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Effects of the non-peptide B_2 antagonist FR173657 on kinin-induced smooth muscle contraction and relaxation, vasoconstriction and prostaglandin release

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1 The non-peptide bradykinin (BK) antagonist (E)-3-(6-acetamido-3-pyridyl)-N-[N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl]phenyl]-N-methylaminocarbonylmethyl]acrylamide (FR173657) was tested in intestinal, uterine, tracheal and vascular *in vitro* preparations. The investigation aimed at determining the antagonistic potency, duration of action, specificity for BK receptors and apparent mode of antagonistic action of FR173657.

2 Contractions of the isolated ileum of the guinea-pig in response to BK were inhibited by FR173657 (10–300 nM) in a concentration-dependent manner. The inhibition lasted for up to 90 min after wash-out of FR173657. Cumulative concentration-response curves to BK were shifted to the right with a concomitant decrease in the maximum effect. A pK_B value of 8.7 was determined. FR173657 had no effect on contractions induced by acetylcholine, histamine, 5-hydroxytryptamine, substance P, angiotensin II or caerulein.

3 The concentration-response curves for B_2 receptor-mediated relaxations of the rat isolated duodenum induced by BK were shifted to the right together with a concomitant reduction of the maximum BK effect in the presence of FR173657 (10–300 nM). A pK_B of 9.0 ± 0.2 was calculated. FR173657 had no effect on B_1 receptor-mediated relaxations in response to des-Arg^9-BK.

4 The concentration-response curves for BK-induced contractions of the rat isolated uterus were shifted to the right by FR173657 (3–300 nM) in a concentration-dependent and parallel manner. The Schild plot for the inhibition caused by FR173657 had a slope of -0.98 indicating a competitive mode of antagonism. A pA_2 value of 9.1 was determined.

5 Contractions of the circular smooth muscles of the guinea-pig isolated trachea in response to BK were concentration-dependently inhibited by FR173657 (10–100 nM). An affinity estimate of 9.3 was calculated for FR173657. Contractions induced by acetylcholine and relaxations in response to isoprenaline remained completely unaffected by FR173657.

6 In the rabbit isolated perfused ear, BK (0.01–10 nmol) produced a dose-dependent vasoconstriction. In the presence of 30 nM FR173657, the effects of BK were reduced by at least 60%, while FR173657 completely abolished the effects of all BK doses at 300 nM. FR173657 did not affect vasoconstriction induced by noradrenaline or angiotensin II.

7 The arterial injection of BK (10 nmol) into the rabbit isolated perfused ear caused an approximately three fold increase in the release of the prostaglandins E_2 and I_2 into the venous effluent. The BK-stimulated prostaglandin release was completely abolished in the presence of FR173657 (300 nM) while the basal prostaglandin release was unchanged.

8 In summary, FR173657 was shown to be a highly potent and selective BK antagonist which was active on B_2, but not B_1, receptors. FR173657 was a competitive antagonist in the rat uterus but showed a deviation from competitive inhibition in the other preparations studied similar to other second generation peptide antagonists. The inhibitory action *in vitro* was long-lasting, but was fully reversible.

Keywords: Bradykinin antagonists; FR173657; bradykinin receptors; smooth muscle contraction; smooth muscle relaxation; vasoconstriction; prostaglandin release

Introduction

Bradykinin (BK) and related peptides are believed to be key mediators of inflammatory diseases since the actions of kinins comprise all classical signs of inflammation including pain. Following the discovery of two receptor subtypes (Regoli & Barabé, 1980) much effort has been concentrated on the production of antagonists for the B_2 receptor. The first B_2 antagonists of sufficient potency (Stewart & Vavrek, 1986) suffered from a very short duration of action *in vivo* (Griesbacher *et al*., 1989). Later peptide B_2 antagonists exhibited a prolonged duration both *in vitro* and *in vivo* (Lembeck *et al*., 1991; Hock *et al*., 1991; Wirth *et al*., 1991). The most potent of these, D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK (Hoe 140, now named icatibant), has already been investigated in initial clinical trials (see Wirth *et al*., 1995). Nevertheless, non-peptide ligands for BK receptors are sought in order to avoid restrictions due to the necessity of parenteral administration. One of the first non-peptide ligands for B_2 receptors, WIN 64338, (Salvino *et al*., 1993) has proved to be a potent B_2 antagonist (Sawutz *et al*., 1994). However, unspecific effects of this compound in certain preparations (Sawutz *et al*., 1994; Wirth *et al*.,

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1994) also highlight the importance of thorough pharmacological investigations of novel compounds. Thus, the *in vitro* effects of FR173657, a recently disclosed non-peptide BK antagonist (Inamura *et al*., 1996), were investigated in intestinal, uterine, airway and vascular preparations. The potency, duration of action, specificity for BK receptors, selectivity for receptor subtypes and apparent mode of antagonistic action of FR17365 were investigated. The *in vivo* actions of FR173657 have been published separately (Griesbacher & Legat, 1997).

Methods

In vitro *preparations*

Guinea-pig ileum Pieces of ileum (about 1.5 cm) from guinea-pigs of either sex (Forschungsinstitut für Versuchstierzucht, Himberg, Austria) were taken starting at least 5 cm proximal to the ileocaecal valve. The tissues were suspended in HEPES-buffered salt solution maintained at 37°C and supplied with 100% O_2. Isotonic contractions were recorded under a resting tension of 20 mN. BK (30 nM) was added to the organ bath for periods of 1 min at regular intervals of 5 min. When the contractile responses of the tissues were stable, FR173657 (3, 10, 30, 100 or 300 nM) was applied to the organ bath 4 min before the next challenge with BK. After the antagonist had been removed together with BK 5 min later, the challenges with BK were continued for at least 30 min or until the responses to BK had returned to values obtained before FR173657. In order to exclude unspecific inhibitory effects, FR173657 (100 nM) was also tested against the contractile effects of caerulein (20 nM), substance P (20 nM), angiotensin II (2 nM), acetylcholine (20 nM), histamine (200 nM) and 5-hydroxytryptamine (200 nM). Acetylcholine and angiotensin II were tested at intervals of 10 min; the intervals for all other agonists were 5 min.

For the construction of concentration-response curves for BK, the ileum was prepared as described above. After an equilibrium period of 15 min, BK was applied to the organ bath in a cumulative manner to obtain concentrations from 1 nM to 9 μM within about 10 min. A further resting period of 30 min was allowed during which the bath solution was replaced with fresh solution 4–6 times. FR173657 (10, 30, 100 or 300 nM) was added to the organ bath 5 min before the construction of a second BK concentration-response curve. In control tissues, FR173657 was replaced by its vehicle, dimethylsulphoxide (DMSO), at a final concentration of 0.03% (v/v). All effects of BK are expressed as % of the maximum effect established during the first concentration-response curve (see Griesbacher & Lembeck, 1992).

Rat duodenum The proximal part of the duodenum of female Sprague-Dawley rats (Forschungsinstitut für Versuchstierzucht, Himberg, Austria) was excised and suspended under a resting tension of 10 mN in HEPES-buffered saline, gassed with pure oxygen at 34°C. After an equilibrium period of 30 min, concentration-response curves to BK (100 pM–1 μM applied in a non-cumulative fashion) were established under control conditions as well as in the presence of FR173657 (10–300 nM). All concentrations of FR173657 were tested, in randomized order, in each individual duodenum preparation. Between the concentration-response curves equilibrium periods of 30–90 min were allowed which included at least 6–7 thorough washing procedures. Isotonic relaxations in response to BK were quantified as % of the maximum BK-induced relaxation obtained under control conditions. In separate tissue preparations, relaxations in response to des-Arg^9-BK (1 nM–1 μM) were obtained after an initial equilibrium period of 2–3 h in the absence and presence of FR173657 (300 nM). The relaxations are expressed as % of the maximum relaxation obtained with 1 μM BK at the beginning of the experiment.

Rat uterus Female Sprague-Dawley rats (230–290 g) were pretreated with diesthylstilbestrol (100 μg kg^{-1}, s.c., dissolved in 1 ml kg^{-1} DMSO) 16 h before the experiment. The distal parts (about 1 cm) of both uterine horns were removed and suspended in De Jalón solution at 34°C. A resting tension of 5 mN was applied to the tissues. Isometric contractions following a 1 min application of BK (10 pM–10 μM) were measured at intervals of 10 min. In each tissue, two concentration-response curves to BK were established, at an interval of 45 min, under control conditions and in the presence of FR173657 (3, 10, 30, 100 or 300 nM). The antagonist was added to the organ bath 5 min before the challenges with BK.

Guinea-pig trachea The tracheae of guinea-pigs of either sex were excised and carefully cleared of the surrounding connective tissue. The epithelial layer was removed by gently rubbing the inside of the trachea with a cotton applicator before the trachea was divided transversally into two pieces of approximately 1 cm. Each piece was incised 3 times, alternately from the left and right side, in order to form a chain of 4 tracheal rings. Each preparation was set up in an organ bath in modified Krebs solution containing mepyramine (1 μM) and was gassed with 95% O_2 and 5% CO_2. Isometric contractions of the tissues were recorded under a resting tension of 12 mN. At the beginning of the experiment, tetrodotoxin (1 μM) was applied to the tissues for 5 min. After an equilibrium period of 15 min, a maximum contraction of the tissues was obtained with acetylcholine (30–100 μM). Following a further resting period of 30 min a concentration-response curve to BK (1 nM–10 μM) was established in the presence of FR173657 (10, 30 or 100 nM) or its solvent (dilute DMSO, final concentration 0.03% v/v). Each concentration of BK was applied to the tissue individually for a period of 2 min. After the removal of BK from the organ bath an interval of 10 min was allowed before the next concentration of BK was tested. Since it was not possible to obtain two comparable concentration-response curves for BK in one tissue due to apparent tachyphylaxis, a separate set of at least 6 preparations was used for each concentration of FR173657 as well as for the solvent control. FR173657 was added to the organ bath 5 min before the subsequent challenge with BK; the washing medium used to remove BK from the organ bath did not contain the antagonist. As a test for the specificity of FR173657, the contractile effects of acetylcholine (1–30 μM) were measured either in the absence or presence of FR173657 (100 nM). Similarly, relaxant effects of isoprenaline (3–30 μM, given in a cumulative fashion) on preparation precontracted with acetylcholine (10 μM) were measured in control tissues and in tissues incubated with FR173657 (100 nM).

Vasoconstriction in the rabbit perfused ear Rabbits of either sex (2.3–3.5 kg body weight; Department of Animal Biology, Graz) were killed by an i.v. injection of an overdose of pentobarbitone sodium. The ears were separated from the body and perfused under constant pressure via the central artery with Tyrode solution (37°C, gassed with 5% CO_2 in O_2) at a flow of 3 ml min^{-1} (Juan & Sametz, 1986). The venous effluent was monitored by means of an electronic drop recorder. Noradrenaline (60 pmol) was injected into the inflow cannula at intervals of 10 min until the reactivity of the preparation had become stable. Dose-response curves for BK (0.03–10.0 nmol), angiotensin II (10–1000 pmol) and noradrenaline (10–1000 pmol) were established in the presence of FR173657 (30 and 300 nM) or of DMSO (final concentration 0.03% v/v). All agonists were injected at intervals of 10 min.

Prostaglandin release from the rabbit ear The ears of rabbits were isolated as described above and perfused at 3 ml min^{-1} for 60 min in a recycling system with Tyrode solution containing 5.55×10^4 Bq [1-^{14}C]-arachidonic acid (Juan & Sametz, 1983). Following a washout-period of 120 min by perfusion with normal Tyrode solution, the ears were perfused for a further 60 min with Tyrode solution containing 0.5 g l^{-1} fatty acid-free bovine serum albumin in order to inhibit the re-incorporation of released [^{14}C]-AA. BK

(10 nmol) was injected into the arterial inflow of the perfusion to stimulate prostaglandin synthesis. The effluent from the ear was collected for 20 min (60 ml) before and after the injection of BK. Prostaglandins were extracted, separated by thin-layer chromatography (t.l.c.) and quantified by scintillation counting at ^{14}C (in Bq) in the respective t.l.c. fractions (Juan & Sametz, 1983).

Solutions

FR173657 ((E)-3-(6-acetamido-3-pyridyl)-N-[N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl]phenyl]-N-methylaminocarbonylmethyl]acrylamide) was dissolved in DMSO and further diluted with a 154 nM solution of NaCl. All solutions were prepared freshly on the day of the experiments. Stock solutions of angiotensin II (Calbiochem, La Jolla, CA, U.S.A.), BK and caerulein (Sigma Chem. Co., St. Louis, MO, U.S.A.) were prepared in a 154 nM solution of NaCl at a concentration of 1 mM. Substance P (Sigma) was dissolved in 0.01 M acetic acid, further dilutions were made in 154 mM NaCl. The stock solutions were stored at $-20^{\circ}C$ and diluted as needed with phosphate-buffered saline before the experiments. Acetylcholine, histamine, 5-hydroxytryptamine, diethylstilbestrol and tetrodotoxin were purchased from Sigma. HEPES-buffered salt solution (mM): NaCl 140.0, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, HEPES 5.0, D-glucose 10.1. De Jalón solution (mM): NaCl 154.0, KCl 5.6, $CaCl_2$ 0.54, $MgCl_2$ 0.05, $NaHCO_3$ 6.0, D-glucose 3.0. Modified Krebs solution (mM): NaCl 118.0, KCl 4.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.0, D-glucose 10.1. Tyrode solution (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.2, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, D-glucose 5.6. Phosphate-buffered saline (mM): NaCl 136.9, KCl 2.7, KH_2PO_4 1.5, Na_2HPO_4 7.7. All salts were of analytical grade and were obtained from Merck (Darmstadt, Germany). Further substances were: DMSO (Merck), HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]) and bovine serum albumin (Sigma). [1-^{14}C]-arachidonic acid (2.04×10^9 Bq $mmol^{-1}$; MedPro, Vienna, Austria).

Data analysis

Values for pK_B as a measure of antagonist affinity were estimated according to the method of Kenakin (1993). Equieffective concentrations of BK in the absence ([A]) and presence ([A′]) of a concentration ([B]) of FR173657 were calculated by linear regression analysis of the BK concentration-response curves followiong logit-log transformation for linearization. The slope (b) of a plot of 1/[A] versus 1/[A′] was used to calculate pK_B by the equation $pK_B = -\log_{10}([B]/(b-1))$. In the rat duodenum, individual pK_B values were calculated in each tissue preparation; the final value of pK_B is given as mean ± s.e.mean. In the guinea-pig ileum and trachea preparations, [A] and [A′] had to be calculated from separate groups of tissues; thus only one estimate of pK_B could be calculated and is given with 95% confidence intervals. Original data for icatibant-induced inhibition of BK in the rat duodenum (Griesbacher, 1992) have been re-analysed accordingly. In the rat uterus, the linear regression lines obtained after logit-log transformation of the concentration-response curves for BK were tested for deviation from parallelarity (Geigy, 1980). The horizontal distances of the concentration-response curves were used to construct a Schild plot (Arunlakshana & Schild, 1959). The pA_2 value as an estimate for the antagonist's affinity in this tissue is given with the 95% confidence interval derived from the linear regression analysis.

Comparisons between different treatment groups were made by non-parametric multiple comparisons for independent data; comparisons of effects in the presence or after the addition of FR173657 to values obtained before were made by non-parametric multiple comparisons for dependent data (Zar, 1984). All values presented in the figures are arithmetical means with s.e.mean; where no s.e.mean is indicated it was smaller than the symbol.

Results

Isolated ileum of the guinea-pig

Addition of 30 nM BK to isolated preparations of the guinea-pig ileum resulted in contractile responses which amounted to 30–60% of the maximum BK-induced contraction. No sign of tachyplaxis was observed when BK was applied at intervals of 5 min and the challenges with BK could be repeated regularly for several hours.

The addition of FR173657 to the organ bath resulted in a concentration-dependent reduction of the contractile effects of BK (Figure 1). While the lowest concentration of FR173657 (3 nM, not shown in the figure) did not produce any inhibitory effect, significant reductions were obtained with concentrations of 10 nM and above. Complete inhibition of the BK-induced contractions was obtained with 300 nM FR173657. After a thorough washing procedure of the organ bath for the removal of FR173657 from the bath solution the responses to BK remained reduced for periods dependent on the concentration of FR173657 that had been applied. While the BK effects returned to pre-antagonist values 20 min after the lowest active concentration of FR173657 (10 nM), the effects of BK remained significantly inhibited for at least 45 min after the highest concentration (300 nM), and at least 90 min were required to obtain responses to BK that were comparable to those observed before the addition of this concentration of FR173657.

The contractile effects in response to the peptides caerulein (20 nM), substance P (20 nM) and angiotensin II (2 nM), as well as the contractions induced by acetylcholine (20 nM) and the biogenic amines, histamine (200 nM) and 5-hydroxytryptamine (200 nM), were completely unaffected by FR173657 (100 nM) (data not shown). In order to exclude unspecific paralytic effects of the solvent, dilute DMSO was added to the organ bath to yield a final concentration of 0.03% (v/v) which corresponds to that produced by the addition of the highest concentration of FR173657. The effects of BK were completely unaffected by this concentration of DMSO.

When concentration-response curves for BK were established in the absence or presence of FR173657 (30–300 nM), the effects of BK were inhibited in a concentration-dependent

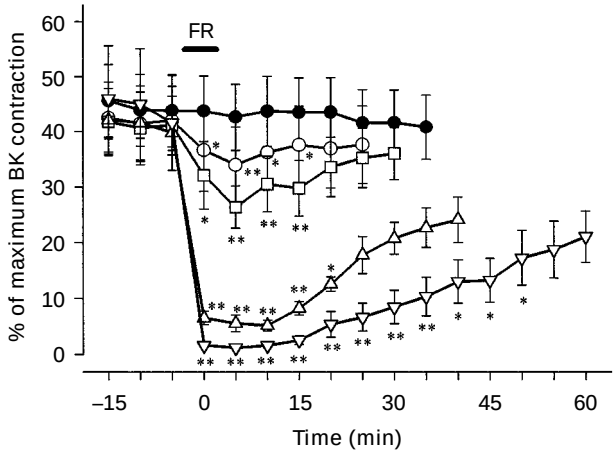

Figure 1 Time-course of FR173657-induced inhibition of contractile responses to bradykinin (BK) on the isolated ileum of the guinea-pig. BK (30 nM) was applied at regular intervals of 5 min and left in contact with the tissues for 1 min. During the time indicated by the horizontal line, FR173657 (FR) was present in the organ bath at concentrations 10 nM (○), 30 nM (□), 100 nM (△) or 300 nM (▽); control tissues (●) were treated with the solvent, DMSO (final concentration 0.03% v/v). BK-induced contractions are expressed as % of the maximum contraction induced by 10 μM BK at the beginning of the experiment. Significance of difference from contractions before FR173657: $*P<0.05$, $**P<0.001$. Means are shown and vertical lines indicate s.e.mean; $n=4-8$.

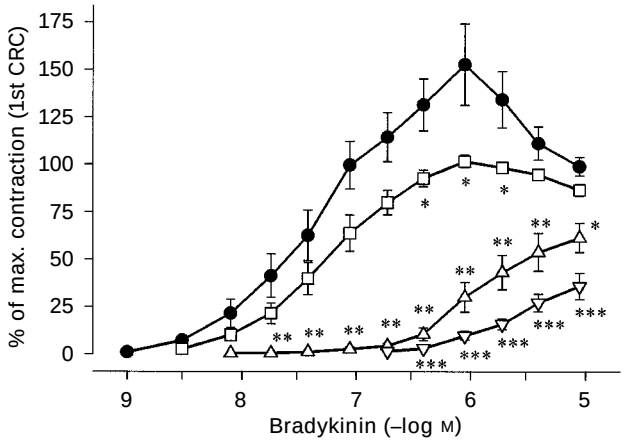

Figure 2 Effect of FR173657 on bradykinin-induced contractions of the isolated ileum of the guinea-pig. Two cumulative concentration-response curves (CRC) for BK were established at an interval of 30 min. Five min before the second test, FR173657 (□: 30 nM; △: 100 nM; ▽: 300 nM) or its vehicle (DMSO, final concentration 0.03% v/v: ●) was added to the organ bath. Contractions during the second CRC are given as % of the maximum obtained in the first CRC. Significance of difference from control tissues: $*P<0.05$, $**P<0.01$, $***P<0.001$. Mean values are shown and vertical lines indicate s.e.mean; $n=6-8$.

manner (Figure 2). The maximum BK-induced effect was significantly ($P<0.05$) reduced already by 30 nM FR173657. The effect of the higher concentrations (100 and 300 nM) of the antagonist on the BK-induced maximum could not be determined since at these concentrations a prominent rightward shift of the concentration-response curve for BK was apparent (Figure 2). From the concentration-response curves of BK in the absence and presence of FR173657 a pK_B value of 8.7 (95% confidence interval 7.8–9.8) was calculated.

Isolated duodenum of the rat

BK caused concentration-dependent relaxations of the isolated duodenum of the rat when applied to the tissues at concentrations of 0.1–1000 nM. The maximum BK-induced effect was observed at about 100 nM. When concentration-response curves to BK were established in the presence of FR173657 (10–300 nM) (Figure 3a), a concentration-dependent rightward shift of the concentration-response curve to BK could be observed. In addition to the rightward shift, FR173657 seemed to cause a reduction of the maximum BK-induced effect. While 30 nM FR173657 reduced the maximum relaxation in response to BK significantly ($P<0.05$) to $74\pm10\%$, the maximum was further decreased to $51\pm9\%$ ($P<0.05$) by 100 nM FR173657. At the highest concentration of FR173657 (300 nM), no maximum BK effect could be established. The apparent pK_B of FR173657 was calculated as 9.0 ± 0.2 ($n=6$).

The B_1 receptor agonist, des-Arg^9-BK, also caused relaxations of the rat duodenum. The effect obtained with the highest concentration of des-Arg^9-BK that was used amounted to about 60% of the maximum BK-induced relaxation. However, higher concentrations of des-Arg^9-BK were not used in the present investigation because of a limited supply of this peptide. When des-Arg^9-BK was tested in the presence of FR173657 (300 nM), the concentration-response curve for the B_1 agonist was identical to that obtained under control conditions (Figure 3b).

Isolated uterus of the rat

BK caused concentration-dependent contractions of uterine smooth muscle preparations taken from rats in oestrus (Figure 4). The maximum tension induced by BK ranged between

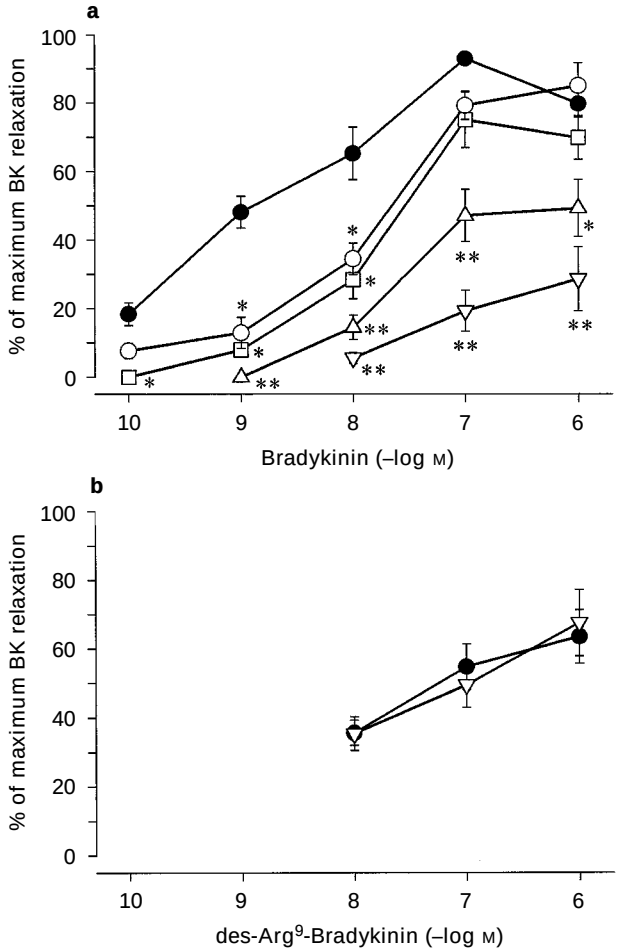

Figure 3 Effect of FR173659 on kinin-induced relaxations of the isolated duodenum of the rat. Concentration-response curves to BK (a) and to des-Arg^9-BK (b) were established under control conditions (●) and in the presence of FR173657 (○: 10 nM; □: 30 nM; △: 100 nM; ▽: 300 nM). All concentrations of FR173657 were tested in each individual preparation in randomized order. Relaxations are given as % of the maximum BK-induced relaxation obtained at the beginning of each experiment. Significance of difference from control values: $*P<0.05$, $**P<0.01$. Mean values are shown and vertical lines indicate s.e.mean; $n=6-10$.

about 60 and 80 mN in the different tissue preparations. When the challenges with BK were repeated in the presence of FR173657, the concentration-response curve for BK was shifted to right by an extent depending on the concentration of FR173657 (3–300 nM). In contrast to the effect of FR173657 in the other smooth muscle preparations, no reduction of the maximum effect of BK could be observed and the concentration-response curves for BK remained strictly parallel (Figure 4). A pA_2 value of 9.1 (95% confidence interval 8.1–10.3) was calculated. The slope of the Schild plot was -0.98 which was not significantly ($P>0.95$) different from the theoretical value of -1 for competitive antagonism. A regression line with the slope constrained to -1 yielded an identical pA_2 value. Therefore, the value of 9.1 can also be interpreted as an estimate for the pK_B of FR173657 in this tissue.

Isolated trachea of the guinea-pig

The maximum contractile force exerted by transversal ring preparations of the guinea-pig isolated trachea in response to 100 μM acetylcholine ranged between about 4 and 6 mN. BK elicited concentration-dependent contractions of the trachea with threshold concentrations of 1 nM. At the highest BK concentration used in these experiments (10 μM), the contrac-

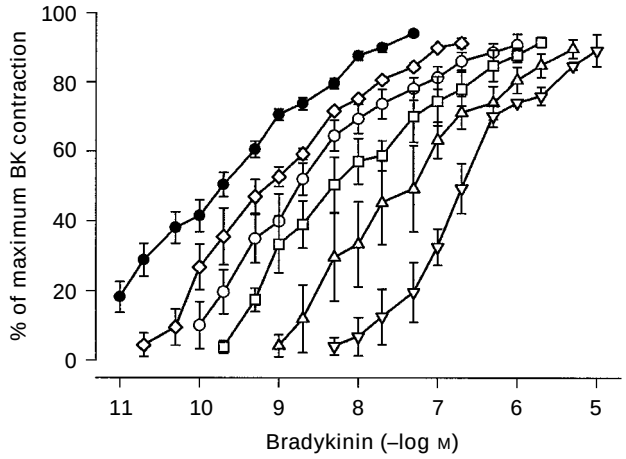

Figure 4 Effect of FR173657 on BK-induced contractions of the isolated uterus of the rat. Concentration-response curves (CRCs) to BK were established under control conditions (●) and in the presence of FR173657 (◇: 3 nM; ○: 10 nM; □: 30 nM; △: 100 nM; ▽: 300 nM) applied 5 min before the challenge with BK. In each individual preparation, two CRCs to BK were established under control conditions and in the presence of one concentration of FR173657. Contractions are given as % of the maximum BK-induced contraction obtained at the beginning of each experiment. Mean values are shown and vertical lines indicated s.e.mean; $n=4-6$.

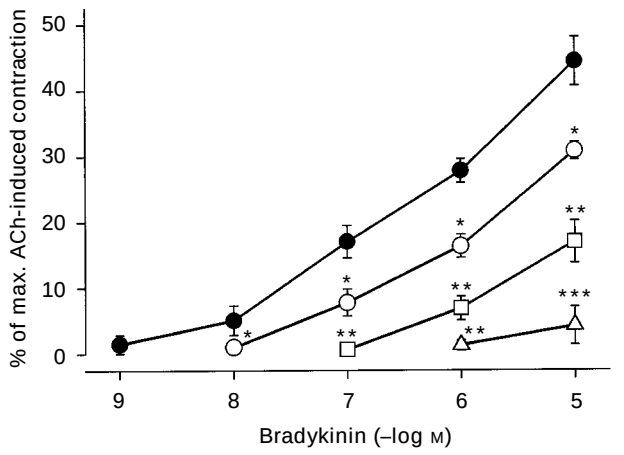

Figure 5 Effect of FR173657 on BK-induced contractions of the isolated trachea of the guinea-pig. Concentration-response curves to BK were established under control conditions (●) and in the presence of FR173657 (○: 10 nM; □: 30 nM; ◇: 100 nM). Each concentration of BK was applied for 2 min at intervals of 10 min. Contractions are given as % of the maximum acetylcholine-induced contraction obtained at the beginning of each experiment. Mean values are shown and vertical lines indicate s.e.mean; $n=6-10$.

tions reached values of $44\pm5\%$ of the acetylcholine-induced maximum (Figure 5). These experiments were performed in the presence of 0.03% (v/v) DMSO in the organ bath medium. This concentration is higher than that reached on addition of 100 nM FR173657 in a DMSO-based solution used in the experiments described below. DMSO at this concentration by itself had no effects on the reactivity of the tissue.

The contractions in response to BK were significantly diminished when the experiments were repeated, in separate tissues, in the presence of FR173657 (10–100 nM; Figure 5). The highest concentration of FR173657 (100 nM) almost abolished the BK-induced effects. However, although the inhibition caused by FR173657 was clearly dependent upon the concentration of the antagonist, no conclusion can be made on the apparent mode of antagonism of FR173657 in this tissue. Since no data were obtained for the upper parts of the concentration-response curve to BK, neither a rightward shift of the concentration-response curve nor a reduction of the maximum BK-induced effect in the presence of FR173657 can be excluded. Although the lack of demonstration of a maximum effect precludes the determination of a pK_B or pA_2 value, we have used the method of Kenakin (1993) to obtain at least a very crude estimate for the affinity of FR173657 in the guinea-pig trachea. The value thus calculated was 9.3 (8.2–10.3, 95% confidence interval).

Contractions induced by acetylcholine (1–30 μM) in the presence of FR173657 at a concentration of 100 nM were identical to those obtained under control conditions (data not shown). Similarly, isoprenaline (30–300 nM)-induced relaxations of tracheal preparations precontracted with 10 μM acetylcholine were completely unaffected by 100 nM FR173657 (data not shown).

Vasoconstriction in the rabbit isolated perfused ear

Injection of BK into the arterial inflow cannula of the isolated perfused ear of the rabbit caused dose-dependent reductions in the venous outflow (Figure 6). The lowest dose of BK used in these experiments (30 pmol) already caused a reduction of the venous outflow by $21\pm3\%$, whereas at 10 nmol the effect amounted to $91\pm3\%$. All effects reached their peak value within 1 min after the injection of BK. The time needed for the normalization of the venous flow was dependent upon the magnitude of the vasoconstrictor effect: while small effects were reversed within 1 min, a period of up to 5 min was required after near-maximal effects of BK.

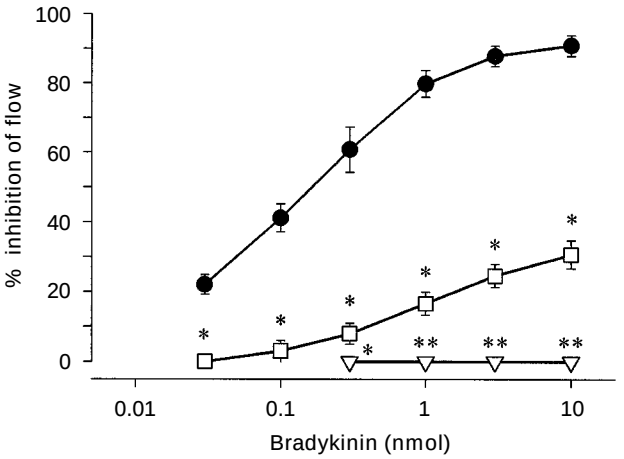

Figure 6 Effect of FR173657 on BK-induced venoconstriction in the rabbit isolated perfused ear. BK was injected into the arterial inflow cannula at intervals of 10 min in doses given on the abscissa scale. All ears were perfused with Tyrode solution under constant pressure at a rate adjusted to 3 ml min^{-1} at the beginning of the experiment. FR173657 was added to the perfusion medium at concentrations of 30 nM (□) or 300 nM (▽), whereas DMSO (final concentration 0.03% v/v) was used in control preparations (●). Vasoconstriction was determined as % reduction in venous outflow. Significance of difference from controls: $*P<0.05$, $**P<0.01$. Mean values are shown and vertical lines indicate s.e.mean; $n=6$ for each treatment group.

In the presence of FR173657 in the perfusion medium, the effects of BK were inhibited significantly dependent upon the concentration of the BK antagonist (Figure 6). At 30 nM, FR173657 abolished the effects of the lowest doses of BK (30 pmol) and reduced the effects of the higher doses by more than 70% ($P<0.05$). The effects of all doses of BK were completely abolished ($P<0.01$) when FR173657 was added to the perfusion medium at a concentration of 300 nM.

In separate experiments, the effects of FR173657 on vasoconstriction induced by angiotensin II (10–1000 pmol) and noradrenaline (10–1000 pmol) were investigated in order to exclude possible unspecific inhibitory effects of the antagonist upon the reactivity of the ear vessels. However, the effects of angiotensin II and noradrenaline remained completely unaffected by FR173657 even at a concentration of 300 nM (data not shown).

Prostaglandin release from the rabbit isolated perfused ear

The release of the prostaglandins E_2 (PGE_2) and I_2 was determined in isolated perfused ears pre-loaded with ^{14}C-labelled arachidonic acid. Under basal conditions, i.e. perfusion with Tyrode solution containing DMSO at a final concentration of 0.03% (v/v) as a control for the solvent of FR173657 used in the experiments described below, similar amounts of PGE_2 and PGI_2 (1.05 ± 0.22 Bq and 0.92 ± 0.11 Bq for PGE_2 and PGI_2, respectively) were released into the venous effluent over a period of 20 min (60 ml) (Figure 7). The injection of BK (10 nmol) into the arterial inflow of the ears was followed by an almost four fold increase in the release of PGE_2 ($P<0.05$; Figure 7a) and a three fold increase in the release of PGI_2 ($P<0.05$; Figure 7b). The net release over basal values was 2.59 ± 0.27 Bq for PGE_2 and 1.97 ± 0.22 Bq for PGI_2, respectively.

When the prostaglandin release from the rabbit isolated perfused ear was determined in preparations perfused with Tyrode solution containing FR173657 at a concentration of 300 nM, the basal release of PGE_2 and PGI_2 (1.05 ± 0.18 Bq and 1.12 ± 0.22 Bq, respectively) was identical to that of the control preparations. However, the increased prostaglandin release elicited by BK (10 nmol) was completely abolished ($P<0.05$) under these conditions (Figure 7a and b).

Discussion

One of the most striking features of the inhibition caused by FR173657 is its very long duration of action. Although the antagonist was present in the organ bath solution for only 5 min, the inhibition lasted for a much longer period of time. A similarly prolonged inhibitory action has already been found previously for second generation peptide antagonists (Lembeck *et al*., 1991). However, the duration of action of FR173657 is even longer than that of the peptide antagonists since a period of up to 90 min was required for a full recovery of the BK effects after FR173657 whereas 30–45 min were sufficient for icatibant (compound I in Lembeck *et al*., 1991).

In the guinea-pig ileum, FR173657 caused a rightward shift of the concentration-response curve for BK together with a reduction of the maximum effect. Thus, neither a typical competitive antagonism nor a simple noncompetitive mode of interaction with the BK receptors of this tissue can be assumed. The same pattern has been described for the actions of icatibant, while first generation antagonists seem to act in a purely competitive manner (Griesbacher & Lembeck, 1992). The inhibitory pattern of FR173657 in the rat duodenum was similar to that in the guinea-pig ileum, whereas not enough data are available to draw definite conclusions in the guinea-pig trachea. The reason for the apparent noncompetitive component of the inhibitory action of FR173657 is assumed to be due to it binding tightly to the receptors; this is exemplified by the extremely long duration of action of the compound *in vitro*. In contrast to the inhibitory pattern of FR173657 in the tissues mentioned above, FR173657 showed a purely competitive antagonism in the rat uterus. By comparison, icatibant has been shown to act competitively in the rat uterus only when the tissue was pre-incubated with the antagonist for periods of 1 min, but not when the pre-incubation time was 5 min (Liebmann *et al*., 1993) as in the present investigation.

The affinity of FR173657 in different smooth muscle preparations from the rat and the guinea-pig has been compared (see Table 1). In the guinea-pig ileum, the present pK_B value for FR173657 of 8.7 is similar to the pK_B or pA_2 values obtained for the peptide antagonist, icatibant. However, it is clearly higher than the affinity estimates found for the non-peptide agent, WIN 64338. In the guinea-pig trachea, the affinity of icatibant seems to be either similar (Field *et al*., 1992) or markedly smaller (Rhaleb *et al*., 1992) compared to the very crude estimate found here for FR173657 (9.3). WIN 64338 is apparently less potent by 2–3 orders of magnitude. The affinity of FR173657 in the rat uterus (pK_B 9.1) is almost identical to that of icatibant obtained by Liebmann *et al*. (1993) but somewhat smaller than the value found by Perkins

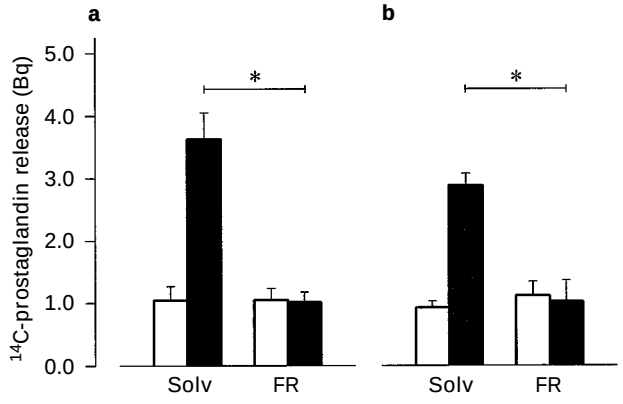

Figure 7 Effect of FR173657 on BK-induced release of prostaglandins (PG) from the rabbit isolated perfused ear. PGE_2 (a) and PGI_2 (b) were quantified by scintillation counting (in Bq) of ^{14}C-prostanoids in the venous effluent from rabbit isolated perfused ears pre-loaded with [^{14}C]-arachidonic acid. The prostaglandins were determined in fractions of 20 min collected before (open columns) and after (solid columns) the injection of BK (10 nmol) into the arterial inflow of the ears. All ears were perfused with Tyrode solution (3 ml min^{-1} initial flow) to which either FR173657 (300 nM; FR) or DMSO (final concentration 0.03% v/v; Solv) was added. Significance of difference: $*P<0.05$. Mean values ± s.e.mean; $n=4$ per treatment group.

Table 1 Comparison of affinity estimates for FR173657 with those of other bradykinin receptor antagonists in smooth muscle preparations from guinea-pigs and rats

Antagonist	*Guinea-pig ileum*	*Guinea-pig trachea*	*Rat uterus*	*Rat duodenum*
FR173657	8.7	(9.3)	9.1	9.0
WIN 64338	7.6 (a) 8.0 (b) 8.2 (c)	7.4 (a) 6.4 (i)		
Icatibant	8.4 (a) 8.8 (d) 8.4 (e) 8.8 (f) 8.9 (g) 8.5 (h)	8.1 (a) 8.9 (k) 7.4 (g)	9.0 (d) 9.7 (f)	8.7 (m)

Underscored numbers are pK_B values, other numbers give estimates for pA_2. Data for FR173657 are from this investigation (for value shown in parentheses see Results section). The values for the other antagonists are taken from: (a) Pruneau *et al*. (1995), (b) Farmer & DeSiato (1994), (c) Sawutz *et al*. (1994), (d) Liebmann *et al*. (1993), (e) Hock *et al*. (1991), (f) Perkins *et al*. (1991), (g) Rhaleb *et al*. (1992), (h) Félétou *et al*. (1995), (i) Scherrer *et al*. (1995), (k) Field *et al*. (1992). The value denoted (m) is derived from a re-analysis of data obtained previously (Griesbacher, 1992).

et al. (1991). No affinity estimates have been published to date for icatibant or WIN 64338 in the rat duodenum. However, a re-analysis of the data published previously for icatibant in the duodenum (Griesbacher, 1992) indicate an almost identical affinity of FR173657 and icatibant.

Practically all of the antagonists available today show a selectivity towards either the B_1 or the B_2 receptor. In the rat proximal duodenum BK and des-Arg^9-BK induce relaxations selectively via B_2 and B_1 receptors, respectively (Griesbacher, 1992). The present finding that FR173657 potently inhibited the relaxation induced by BK but had no effect on the relaxations in response to des-Arg^9-BK proves that FR173657 is a selective inhibitor of B_2, but not B_1, receptors. It has been proposed that a subtype of B_2 receptors exists in the rat uterus preparation, based on the observation that a number of peptide BK antagonists had markedly higher pK_B or pA_2 values in this tissue as compared to the guinea-pig ileum (Birch *et al*., 1991; Perkins *et al*., 1991). However, the data obtained with the present BK antagonist FR173657 do not support such tissue-specific differences between B_2 receptors, because the pK_B values obtained in the rat uterus and the guinea-pig ileum were similar. A third BK receptor, termed B_3, has been suggested for the actions of BK in the guinea-pig airways, particularly the trachea, because BK-induced contractions of these tissues were only weakly reduced by certain peptide B_2 antagonists (Farmer *et al*., 1989; Field *et al*., 1992). The inhibitory profile of FR173657 in the present experiments suggests that the potency of this novel antagonist is similar to that in the other preparations tested. Thus, a difference between the BK receptors in the guinea-pig trachea and the B_2 receptors of other tissues was not apparent with FR173657. For the same reason, species variants of the B_2 receptor (see Hall, 1992; Regoli *et al*., 1993) could be detected with FR173657.

Any new compound that is suggested to be a receptor antagonist must exhibit both a selective inhibition of the receptor-mediated actions of the agonist in question and a lack of effect at other receptor systems. Indeed, the first non-peptide BK antagonist of sufficient potency, WIN 64338 also exhibited ancillary pharmacological actions at other receptor systems (Sawutz *et al*., 1994). However, FR173657 did not inhibit the actions of histamine, 5-hydroxytryptamine, acetylcholine, noradrenaline, isoprenaline, substance P, angiotensin II or the cholecystokinin agonist, caerulein. Residual agonist-like effects have been found with some of the early peptide BK antagonists (Stewart & Vavrek, 1986). In the present investigation, FR173657 proved to have no agonist activity in all the *in vitro* assays studied, even at the highest concentrations used.

Isolated smooth muscle preparations, basically, are mainly importantly used for the pharmacological characterization of agonists and antagonists. However, the isolated perfused rabbit ear allows the investigation of the possible modulatory functions of kinins that clearly are involved in pathophysiological states. The vasoconstriction observed in this preparation is most probably due to a constrictor action at the venous side of the vasculature (Guth *et al*., 1966). Venoconstriction, especially in small and postcapillary veins contributes, together with praecapillary/arterial vasodilatation and capillary increase in vascular permeability, to the oedema formation induced by kinins in inflammatory conditions. The actions of FR173657 in *in vivo* models on the effects of BK on blood pressure, bronchoconstriction, and visceral and peripheral oedema formation are presented in a separate study (Griesbacher & Legat, 1997). Prostaglandins are secondary mediators which are released by BK in many tissues and contribute to, or modulate, the direct actions of kinins. One of the most important roles of this prostanoid release is a facilitatory effect on afferent neurones involved in nociception (Juan, 1978).

In conclusion, the non-peptide compound FR173657 was demonstrated to be a highly selective BK antagonist which is active at B_2, but not B_1 receptors. The potency and apparent type of antagonistic action of the compound *in vitro* is similar to that of current, second generation peptide BK antagonists. Although the inhibitory action lasted even longer than that of present antagonists, the effects were fully reversible. Thus, it can be expected that FR173657 will be a major advance for the development of agents that can be used as tools for the elucidation of pathological and physiological roles of the kinin system.

Note added in proof

Data on the identification, receptive binding and oral activity of FR173657 have been published recently by Asano *et al*., (1997). *Br. J. Pharmacol*., **120,** 617 – 624.

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