ELSEVIER

European Journal of Pharmacology 409 (2000) 185–194

ejp

www.elsevier.nl/locate/ejphar

Peptide and non-peptide bradykinin B_2 receptor agonists and antagonists: a reappraisal of their pharmacology in the guinea-pig ileum

Stefania Meini [a,*], Riccardo Patacchini [a], Alessandro Lecci [a], Laura Quartara [b], Carlo Alberto Maggi [a]

[a] *Pharmacology Department, Menarini Ricerche S.p.A., via Rismondo 12 A, 50135 Florence, Italy*
[b] *Chemistry Department, Menarini Ricerche S.p.A., Florence, Italy*

Received 27 July 2000; received in revised form 26 October 2000; accepted 31 October 2000

Abstract

We have compared the pharmacology of different antagonists, Icatibant (H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DTic-Oic-Arg-OH), MEN 11270 (H-DArg-Arg-Pro-Hyp-Gly-Thi-c(Dab-DTic-Oic-Arg)c(7 γ–10 α)), and FR173657 ((*E*)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl]phenyl]-*N*-methylaminocarbonylmethyl]acrylamide) at bradykinin B_2 receptors expressed in the guinea-pig ileum by using bradykinin and the non-peptide FR190997 ((8-[2,6-dichloro-3-[*N*-[(*E*)-4-(*N*-methylcarbamoyl)cinnamidoacetyl]-*N*-methylamino]benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline) as agonists. In organ bath experiments, Icatibant and FR173657 exerted a non-competitive antagonism (pK_B 9.5 and 9.2, respectively) of the contractile response to bradykinin, whereas MEN 11270 showed competitive antagonism (pK_B 8.3, slope −0.90). The profile of action and apparent affinities of the three antagonists did not change if contact time was prolonged. The inhibition by the three antagonists of the contractile response to bradykinin was differently reverted by washout (MEN 11270 < 30 min, Icatibant < 60 min, FR173657 > 60 min). The non-peptide ligand FR190997 acted as partial agonist if applied cumulatively to the bath (pD_2 8.06, E_{max} 43% of maximal contractility), but as a full agonist when a maximally effective concentration was added (E_{max} 83%). FR173657 produced non-competitive antagonism of the response to FR190997 with apparent affinity similar to that measured toward bradykinin. On the contrary, Icatibant and MEN 11270 (300 nM both) competitively antagonized the contractile activity exerted by FR190997 with lower apparent pA_2 value (6.9 and 7.2, respectively). In radioligand binding experiments, MEN 11270 and Icatibant displaced the [^{3}H]bradykinin binding with pK_i of 10.2 and 10.5 (Hill slope not different from unity), respectively. The non-peptide ligands displaced the [^{3}H]bradykinin binding with similar affinity, their pK_i being 8.7 and 8.6 for FR173657 and FR190997, respectively (both Hill slopes < 1). The present study indicates the difference in the antagonism type (competitive vs. non-competitive) by Icatibant, MEN 11270, and FR173657, as mainly ascribable to their different reversibility from the bradykinin B_2 receptor, and affected by the kinetic of the response induced by the different agonists. Results are discussed in view of a different interaction of peptide and non-peptide agonist at the receptor. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Antagonism; Bradykinin B_2 receptor; FR173657; FR190997; MEN 11270; Non-peptide

1. Introduction

The biological effects of kinins are mediated by two different types of G protein-coupled receptors, namely B_1 and B_2, which have recently been cloned from various species including humans (Eggerickx et al., 1992; Hess et al., 1992; Menke et al., 1994). Before their molecular identification, the existence of B_1 and B_2 receptors was postulated on the basis of pharmacological studies (Regoli and Barabé, 1980).

A number of potent and selective antagonists for bradykinin B_2 receptor has been identified in recent years (Altamura et al., 1999, for review): these include the metabolically stable, linear peptide antagonist Icatibant (H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DTic-Oic-Arg-OH) (Hock et al., 1991; Wirth et al., 1991), its cyclic constrained derivative MEN 11270 (H-DArg-Arg-Pro-Hyp-Gly-Thi-c(Dab-DTic-Oic-Arg)c(7 γ–10 α)) (Meini et al., 1999), and a number of non-peptide receptor antagonists

* Corresponding author. Tel.: +39-55-5680-736; fax: +39-55-5680-419.
E-mail address: camaggi@menarini-ricerche.it (S. Meini).

0014-2999/00/$ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0014-2999(00)00850-5

such as FR173657 ((*E*)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl] phenyl]-*N*-methylaminocarbonylmethyl]acrylamide) (Aramori et al., 1997a; Asano et al., 1997).

In the guinea-pig ileum assay, which has been the object of several studies on the bradykinin B_2 receptor pharmacology (Hall, 1992, for review), some conflicting results have been reported with regard to the nature of antagonism exerted in antagonizing bradykinin-induced contraction: Icatibant has been reported to act as a non-competitive antagonist (Griesbacher and Lembeck, 1992; Pruneau et al., 1995), whereas it has been reported to act as a competitive or non-competitive antagonist for different preparations expressing B_2 receptor in the guinea-pig species (Pruneau et al., 1995). In the same assay, FR173657 was reported to act both as a non-competitive (Griesbacher et al., 1997; Rizzi et al., 1997) and competitive antagonist (Asano et al., 1997).

In the present study, we have compared the activity of the three bradykinin B_2 receptor antagonists, Icatibant, FR173657 and MEN 11270, under comparable experimental conditions in the guinea-pig ileum longitudinal smooth muscle. In particular, we first verified whether contact time and reversibility of the action of the antagonists could be factors important in determining the exerted antagonism type. Second, in order to assess whether the antagonism type could be dependent from the agonist, we tested their antagonist activity not only against bradykinin, the natural peptide ligand for B_2 receptors, but also against the non-peptide agonist FR190997 (8-[2,6-dichloro-3-[*N*-[(*E*)-4-(*N*-methylcarbamoyl) cinnamidoacetyl]-*N*-methylamino]-benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline) (Aramori et al., 1997b). Finally, we also determined the affinity of all the studied ligands in competing for the [^{3}H]bradykinin binding sites in guinea-pig ileum longitudinal smooth muscle membranes.

2. Materials and methods

2.1. Functional in vitro experiments

Male albino guinea-pigs (400–450 g) were stunned and bled. The small intestine was removed, placed in oxygenated and gassed (95% O_2, 5% CO_2) Krebs' Henseleit solution containing indomethacin, guanethidine (both 3 μM), clorpheniramine and atropine (both 1 μM). Longitudinal smooth muscle-myenteric plexus preparations were obtained from the guinea-pig ileum as described by Paton and Vizi (1969). The composition of Krebs' solution was as follows (mM): NaCl (119); $NaHCO_3$ (25); KH_2PO_4 (1.2); $MgSO_4$ (1.5); $CaCl_2$ (2.5); KCl (4.7) and glucose (11). The strips were placed in organ baths (5 ml capacity) and prepared for isotonic recording (load 5 mN) of mechanical activity (via Basile transducers) which was displayed on Basile 7050 pen recorder.

After an equilibration period of 1 h, bradykinin (1 μM) was administered 3–4 times to preparations, every 20-min interval, to assay sensibility and reproducibility of the contractile response. Afterwards, a cumulative concentration–response curve to bradykinin (1 nM–1 μM) was constructed. At the end of each curve, the maximal contractile response of the preparation was evaluated by administration of KCl (80 mM). After washout and recovery of basal tone, the concentration–response curve to bradykinin was repeated in the presence of one of the receptor antagonists under study. Control experiments revealed no significant changes in the responses over three consecutive concentration–response curves to bradykinin (data not shown) constructed at 45-min intervals.

Peptidase inhibitors (thiorphan, bestatin, and captopril, 1 μM) were added 15 min prior to determination of the bradykinin-induced concentration–response curve. Antagonists contact time was 15 min, when not stated otherwise.

The reversibility of B_2 receptor blockade produced by the antagonists under study was evaluated as follows: bradykinin (100 nM) was administered to the preparations at 30-min intervals, until reproducible contractile responses were obtained (generally after three administrations). At this time, MEN 11270 (100 nM) or Icatibant (10 nM) or FR173657 (10 nM) or vehicle were added to the bath solution, 15 min before the next challenge with the agonist. The preparations were then thoroughly washed with Krebs' solution, which was renewed every 5 min. Administration of the agonist was repeated 30, 60 and 90 min after washout of the antagonist, and the responses were compared to those obtained in control time-matched preparations.

When experiments with the non-peptide agonist were carried out, a different protocol was used because of the difficult reversibility of its contractile response through the washout-over the time. Cumulative concentration–response curve to FR190997 (1 nM–3 μM) were constructed in the absence or in the presence of the antagonist in different preparations belonging to the same animal.

2.2. Membrane preparation

Segments of guinea-pig small intestine were excised as described above, and placed in *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulphonic acid (TES, 10 mM, pH 7.4, at 4°C) added with a cocktail of peptidase inhibitors: 1,10 phenanthroline (1 mM), ethylene glycol *bis* (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (1 mM), captopril, leupeptin, soybean trypsin inhibitor, DL-2-mercaptomethyl-3-guanidoethylthiopropanoic acid (1 μM each), chymostatin (3.3 μM), phenylmethyl–sulphonyl fluoride (0.1 mM), and bacitracin (140 μg ml^{-1}). The longitudinal smooth muscle was dissected as for functional

experiments, minced and homogenized with a Polytron (PT 3000, Kinematica), set at 15 000 r.p.m. for 30 s in 10 ml g^{-1} of the above buffer. The homogenate was centrifuged at $2500 \times g$ for 10 min to remove cellular debris. The supernatant was homogenized and centrifuged at $45\,000 \times g$ (4°C) for 30 min. The pellet was resuspended in binding buffer and frozen immediately in 2 ml aliquots by immersion in liquid nitrogen, and then stored at −80°C until use.

The protein concentration was determined by the method of Bradford (1976) using a Bio-Rad kit, using bovine serum albumin as reference standard.

Immediately prior to use, frozen membrane aliquots were thawed in binding buffer (see below) and mixed to give a homogeneous membrane suspension.

2.3. Binding experiments

The buffer used for binding experiments was TES (10 mM, pH 7.4) containing 1,10 phenanthroline (1 mM), bacitracin (140 μg ml^{-1}), and bovine serum albumin (1 g l^{-1}). Binding assay was performed in polypropylene tubes in a final volume of 0.5 ml. Non-specific binding was defined as the amount of labelled ligand bound in the presence of 1 μM bradykinin. An incubation time of 90 min at 25°C was used for both saturation and competition studies (Manning et al., 1986).

All incubations were terminated by rapid filtration through Whatman GF/B glass fibre filtermats that had been pre-soaked for at least 2 h in polyethylenimine 0.6%, using a Brandel 48 cell harvester. The tubes and filters were then washed four times with 3 ml aliquots of Tris buffer (50 mM, pH 7.4, 4°C). Filters were soaked in CytoScint scintillation fluid (ICN Biomedicals) overnight, and bound radioactivity was counted in a β-scintillation counter (2200 CA, Packard).

In preliminary experiments, we observed that the specific binding of [^{3}H] bradykinin was directly proportional to membrane concentration (data not shown). A membrane concentration of 100 μg ml^{-1} was chosen for both saturation and competition studies. At this protein concentration, the specific binding represented approximately the 70–80% of the total binding for [^{3}H]bradykinin. [^{3}H]bradykinin (0.02–4 nM) saturation isoterm fitted with a one-site model with a K_D of 0.29 ± 0.01 nM ($n = 3$) and a B_{max} of 200 ± 6.5 fmol mg^{-1} of protein. On each batch of membranes a saturation curve to the [^{3}H]bradykinin was performed in order to elaborate competition studies. The chosen [^{3}H]bradykinin concentration for competition studies was 0.25 nM. All the bradykini B_2 antagonists were tested in a range of 14 concentrations (1 pM–1 μM).

2.4. Analysis of data

Functional responses to bradykinin either in the absence or presence of antagonist were normalized towards the maximal effect of bradykinin itself reached with the first curve. Responses to FR190997 were normalized as percentage of the maximal contractile response obtained with the administration of KCl (80 mM) on each preparation.

The agonist concentration producing the 50% of the maximal response for that agonist was calculated as negative logarithm to base 10, and indicated as pD_2 and 95% confidence limits (c.l.) were calculated by non-linear regression of the concentration–response curve.

The nature of the interaction of antagonists with the receptors was checked by the Schild regression as follows: antagonist-induced parallel shifts of concentration–response curves to the agonist were calculated graphically at the level of the half-maximal response as the ratio (concentration-ratio, CR) of equieffective concentrations of agonist. Estimates of log [CR − 1] were plotted against log [antagonist concentration] (Arunlakshana and Schild, 1959). Antagonists providing plots with linear regression lines and slopes not significantly different from unity were considered to act in a competitive manner. The affinity of competitive antagonists was expressed in terms of pK_B calculated from the equation: $pK_B = \log [CR - 1] - \log$ [antagonist concentration] (Kenakin, 1997a).

When antagonists caused non-parallel rigthward shifts of the concentration–response curve to bradykinin, and decreased the E_{max}, the estimate of the affinity was calculated by a method for non-competitive and/or pseudoirreversible antagonists as described by Kenakin (1997b). In practice, a double-reciprocal plot of equieffective concentrations of agonist (A) in the absence (1/A) and in the presence (1/A′) of the antagonist (B) was constructed, and K_B derived from the equation:

$$K_B = [B]/\text{slope} - 1.$$

In order to obtain more accurate estimates of K_B, we selected the experiments in which E_{max} to the agonist was depressed to or less than 50% of control by the antagonist.

Saturation and displacement binding data were processed by Prism 2.0 (GraphPad, San Diego, CA), to determine the maximum binding site density (B_{max}), affinity constant (K_D), −log of equilibrium inhibition constants (pK_i), Hill slope, and to test significance of site binding models. All values are given as mean ± s.e. of the mean from the stated number of experiments (n), each one performed in duplicate.

2.5. Materials

[^{3}H]bradykinin (specific activity 114 Ci $mmol^{-1}$) was provided by Du Pont NEN (Hertfordshire, UK). Bestatin and bradykinin were obtained from Peninsula (St. Helens, UK). Leupeptin was obtained from Boehringer-Mannheim (Germany), Thiorphan from Bachem (Essex, UK), and DL-2-mercaptomethyl-3-guanidoethylthiopropanoic acid from Calbiochem (La Jolla, CA, USA). GF/B glass fibre filtermats were provided by Brandel (Semat, St. Albans, Herts. UK). All salts used were purchased from Merck

(Darmstadt, Germany). All other materials were obtained from Sigma (St. Louis, MO, USA). All bradykinin B_2 receptor antagonists used were synthesized in Menarini Ricerche (Florence, Italy). FR190997 was a kind gift from Fujisawa Pharmaceuticals. FR173657 and FR190997 were dissolved in dimethylsulphoxide up to 1 mM, whereas Icatibant and MEN 11270 were dissolved in distilled water. All compounds were stored at −25°C.

3. Results

3.1. Organ bath experiments

3.1.1. Antagonist activity of Icatibant, FR173657, and MEN 11270 towards bradykinin

Bradykinin (1 nM–1 μM) produced concentration-dependent contraction of the longitudinal smooth muscle of guinea-pig ileum the pD_2 being 7.72 (95% c.l. 7.57–7.87) ($n = 23$). KCl (80 mM) administered on the plateau of the response to bradykinin, did not produce any further contraction, showing that the E_{max} of bradykinin corresponds to the maximal contractility of this muscle. The effects of each concentration of bradykinin developed in less than 1 min and a full concentration–response curve was constructed in less than 10 min through the cumulative protocol of administration of the agonist (not shown).

None of the antagonists under study produced agonist effect, up to the highest concentration tested. Icatibant and FR173657 (3–300 nM) produced a concentration-dependent insurmountable antagonism of bradykinin-induced contraction (Fig. 1A and B), while leaving the maximal response to KCl (80 mM) unaffected. The apparent pK_B values were: 9.5 ± 0.1 ($n = 8$) for Icatibant and 9.2 ± 0.2 ($n = 8$) for FR173657.

In contrast, MEN 11270 (10 nM–1 μM) produced concentration-dependent and parallel rightward shifts of

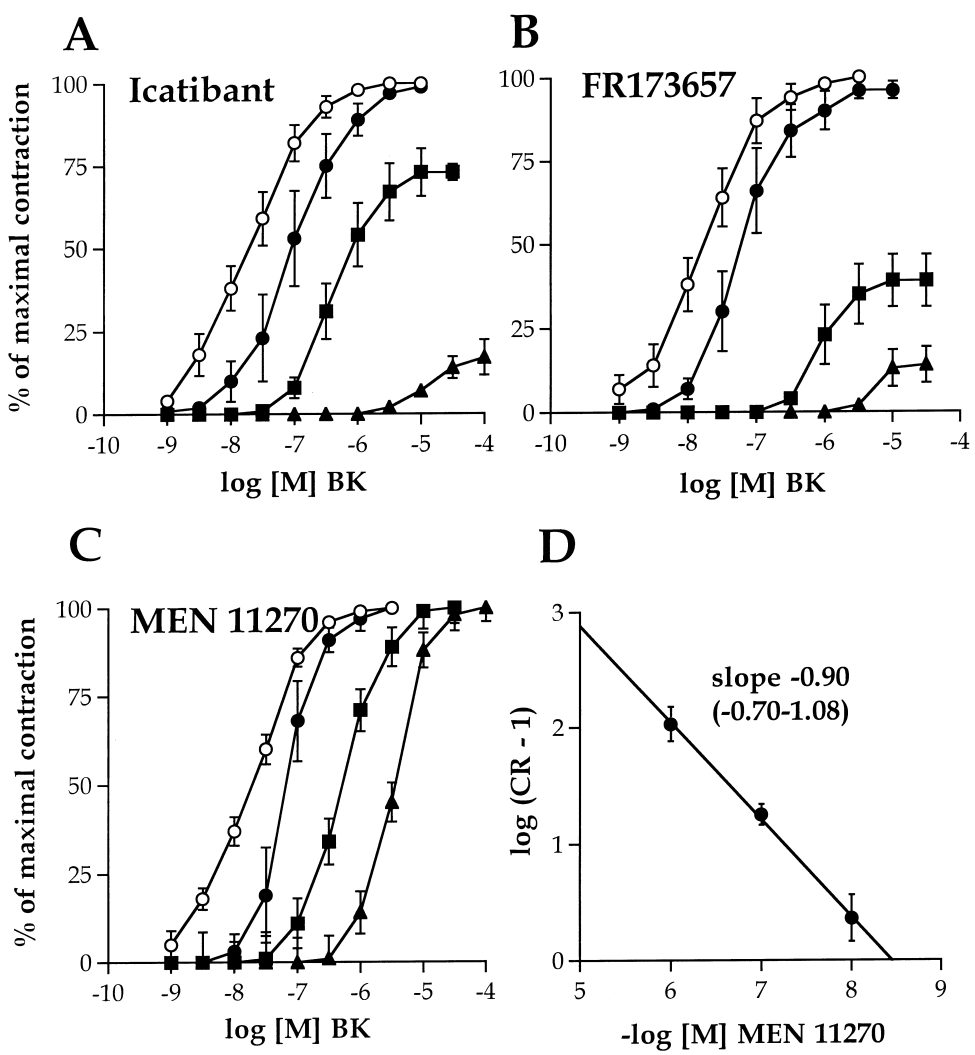

Fig. 1. Concentration–response curves to bradykinin in the guinea-pig ileum longitudinal smooth muscle in the absence (control, ○) and presence of various concentrations of bradykinin B_2 receptor antagonists: (A) Icatibant 3 nM (●), 30 nM (■), and 300 nM (▲); (B) FR173657 3 nM (●), 30 nM (■), and 300 nM (▲); (C) MEN 11270 10 nM (●), 100 nM (■), and 1000 nM (▲). Contact time of antagonists was 15 min. (D) Schild plot of MEN 11270 against bradykinin. Values represent the mean ± S.E.M. of 5–8 experiments.

the concentration–response curve to bradykinin without producing depression of E_{max} (Fig. 1C). Schild plot analysis was consistent with a competitive antagonism, the slope being −0.90 (95% c.l. −0.70 to −1.08) (Fig. 1D). The pK_B value of MEN 11270 was 8.3 ± 0.1 ($n = 24$).

In order to evaluate a possible time-dependency of the interaction with bradykinin B_2 receptor, the effect of the three antagonists was evaluated after 15 or 60 min contact time. MEN 11270 at 300 nM produced a rightward shift of the agonist concentration–response curve and its effect

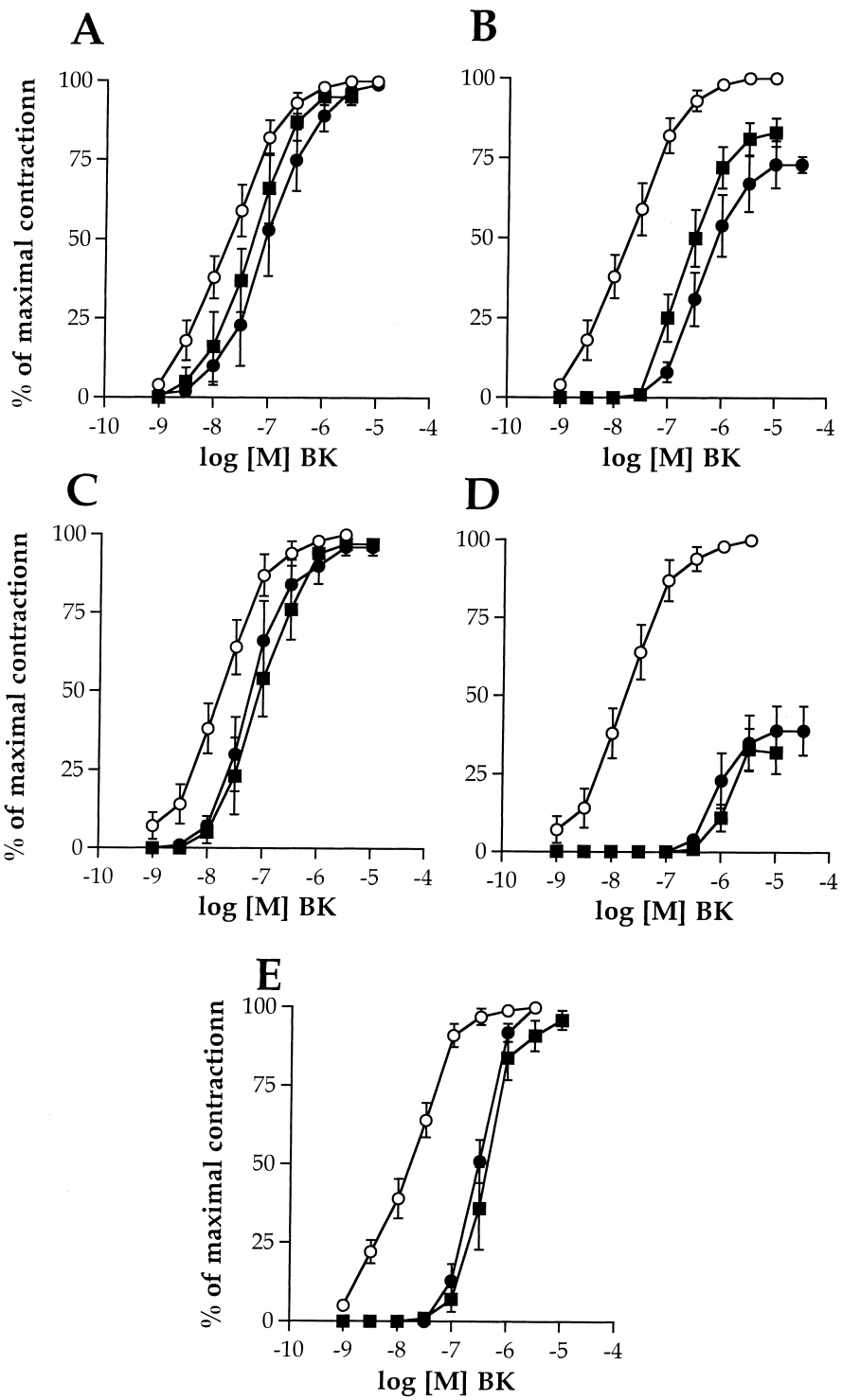

Fig. 2. Concentration–response curves to bradykinin in the guinea-pig ileum longitudinal smooth muscle in the absence (control, ○) and presence of bradykinin B_2 receptor antagonists, after 15 min (●) or 60 min (■) contact time: (A) Icatibant 3 nM; (B) Icatibant 30 nM; (C) FR173657 3 nM; (D) FR173657 30 nM; (E) MEN 11270 300 nM. Values represent the mean ± S.E.M. of five experiments.

was just superimposable after 15 or 60 min contact time (Fig. 2E). In the same set of experiments, Icatibant and FR173657 were tested at two concentrations: 3 nM (which for both antagonists did not depress the E_{max} of bradykinin at 15 min contact time) and 30 nM. The antagonist activities of both Icatibant (Fig. 2A and B) and FR173657 did not change after 15 or 60 min of incubation (Fig. 2C and D).

3.1.2. Reversibility of bradykinin B_2 receptor blockade produced by Icatibant, FR173657, and MEN 11270

The reversibility of bradykinin B_2 receptor blockade produced by the antagonists was evaluated as the capacity of the longitudinal smooth muscle to recover the contraction produced by a single dose of bradykinin (100 nM) after a 15-min contact time with the antagonists (see Section 2). MEN 11270 (100 nM), Icatibant (10 nM) and FR173657 (10 nM) produced a comparable degree of inhibition ($78 \pm 7\%$, $65 \pm 12\%$, $76 \pm 2\%$ inhibition, $n =$ 4–6, respectively) of the response to bradykinin (0.1 μM). As shown in Fig. 3, the inhibition exerted by MEN 11270 was fully and quickly reversed by washout since 30 min after the removal of the antagonist the response to bradykinin matched that obtained in vehicle-treated preparations (Fig. 3). Bradykinin B_2 receptor blockade produced by Icatibant was more slowly reversed by washout, but a full recovery of the response to the agonist occurred within 60 min from removal of the antagonist (Fig. 3). On the contrary, FR173657 produced a persistent antagonism at bradykinin B_2 receptors, which was not fully reversed up to 90 min from removal of the antagonist from the bath solution (Fig. 3).

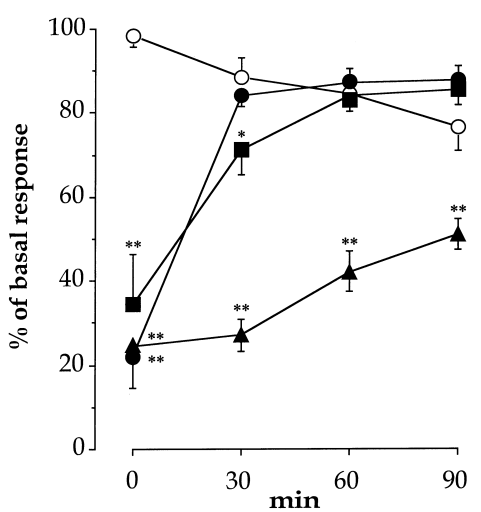

Fig. 3. Reversibility of bradykinin B_2 receptor blockade produced by MEN 11270, Icatibant and FR173657 in the guinea-pig ileum longitudinal smooth muscle preparation. The contractile response to single concentrations of bradykinin (100 nM) is shown in the absence (○) and in the presence (time = 0) of MEN 11270 100 nM (●), Icatibant 10 nM (■), and FR173657 10 nM (▲), and after 30, 60, and 90 min from washout of the antagonists (see Experimental Procedures). Antagonist contact time was 15 min. Each value is the mean ± S.E.M. of 4–6 determinations. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. matched vehicle responses.

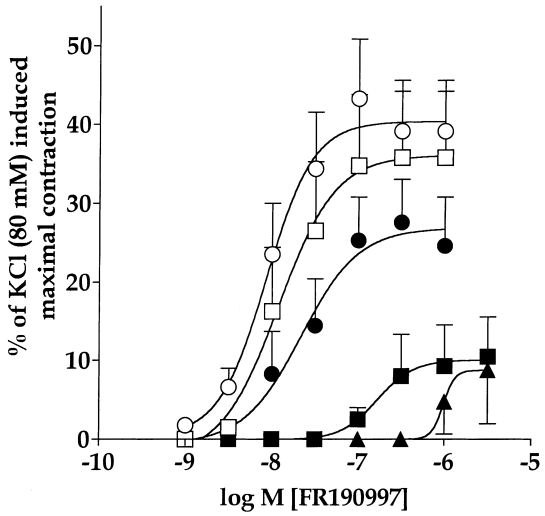

Fig. 4. Concentration–response curves to FR190997 in the guinea-pig ileum smooth muscle in the absence (control, ○) or presence of the bradykinin B_2 receptor antagonist FR173657 at 1 nM (□), 3 nM (●), 30 nM (■), and 300 nM (▲). Antagonist contact time was 15 min. Each value is the mean ± S.E.M. of 4–7 determinations.

3.1.3. Antagonist activity of Icatibant, FR173657, and MEN 11270 towards the contractile effect elicited by the non-peptide agonist FR190997

The activity of the non-peptide bradykinin B_2 receptor agonist FR190997 was tested in the concentration range 1 nM–3 μM. At the end of cumulative administration of the agonist, the maximal contractile response to FR190997, expressed as percentage of the maximal contraction elicited by 80 mM of KCl, averaged $43 \pm 8\%$ ($n = 8$, $P < 0.05$ vs. bradykinin maximal effect) and the pD_2 value resulted 8.06 (95% c.l. 7.63–8.48) (Fig. 4). Contrary to the effect of bradykinin, the response to FR190997 was much slower in onset, and the effect of each added concentration required 10–15 min to achieve a steady state. However, when a maximally effective concentration of FR190997 (0.1 μM) was administered as a single concentration to the bath, the resulting contractile response averaged $83 \pm 6\%$ of bradykinin produced E_{max} ($n = 5$). It should be noted that FR190997, either when administered in a single or in a cumulative manner to the bath, produced a contraction which did not show any fading, even after long period of contact (> 45 min), and that independently from the contact time, it was resistant to extensive and repeated washouts.

As shown in Fig. 4, FR173657 (1–300 nM) antagonized in a concentration-dependent manner the contractile response produced by FR190997; the antagonism was insurmountable, and yielded apparent pK_B values of 9.11 (95% c.l. 8.74–9.30) and 8.82 (95% c.l. 8.5–9.0) at 3 and 30 nM antagonist concentration, respectively.

Both Icatibant and MEN 11270, at 300 nM concentration, antagonized the contractile effects produced by FR190997 (a 10-fold lower antagonist concentration was ineffective, data not shown). As shown in Fig. 5, the maximal contractile effect produced by the non-peptide agonist FR190997 averaged $37 \pm 5\%$ ($n = 7$) of the maximal contraction elicited by KCl (80 mM) in the absence of antagonists: it was 39 ± 4 ($n = 7$) and 33 ± 6 ($n = 7$) in the presence of MEN 11270 and Icatibant (300 nM each), respectively. From the ratios of the EC_{50} values of concentration–response curves of FR190997 obtained in the presence and absence of the two antagonists, apparent pA_2 values of 6.9 and 7.2 were calculated for Icatibant and MEN 11270, respectively.

3.2. Binding experiments

Icatibant and MEN 11270 competed for [^{3}H]bradykinin binding site with high affinity, their pK_i being 10.5 ± 0.05 (pIC_{50} 10.1 ± 0.04, $n = 3$, $R = 0.974$) and 10.2 ± 0.06 (pIC_{50} 9.9 ± 0.05, $n = 3$, $R = 0.984$), respectively (Fig. 6). On the other hand, both non-peptide ligands, FR173657 and FR190997, displayed similar affinities, their pK_i values being 8.7 ± 0.01 (pIC_{50} 8.5 ± 0.05, $n = 3$, $R = 0.979$), and 8.6 ± 0.05 (pIC_{50} 8.3 ± 0.06, $n = 3$, $R = 0.964$), respectively (Fig. 6). Differences between peptide and non-peptide ligands were evident when analysing the slopes of the curves: competition curve had slopes not different from unity, being -1.13 (95% c.l. -1.34 to -0.92) and -0.95 (95% c.l. -1.17 to -0.74), for peptide ligands, Icatibant and MEN 11270, respectively. On the contrary, the competition curves of the non-peptide ligands at the [^{3}H]bradykinin binding site, appeared more shallow, showing Hill slope values significantly less than unity: -0.72 (95% c.l. -0.81 to -0.64) and -0.73 (95% c.l. -0.85 to -0.60), for FR173657 and FR190997, respectively. The analysis of curves by a two-site competitive model brought to a significant improvement: for FR173657 the two affinity (pIC_{50}) values were 9.4 and 8.1 ($R = 0.992$, F 7.938, P 0.00046 vs. one-site fit), and for FR190997 9.3 and 8.1 ($R = 0.981$, F 3.878, P 0.020 vs. one-site fit).

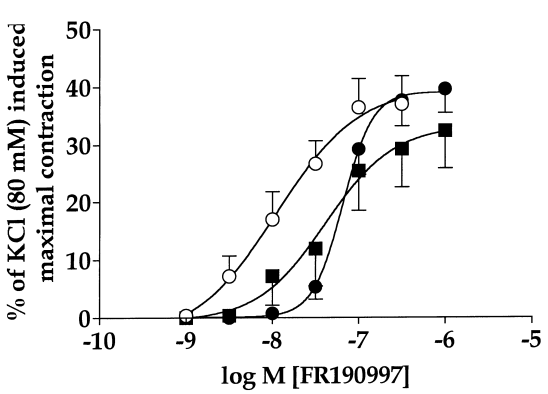

Fig. 5. Concentration–response curves to FR190997 in the guinea-pig ileum longitudinal smooth muscle in the absence (control, ○) or presence of the bradykinin B_2 receptor antagonist Icatibant (■), or MEN 11270 (●), both at 300 nM concentration. Antagonist contact time was 15 min. Each value is the mean ± S.E.M. of seven determinations.

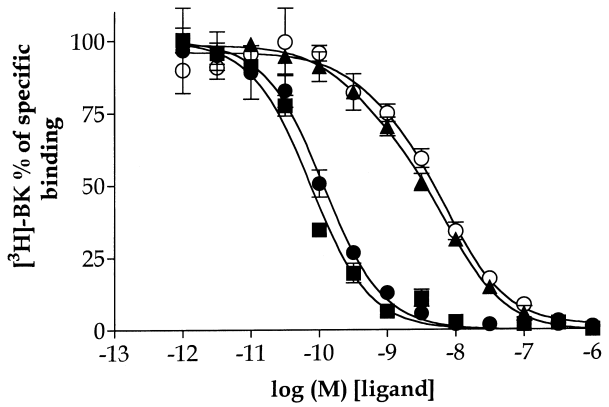

Fig. 6. Inhibition of [^{3}H]bradykinin binding to guinea-pig longitudinal smooth muscle membranes by Icatibant (■), MEN 11270 (●), FR173657 (▲), and FR190997 (○). Specific [^{3}H]bradykinin binding was plotted against increasing concentration of the above ligands ranging between 1 pM and 1 μM. [^{3}H]bradykinin (0.25 nM) binding and membranes (100 μg ml^{-1}) were incubated as described in Experimental Procedures. The data shown represent the mean ± S.E.M. of three separate determinations in duplicate.

4. Discussion

In the present study, we re-addressed the pharmacological profile of various agonists and antagonists for the bradykinin B_2 receptor in the guinea-pig ileum longitudinal smooth muscle. This preparation has been extensively studied, both in functional and in binding studies (Hall, 1992, for review), besides to be used for testing the activity of new compounds at the bradykinin B_2 receptor (Griesbacher and Lembeck, 1992; Pruneau et al., 1995; Rhaleb et al., 1992; Stewart et al., 1996; Hall, 1992, for review).

MEN 11270 is a selective bradykinin B_2 receptor antagonist obtained by introducing a diaminobutiric (Dab) residue in the place of the serine in the sequence of Icatibant. The cyclization between the side chain of Dab and the carboxyl group of the C-terminal arginine residue (Meini et al., 1999) would fix the C-terminal sequence of the peptide into a β-turn conformation, proved to be critical for the high affinity interaction of Icatibant with the bradykinin B_2 receptor (Guba et al., 1994). Owing to the structural similarity with Icatibant, we can assume that the mode of interaction of these two peptide antagonists with bradykinin B_2 receptor is similar if not overlapping (Meini et al., 1999). In keeping with this concept, both MEN 11270 and Icatibant displaced [^{3}H]bradykinin binding to guinea-pig ileal membranes with high affinities (pK_i 10.5 and 10.2, respectively).

The binding affinity of MEN 11270 was significantly higher (pK_i 10.2) than the apparent affinity measured in

functional experiments (pK_B 8.3). This phenomenon has been repeatedly reported to occur in pharmacological studies on bradykinin B_2 receptors (defined 'binding paradox' by Hall, 1992 for review), and seems to be at least in part ascribable to the lower ionic strength buffer used in binding studies (Ransom et al., 1992; Paquet et al., 1999). In functional experiments, MEN 11270 behaved as a competitive antagonist (slope from Schild analysis not different from unity), whereas Icatibant behaved as an insurmountable antagonist of bradykinin-induced contraction. This difference is not ascribable to non-equilibrium conditions, since extending the contact time from 15 to 60 min did not influence the type of antagonism. The difference in the type of antagonism produced by MEN 11270 and Icatibant can be explained, at least in part, by the results of reversibility studies which show that receptor blockade produced by Icatibant is reversed more slowly than that produced by MEN 11270. Moreover, the functional response to bradykinin develops quickly and undergoes fading: this, coupled with the relatively slow dissociation of Icatibant from the bradykinin B_2 receptor, yields non-equilibrium conditions in the time frame of the concentration–response curve in organ bath experiments, leading to the apparent non-competitive behaviour of this antagonist. In favour of this interpretation is the observation that antagonists that bind tightly to, and dissociate slowly from, the receptor produce insurmountable antagonism, irrespective of the mechanism of interaction (competitive/non-competitive) with the receptor (Kenakin, 1997b; Fierens et al., 1999). In support of this interpretation, when tested against FR190997, an agonist with onset/offset kinetic markedly slower than bradykinin, both MEN 11270 and Icatibant displayed a competitive-like antagonist behaviour.

Asano et al. (1997) showed that FR173657, in the concentration range 1–10 nM, antagonized bradykinin induced contractions in a competitive manner (pA_2 9.2), although the Schild plot indicated a slope greater than unity. Others (Griesbacher et al., 1997; Rizzi et al., 1997) showed that this antagonist, in a higher concentration range (10–1000 nM), had a non-competitive behaviour, but the same apparent affinity. Our data reconcile the results presented in the above mentioned studies, since we found that at 3 nM (a concentration in the range of those studied by Asano et al., 1997) FR173657 determines a rightward shift of the concentration–response curve to bradykinin without depressing E_{max}, whereas a non-competitive behaviour was evident at higher concentrations.

Contrary to the peptide antagonists, FR173657 displaced the binding of [^{3}H]bradykinin with an affinity comparable to that measured in the functional assay (Paquet et al., 1999), but the competition binding curves had a Hill slope significantly less than unity, indicating a two-sites competitive binding model as significant.

The non-peptide bradykinin B_2 receptor agonist, FR190997, has been reported to act as a partial agonist in this and other assays (Aramori et al., 1997b; Asano et al., 1998; Rizzi et al., 1999): in these studies the peak response produced by FR190997 has been used to provide an estimate of its agonist efficacy. However, if measuring events (such as contraction of smooth muscle) which are distant from the initial agonist–receptor interaction, it appears likely that kinetic factors could heavily influence the estimate of the properties of the agonist. We showed that the estimate of agonist efficacy of FR190997 in the guinea-pig ileum is markedly influenced by the mode of administration: depending on the cumulative or single dose application, FR190997 could be labeled either as a partial or a full agonist, respectively. The reasons for this difference are not immediately evident. Moreover, since the contractile response to FR190997 does not show significant fading, either after cumulative and single dose application, it does not appears that desensitization of bradykinin B_2 receptor is involved. From a kinetic point of view, the responses induced by FR190997 are much slower, in both onset and offset, as compared to those produced by bradykinin, besides to be persistent and extremely resistant to the washout, such a different protocol was used (see Section 2) to study the activity of antagonists towards FR190997. It may be speculated that qualitative and/or quantitative differences exist between bradykinin and FR190997 in terms of activation of different second messenger pathways (as also suggested by Rizzi et al., 1999) which eventually determine the final response under study. Notably, also the non-peptide agonist FR190997, which is a close structural analog of FR173657, displayed a similar behaviour in the binding assay: in this perspective, the fact that the analysis of curves of both non-peptide ligands, with a two-site competitive binding model resulted significant, strongly indicates the ability of these ligands to recognize differently the [^{3}H]bradykinin binding sites.

When FR173657 was tested towards the agonist responses induced by FR190997, it displayed a non-competitive behaviour even at the lowest effective concentration (3 nM), and its apparent affinity was close to that determined in antagonism of bradykinin. On the other hand, the fact that the peptide antagonists, MEN 11270 and Icatibant, antagonize the functional responses of the non-peptide agonist FR190997 with much lower potency (apparent pA_2 values 7.2 and 6.9, respectively), may be alternatively (see above) explained by a different binding mode at the receptor, which results in a less capability to hamper the receptor conformational changes induced by FR190997.

A minor potency of FR173657 as compared to that of Icatibant (Griesbacher and Legat, 1997; Griesbacher et al., 1998) and MEN 11270 (Meini et al., 2000) in different in vivo models has been reported. Nevertheless, the reasons for the less in vivo potency of FR173657 cannot be ascribable to the estimated affinity obtained in in vitro functional studies, but rather to differences due to pharmacokinetic factors (Meini et al., 2000).

In conclusion, both non-peptide ligands, agonist and antagonist, despite their opposite behaviour in terms of

receptor activation, share some common pharmacological features: (i) displace with a similar affinity and analogue behaviour (shallow/two-sites displacement curves) of the [^{3}H]bradykinin binding, (ii) show a similar kinetic profile of interaction with bradykinin B_2 receptors (slow reversibility of the antagonist, slow onset and offset of contraction of the agonist). These observations and the much lower antagonist effect of MEN 11270 and Icatibant, but not FR173657 (which is chemically related to FR190997) against FR190997, than bradykinin-induced contractions, together with the different pharmacological profile of the two agonists, suggest that the peptide (bradykinin) and the non-peptide (FR190997) agonists stabilize active receptor conformations which are different. It may be speculated that the MEN 11270 and Icatibant bind at a receptor epitope which is less involved in the conformational changes induced by FR190997 than those induced by bradykinin. In contrast, the binding site of FR173657 may be such that both the bradykinin- and FR190997-induced preferential conformations are hampered.

References

Altamura, M., Meini, S., Quartara, L., Maggi, C.A., 1999. Nonpeptide antagonists for kinin receptors. Regul. Peptides 80, 13–26.

Aramori, I., Zenkoh, J., Morikawa, N., O'Donnell, N., Asano, M., Nakamura, K., Iwami, M., Kojo, H., Notsu, Y., 1997a. Novel subtype-selective nonpeptide bradykinin receptor antagonists FR167344 and FR173657. Mol. Pharmacol. 51, 171–176.

Aramori, I., Zenkoh, J., Morikawa, N., Asano, M., Hatori, C., Sawai, H., Kayakiri, H., Satoh, S., Inoue, T., Abe, Y., Sawada, Y., Mizutani, T., Inamura, N., Nakahara, K., Hitoshi, K., Oku, T., Notsu, Y., 1997b. Nonpeptide mimic of bradykinin with long-acting properties at the bradykinin B_2 receptor. Mol. Pharmacol. 52, 16–20.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. Chemother. 14, 48–58.

Asano, M., Inamura, N., Hatori, C., Sawai, H., Fujiwara, T., Katayama, A., Kayakiri, H., Satoh, S., Abe, Y., Inoue, T., Sawada, Y., Nakahara, K., Oku, T., Okuhara, M., 1997. The identification of an orally active, nonpeptide bradykinin B_2 receptor antagonist, FR173657. Br. J. Pharmacol. 120, 617–624.

Asano, M., Hatori, C., Sawai, H., Johki, S., Inamura, N., Kayakiri, H., Satoh, S., Abe, Y., Inoue, T., Sawada, Y., Mizutani, T., Oku, T., Nakahara, K., 1998. Pharmacological characterization of a nonpeptide bradykinin B2 receptor antagonist FR165649, and agonist FR190997. Br. J. Pharmacol. 124, 441–446.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 75, 248–254.

Eggerickx, D., Raspe, E., Bertrand, D., Vassart, G., Parmentier, M., 1992. Molecular cloning, functional expression and pharmacological characterisation of a human bradykinin B_2 receptor gene. Biochem. Biophys. Res. Commun. 187, 1306–1313.

Fierens, F.L.P., Vanderheyden, P.M.L., De Backer, J-P., Vauquelin, G., 1999. Insurmountable angiotensin AT_1 receptor antagonists: the role of tight antagonist binding. Eur. J. Pharmacol. 372, 199–206.

Griesbacher, T., Legat, F.J., 1997. Effects of FR173657, a non-peptide B_2 antagonist, on kinin-induced hypotension, visceral and peripheral oedema formation and bronchoconstriction. Br. J. Pharmacol. 120, 933–939.

Griesbacher, T., Lembeck, F., 1992. Analysis of the antagonistic actions of HOE 140 and other novel bradykinin analogues on the guinea-pig ileum. Eur. J. Pharmacol. 211, 393–398.

Griesbacher, T., Sametz, W., Legat, F.J., Diethart, S., Hammer, S., Juan, H., 1997. Effects of the non-peptide B_2 antagonist FR173657 on kinin-induced smooth muscle contraction and relaxation, vasoconstriction and prostaglandin release. Br. J. Pharmacol. 121, 469–476.

Griesbacher, T., Amann, R., Sametz, W., Diethart, S., Juan, H., 1998. The nonpeptide B_2 receptor antagonist FR173657: inhibition of effects of bradykinin related to its role in nociception. Br. J. Pharmacol. 124, 1328–1334.

Guba, W., Haessner, R., Breiphol, G., Henke, S., Knolle, J., Santagada, V., Kessler, H., 1994. Combined approach of NMR and molecular dynamics within a biphasic membrane mimetic: conformation and orientation of the bradykinin antagonist Hoe 140. J. Am. Chem. Soc. 116, 7532–7540.

Hall, J.M., 1992. Bradykinin receptors: pharmacological properties and biological roles. Pharmacol. Ther. 56, 131–190.

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.T., Breipohl, G., König, W., Knolle, J., Schölkens, B.A., 1991. Icatibant a new potent and long acting bradykinin antagonist: in vitro studies. Br. J. Pharmacol. 102, 769–773.

Kenakin, T.P., 1997a. Competitive antagonism. Pharmacologic Analysis of Drug–Receptor Interaction. 3rd edn. Lippincott-Raven Press Publishers, Philadelphia, pp. 331–373.

Kenakin, T.P., 1997b. Allotopic, noncompetitive, and irreversible antagoinism. Pharmacologic Analysis of Drug–Receptor Interaction. 3rd edn. Lippincott-Raven Press Publishers, Philadelphia, pp. 374–395.

Manning, D.C., Vavrek, R., Stewart, J.M., Snyder, S.H., 1986. Two bradykinin binding sites with picomolar affinities. J. Pharmacol. Exp. Ther. 237, 504–512.

Meini, S., Quartara, L., Rizzi, A., Patacchini, R., Cucchi, P., Giolitti, A., Calò, G., Regoli, D., Criscuoli, M., Maggi, C.A., 1999. MEN 11270, a novel selective constrained peptide antagonist with haigh affinity at the human B_2 kinin receptor. J. Pharmacol. Exp. Ther. 289, 1250–1256.

Meini, S., Patacchini, R., Giuliani, S., Lazzeri, M., Turini, D., Maggi, C.A., Lecci, A., 2000. Characterization of bradykinin B_2 receptor antagonists in human and rat urinary bladder. Eur. J. Pharmacol. 388, 177–182.

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. Biochem. Chem. 269, 21583–21586.

Paquet, J-L., Luccarini, J-M., Fouchet, C., Defrene, E., Loillier, B., Robert, C., Belichard, P., Cremers, B., Pruneau, D., 1999. Pharmacological characterization of the bradykinin B_2 receptor: inter-species variability and dissociation between binding and functional responses. Br. J. Pharmacol. 126, 1083–1090.

Paton, W.D.M., Vizi, E.S., 1969. The inhibitory action of noradrenaline on acetylcholine output by guinea-pig ileum longitudinal smooth muscle. Br. J. Pharmacol. 35, 10–28.

Pruneau, D., Luccarini, J.M., Defrêne, E., Paquet, J.L., Bélichard, P., 1995. Pharmacological evidence for a single bradykinin B_2 receptor in the guinea-pig. Br. J. Pharmacol. 116, 2106–2112.

Ransom, R.W., Goodman, C.B., Young, G.S., 1992. Bradykinin stimulation of phosphoinositide hydrolysis in guinea-pig ileum longitudinal muscle. Br. J. Pharmacol. 105, 919–924.

Regoli, D., Barabé, J., 1980. Pharmacology of bradykinin and related kinins. Pharmacol. Rev. 32, 1–46.

Rhaleb, N-E., Rouissi, N., Jukic, D., Regoli, D., Henke, S., Breipohl, G.,

Knolle, J., 1992. Pharmacological characterization of a new highly potent B2 receptor antagonist (HOE 140: D-Arg-[Hyp3, Thi5, D-Tic7, Oic8]bradykinin). Eur. J. Pharmacol. 210, 115–120.

Rizzi, A., Gobeil, F., Bogoni, G., Calò, G., Campobasso, C., Inamura, N., Regoli, D., 1997. Antagonistic effects of FR173657 on human, pig, rabbit, and guinea pig kinin receptors: an in vitro study. Can. J. Physiol. Pharmacol. 75, 601–608.

Rizzi, A., Rizzi, C., Amadesi, S., Calò, G., Varani, K., Inamura, N., Regoli, D., 1999. Pharmacological characterisation of the first non-peptide bradykinin B2 receptor agonist FR190997: an in vitro study on human, rabbit and pig vascular B2 receptors. Naunyn-Schmiedeberg's Arch. Pharmacol. 360, 361–367.

Stewart, J.M., Gera, L., Hanson, W., Zuzack, J.S., Burkard, M., McCullough, R., Whalley, E.T., 1996. A new generation of bradykinin antagonists. Immunopharmacology 33, 51–60.

Wirth, K., Hock, F.J., Albus, U., Linz, W., Alpermann, H.G., Anagnostopoulos, H., Henke, S., Breipohl, G., Konig, 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.