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A different molecular interaction of bradykinin and the synthetic agonist FR190997 with the human B_2 receptor: evidence from mutational analysis

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1 Binding affinity at the [^{3}H]-BK binding site and activity as inositol phosphate (IP) production by the peptide bradykinin (BK) and the nonpeptide FR190997 were studied at wild-type or point-mutated human B_2 receptors (hB_2R) expressed in CHO cells.

2 The effect of the following mutations were analyzed: E47A (TM1), W86A and T89A (TM2), I110A, L114A and S117A (TM3), T158A, M165T and L166F (TM4), T197A and S211A (TM5), F252A, W256A and F259A (TM6), S291A, F292A, Y295A and Y295F (TM7), and the double mutation W256A/Y295F.

3 As the wild-type receptor-binding affinity of FR190997 was 40-fold lower than BK, whereas their agonist potency was comparable, both agonists produced similar maximal effects (E_{max}). Mutations were evaluated as affecting the affinity and/or efficacy of FR190997 compared with BK.

4 Two mutations were found to impair the agonist affinity of both agonists drastically: W86A and F259A. BK agonist affinity (pEC_{50}) was reduced by 1400- and 150-fold, and that of FR190997 was reduced by 400- and 25-fold, at the W86A and F259A mutant B_2 receptors, respectively.

5 Contrary to BK, the affinity of FR190997 was selectively decreased at I110A, Y295A, and Y295F mutants ($>10^3$-fold), and a different efficacy was measured at the Y295 mutants, FR190997 being devoid of the capability to trigger IP production at Y295A mutant.

6 L114A, F252A, and W256A selectively impaired the efficacy of FR190997, whereas its binding affinity was not affected. As a consequence, FR190997 behaved as a high-affinity antagonist in blocking the IP production induced by BK.

7 The lack of capability of FR190997 to activate or to bind the double mutant W256A/Y295F suggests that these residues are part of the same binding site, which is also important for receptor activation by the nonpeptide ligand.

8 Overall, by means of mutational analysis, we indicate an hB_2R recognition site for the nonpeptide agonist FR190997 (between TM3, 6, and 7), different from that of BK, and show that in the same binding crevice some mutations (L114, W256, and F252) are selectively responsible for the agonist properties of only FR190997.

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Abbreviations: BK, bradykinin; CHO, Chinese hamster ovary cells; hB_2R, human B_2 receptor; IPs, inositol phosphates

Introduction

Bradykinin (BK) is a nonapeptide (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH), whose actions are mediated through the activation of the B_2 receptor (Regoli & Barabé, 1980) which belongs to the family of G-protein-coupled receptors (GPCRs) with seven transmembrane (TM) helices (Eggerickx *et al*., 1992; Hess *et al*., 1992). BK is involved in inflammatory processes, such as smooth muscle contraction, vasodilation, increased vascular permeability, recruitment of inflammatory cells, and proalgesic effects (Hall, 1992). On the other hand, BK is involved in the cardioprotective effects of ACE inhibitors and AT1 receptor antagonists (Baxter & Ebrahim, 2002), and its proteolytically resistant analogs have been proposed for promoting the delivery of chemotherapeutic drugs to brain tumors (Bartus *et al*., 1996; Emerich *et al*., 2001).

The discovery of nonpeptide receptor agonists able to mimic the biological activity of peptide hormones, which have limitations in terms of bioavailability and stability, has become one of the pharmaceutical goals of the last decade (Beeley,

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2000). The first nonpeptide agonist discovered at the B_2 receptor has been 8-[2,6-dichloro-3-[*N*-methylcarbamoyl)cinnamidoacetyl]-*N*-methylamino] benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline (FR190997) (Aramori *et al*., 1997). *In vivo* FR190997 is as potent as the endogenous ligand BK in inducing hypotension (Ueno *et al*., 1999) and proinflammatory effects (Ueno *et al*., 1998; Hayashi *et al*., 2001), but with a longer duration of action as a consequence of a greater resistance to proteolytic degradation. On the other hand, in *in vitro* studies, a long-lasting receptor activation by FR190997 has been reported (Altamura *et al*., 1999; Cuthbert, 1999; Gobeil *et al*., 1999). Differences between BK and FR190997 have been described with respect to their efficacy pattern (full *versus* partial agonism or antagonism), coupling mechanism (Rizzi *et al*., 1999; Meini *et al*., 2000; Santicioli *et al*., 2001), or receptor phosphorylation (Blaukat *et al*., 2001).

By site-directed mutagenesis at the human B_2 receptor (hB_2R), the present study aimed to identify molecular determinants that may be responsible for the complex pharmacological profile of the nonpeptide agonist FR190997 and the peptide hormone BK. BK and FR190997 were compared in inhibiting the [^{3}H]-BK binding, and in triggering the inositol phosphate (IP) production at the wild-type and a 19-point-mutated hB_2R. The receptor amino-acidic residues were selected in the TM portion of the receptor. Most of them were substituted into alanine, whereas some were mutated into the corresponding residue of the B_1 receptor sequence (M165T, L166F, S291N, Y295F; Menke *et al*., 1994).

Overall, by means of mutational analysis, we support a different binding mode of BK and FR190997 with the hB_2R, and indicate some mutations that specifically affect only the efficacy of the nonpeptide molecule FR190997.

Methods

Stable transfection in CHO cells of pooled B_2 receptor clones

Large-scale preparation of vector DNA for transfection experiments was carried out using a Qiagen maxi-preparation column (Qiagen). Human wild-type and mutated B_2 receptor cDNAs in pmCMVβSV1dhfr were introduced by lipofection into DHFR-deficient CHO DUKX-B11 cells. Stable DHFR$^+$ transformants were selected in nucleoside-free α-MEM containing fetal bovine serum (FBS) dialyzed 5%; 12–14 days after transfection, more than 100 individual DHFR$^+$ clones stably expressing the human B_2 receptor were pooled, and cultured in Iscove's modified Dulbecco's Medium (IMDM) with L-glutamine (2 mM) and FBS dialyzed 10%. The cells were subcultured by using trypsin 0.25% and ethylenediaminetetraacetate (EDTA, 1 mM) to detach them, and then cultured in 175 cm^2 flasks and maintained in a humidified atmosphere at 37°C with 5% CO_2.

Membrane preparation

Cells at confluence were washed out of the medium by Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium (Ca/Mg free), and harvested by incubating at 37°C with Hanks buffered salt solution (HBSS, pH 7.4) supplemented with *N*-[2-hydroxy-ethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES, 10 mM), EDTA (1 mM), and a cocktail of peptidase inhibitors: 1,10-phenanthroline (1 mM), ethylene glycol *bis*(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA, 1 mM), captopril, leupeptin, soybean trypsin inhibitor, DL-2-mercaptomethyl-3-guanidoethylthiopropanoic acid (MERGETPA) (1 μM each), chymostatin (3.3 μM), phenylmethyl sulfonyl fluoride (PMSF, 0.1 mM), and bacitracin (140 μg ml^{-1}). Cells were then washed in *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES, 10 mM, pH 7.4, at 4°C), containing the above-described peptidase inhibitors' cocktail, and homogenized with a Polytron (PT 3000, Kinematica), set at 15,000 r.p.m. for 30 s. The homogenate was centrifuged at 45,000 × *g* for 45 min (4°C). The pellet was resuspended to obtain 7.5 mg ml^{-1} membrane protein concentration, and frozen immediately in 1 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) with a Bio-Rad kit, using bovine serum albumin (BSA) as the reference standard. Immediately prior to use, frozen membrane aliquots were thawed in binding buffer (see below) and mixed to yield a homogeneous membrane suspension.

Radioligand binding

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 BSA (1 g l^{-1}). Binding assay was performed in a final volume of 0.5 ml. An incubation time of 60 min at room temperature was used. Competition-binding experiments were carried out at the [^{3}H]-BK radioligand-binding site of 0.15–0.2 nM. At this concentration, radioligand bound less than 10% of the total added concentration. Preliminary experiments were performed to determine membrane protein concentration (90–200 μg ml^{-1}) of each receptor, in order to obtain a signal of 1500–3000 dpm per assay of specific binding. Nonspecific binding was defined as the amount of labelled radioligand bound in the presence of 1 μM of cold BK, and represented less than 10 and 30% of total bound [^{3}H]-BK. The agonist ligand inhibition curves were tested in a wide range of concentrations (1 pM–10 μM). Each experiment was performed in duplicate. All incubations were terminated by rapid filtration through UniFilter-96 plates (Packard) that had been presoaked for at least 2 h in polyethylenimine (PEI) 0.6%, using a MicroMate 96 Cell Harvester (Packard Instrument Company). The tubes and filters were then washed five times with 0.5 ml aliquots of Tris buffer (50 mM, pH 7.4, 4°C). Filters were dried and soaked in 50 μl per well of Microscint 40 (Packard Instrument Company), and the bound radioactivity was counted by a TopCount Microplate Scintillation Counter (Packard Instrument Company).

IP determination

Cells were grown in 24-well tissue culture clusters, and labelled for 24 h with *myo*-[1,2-^{3}H] inositol (0.5 ml per well, 1 μCi ml^{-1}) in IMDM and Ham's F12 medium (F12) (1 : 1) containing FBS dialyzed 1% and L-glutamine (2 mM). After a 15-min preincubation period at 37°C in a buffer consisting of Ca-/Mg-free PBS (135 mM), HEPES (20 mM), $CaCl_2$ (2 mM), $MgSO_4$ (1.2 mM), EGTA (1 mM), glucose (11.1 mM), BSA 0.05%, and LiCl (25 mM) (IP buffer), cells were incubated

for 30 min at 37°C in 0.5 ml of IP buffer added with different concentrations of agonist. In experiments that investigated the effect of FR190997 as an antagonist, it was added 15 min prior to stimulation with BK. The reaction was stopped by 1 ml of ice-cold mixture methanol and HCl 0.1 N (1 : 1, v v^{-1}), and samples were applied to a Bio-Rad AG1X8 column. The columns were washed twice with 6 ml of ammonium formate (0.06 M) in sodium tetraborate (0.005 M) to remove free inositol. After these washing steps, the total [^{3}H]IPs were eluted with two 3 ml aliquots of ammonium formate (1.2 M) in formic acid (0.1 M). The radioactivity in the eluates was determined by a liquid β-scintillation counter (2200 CA, Packard). Determinations were carried out in triplicate.

Data analysis

All values in the text, tables, or figures are means and 95% confidence limits of the means (c.l.), or mean ± s.e.m., of the given number of experiments (n). Radioligand-binding competition data and concentration–response curves for phosphoinositide hydrolysis were analyzed by fitting the data with the GraphPad Prism program (San Diego, CA, U.S.A.) in order to determine the −log of the molar concentration of the ligand producing 50% of the inhibition of radioligand binding (pIC_{50}), or of the agonist producing 50% of its maximal effect (pEC_{50}). When FR190997 was evaluated as an antagonist at those mutants where it did not show agonist activity, the antagonist affinity was expressed in terms of pA_2, calculated from the equation $pA_2 = \log[CR-1] - \log[\text{antagonist concentration}]$, where CR (concentration ratio) is the ratio between the EC_{50} values of BK in the presence and absence of FR190997 (Kenakin, 1997).

Drugs

[^{3}H]-BK (specific activity 90 Ci mmol^{-1}) and *myo*-[1,2-^{3}H] inositol (specific activity 74.7 Ci mmol^{-1}) were provided by Perkin-Elmer New England Nuclear (Boston, MA, U.S.A). BK was obtained from Peninsula (St Helens, U.K.). FR190997 was a kind gift from Fujisawa Pharmaceuticals. FR190997 was dissolved in dimethylsulfoxide up to 100 μM. Leupeptin was obtained from Boehringer Mannheim (Germany) and Thiorphan was obtained from Bachem (Essex, U.K.). MERGEPTA was obtained from Calbiochem (La Jolla, CA, U.S.A.). All salts used were purchased from Merck (Darmstadt, Germany). All other materials were obtained from Sigma (St Louis, LA, U.S.A.). All the compounds were stored at −25°C.

Results

Competition-binding experiments performed at the wild-type hB_2R [^{3}H]-BK-binding site indicated that the affinity of FR190997 was 40-fold lower than that measured with BK itself (Table 1). On the contrary, BK and FR190997 induced comparable concentration-dependent responses (0.1 nM–1 μM concentration range) in the IP production assay (Figure 1, Table 2).

The ligand-independent spontaneous activity of the mutant receptors was analyzed and compared to that of the wild-type hB_2R. No significant differences could be detected in terms of basal cellular IP hydrolysis (data not shown). The saturating concentration of [^{3}H]-BK (2 nM) indicated variable maximal specific binding (B_{max}) among receptors varying between 80 and 600 fmol mg^{-1} of proteins (Table 1). On the other hand, the variability of the maximal response in terms of IP production (E_{max}) obtainable with BK at the different

Table 1 Binding affinities of BK and the nonpeptide ligand FR190997 for the wild-type and mutant human B_2 receptors

hB_2R	*TM*	*pIC_{50} (95% c.l.)*		B_{max} (fmol mg^{-1} *proteins*)
		BK	*FR190997*	
Wild type		9.5 (9.5–9.6)	7.9 (7.7–8.0)	393 (385–402)
E47A	1	9.6 (9.6–9.7)	7.1 (7.0–7.3)	582 (576–590)
W86A	2	n.d.	—	—
T89A	2	9.6 (9.4–9.7)	8.2 (8.0–8.4)	344 (337–351)
I110A	3	9.8 (9.7–9.8)	5.2 (5.1–5.3)	414 (396–433)
L114A	3	9.5 (9.5–9.6)	8.5 (8.4–8.6)	216 (209–222)
S117A	3	9.0 (8.9–9.1)	8.0 (7.8–8.1)	84 (80–88)
T158A	4	9.4 (9.2–9.5)	7.6 (7.4–7.8)	471 (463–479)
M165T	4	9.0 (8.7–9.3)	8.4 (8.2–8.6)	223 (216–230)
L166F	4	9.3 (9.3–9.4)	7.9 (7.8–8.1)	589 (507–616)
T197A	5	9.3 (9.1–9.5)	8.5 (8.3–8.7)	496 (482–510)
S211A	5	9.0 (8.6–9.4)	8.0 (7.8–8.2)	470 (468–472)
F252A	6	9.5 (9.5–9.6)	8.5 (8.4–8.5)	218 (214–222)
W256A	6	9.6 (9.4–9.8)	8.5 (8.4–8.6)	138 (135–141)
F259A	6	n.d.	—	—
W256A/Y295F	6/7	9.6 (9.3–9.9)	<6	441 (437–445)
S291A	7	9.3 (9.1–9.6)	8.7 (8.6–8.8)	365 (360–370)
F292A	7	9.4 (9.3–9.5)	8.6 (8.5–8.6)	197 (194–200)
Y295A	7	9.2 (9.1–9.2)	<5	210 (199–221)
Y295F	7	9.5 (9.5–9.6)	<5	302 (291–314)

The affinity values of BK and FR190997 were measured at [^{3}H]-BK binding site to membrane preparations of CHO cells stably expressing the wild-type or mutant receptors, as described under Methods. The reported pIC_{50} values are obtained from homologous and heterologous competition curves, and represent three independent experiments, each one performed in duplicate. Maximal receptor density (B_{max}) was expressed as [^{3}H]-BK specifically bound as fmol mg^{-1} of protein, at a saturating radioligand concentration (2 nM). n.d.: not detectable binding. TM: transmembrane helix.

receptors spanned between 44 and 116% of the wild type (Table 2). The analysis of the B_{max} *versus* E_{max} data obtained at the different receptors did not give any significant correlation.

Results are presented by grouping mutations that have a similar or different effect on affinity and/or efficacy of the two agonists, FR190997 being referred to BK. The investigated receptor residues are represented in Figure 2.

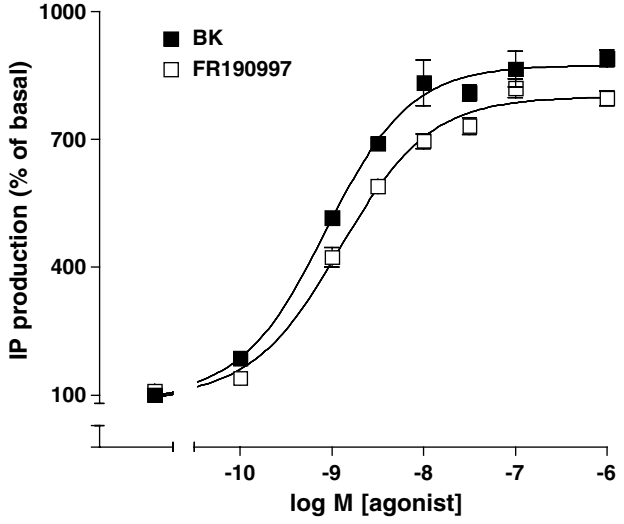

Figure 1 Agonist concentration–response curves on IP hydrolysis in cells expressing the human wild-type B_2 receptor. Cells were incubated for 30 min at 37°C with BK and FR190997 in the concentration range from 0.1 nM to 1 μM, as described under Methods. Results are expressed as percentage of the basal (no added drugs) IP production. Each point represents the mean ± s.e.m. of three independent experiments, each one performed in triplicate.

Mutations that drastically impair the affinity of both BK and FR190997 (W86A (TM2) and F259A (TM6))

[^{3}H]-BK was not able to bind the W86A (TM2) and F259A (TM6) mutant receptors significantly and specifically, but the concentration–response curves (10 nM–10 μM) in IP accumulation indicated a reduction in BK agonist affinity by 1400- and 150-fold, at the W86A and F259A mutant B_2 receptors, respectively (Table 2). Even the potency of FR190997 in terms

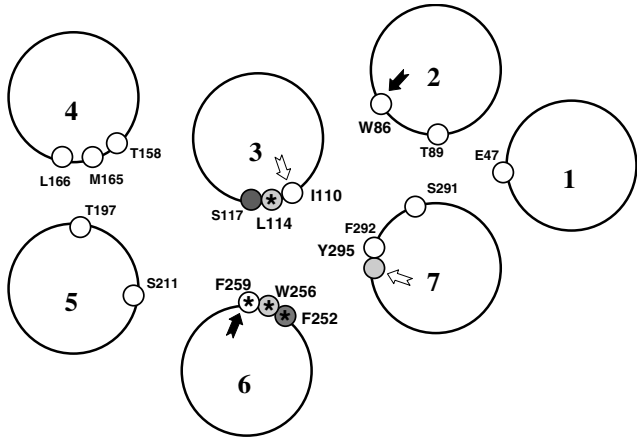

Figure 2 Cross-sectional schematic sight of the seven TM domains of the human B_2 receptor. The TM domains (large circles 1–7) bear the investigated amino-acid residues (small circles). Amino-acid residues that lie one helix turn down from each other are filled with grey color. Arrows indicate residues which when mutated affected the affinity of both BK and FR190997 (black); amino acids that are important determinants for FR190997 affinity (open), and those which when mutated reduce the receptor-activating properties of FR190997 or convert it into an antagonist (asterisk).

Table 2 Potency and activation properties by BK and FR190997 ligands to wild-type and mutant human B_2 receptors

		BK		*FR190997*	
hB_2R	*TM*	*pEC_{50} (95% c.l.)*	*E_{max} (% of wild type)*	*pEC_{50} (95% c.l.)*	*E_{max} (% of BK E_{max})*
Wild type		9.1 (8.9–9.2)	100	8.9 (8.8–9.0)	89±3
E47A	1	9.2 (9.0–9.4)	91±3	8.3 (8.2–8.4)	109±3
W86A	2	5.9 (5.9–6.0)*	66±2	6.3 (6.2–6.4)*	98±3
T89A	2	9.2 (8.9–9.5)	78±4	9.1 (8.0–9.4)	92±3
I110A	3	9.2 (9.2–9.3)	85±2	7.2 (7.1–7.3)*	54±1
L114A	3	9.2 (9.1–9.2)	83±2	n.e.	10±2
S117A	3	9.3 (9.0–9.7)	60±4	9.0 (8.8–9.2)	69±1
T158A	4	9.2 (8.9–9.4)	114±2	9.1 (9.0–9.3)	106±2
M165T	4	8.9 (8.7–9.1)	79±2	9.2 (8.8–9.7)	117±3
L166F	4	9.0 (8.9–9.2)	97±1	9.3 (9.2–9.4)	81±1
T197A	5	9.0 (8.8–9.2)	116±1	9.0 (8.9–9.2)	85±2
S211A	5	9.2 (8.9–9.4)	110±1	9.2 (9.0–9.4)	93±2
F252A	6	9.0 (8.9–9.2)	92±4	n.e.	21±1
W256A	6	8.9 (8.7–9.2)	68±4	n.e.	10±1
F259A	6	6.9 (6.8–7.1)*	72±2	7.5 (6.9–8.1)*	33±4
W256A/Y295F	6/7	8.9 (8.5–9.2)	67±2	n.e.	10±2
S291A	7	8.6 (8.0–9.2)	85±7	9.3 (9.0–9.5)	70±2
F292A	7	8.9 (8.6–9.1)	101±2	9.0 (8.8–9.1)	103±3
Y295A	7	8.4 (7.9–8.9)	82±1	n.e.	—
Y295F	7	9.0 (8.7–9.4)	44±2	7.1 (6.9–7.4)*	94±5

Concentration–response curves to BK and FR190997 in producing inositol phosphate accumulation were carried out as described under Methods, the reported pEC_{50} values are calculated from fitting the curves obtained from 3–4 experiments, each one performed in triplicate. Basal (in the absence of ligand) IP production levels were comparable for wild-type and mutant receptors. The maximal effect (E_{max}) of BK in each mutant receptor is expressed as percentage of wild type (100%). The E_{max} produced by FR190997 is expressed according to the E_{max} produced by BK (100%) in each mutant receptor. *$P<0.05$ *versus* wild-type receptor. n.e.: not evaluable from nonlinear regression curve fit.

of EC_{50} was reduced compared to the wild-type receptor by 400-fold at the W86A and 25-fold at the F259A mutant receptors (Table 2). The E_{max} produced by the two agonists was comparable at the W86A mutant, whereas the E_{max} of FR190997 (10 μM) measured at the F259A mutant amounted to 33% of the E_{max} produced by BK (Table 2).

Mutations crucial for the FR190997-receptor interaction only (I110A (TM3), Y295A, and Y295F (TM7))

No significant differences in BK affinity and potency was observed at I110A (TM3), Y295A, and Y295F (TM7) mutant receptors. On the contrary, the I110A mutation caused a marked reduction (>100-fold) of the FR190997-binding affinity (Table 1). In agreement with binding data, the concentration–response curve to FR190997 was rightward shifted (about 50-fold) as compared to that obtained at the wild-type receptor, and the maximal effect was depressed as compared to that of BK (Table 2).

FR190997, at 10 μM concentration, barely inhibited the [^{3}H]-BK-specific binding at the Y295A (43±5%) and Y295F (44±7%) mutant receptors. IP production experiments revealed that the concentration–response curve to FR190997 at the Y295F mutant was rightward shifted (75-fold) as compared to that of BK, but the maximal effect was comparable to that produced by BK. On the contrary, at the Y295A mutant, FR190997 was almost ineffective in inducing an IP production up to 10 μM concentration. Therefore, FR190997 was tested as an antagonist at the Y295A mutant; the concentration–response curves to BK, in the absence and presence of 10 μM FR190997, were overlapping (data not shown).

Mutations of hydrophobic residues in TM3 (L114A) and TM6 (W256A and F252A) selectively impair the FR190997 agonist efficacy

The Ala substitution of the L114 residue, which lies in the middle of TM3, and between W256 and F252 residues, located in the middle of TM6, did not influence either the binding or agonist affinity of BK (Tables 1 and 2). Moreover, the affinity of FR190997 evaluated at these mutant receptors rather increased as compared to the wild-type receptor (Table 1). Despite this, FR190997 up to 10 μM barely induced IP production at the L114A, W256A, and F252A mutants. The activity of FR190997 was evaluated in inhibiting the concentration–response curves to BK in these mutants. In the L114A mutant, FR190997 was able to shift rightward the concentration–response curve to BK, the EC_{50} values being 0.7 nM (95% c.l. 0.6–0.8) and 49.5 nM (95% c.l. 37.3–65.7) in the absence and in the presence of FR190997 (100 nM), respectively. The E_{max} produced by BK was not reduced in the presence of FR190997, and the calculated pA_2 value was 8.8±0.1.

FR190997 blocked BK-induced IP turnover also at the W256A mutant: the BK concentration–response curve was shifted to the right, the EC_{50} values being 1.2 nM (95% c.l. 0.7–2.0) and 236.4 nM (95% c.l. 148.2–377.1) in the absence and presence of FR190997 (100 nM), respectively. BK E_{max} was not altered in the presence of FR190997, and the estimated pA_2 value was 9.3±0.1.

The concentration–response curve to BK was shifted to the right also at the F252A mutant receptor, the EC_{50} values being 0.9 nM (95% c.l. 0.6–1.3) and 187.2 nM (95% c.l. 64.0–547.6) in the absence and presence of FR190997 (100 nM). In the presence of FR190997, the BK E_{max} was depressed to 65±2% of the control curve.

The W256A/Y295F double mutant impairs both the affinity and the efficacy of FR190997

BK affinity was not affected by the double-point mutation both in radioligand-binding and IP measurement assays (Tables 1 and 2). FR190997 at 10 μM concentration scarcely inhibited the binding of [^{3}H]-BK at the W256A/Y295F mutant (48±4%). In the functional experiment, FR190997 was not able to induce a significant IP production up to 10 μM concentration (Table 2).

Discussion

The present receptor mutational analysis shows that differences in the pharmacology by the two agonists BK and FR190997 can rely on a different recognition site with the hB_2R. Mutations that impair the affinity of both the natural and synthetic agonists lie in the outer TM portions of the receptor (W86 in TM2 and F259A in TM6). All the other residues found to be involved both in the recognition site and/or the receptor activation of only the nonpeptide ligand were postulated to project at the level of the TM helices, into a putative receptor pocket comprised among TM3, 6, and 7 (Meini *et al*., 2002). Our experimental evidence supports the concept that alterations in apparent affinity and relative efficacy may vary in opposite directions (Blaker *et al*., 2000), and that any given ligand may stabilize an ensemble of receptor conformers (Colquhoun, 1998; Kenakin & Onaran, 2002), which are responsible for different transduction/coupling mechanisms (Holst *et al*., 2001), as it has been shown for other available nonpeptide *versus* peptide agonist ligands for GPCRs (Perlman *et al*., 1997; Blaker *et al*., 1998; Thibonnier *et al*., 2000). In particular, our data suggest a core of amino-acidic residues in the middle of TM3 (L114) and TM6 (F259, W256, F252), which selectively affect the efficacy of FR190997, leaving that of BK unaltered. F259 was previously shown to be involved in the binding affinity of BK when mutated to Ala (F259A) (Jarnagin *et al*., 1996; Marie *et al*., 2001). This mutation, besides affecting the affinity of FR190997, makes it a partial agonist. One and two helix turns down the F259 residue lie W256 and F252, and in close proximity to L114. All these residues when mutated into Ala do not impair the affinity of FR190997 at the [^{3}H]-BK-binding site, but its capability to activate the IPs cascade. As a consequence, FR190997 behaves as a high-affinity antagonist in blocking the IP production induced by BK at these mutants. It is worth mentioning that the above amino-acidic residues are indeed maintained in the sequences of different species of B_2 receptor (Farmer *et al*., 1998); therefore their involvement in the observed species-dependent pharmacological differences with FR190997 (Rizzi *et al*., 1999) can be excluded.

It might be speculated that FR190997 affects the receptor (differently from BK) by interacting with aromatic residues in the TM6 and TM3, which have been recently indicated as belonging to a network of amino-acidic residues involved in the maintenance of the inactive/active conformational

equilibrium of the hB_2R (Marie *et al*., 2001). It is well established that the GPCR activation process requires helix motions, in particular movements of TM3, TM6, and TM7 (Gether, 2000; Meng & Bourne, 2001).

Other mutations were found to reduce ($>10^3$-fold) the high-affinity interaction of the only synthetic agonist FR190997 critically: I110A (TM3), and Y295A or Y295F (TM7). Data obtained with these mutants support a different role for the hydroxilic and the aromatic functions of Y295, the first being involved in the FR190997 receptor binding, and the aromatic function in its capability to activate the receptor. The double-mutant W256A/Y295F was constructed: W256A mutation was selected because it maintained the high binding affinity of FR190997, but completely affected its capability to trigger IP production. On the contrary, the Y295F mutation was chosen because it was crucial for FR190997-binding affinity, but not for its capability to activate the mutant receptor. The lack of capability by FR190997 to activate or to bind the double-mutant W256A/Y295F suggests that these residues are part of a same binding area that is also important for receptor activation by the nonpeptide ligand.

As a whole, the presented pharmacological analysis of point-mutated hB_2R indicates that the synthetic agonist FR190997 and the endogenous peptide BK activate the hB_2R by molecular mechanisms which are, at least in part, distinct. In particular, receptor discriminants that are responsible for the different mode of activation of the B_2 receptor by FR190997 *versus* the natural agonist BK (L114 in TM3, and F259, W256, F252 in TM6), and some residues which are important in maintaining a high-affinity interaction of only the synthetic agonist (I110 in TM3, and Y295 in TM7), are described.

Recent experimental evidence that BK B_2 receptor agonism may offer therapeutic opportunities for the treatment and even prevention of cardiovascular disorders (Heitsch, 2003; Ito *et al*., 2003) drives the need to deepen the knowledge of FR190997 molecular pharmacology.

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