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SPECIAL REPORT

Bradykinin B_2 and GPR100 receptors: a paradigm for receptor signal transduction pharmacology

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The aim of the present report was to investigate the ligand selectivity of the human orphan G-protein-coupled receptor GPR100 (hGPR100), recently identified as a novel bradykinin (BK) receptor, as compared with that of the human B_2 receptor (hB_2R) stably transfected in Chinese hamster ovary cells. BK was able to inhibit the cAMP production induced by forskolin with a potency 100-fold lower at the hGPR100 ($pEC_{50} = 6.6$) than that measured at the hB_2R ($pEC_{50} = 8.6$). Both effects were inhibited by the B_2 receptor antagonist Icatibant (1 μM). The nonpeptide B_2 receptor agonist FR190997 (8-[2,6-dichloro-3-[*N*-methylcarbamoyl)cinnamidoacetyl]-*N*-methylamino]benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline) did inhibit the forskolin-induced cAMP production ($pEC_{50} = 7.7$) at the hB_2R, whereas it was not able to exert any effect at the hGPR100. The human insulin-like peptide relaxin 3 did inhibit the cAMP production at the hGPR100 ($pEC_{50} = 7.3$) at a greater extent than BK, and was devoid of any effect at the hB_2R. FR190997 and relaxin 3 responses at the hB_2R and hGPR100, respectively, were not inhibited by Icatibant (1 μM). These data indicate FR190997 and relaxin 3 as selective agonists for hB_2R and hGPR100, respectively, and support the concept that different agonists may specifically bias the conformational states of a receptor to result in a final common G protein coupling, which is differentially recognized by antagonists.
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Keywords: GPCRs; GPCR142; FR190997; Icatibant; nonpeptide ligands; relaxin 3; adenylyl cyclase

Abbreviations: BK, bradykinin; CHO, Chinese hamster ovary; hB_2R, human B_2 receptor; hGPR100, human G-protein-coupled receptor 100; IP, inositol phosphates

Introduction Two types of kinin receptors have been pharmacologically characterized and cloned: B_2 receptors, which are constitutively expressed by a variety of cells, are stimulated by kinins such as bradykinin (BK) and Lys-BK (kallidin), and B_1 receptors, which are upregulated or *de novo* expressed following tissue injury and inflammation, and are stimulated by metabolites of kinins lacking the C-terminal arginine (Couture *et al*., 2001). Both kinin B_1 and B_2 receptors have been involved in inflammation and hyperalgesia, and more recently, these latter receptors have been implicated in tumor growth (Drube & Liebmann, 2000).

In a recent investigation, Boels & Schaller (2003) have identified an orphan G-protein-coupled receptor, termed GPR100, as a novel kinin receptor. Although the two receptors, hB_2 and human G-protein-coupled receptor 100 (hGPR100), share only 27% sequence identity, B_2 receptor agonists, such as BK or Lys-BK, were described to activate the human GPR100 (hGPR100), stably expressed in Chinese hamster ovary (CHO) cells together with the apoaequorine and Gα16 proteins to improve calcium mobilization signalling (Boels & Schaller, 2003).

On the other hand, Liu *et al*. (2003), while working on insulin-like peptides, have recognized the peptide relaxin 3 as the ligand for the orphan receptor GPCR142 (which has the same receptor sequence as GPR100). Intriguingly, relaxin peptides have widespread effects, and at the cardiovascular level are able to evoke responses similar to BK (Samuel *et al*., 2003).

The aim of the present study was to further characterize the pharmacology of the hGPR100, as compared with that of hB_2R, by means of the peptide agonist BK, the nonpeptide B_2 receptor agonist FR190997 (8-[2,6-dichloro-3-[*N*-methylcarbamoyl)cinnamidoacetyl]-*N*-methylamino]benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline) (Aramori *et al*., 1997), the human peptide relaxin 3 (Liu *et al*., 2003), and the B_2 receptor antagonist Icatibant (Hock *et al*., 1991).

Methods *Stable transfection in CHO cells of pooled B_2 and GPR100 receptor clones* The hB_2R or hGPR100 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 5% dialysed foetal bovine serum (FBS); 12–14 days after transfection, more than 100 individual $DHFR^+$ clones were pooled, and cultured in Iscove's modified Dulbecco's medium (IMDM) with L-glutamine (2 mM) and 10% dialysed FBS.

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Radioligand binding Binding experiments were performed in *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES 10 mM, pH 7.4) containing 1,10 phenanthroline (1 mM), bacitracin (140 $\mu g\,ml^{-1}$), and bovine serum albumin (BSA, $1\,g\,l^{-1}$) on membranes prepared as described previously (Bellucci *et al*., 2003). Briefly, the binding assay was performed in a final volume of 0.5 ml. For binding at the hB_2R, an incubation time of 60 min at room temperature was used (the final protein concentration was 100 $\mu g\,ml^{-1}$). Nonspecific binding was defined as the amount of radioligand bound in the presence of 1 μM of unlabelled BK, and represented less than 10% total bound [^{3}H]BK. Competition-binding experiments were carried out at [^{3}H]BK radioligand concentration comparable with the calculated K_d value (see Results). Determinations were made in duplicate.

Inositol phosphates (IP) determination Cells were grown in 24-well tissue culture plates and labelled for 24 h with *myo*-[1,2-^{3}H]inositol (0.5 ml, 1 $\mu Ci\,ml^{-1}$) in IMDM and Ham's F12 medium (F12) (1 : 1) containing 1% dialysed FBS and L-glutamine (2 mM). Different concentrations of agonists were incubated for 30 min at 37°C in the stimulation buffer (PBS Ca/Mg free 135 mM, HEPES 20 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1.2 mM, EGTA 1 mM, glucose 11.1 mM, captopril 10 μM, BSA 0.05%) added with LiCl (25 mM). Total IP levels were determined as described previously (Bellucci *et al*., 2003). Determinations were made in triplicate.

cAMP determination Cells were grown to confluence in 96-well plates as described for the IP assay. Agonists were incubated at the indicated concentrations for 10 min at 37°C in the stimulation buffer, to which IBMX 100 μM was added. The cAMP cell content was measured by using a commercially available ELISA (Amersham Biosciences, Buckinghamshires, U.K.) according to the manufacturer's instructions.

Data analysis All values in the text and figures are expressed as mean ± s.e.m., and the number (n) of experiments is given. Radioligand binding data and concentration–response curves for phosphoinositide and cAMP determination were analysed by fitting the data with GraphPad Prism program (San Diego, CA, U.S.A.). The −log of agonist concentration producing 50% of its maximal effect (pEC_{50}) or the −log of ligand concentration inhibiting the 50% of radioligand-specific binding (pIC_{50}) was calculated.

Drugs [^{3}H]BK (specific activity 90 $Ci\,mmol^{-1}$) and *myo*-[1,2-^{3}H]inositol (specific activity 75 $Ci\,mmol^{-1}$) were provided by Perkin-Elmer New England Nuclear (Boston, MA, U.S.A.). BK was obtained from Peninsula (St Helens, U.K.) and human relaxin 3 from PhoenixEurope (Karlsruhe, Germany). FR190997 was synthesized in Fujisawa Pharmaceuticals (Tsukuba, Japan), and Icatibant in Menarini Ricerche (Florence, Italy). Leupeptin was obtained from Boehringer Mannheim (Germany), Thiorphan from Bachem (Essex, U.K.). MERGEPTA was 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.).

Results *Binding experiments at the human B_2 (hB_2R) and GPR100 receptors* Binding experiments were performed in membranes of CHO cells transfected with the hB_2R or GPR100.

[^{3}H]BK (10 pM–9 nM) saturation experiments at the hB_2R indicated a K_d value of 0.08 nM and a B_{max} of 350 $fmol\,mg^{-1}$ of protein. Competition curves performed with Icatibant and FR190997 (1 pM–1 μM) fitted with a one-site competition model yielding pIC_{50} values of 10.01 ± 0.03 and 8.01 ± 0.04 ($n = 3$), respectively. Experiments at [^{3}H]BK binding performed with relaxin 3 did not produce inhibition up to 1 μM.

When experiments were carried out at the hGPR100, a specific binding was observable, only at higher [^{3}H]BK concentrations (5–9 nM) reaching a value of 19 ± 2% of total bound. A greater specific binding could not be obtained even if different experimental conditions were used, that is, different time-course association (up to 3 h) carried out at different temperatures (4, 20, and 37°C).

Functional characterization of hB_2R and GPR100 receptor by IP accumulation and cAMP inhibition assays In cells expressing the hB_2R BK (0.1–100 nM) induced a concentration-dependent IP increase, the pEC_{50} being 9.1 ± 0.07. On the contrary, BK did not evoke any response up to 10 μM concentration, when the same assay was carried out in cells expressing the hGPR100. The peptide relaxin 3 was devoid of any activity up to 1 μM concentration both in cells expressing the hB_2R or hGPR100.

According to Liu *et al*. (2003), the activation of the GPR100 receptor leads to inhibition of adenylyl cyclase activity. In the CHO cell systems expressing the hB_2R or hGPR100, forskolin (0.5 μM) induced an activation of adenylyl cyclase, which resulted in a similar cAMP output of 7.1 ± 0.44 and 6.8 ± 0.46 nM, respectively. None of the studied compounds had any effect on the cAMP response (basal or forskolin stimulated) measured in untransfected CHO cells.

In the CHO cells expressing the hB_2R, both BK and FR190997 (0.1 nM–10 μM) concentration dependently inhibited the cAMP production with pEC_{50} values of 8.6 ± 0.29 and 7.7 ± 0.2, respectively, whereas the peptide relaxin 3 did not evoke any effect up to 1 μM (Figure 1).

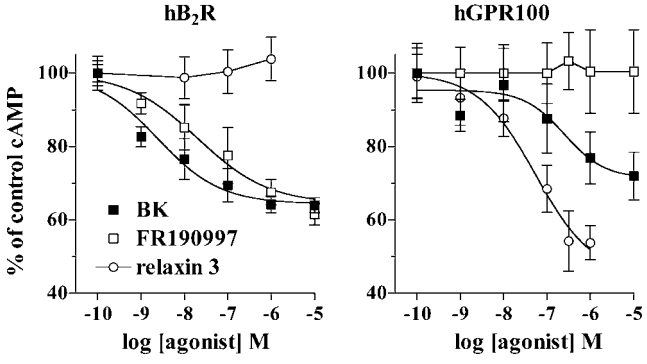

Figure 1 Concentration-dependent inhibition of cAMP by BK, FR190997, and relaxin 3 in cells expressing the human B_2 (left panel) or the human GPR100 receptor (right panel). Data are expressed as the percentage of control cAMP stimulated with forskolin (0.5 μM) and are the mean ± s.e.m. of four to six experiments, each one performed in duplicate.

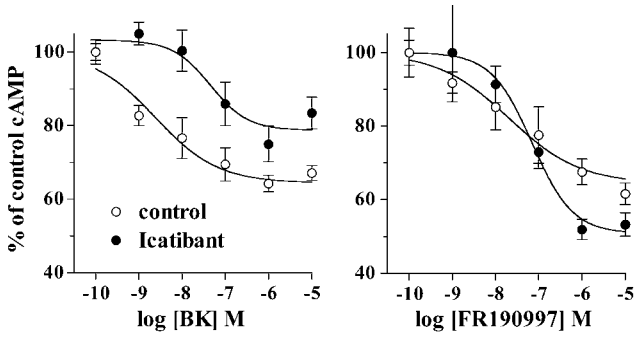

Figure 2 Concentration-dependent inhibition of cAMP output by BK and FR190997 in cells expressing the human B_2 receptor, in control conditions, or after 5 min incubation with the B_2 receptor antagonist Icatibant (1 μM). Data are expressed as the percentage of control cAMP stimulated with forskolin (0.5 μM), and are the mean $\pm$ s.e.m. of four to six experiments, each one performed in duplicate.

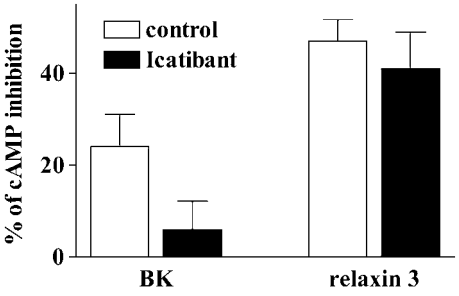

Figure 3 In cells expressing the human GPR100, Icatibant (1 μM, 5 min preincubation) is able to revert the moderate inhibition of cAMP output induced by BK (1 μM) but not that of relaxin 3 (1 μM). Data are the mean $\pm$ s.e.m. of four experiments, each one performed in duplicate.

Pretreatment with Icatibant 1 μM did shift the concentration response curve to BK, the fit giving a pEC_{50} value of 7.3 ± 0.4, and reduced the BK maximal effect (Figure 2). On the contrary, the effect produced by FR190997 was not reduced in the presence of Icatibant, the sigmoidal curve slope being rather steepened from shallow (Hill slope 0.53 ± 0.1) to unity (Hill slope 0.92 ± 0.26) (Figure 2).

In the CHO cells expressing the hGPR100, BK inhibited the cAMP production induced by forskolin, the pEC_{50} being 6.6 ± 0.7. On the contrary, FR190997 was devoid of any effect up to 10 μM. The peptide relaxin 3 did inhibit the cAMP production at a greater extent and potency (Figure 1), the pEC_{50} being 7.3 ± 0.3. Icatibant (1 μM) did reduce the moderate effect induced by BK, whereas it left unaffected that produced by relaxin 3 (Figure 3).

Icatibant, at the tested concentration (1 μM), did not modify either the basal cAMP output or the forskolin-stimulated cAMP production of both cell expressing the hB_2R or hGPR100.

Discussion From the present analysis of the pharmacology of the hB_2R and hGPR100, expressed in the same cell system (CHO), we show that BK is able to activate both receptors, in terms of inhibition of cAMP production, but with different potencies, being 100-fold less potent at the GPR100 receptor. The significantly lower agonist potency of BK determined at the hGPR100 may explain the fact that a specific [^{3}H]BK binding cannot be detected up to nM concentrations of radioligand in membranes from cells expressing this receptor.

It has been shown that a cell system, which is enriched with Gα16 protein, links coexpressed receptors functionally to endogenous phospholipase C (Offermanns & Simon, 1995). In agreement, previous data indicated that BK can induce calcium mobilization in a cell system cotransfected with both GPR100 and Gα16 protein (Boels & Schaller, 2003). In the present cell system, CHO cells transfected with the hGPR100 only, BK is not able to induce any IP stimulation. The same observation was highlighted by Liu *et al.* (2003) with the human insulin-like peptide relaxin 3.

Our data show that, in terms of inhibition of adenylyl cyclase activity, BK can indeed activate the GPR100 receptor, but with lower efficacy and potency as compared with the human insulin like peptide relaxin 3. Contrary to BK, the nonpeptide B_2 receptor agonist FR190997 is uneffective at the hGPR100. On the other hand, we show that relaxin 3 is devoid of any affinity and activity at the hB_2R. The fact that Icatibant is able to block the effect produced by BK at the hGPR100, but not that by relaxin 3, suggests that the two peptide agonists activate this receptor at different sites.

Another aspect arising from this study is the discrepancy in the pharmacology of BK and FR190997. By means of mutational analysis at the hB_2R, we have previously shown that these two agonists bind to the receptor and activate the IP cascade through different receptor determinants (Bellucci *et al.*, 2003). Moreover, whereas Icatibant was able to antagonize competitively with similar potency their IP production (pA_2 values 8.5 and 8.2 towards BK or FR190997, respectively) (Bellucci *et al.*, 2004), the present data indicate that Icatibant cannot prevent the cAMP inhibition produced by the nonpeptide agonist FR190997, while being able to block the effect induced by BK. The analysis of BK concentration response curves in the absence and presence of Icatibant, which *per se* at the used concentration does not affect the control cAMP output (both basal and forskolin stimulated), suggests an insurmountable antagonism. At present, it is difficult to ascertain the mechanism responsible for the diverging behaviour of Icatibant with respect to the different transduction pathways activated by FR190997. A different recognition site for Icatibant and FR190997 on the hB_2R macromolecule, as supported by their affinity values measured at [^{3}H]BK binding (pIC_{50} 10.01 and 8.01, respectively), could result in a distinct modulation of IP and cAMP responses. A difference in Icatibant potency towards contractile effects elicited by BK and FR190997 had been previously observed in the guinea-pig ileum smooth muscle (Meini *et al.*, 2000). Since the possibility of a regulation between adenylyl cyclase and phospholipase C activity has been reported (Campbell *et al.*, 1990; Boyajian *et al.*, 1991; Hanke *et al.*, 2001), it remains to be established whether this inhibitory activity of FR190997, which is not blocked by Icatibant, is responsible for the tissue/cell-dependent agonist/antagonist profile of FR190997 (Rizzi *et al.*, 1999). To our knowledge, this is the first evidence showing that the synthetic ligand FR190997 can activate an inhibitory adenylyl cyclase pathway, which although not antagonized by Icatibant can be considered B_2 receptor dependent, since it is not observable in cells expressing GPR100 or in not transfected CHO cells.

Evidence about an inhibitory coupling to adenylyl cyclase by the BK B_2 receptor have been previously reported in different bioassays, such as guinea-pig colon and ileum smooth muscles and rat myometrium (Liebmann *et al*., 1990; 1994; Hasler *et al*., 1995), and in neuronal NCB-20 or N1E115 cells (Bozou *et al*., 1989; Boyajian *et al*., 1991). Moreover, BK is able to produce a mitogenic effect by stimulating the mitogen-activated protein kinase *via* Gαi coupling. This Gαi-coupled (pertussis toxin sensitive) B_2 receptor activity has been shown to be specific for certain tumor cell lines, such as the human breast carcinoma EFM-192A and the small-cell lung carcinoma H-69 (Drube & Liebmann, 2000), or the human endometrium carcinoma cell line EFE-184 (Liebmann, 2001), and also in COS-7 cells transfected with the hB_2R (Hanke *et al*., 2001). It is worth mentioning that Icatibant behaved as BK in activating the mitogenic signal in cell systems both coupled to Gαi or Gαq protein (Drube & Liebmann, 2000), although in the presented cell system it did not produce any agonist effect.

In conclusion, the present findings point out that BK can activate GPR100 (or GPCR142), but with a lower potency than relaxin 3. Moreover, although Icatibant can prevent the effects produced by BK, both at the B_2 and the GPR100 receptor, it does not affect the adenylyl cyclase inhibitory activity produced by FR190997 or relaxin 3 at the hB_2R and hGPR100, respectively, thus suggesting that the activation mode by BK and FR190997 on the one hand, and BK and relaxin 3 on the other is different. Overall this study supports the general concept that different agonists may specifically bias the conformational states of a receptor to result in a final common G protein coupling, but not equally recognized by antagonists.

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