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Pharmacological characterization of a nonpeptide bradykinin B_2 receptor antagonist, FR165649, and agonist, FR190997

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1 The nonpeptide bradykinin (BK) B_2 receptor antagonist, FR165649 (8-[2,6-dichloro-3-[N-[(E)-4-(N-methylcarbamoyl)cinnamidoacetyl]-N-methylamino]benzyloxy]-2-methylquinoline), and agonist, FR190997 (8-[2,6-dichloro-3-[N-[(E)-4-(N-methylcarbamoyl) cinnamidoacetyl]-N-methylamino]benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline) have been identified. These compounds have a common chemical structure, and the 2-pyridylmethoxy group is the only structural difference between them.

2 Both FR165649 and FR190997 displaced [^{3}H]-BK binding to B_2 receptors in guinea-pig ileum membranes, with an IC_{50} of 4.7×10^{-10} M and 1.5×10^{-9} M, respectively. They also displaced [^{3}H]-BK binding to B_2 receptors in human lung fibroblast IMR-90 cells, with an IC_{50} of 1.6×10^{-9} M and 9.8×10^{-10} M, respectively.

3 In guinea-pig isolated ileum-preparations, FR165649 had no agonistic effect on contraction and caused parallel rightward shifts of the concentration-response curves to BK on contraction. Analysis of the data produced a nominal pA_2 value of 9.2 ± 0.1 ($n=5$) and a slope of 1.4 ± 0.1 ($n=5$). On the other hand, FR190997 induced concentration-dependent contraction of guinea-pig ilea with a pD_2 of 7.9 ± 0.2 and the contraction was inhibited by a specific peptide bradykinin B_2 receptor antagonist, Hoe 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK) in a non-competitive manner.

4 In IMR-90 cells, FR165649 had no agonistic effect on phosphatidyl inositol (PI) hydrolysis and caused parallel rightward shifts (approximately 200 fold shift at 10^{-7} M) of the concentration-response curves to BK on PI hydrolysis. FR190997 induced concentration-dependent PI hydrolysis in IMR-90 cells with a pD_2 of 8.4 ± 0.1, and this effect was inhibited by Hoe 140.

5 These results indicate that FR165649 and FR190997 are, respectively, a potent bradykinin B_2 receptor antagonist and agonist, and that the agonistic activity depends on the small part of the nonpeptide ligand. FR165649 and FR190997 may be useful tools for studying the relationship between ligands and receptors.

Keywords: Bradykinin; nonpeptide antagonist; nonpeptide agonist; B_2 receptor; FR165649; FR190997

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 a potentially important role 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 BK B_2 receptors (Bhoola *et al*., 1992).

To investigate the pathophysiological role of BK and to develop a drug for inflammatory diseases, many BK receptor antagonists have been synthesized (Hock *et al*., 1991; Cheronis *et al*., 1992; Sawutz *et al*., 1994; Stewart, 1995). Recently, we have described nonpeptide BK B_2 receptor antagonists (Aramori *et al*., 1997b; Asano *et al*., 1997a, b; Inamura *et al*., 1997). They are potent, specific, orally active and long-acting. Therefore, they will be not only good tools for studying the pathophysiological role of BK but also useful for the treatment of asthma and other inflammatory diseases. Moreover, we have found a nonpeptide B_2 receptor agonist, FR190997 (8-[2-6-dichloro-3-[N-[(E)-4-(N-methylcarbamoyl)-cinnamidoacetyl]-N-methylamino]benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline) (Aramori *et al*., 1997a) (Figure 1b) in the process of the screening for B_2 receptor antagonists. This compound stimulated phosphatidyl inositol (PI) hydrolysis in Chinese hamster ovary cells expressing the human BK B_2 receptor and induced hypotensive responses in rats (Aramori *et al*., 1997a). However, the antagonistic effect of FR190997 on normal tissues and normal cells remains to be clarified. On the other hand, we also identified another potent BK B_2 receptor antagonist, FR165649 (8-[2,6-dichloro-3-[N-[(E)-4-(N-methylcarbamoyl)cinnamidoacetyl]-N-methylamino]benzyloxy]2-methylquinoline) (Figure 1a) that has a similar chemical structure to FR190997.

In the present study, we examined the pharmacological effects of FR165649 and FR190997 on the same normal tissues and cells in order to elucidate the difference and similarity between nonpeptide antagonists and agonists. Also, these compounds may present useful tools for studying the relationship between ligands and receptors.

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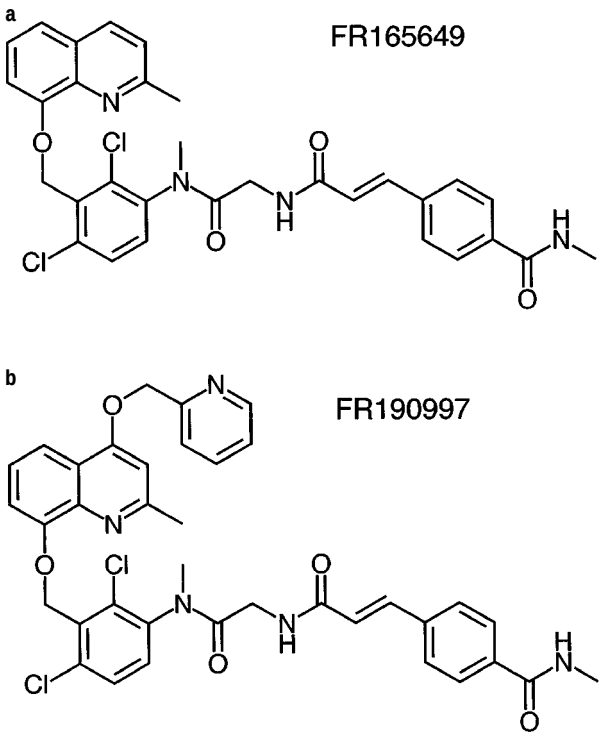

Figure 1 Chemical structure of FR165649 (a) (8-[2,6-dichloro-3-[N-[(E)-4-(N-methylcarbamoyl)cinnamidoacetyl]-N-methylamino]benzyloxy]-2-methylquinoline) and FR190997 (b) (8-[2,6-dichloro-3-[N-[(E)-4-(N-methylcarbamoyl)cinnamidoacetyl]-N-methylamino]benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline).

Methods

Receptor binding

Guinea-pig ileum The specific binding of [^{3}H]-BK (a high affinity B_2 ligand) was assayed according to the method previously described by Asano *et al.* (1997b). 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 N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) and 1 mM 1,10-phenathroline, pH 6.8) with a 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 used.

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 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 unlabeled BK) from total binding. The experiments were performed three times in duplicate.

Human fibroblast cells The human fibroblast binding assay was performed as previously described by Asano *et al.* (1997b). IMR-90 cells, 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 a concentration of 10^5 cells per well before the assay. The cells were washed twice with phosphate-buffered saline (PBS) containing 0.1% BSA, then incubated with [^{3}H]-BK (final concentration 1 nM) and test compounds 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 determined in the presence of 1 μM unlabelled BK. At the end of the incubation, the buffer was aspirated and the cells were washed three times with PBS containing 0.1% BSA. Bound radioactivity was determined by solubilizing the cells with 1% sodium dodecyl sulphate containing 0.05 M NaOH and quantitating in a liquid scintillation counter. The experiments were performed three times in duplicate.

Smooth muscle contraction in guinea-pig ileum

Guinea-pig ileum contraction by BK 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 mM: NaCl 137, KCl 2.7, $MgCl_2$ 1.1, $CaCl_2$ 1.8, $NaHCO_3$ 12, $NaHPO_4$ 0.4 and glucose 5.6), maintained at 37°C 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. After an equilibration period of about 30 min, a stable baseline tone was reached and two or three contractions were induced by BK (6×10^{-8} M); then 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 were used. The strips were contracted with BK in a cumulative manner, 9 concentrations between 10^{-9} M and 10^{-5} M being used. A further resting period of 30 min was allowed after the strips had been washed 5 times with Tyrode solution. FR165649 (5.6×10^{-10}, 1.8×10^{-9} or 5.6×10^{-9} M) was added to the organ bath 10 min before the construction of a second BK concentration-response curve. In control tisues, FR165649 was replaced by its vehicle, dimethylsulphoxide (DMSO), at a final concentration of 0.1% (v/v). All responses to BK either in absence or presence of FR165649 are expressed as a percentage of the maximal effect of BK established during the first concentration-response curve (Griesbacher, 1992). The experiment was performed in 4 different strips from the same animal (for three concentrations of FR165649 and control), and was repeated 4 times in different animals.

Acetylcholine (ACh) or histamine-induced contraction was measured by the same method as described above with minor modifications. Initial tension was set at 0.5 g. The temperature of the organ baths was maintained at 27°C. ACh or histamine was added at the concentration of 1×10^{-6} or 5×10^{-7} M, respectively. The last control response was taken as 100% and subsequent responses to ACh or histamine obtained in the presence of FR165649 were expressed as a percentage of this. The segments were incubated with the BK antagonists for 10 min before ACh or histamine was added.

For the measurement of the agonistic activity of FR190997, the strips of guinea-pig ileum were contracted with FR190997

in a cumulative manner, 5 concentrations between 10^{-9} M and 10^{-5} M in the absence and presence of a specific B_2 receptor antagonist, Hoe 140 (10^{-7} M) with the same methods as described above. All responses to FR190997 either in absence or presence of Hoe 140 are expressed as a percentage of the maximal effect of BK established during the first concentration-response curve.

PI hydrolysis in IMR-90-cells

Phosphatidyl inositol (PI) hydrolysis was measured according to the method previously described (Aramori *et al*., 1997b). IMR-90 cells were seeded in 12-well tissue culture plates at a concentration of 10^5 cells per well and cultured overnight. The cells were labelled with [^{3}H]-inositol (1 μCi ml^{-1}) for 24 h. The cells were washed twice with PBS containing 0.2% BSA and incubated with the same solution for 30 min, and then incubated with PBS containing 0.2% BSA and 10 mM LiCl for 30 min at 37°C. Agonist stimulation was started by replacing the medium with fresh PBS containing 0.2% BSA, 10 mM LiCl and test compounds. In control wells, the test compounds were replaced by its vehicle, DMSO, at a final concentration of 0.1% (v/v). The reaction was terminated by 5% (w/v) trichloroacetic acid after incubation for 30 min at 37°C. Separation of [^{3}H]-inositol phosphates was carried out by BioRad (Richmond, U.S.A.) AG1X8 chromatography as previously described (Berridge *et al*., 1983). Inositol phosphates (IPs) were eluted with 0.1 M formic acid and 1.0 M ammonium formate. The radioactivity in the elutes was determined by a liquid scintillation counter. All responses to the compounds are expressed as a percentage of the radioactivity of control (0.1% DMSO).

Materials

FR165649, FR190997 and Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7, Oic^8]BK) wre chemically synthesized in Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). BK, BSA, DMEM, penicillin, streptomycin, N-methyl-D-glucamine and captopril were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). [^{3}H]-BK was purchased from Dupont/NEN Research Products (Wilmington, U.S.A.) or Zeneca (Cheshire, U.K.). [^{3}H]-inositol was purchased from Amersham (Arlington Heights, U.S.A.). All other compounds were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). FR165649 and FR190997 were dissolved in DMSO and diluted with appropriate buffer.

Data analysis

The results are expressed as the mean ± s.e.mean. After the data were tested for differences in variations with the *F*-test and for normality of distribution, appropriate statistical analysis was chosen. Statistical significance of difference between groups was analysed by Kruskal-Wallis test (three or more groups with unequal variations), Student's *t* test (two groups with equal variations) or Welch's *t* test (two groups with unequal variations). IC_{50} or ED_{50} values were 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 (1973). A dose-ratio (DR) was calculated from the ED_{50} of the concentration-response curve in the presence of FR165649 divided by the ED_{50} for the individual concentration-response curve of bradykinin alone. The nominal pA_2 and slope were calculated by Schild plot (Schild, 1947) from three DR in the same animal and the mean values were calculated from 5 different nominal pA_2s and slopes obtained by repeated experiments.

Results

Receptor binding

Both FR165649 and FR190997 potently displaced [^{3}H]-BK binding to B_2 receptors in guinea-pig ileum membranes with an IC_{50} of 4.7×10^{-10} M and 1.5×10^{-9} M, respectively ($K_i = 0.9$ and 2.9×10^{-10} M, respectively) (Figure 2a). They also potently displaced [^{3}H]-BK binding to B_2 receptors in human lung fibroblast, IMR-90 cells with an IC_{50} of 1.6×10^{-9} M and 9.8×10^{-10} M, respectively ($K_i = 2.0$ and 1.2×10^{-10} M, respectively) (Figure 2b). There was no significant difference between the IC_{50} or K_i of FR165649 and that of FR190997 in guinea-pig ilea and human fibroblasts (Student's *t* test).

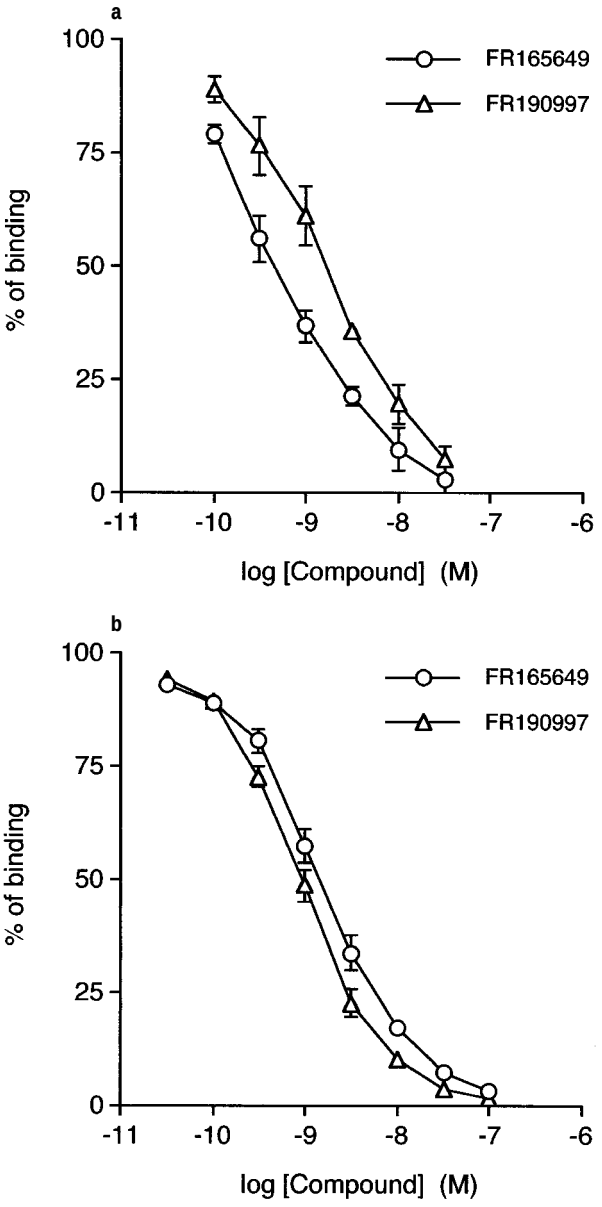

Figure 2 Effects of FR165649 and FR190997 on [^{3}H]-BK binding to B_2 receptors in guinea-pig ileum membranes (a) and IMR-90 cells (b). Guinea-pig ileum membranes or IMR-90 cells were incubated with [^{3}H]-BK and increasing concentrations of FR165649 or FR190997. Data are expressed as mean and vertical lines show s.e.mean ($n = 3$).

Smooth muscle contraction in guinea-pig ileum

In guinea-pig isolated ileum-preparations, FR165649 had no agonistic effect on contraction, and it caused parallel rightward shifts of the concentration-contractile response curves for BK at concentrations of 5.6×10^{-10}, 1.8×10^{-9} and 5.6×10^{-9} M (Figure 3a). Analysis of the data produced a nominal pA_2 value of 9.2 ± 0.1 ($n=5$) and a slope of 1.4 ± 0.1 ($n=5$) (Figure 3b). The slope was significantly different from unity. The compound had almost no effect on ACh or histamine-induced guinea-pig ileum contraction even at 10^{-6} M (% inhibition was 6.9 ± 5.4 ($n=3$) or 5.6 ± 2.1 ($n=3$), respectively). After the removal of FR165649 (5.6×10^{-9} M) by washing, the contractile response to bradykinin was partially restored (data not shown).

On the other hand, FR190997 induced concentration-dependent contraction of guinea-pig ilea ($P<0.01$ by Kruskal-Wallis test), and the contraction was inhibited by a specific BK B_2 receptor antagonist, Hoe 140, in a non-competitive manner (Figure 4). The pD_2 of FR190997 was 7.9 ± 0.2 in this assay. The maximal contraction induced by FR190997 was approximately 60% of that induced by BK ($P<0.01$ by Student's t test). The pD_2 of BK was 7.6 ± 0.1. There was no significnt difference between the pD_2 of FR190997 and that of BK (Student's t test).

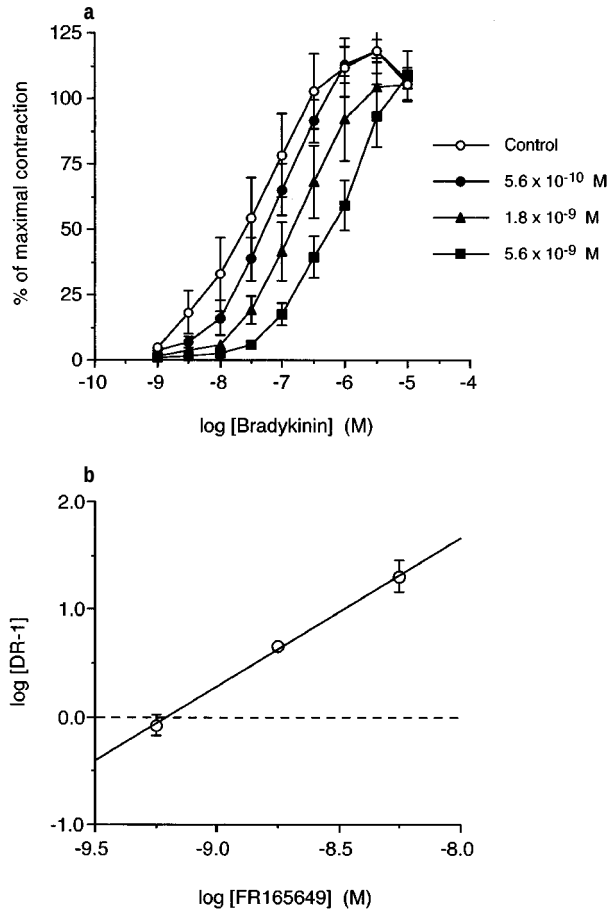

Figure 3 Effects of FR165649 (5.6×10^{-10}, 1.8×10^{-9} and 5.6×10^{-9} M) on concentration-response curves to BK in guinea-pig isolated ileum contraction and Schild analysis. (a) Dose-response curves; (b) Schild analysis. Data are expressed as mean and vertical lines show s.e.mean ($n=5$). All responses to BK either in absence or presence of FR165649 are expressed as a percentage of the maximal effect of BK established during the first concentration-response curve. Analysis of the data produced a nominal pA_2 value of 9.2 ± 0.1 ($n=5$) and a slope of 1.4 ± 0.1 ($n=5$).

PI hydrolysis in IMR-90 cells

BK induced concentration-dependent PI hydrolysis in IMR-90 cells with a pD_2 of 8.9 ± 0.1, and the reaction was inhibited by Hoe 140 (10^{-7} M). The maximal effect of BK on formation of inositol phosphates (IPs) was approximately 18 times as much as control (Figure 5). FR165649 had no agonistic effect on PI hydrolysis, and this compound inhibited BK-induced PI hydrolysis ($P<0.01$ by Student's or Welch's t test) with a parallel rightward shift (approximately 200 fold shift at 10^{-7} M) of the concentration-response curve to BK (Figure 5a). Hoe 140 (10^{-7} M) also caused a similar parallel rightward shift (approximately 200 fold shift) of the concentration response curve to BK.

FR190997 also induced concentration-dependent PI hydrolysis in IMR-90 cells ($P<0.05$ by Kruskal-Wallis test), and this PI hydrolysis was also inhibited by 10^{-7} M Hoe 140 with a parallel rightward shift of the concentration-response curve (Figure 5b). The pD_2 of FR190997 was 8.4 ± 0.1 in this assay. The maximal effect of FR190997 on formation of IPs was approximately 50% of that of BK ($P<0.05$ by Student's t test) (Figure 5b). There was a significant difference between the pD_2 of FR190997 and that of BK ($P<0.01$ by Student's t test).

Discussion

In the present study, we defined the pharmacological characterization of a nonpeptide BK B_2 receptor antagonist (FR165649) and agonist (FR190997) which have a common chemical structure. The structural difference between these compounds is only a '2-pyridylmethoxy' group. However, it was demonstrated that FR165649 is a potent B_2 receptor antagonist and FR190997 is a potent B_2 receptor agonist in guinea-pig ilea and human fibroblasts.

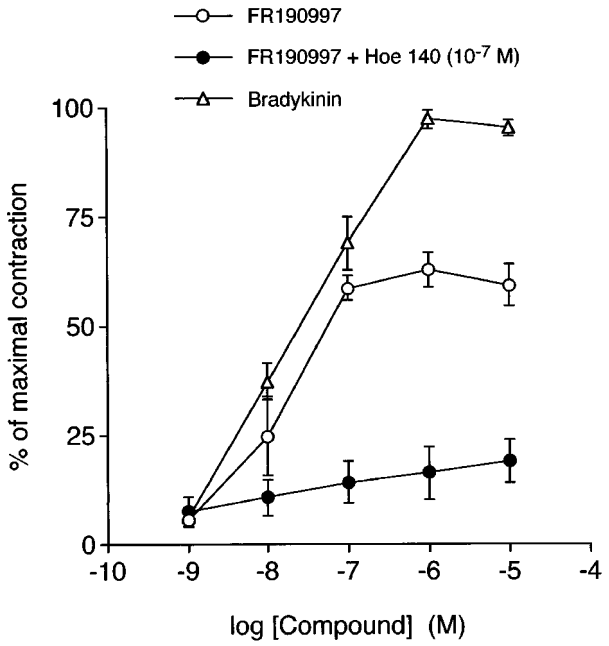

Figure 4 Effects of FR190997 on smooth muscle contraction in guinea-pig ileum. Data are expressed as mean and vertical lines show s.e.mean ($n=5$). All responses to FR190997 either in absence or presence of Hoe 140 (10^{-7} M) are expressed as a percentage of the maximal effect of BK established during the first concentration-response curve.

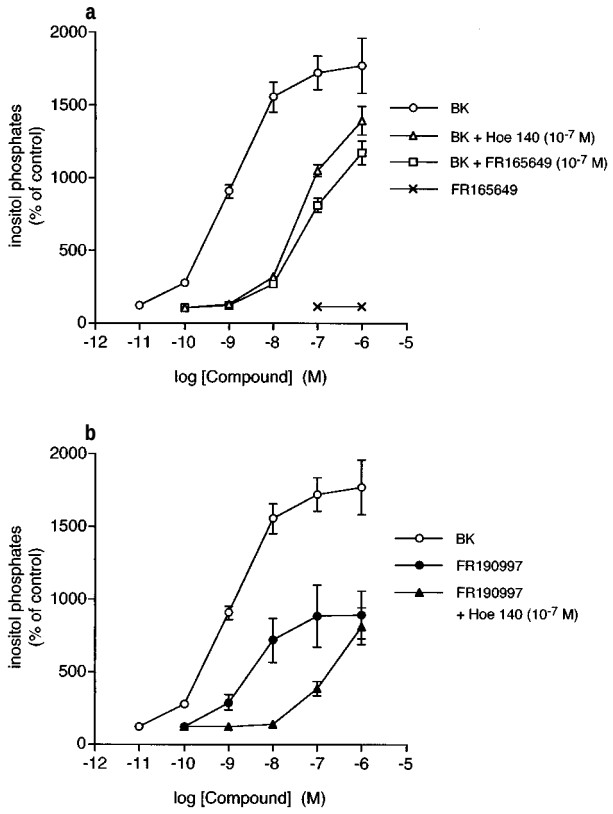

Figure 5 Effects of FR165649 (a) and FR190997 (b) on PI hydrolysis in IMR-90 cells. Data are expressed as mean and vertical lines show s.e.mean ($n=3$). All responses to the compounds are expressed as a percentage of the radioactivity of control (0.1% DMSO).

Antagonists and agonists usually have a similar structure and bind to the same binding site in receptors, but only agonists stimulate the receptors. In the two-state model of receptor activation, G-protein-coupled receptors are thought to be in an equilibrium between an inactive (R) and an active (R*) conformation. The binding of agonist to receptor shifts the equilibrium toward the active conformation resulting in productive receptor G-protein coupling (Gilman, 1987; Levitzki, 1988). FR190997, which has a structure quite different from the natural peptide ligand, may also shift the equilibrium toward the active conformation. Although a variety of nonpeptide receptor antagonists have been described (Moore *et al*., 1995), only a few nonpeptide agonists have been found such as an AT_1 agonist (Perlman *et al*., 1995), a CCK_A agonist (Aquino *et al*., 1996) and a κ-opioid agonist (Hunter *et al*., 1990), but, until now, a nonpeptide BK receptor agonist has not yet been determined. Thus, FR190997 is the first nonpeptide BK B_2 receptor agonst described (Aramori *et al*., 1997a). This study also demonstrates that this compound is a potent BK B_2 receptor agonist in normal tissues. To our knowledge, FR190997 is one of the most potent nonpeptide agonists in human cells.

Bradykinin B_2 receptors were expressed in guinea-pig ilea (Manning *et al*., 1986) and IMR-90 cells, human fibroblasts (Baenzinger *et al*., 1992; Sawutz *et al*., 1992). Our data demonstrating the displacement of [^{3}H]-BK indicate either that FR165649 and FR190997 bind to the BK binding site on B_2 receptor with high affinity, or that they bind to the allosteric binding site interfering with the binding of BK (Gether *et al*., 1993; Elling *et al*., 1995). Other results in this study suggest that the former may be more likely, but the possibility of the latter cannot be excluded. FR165649 caused parallel rightward shifts of the concentration-response curves to BK with no depression of the maximal response, suggesting that this compound may be a competitive antagonist in guinea-pig ileum. However, the slope of the Schild plot differed from unity, suggesting that other modes of antagonism cannot be excluded. FR190997 had a BK mimicking effect, and this activity was inhibited by Hoe 140 which has a similar structure to BK. These results suggest that FR190997 binds to the same binding site as BK.

FR165649 and FR190997 have almost the same IC_{50} for the displacement of [^{3}H]-BK in guinea-pig ilea and human fibroblasts. These data indicate that the affinity is not the determinant for agonists activity. The interaction between the '2-pyridylmethoxy' group and the binding site of B_2 receptor may stimulate the receptor to change into an active conformation. A similar result was obtained from a study on the angiotensin receptor (Perlman *et al*., 1997).

We observed that the maximal response induced by FR190997 was only 50–60% of that induced by BK in guinea-pig and human tissues *in vitro*. This indicates that the intrinsic activity of FR190997 is lower than BK. It is suggested that the interaction between FR190997 and the binding site of the B_2 receptor cannot completely alter the receptor into an active conformation. The pD_2 values of FR190997 were considerably higher in guinea-pig ilea and human fibroblasts. Therefore, FR190997 is found to be a fairly potent agonist, in agreement with data from a previous study (Aramori *et al*., 1997a). FR190997 seems to be more resistant to proteolytic degradation than BK, although we have no evidence for this. In fact this compound was long-acting *in vivo* (Aramori *et al*., 1997a). Long-activity may be useful for studying the pathophysiological role of BK. It is well-known that BK acts as a protective agent against renal failure (Uehara *et al*., 1994), myocardial ischaemia (Goto *et al*., 1995) and hypertension (Majima & Katori, 1995). FR190997 may have therapeutic potential against these diseases.

Another nonpeptide B_2 receptor antagonist, FR173657, has also recently been described (Asano *et al*., 1997b; Griesbacher *et al*., 1997; Rizzi *et al*., 1997). FR165649 has a similar chemical structure and almost the same activity as FR173657. However, FR165649 is more resistant to oxidative metabolism with liver homogenate (data not shown).

In conclusion, the present study showed the pharmacological effects of FR165649 and FR190997 on guinea-pig ilea and human fibroblasts, and demonstrated the difference and similarity between nonpeptide antagonists and agonists. The findings of the study suggest that these compounds may be useful tools for studying the relationship between ligands and receptors.

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