vitro* and *in vivo*. To our knowledge, this compound is the most potent B_2 antagonist of the nonpeptide compounds.

Human B_2 receptors are expressed in IMR-90, human fibroblast cells (Baenzinger *et al*., 1992; Sawutz *et al*., 1992), and B_1 receptors can be induced by IL-1β in these cells (Menke *et al*., 1994). We obtained similar data to those found in previous studies. FR173657 antagonized BK binding to B_2 receptors in IMR-90 cells. This compound showed similar antagonistic activity in WI-38 (human fibroblasts) and A431 (human epidermoid carcinoma) (data not shown) which express B_2 receptors (Roberts & Gullick, 1989; Jong *et al*., 1993). These results suggest that this compound may be clinically effective. In the B_1 receptor assay, FR173657 had little effect on the

binding of the B_1 radioactive ligand to human B_1 receptors even at a concentration of 10^{-5} M, suggesting FR173657 is B_2 selective. Furthermore, the data in Table 2 indicate that FR173657 has considerable selectivity. In Scatchard analysis, it increased the K_d value without changing the B_{max} value. This suggests that FR173657 may competitively inhibit [^{3}H]-BK binding B_2 receptors in human cells. Hoe 140 was much more potent than FR173657 in the guinea-pig and rat binding assay, but it had almost the same potency as FR173657 in the human binding assay. This may be due to species specificity or the different experimental methods used.

In guinea-pig isolated ileum, FR173657 inhibited BK-induced contractions at low concentrations, and showed no agonistic activity. It did not inhibit ACh or histamine-induced guinea-pig ileum contractions even at a concentration of 10^{-6} M. These results confirm that FR173657 is a potent and selective B_2 antagonist. Although FR173657 was less potent than Hoe 140 in inhibiting BK binding to B_2 receptors in guinea-pig ileum, it had the same potency as Hoe 140 in inhibiting BK-induced contractions of guinea-pig ileum. The reason for this discrepancy could be that FR173657 is more resistant to peptidases and more penetrative of the tissue than Hoe 140, because FR173657 is a nonpeptide and smaller molecular compound.

As shown in Figure 6, there are apparently parallel rightward shifts of concentration-response curves with no depression of the maximal response in the presence of FR173657. Our data indicate that this compound may be a competitive antagonist in guinea-pig ileum, albeit the slope of the Schild plot is larger than 1. Negative values were obtained for some BK concentrations (1–32 nM) in the presence of FR173657 (3.2–10 nM). This phenomenon suggests that there may be another subtype of BK receptor in guinea-pig ileum. BK may have caused a little relaxation of the ileum through this second subtype, but FR173657 may not have antagonized this response.

In anaesthetized guinea-pigs, FR173657 showed potent inhibitory activity against BK-induced bronchoconstriction when administered orally. This demonstrates that FR173657 is orally active and the plasma concentration of FR173657 (10^{-6} M) was sufficient to inhibit BK-induced response *in vitro*.

BK elicits contraction of tracheal smooth muscle in guinea-pigs *in vitro* (Bramley *et al*., 1990) as well as *in vivo* (Ichinose *et al*., 1990). BK induces the release of tachykinins (Saria *et al*., 1988) and histamine (Ishizaka *et al*., 1985) which cause bronchoconstriction and microvascular leakage (Barnes *et al*., 1988). In asthmatic patients, BK inhalation causes bronchoconstriction (Fuller *et al*., 1987) and kininogenase activity and immunoreactive kinins are increased in bronchoalveolar lavage fluid of asthmatic patients (Christiansen *et al*., 1987). From these findings, it has been proposed that BK is a pivotal mediator in asthma (Proud & Kaplan, 1988; Farmer, 1991). Therefore, it is speculated that BK antagonists may have therapeutic potential against asthma.

The oral administration of FR173657 significantly inhibited carrageenin-induced paw oedema in rats. It has already been shown that several BK antagonists reduce this inflammatory reaction (Costello & Hargreaves, 1989; Wirth *et al*., 1991; Félétou *et al*., 1995a). Our results confirm that BK plays an important role in this model. Inhibition of carrageenin-induced inflammation has been shown to be highly predictive of anti-inflammatory drug activity in human inflammatory diseases (Wirth *et al*., 1991). Furthermore, BK levels are increased in plasma of patients with rheumatoid arthritis (Hargreaves *et al*., 1988). These findings suggest that BK antagonists may also have therapeutic potential against rheumatoid arthritis and other inflammatory diseases. In the case of a chronic disease such as rheumatoid arthritis or asthma, oral activity is a prerequisite for FR173657 for patient's compliance with therapy.

Injection of BK or its application to a blister base causes pain in human volunteers (Armstrong *et al*., 1957; Whalley *et al*., 1987). BK also induces hyperalgesia to heat stimuli in human skin (Manning *et al*., 1991). Exogenous BK produces activation and sensitization of nociceptive neurones (Dray *et al*., 1988; Mizumura *et al*., 1990; Rang *et al*., 1991). In animal models, BK antagonists inhibit the pain and hyperalgesia induced by various irritant substances such as BK, carrageenin, kaolin, acetic acid and Freund's adjuvant (Steranka *et al*., 1988; Costello & Hargreaves, 1989; Heapy *et al*., 1993; Perkins *et al*., 1993). These findings suggest that BK antagonists (B_2 receptor antagonists) including FR173657 may be useful for the relief of pain. Compared with B_2 receptors, B_1 receptors do not seem to be involved in pain and hyperalgesia (Whalley *et al*., 1987; Mizumura *et al*., 1990), but it has recently been shown that the B_1 receptor is involved in chronic inflammatory hyperalgesia (Perkins *et al*., 1993; Perkins & Kelly, 1993). Potent B_1 receptor antagonists may therefore be useful for the relief of chronic pain.

In conclusion, this study shows that the nonpeptide B_2 receptor antagonist, FR173657, is potent, selective, and orally active. It seems that this compound may not only be a good tool for studying the pathophysiological role of BK but also a useful drug for inflammatory diseases.

References

AMBAR, I., KLOOG, Y., SCHVARTZ, I., HAZUM, E. & SOKOLOVSKY, M. (1989). Competitive interaction between endothelin and sarafotoxin: Binding and phosphoinositides hydrolysis in rat atria and brain. *Biochem. Biophs. Res. Commun*., **158,** 195–201.

ARMSTRONG, D., JEPSON, J.B., KEELE, C.A. & STEWART, J.M. (1957). Pain producing substance in human inflammatory exudates and plasma. *J. Physiol*., **135,** 350–370.

ASANO, M., INAMURA, N., NAKAHARA, K., NAGAYOSHI, A., ISONO, T., HAMADA, K., OKU, T., NOTSU, Y., KOHSAKA, M. & ONO, T. (1992). A 5-lipoxygenase inhibitor, FR110302, suppresses airway hyperresponsiveness and lung eosinophilia induced by sephadex particles in rats. *Agents Actions*, **36,** 215–221.

BAENZINGER, N.L., JONG, Y.J., YOCUM, S.A., DALEMAR, L.R., WILHELM, B., VAVREK, R., & STEWART, J.M. (1992). Diversity of B_2 bradykinin receptors with nanomolar affinity expressed in passaged IMR90 human lung fibroblasts. *Eur. J. Cell Biol*., **58,** 71–80.

BARNES, P.J., CHUNG, F. & PAGE, C.P. (1988). Inflammatory mediators and asthma. *Pharmacol. Rev*., **40,** 49–83.

BHOOLA, K.D., FIGUEROA, C.D. & WORTHY, K. (1992). Bioregulation of kinins: Kallikreins, kininogens, and kininases. *Pharmacol. Rev*., **44,** 1–80.

BRAMLEY, A.M., SAMHOUN, M.N. & PIPER, P.J. (1990). The role of the epithelium in modulating the responses of guinea-pig trachea induced by bradykinin *in vitro Br. J. Pharmacol*., **99,** 762–766.

BRUNS, R.F., LU, G.H. & PUGSLEY, T.A. (1986). Characterization of the A_2 adenosine receptor labeled by [^{3}H]-NECA in rat striatal membranes. *Mol. Pharmacol*., **29,** 331–346.

BURCH, R.M. & AXELROD, J. (1987). Dissociation of bradykinin-stimulated arachidonic acid release from inositol phosphate formation in Swiss 3T3 fibroblasts. Evidence for a G protein-coupled phospholipase A_2. *Proc. Natl. Acad. Sci. U.S.A*., **85,** 6374–6378.

BURCH, R.M., FARMER, S.G. & STERANKA, L.R. (1990). Bradykinin receptor antagonists. *Med. Res. Rev*., **10,** 237–269.

CHERONIS, J.C., WHALLEY, E.T., NGUYEN, K.T., EUBANKS, S.R., ALLEN, L.G., DUGGAN, M.J. LOY, S.D. BONHAM, K.A. & BLODGETT, J.K. (1992). A new class of bradykinin antagonists: synthesis and *in vitro* activity of bissuccinimidoalkane peptide dimers. *J. Med. Chem*., **35,** 1563–1572.

CHRISTIANSEN, S.C., PROUD, D. & COCHRANE, C.G. (1987). Detection of tissue kallikrein in bronchoalveolar lavage fluid of asthmatic subjects. *J. Clin. Invest*., **79,** 188–197.

COSTELLO, A.H. & HARGREAVES, K.M. (1989). Suppression of carrageenin-induced hyperalgesia, hyperthermia and oedema by a bradykinin antagonist. *Eur. J. Pharmacol.*, **171,** 259–263.

DRAY, A., BETTANEY, J., FORSTER, P. & PERKINS, M.N. (1988). Activation of a bradykinin receptor in peripheral nerve and spinal cord in the neonatal rat *in vitro*. *Br. J. Pharmacol.*, **95,** 1008–1010.

FARMER, S.G. (1991). Role of kinins in airway diseases. *Immunopharmacology*, **22,** 1–20.

FÉLÉTOU, M., LONCHAMPT, M., ROBINEAU, P., JAMONNEAU, I., THURIEAU, C., FAUCHERE, J.L., VILLA, P., GHEZZI, P., PROST, J.F. & CANET, E. (1995a). Effects of the bradykinin B_2 receptor antagonist S 16118 (p-guanidobenzoyl-[Hyp^3, Thi^5, D-Tic^7, Oic^8]bradykinin) in different in vivo animal models of inflammation. *J. Pharmacol. Exp. Ther.*, **273,** 1078–1084.

FÉLÉTOU, M., ROBINEAU, P., LONCHAMPT, M., BONNARDEL, E., THURIEAU, C., FAUCHERE, J.L., WIDDOWSON, P., MAHIEU, J.P., SERKIZ, B., VOLLAND, J.P., MARTIN, C., NALINE, E., ADVENIER, C., PROST, J.F. & CANET, E. (1995b). S 16118 (p-guanidobenzoyl-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin) is a potent and long-acting bradykinin B_2 receptor antagonist, *in vitro* and *in vivo*. *J. Pharmacol. Exp. Ther.*, **273,** 1071–1077.

FULLER, R.W., DIXON, C.M.S., CUSS, F.M.C. & BARNES, P.J. (1987). Bradykinin-induced bronchoconstriction in humans. *Am. Rev. Respir. Dis.*, **135,** 176–180.

HAAKSMA, E.E., LEURS, R. & TIMMERMAN, H. (1990). Histamine receptors: subclasses and specific ligands. *Pharmacol. Ther.,* **47,** 73–104.

HARGREAVES, K.M., TROULLOS, E.S., DIONNE, R.A., SCHMIDT, E.A., SCHAFER, S.C. & JORIS, J.L. (1988). Bradykinin is increased during acute and chronic inflammation: Therapeutic implications. *Clin. Pharmacol. Ther.*, **44,** 613–621.

HEAPY, C.G., SHAW, J.S. & FARMER, S.C. (1993). Differential sensitivity of antinociceptive assays to the bradykinin antagonist Hoe 140. *Br. J. Pharmacol.*, **108,** 209–213.

HESS, J.F., BORKOWSKI, J.A., YOUNG, G.S., STRADER, C.D. & RANSOM, R.W. (1992). Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. *Biochem. Biophys. Res. Commun.*, **184,** 260–268.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., HENKE, S., BREIPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.*, **102,** 769–773.

ICHINOSE, M., BELVISI, M.G. & BARNES, P.J. (1990). Bradykinin-induced bronchoconstriction in guinea pig *in vivo*: role of neural mechanims. *J. Pharmacol. Exp. Ther.*, **253,** 594–599.

ISHIZAKA, S.T., IWATA, M. & ISHIZAKA, K. (1985). Release of histamine and arachidonate from mouse mast cells induced by glycosylation-enhancing factor and bradykinin. *J. Immunol.*, **134,** 1880–1887.

JONG, Y.J., DALEMAR, L.R., WILHELM, B. & BAENZIGER, N.L. (1993). Human bradykinin B_2 receptors isolated by receptor-specific monoclonal antibodies are tyrosine phosphorylated. *Proc. Natl. Acad. Sci. U.S.A.*, **90,** 10994–10998.

LUTHIN, G.R. & WOLFE, B.B. (1984). Comparison of [^{3}H]Pirenzepine and [^{3}H]Quinuclidinylbenzilate binding to muscarinic cholinergic receptors in rat brain. *J. Pharmacol. Exp. Ther.*, **228,** 648–655.

MANNING, D.C., RAJA, S.N., MEYER, R.A. & CAMPBELL, J.N. (1991). Pain and hyperalgesia after intradermal injection of bradykinin in humans. *Clin. Pharmacol. Ther.*, **50,** 721–729.

MANNING, D.C., VAVREK, R., STEWART, J.M. & SYNDER, S.H. (1986). Two bradykinin binding sites with picomolar affinities. *J. Pharmacol. Exp. Ther.*, **237,** 504–512.

MCLEAN, S., GANONG, A., SEYMOUR, P.A., SNIDER, R.M., DESAI, M.C. & ROSEN, T. (1993). Pharmacology of CP-99,994; a nonpeptide antagonist of the tachykinin NK-1 receptor. *J. Pharmacol. Exp. Ther.*, **267,** 472–479.

MENKE, J.G., BORKOWSKI, J.A., BIERILO, K.K., MACNEIL, T., DERRICK, A.W., SCHNECK, K.A., RANSOM, R.W., STRADER, C.D., LINEMEYER, D.L. & HESS, J.F. (1994). Expression cloning of a human B_1 bradykinin receptor. *J. Biol. Chem.*, **269,** 21583–21586.

MIZUMURA, K., MINAGAWA, M., TSUJII, T. & KUMAZAWA, T. (1990). The effects of bradykinin agonists and antagonists on visceral polymodal receptor activities. *Pain*, **40,** 221–227.

NORMAN, P., ABRAM, T.S., CUTHBERT, N.J. & GARDINER, P.J. (1990). The inhibition of [^{3}H]Leukotriene D_4 binding to guinea-pig lung membranes. The correlation of binding affinity with activity on the guinea-pig ileum. *Eur. J. Pharmacol.*, **182,** 301–312.

PERKINS, M.N., CAMPBELL, E. & DRAY, A. (1993). Antinociceptive activity of the bradykinin B_1 and B_2 receptor antagonists, des-Arg^9, [Leu^8]-BK and HOE 140, in two models of persistent hyperalgesia in the rat. *Pain*, **53,** 191–197.

PERKINS, M.N. & KELLY, D. (1993). Induction of bradykinin B_1 receptors *in vivo* in a model of ultra-violet irradiation-induced thermal hyperalgesia in rat. *Br. J. Pharmacol.*, **110,** 1441–1444.

PROUD, D. & KAPLAN, A. (1988). Kinin formation: mechanisms and role in inflammatory disorders. *Ann. Rev. Immunol.*, **6,** 49–83.

RANG, H.P., BEVAN, S. & DRAY, A. (1991). Chemical activation of nociceptive peripheral neurons. *Br. Med. Bull.*, **47,** 534–548.

READER, T.A., BRIERE, R. & GRONDIN, L. (1987). Alpha$_1$ and alpha$_2$ adrenoceptor binding in cerebral cortex: Competition studies with [^{3}H]Prazosin and [^{3}H]Idazoxan. *J. Neural. Transm.*, **68,** 79–95.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related peptides. *Pharmacol. Rev.*, **32,** 1–46.

ROBERTS, R.A. & GULLICK, W.J. (1989). Bradykinin receptor number and sensitivity to ligand stimulation of mitogenesis is increased by expression of a mutant ras oncogene. *J. Cell Sci.*, **94,** 527–535.

SALVINO, J.M., SEOANE, P.R., DOUTY, B.D., AWAD, M.M., DOLLE, R.E., HOUCK, W.T., FAUNCE, D.M. & SAWUTZ, D.G. (1993). Design of potent non-peptide competitive antagonists of the human bradykinin B_2 receptor. *J. Med. Chem.*, **36,** 2583–2584.

SARIA, A., MARTLING, C.R., YAN, Z., THEODORSSON–NORHEIM, E., GAMSE, R. & LUNDBERG, J.M. (1988). Release of multiple tachykinins from capsaicin-sensitive sensory nerves in the lung by bradykinin, histamine, dimethylphenyl piperazinium, and vagal nerve stimulation. *Am. Rev. Respir. Dis.*, **137,** 1330–1335.

SAWUTZ, D.G., FAUNCE, D.M., HOUCK, W.T. & HAYCOCK, D. (1992). Characterization of bradykinin B_2 receptors on human IMR-90 lung fibroblasts: stimulation of $^{45}Ca^{2+}$ efflux by D-Phe substituted bradykinin analogues. *Eur. J. Pharmacol.*, **227,** 309–315.

SAWUTZ, D.G., SALVINO, J.M., DOLLE, R.E., CASIANO, F., WARD, S.J., HOUCK, W.T., FAUNCE, D.M., DOUTY, B.D., BAIZMAN, E., AWAD, M.M., MARCEAU, F. & SEOANE, P.R. (1994). The nonpeptide WIN 64338 is a bradykinin B_2 receptor antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 4693–4697.

SCHILD, H.O. (1947). pA_2, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol.*, **2,** 189–206.

STERANKA, L.R., MANNING, D.C., DE HAAS, C.J., FERKANY, J.W. & BOROSKY, S.A. (1988). Bradykinin as pain mediator: receptors are localized to sensory neurones, and antagonists have analgesic actions. *Proc. Natl. Acad. Sci. U.S.A.*, **85,** 3245–3249.

STEWART, J.M. (1995). Bradykinin antagonists: development and applications. *Biopolymers,* **37,** 143–155.

VAVREK, R. & STEWART, J.M. (1985). Competitive antagonists of bradykinin. *Peptides*, **6,** 161–164.

WHALLEY, E.T., CLEGG, S., STEWART, J.M. & VAVREK, R.J. (1987). The effect of kinin agonists and antagonists on the pain response of the human blister base. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **336,** 652–655.

WINTER, C.A., RISLEY, E.A. & NUSS, G.W. (1962). Carrageenin-induced oedema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc. Soc. Exp. Biol.*, **111,** 544–547.

WIRTH, K., HOCK, F.J., ALBUS, U., LINZ, W., ALPERMANN, H.G., ANAGNOSTOPOULOS, H., HENK, S., BREIPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vivo* studies. *Br. J. Pharmacol.*, **102,** 774–777.

(Received June 21, 1996
Revised October 2, 1996
Accepted November 4, 1996)