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Short communication

The N-terminal of Icatibant and bradykinin interact with the same Asp residues in the human B_2 receptor

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Abstract

The pharmacology of peptide and non-peptide bradykinin B_2 receptor ligands was evaluated in the inositol phosphate (IP) production assay in CHO cells expressing the human bradykinin B_2 receptor. The effect of single and double alanine mutation of D266 and D284 residues at the human bradykinin B_2 receptor was evaluated on the agonist profile of bradykinin (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) and the synthetic agonist FR190997 (8-[2,6-dichloro-3-[*N*-methylcarbamoyl)cinnamidoacetyl]-*N*-methylamino]benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline). Bradykinin potency (EC_{50} 0.5 nM at the wild-type receptor) was reduced by 16-fold at D266A and D284A mutants and by 2300-fold at the D266A/D284A double mutant. None of the mutants affected the potency or the efficacy of FR190997. Peptide antagonists, Icatibant (H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-Dtic-Oic-Arg-OH) and MEN11270 (H-DArg-Arg-Pro-Hyp-Gly-Thi-c(Dab-DTic-Oic-Arg)c(7γ-10α)) (100 nM) similarly antagonized the concentration–response curve to bradykinin or FR190997 (pA_2 values 8.5 and 8.4 versus bradykinin and 8.2 and 8.4 versus FR190997) at the wild-type receptor. Non-peptide antagonists FR173657 ((E)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl]phenyl]-*N*-methylaminocarbonyl methyl]acrylamide) and LF16-0687 (1-[[2,4-dichloro-3-[(2,4-dimethylquinolin-8-yl)oxy] methyl]-phenyl]sulfonyl]-*N*-[3-[[4-(aminoiminomethyl)-phenyl]carbonylamino]propyl]-(S)-pyrrolidine carboxamide) (100 nM) showed an equivalent potency values in blocking the IP production induced by bradykinin or FR190997 (pA_2 values 8.7 and 8.8 versus bradykinin and 8.8 and 8.6 versus FR190997). Whilst the antagonist potency of FR173657 and LF16-0687 was not affected by D266A/D284A double mutation (IP production induced by the synthetic agonist), that of Icatibant and MEN11270 was reduced by 50- and 200-fold. The antagonist potency of [Ala^1]-Icatibant and [Ala^2]-Icatibant (pA_2 values at wild-type 7.7 and 6.4) was significantly less reduced (20-fold and 13-fold, respectively) by the D266A/D284A double mutation.

Our results highlight a crucial role for two aspartic residues, D266 and D284, located at the top of transmembrane segments 6 and 7, in the high-affinity interaction of peptide antagonists with the human bradykinin B_2 receptor. An interaction of these receptor residues with the N-terminal basic residues of Icatibant is hypothesized.

Keywords: Antagonism; FR190997; G-protein coupled receptor; Inositol phosphate; Non-peptide; Mutagenesis

1. Introduction

The bradykinin B_2 receptor belongs to the G-protein-coupled seven-transmembrane (TM) receptor superfamily (Hess et al., 1992) and mediates the bradykinin biological effects. A number of studies have provided a model of ligand–receptor interaction. It has been suggested that bradykinin sequence, when bound to the B_2 receptor, positions its N-terminal end in proximity of the fourth extracellular loop (Herzig and Leeb-Lundberg, 1995; Herzig et al., 1996) stretching the molecule along the interior face of the TM 6, 5, 4, and 3 domains (Nardone and Hogan, 1994; Leeb et al., 1997), and locating its C-terminal end, in a β-turn conformation, adjacent to the S111 residue in TM3 domain (Fathy et

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al., 1998). The binding pocket of antagonist ligands has been shown not to be completely overlapping that of bradykinin, as evaluated with the peptide antagonists NPC17731 (H-Darg-Arg-Pro-Pro-Gly-Phe-Ser-D-Hype(trans-propyl)-Oic-Arg-OH) (Novotny et al., 1994) and Icatibant (H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-Dtic-Oic-Arg-OH) (Hock et al., 1991; Kyle, 1995; Abd Alla et al., 1996; Jarnagin et al., 1996) or non-peptide antagonist ligands such as FR173657 ((E)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl] phenyl]-*N*-methyl aminocarbonyl methyl]acrylamide) (Asano et al., 1997) and LF16-0687 (1-[[2,4-dichloro-3-[(2,4-dimethylquinolin-8-yl)oxy] methyl]-phenyl] sulfonyl]-*N*-[3-[[4-(aminoiminomethyl)-phenyl]carbonylamino]propyl]-2(S)-pyrrolidine carboxamide) (Pruneau et al., 1999), which recognition site on the receptor is in a binding crevice amongst TM 3, 6, and 7 (Meini et al., 2002, 2004). In the present study, we have investigated the role of two aspartate residues (D266 and D284), present in the fourth extracellular loop of the human bradykinin B_2 receptor sequence. Interestingly, a negative charge at the top of TM 6 and/or 7 of peptide G-protein-coupled receptors has been involved for interaction with cationic group of high affinity ligands of peptide and non-peptide nature (Hjorth et al., 1994; Feng et al., 1995; Fathy et al., 2000; Fromme et al., 2001; Larson et al., 2000). The receptor activation properties of bradykinin and the synthetic non-peptide agonist FR190997 (8-[2,6-dichloro-3-[*N*-methylcarbamoyl)cinnamidoacetyl]-*N*-methylamino] benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline) (Aramori et al., 1997) were compared in the IP production. Moreover, the antagonist profiles of the peptide MEN11270 (H-DArg-Arg-Pro-Hyp-Gly-Thi-c(Dab-DTic-Oic-Arg)c(7γ-10α)) (Meini et al., 1999) and Icatibant were compared with those of the non-peptide FR173657 and LF16-0687. Two substituted analogues of Icatibant ([Ala^1]-Icatibant and [Ala^2]-Icatibant) were used as tools to evaluate a possible role of N-terminal basic charges in the interaction with aspartic residues in the receptor.

2. Materials and methods

2.1. Inositol phosphate determination

Pools of CHO cell clones stably expressing the human wild-type or mutant receptors were obtained as previously described (Meini et al., 2002), and cultured in Iscove's modified Dulbecco's Medium (IMDM) with L-glutamine (2 mM) and fetal bovine serum dialyzed 10%. Cells grown in 24-well were labelled for 24 h with *myo*-[1,2-^{3}H]inositol (0.5 ml/well, 1 μCi/ml) in IMDM and Ham's F12 Medium (F12) (1:1) containing fetal bovine serum dialyzed 1% and L-glutamine (2 mM). After a 15-min preincubation period at 37 °C in a buffer consisting of Dulbecco's phosphate-buffered saline (PBS) Ca^{2+}/Mg^{2+}-free (135 mM), HEPES (20 mM), $CaCl_2$ (2 mM), $MgSO_4$ (1.2 mM), EGTA (1 mM), glucose (11.1 mM), bovine serum albumin 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. Antagonists were added 15 min prior to stimulation with the agonist. IP were then extracted and isolated with anion exchange chromatography as previously described (Bellucci et al., 2003).

2.2. Data analysis

All values in the text, table or figure are mean and 95% confidence limits (c.l.) or mean ± S.E. as indicated. Concentration–response curves were analysed by fitting the data with GraphPad Prism program (San Diego, CA) in order to determine the agonist molar concentration producing 50% (EC_{50}) of its maximal effect (E_{max}). The IP production is expressed as percentage of the E_{max} induced by agonist. The antagonist affinity was expressed in terms of pA_2 calculated from the equation: $pA_2 = \log [CR - 1] - \log$ [antagonist concentration] where CR (concentration ratio) is the ratio between the EC_{50} values of the agonist determined in the presence and absence of antagonist (Kenakin, 1997).

2.3. Drugs

Myo-[1,2-^{3}H]inositol (specific activity 74.7 Ci·mmol^{-1}) was provided by PerkinElmer New England Nuclear (Boston, MA, USA). Bradykinin was obtained from Neosystem (Strasbourg, France). FR190997 was a kind gift from Fujisawa Pharmaceuticals. All other materials were obtained from Sigma (St. Louis, LA, USA). All bradykinin B_2 receptor antagonists used were synthesized in Menarini Ricerche (Florence and Rome, Italy). Non-peptide ligands were dissolved in dimethilsulphoxide up to 100 μM. All compounds were stored at −25 °C.

3. Results

No differences in basal outputs of IP production were detectable among the wild-type and D266A, D284A and D266A/D284A mutant receptors expressed in stably transfected CHO cells.

Bradykinin elicited a concentration-dependent increase in IP formation at the wild-type receptor (EC_{50} = 0.5 nM, 95% c.l. 0.4–0.7). The potency (EC_{50}) of bradykinin was reduced by 16-fold at both D266A and D284A mutant receptors, being 8.0 nM (95% c.l. 6.4–10.1) and 7.6 nM (95% c.l. 6.3–9.2), respectively, and by 2300-fold at D266A/D284A double mutant, the EC_{50} being 1145 nM (95% c.l. 801–1636).

No differences were observed in terms of E_{max} elicited by bradykinin at the mutant receptors as compared to the wild-type (Fig. 1A).

Contrary to bradykinin, the single or double mutations did not affect the potency of FR190997, the EC_{50} values being 0.9 nM (95% c.l. 0.7–1.2) for both D266A and

D284A mutants and of 0.7 nM (95% c.l. 0.5–0.9) for D266A/D284A mutant similar to that resulted at the wild-type receptor, being 1.0 nM (95% c.l. 0.6–1.6), and comparable E_{max} observed were obtained in mutant and wild-type receptors (Fig. 1B).

Because of the large decrease of bradykinin potency at the D266A/D284A mutant, we studied the influence of this double mutation on the antagonist properties of peptide (Icatibant and MEN11270) and non-peptide (LF16-0687 and FR173657) ligands by measuring their ability to inhibit FR190997-stimulated IP formation.

Both Icatibant and MEN11270, at 100 nM concentration, showed a similar antagonist potency in inhibiting the stimulatory effect of bradykinin at the wild-type receptor, and appeared less potent than LF16-0687 and FR173657 (Table 1).

The D266A/D284A double mutation did not affect the pA_2 values of LF16-0687 and FR173657 (100 nM each) (Table 1). On the other hand, pA_2 values of Icatibant and MEN11270 (10 μM each), resulted in a decrease of about 200-fold and 50-fold, respectively, as compared to the wild-type receptor (Table 1).

The antagonist potency of the two Icatibant derivates [Ala^1]-Icatibant and [Ala^2]-Icatibant was investigated at 1 and 10 μM, respectively. They antagonized the IP production elicited by bradykinin or FR190997 at the wild-type receptor with a similar potency, but they result less potent than Icatibant itself (Table 1). [Ala^1]-Icatibant and [Ala^2]-Icatibant (10 μM each) were compared at the D266A/D284A mutant, and their antagonist potency was reduced of about 20-fold and 13-fold, respectively (Table 1).

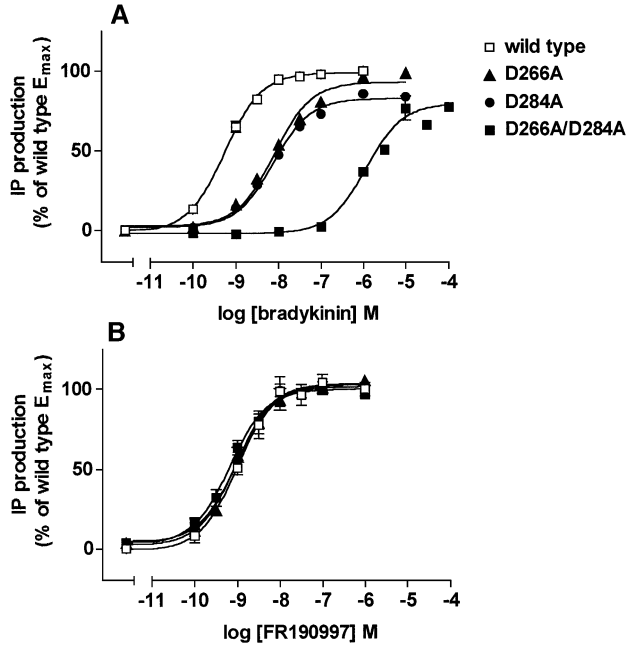

Fig. 1. Concentration–response curve to bradykinin (panel A) and FR190997 (panel B) in the IP production assay performed in cells expressing the wild-type and mutant human bradykinin B_2 receptors. Cells were incubated for 30 min at 37 °C with the indicated agonist concentrations as described under Materials and methods. Results are expressed as percentage of the bradykinin E_{max} obtained at the wild-type. Each point represents the mean ± S.E. mean of three independent experiments, each one performed in triplicate.

Table 1
Antagonist potencies of peptides and non-peptides at the wild-type and D266A/D284A mutant human bradykinin B_2 receptors on agonist-stimulated IP production

	pA_2	
	Wild-type	D266A/D284A
Bradykinin-induced stimulation		
FR173657	8.7 ± 0.1	n.e.
LF16-0687	8.8 ± 0.1	n.e.
MEN11270	8.4 ± 0.2	n.e.
Icatibant	8.5 ± 0.1	n.e.
[Ala^1]-Icatibant	7.6 ± 0.2	n.e.
[Ala^2]-Icatibant	6.3 ± 0.1	n.e.
FR190997-induced stimulation		
FR173657	8.8 ± 0.1	8.8 ± 0.1
LF16-0687	8.6 ± 0.1	8.5 ± 0.1
MEN11270	8.4 ± 0.1	6.1 ± 0.2
Icatibant	8.2 ± 0.2	6.5 ± 0.2
[Ala^1]-Icatibant	7.7 ± 0.1	6.4 ± 0.1
[Ala^2]-Icatibant	6.4 ± 0.1	5.3 ± 0.1

n.e.—Not evaluated.

4. Discussion

We recently reported a mutational analysis of the human bradykinin B_2 receptor aimed to investigate whether pharmacological differences observed with the natural agonist bradykinin and the synthetic agonist FR190997 could rely on a different recognition site with the receptor (Bellucci et al., 2003). Indeed, we identified a set of residues, all located in the TM portions (TM3, 6, and 7), which when mutated, impaired the affinity and efficacy of the agonist FR190997 but not bradykinin. The present study highlights a further difference since the mutation of the Asp residues, located at top of TM6 and 7 (D266 and D284) into Ala residues, drastically impaired the bradykinin agonist potency (2300-fold) measured in activating the IP cascade. A comparable shift of bradykinin agonist response, in terms of Cl^- currents, was described by the equivalent amino acid substitutions (D268A/286A) in the rat bradykinin B_2 receptor sequence (Novotny et al., 1994). On the contrary, the profile of the synthetic agonist FR190997, both in terms of efficacy and potency in inducing IP production, was not affected by these mutations (the present study). Moreover, the observed homogeneous basal activity (IP assay) and the absence of significant difference in the activation of the signal transduction by the mutants versus the wild-type human bradykinin B_2 receptors, indicate that the overall conformational states of the considered mutants are not altered.

The further investigation concerns the antagonist profile of peptide ligands (Icatibant and MEN11270) versus non-

peptide ones (LF16-0687 and FR173657). All antagonists displayed similar potency (as pA_2) in antagonizing both bradykinin and FR190997 in cells expressing the wild-type receptor. The capability of FR190997 to produce equivalent responses in cells expressing both the wild-type and mutant receptors, let us to detect the differences of antagonist potency by MEN11270 and Icatibant at the double-mutant D266/D284 receptor, and to evidence that the high-affinity peptide antagonist-receptor complex is hampered by the D266/D284 mutation. On the contrary, the antagonist potency of LF16-0687 and FR173657 was not influenced by the D266/D284 mutation, in agreement with previous mutagenesis and modelling studies which located the binding pocket for non-peptide bradykinin B_2 receptor antagonists mainly within TM domains (Marie et al., 2001; Meini et al., 2002, 2004). Our data do not support the hypothesis of a possible interaction between D266 and D284 residues and the basic charge of benzamidine group present in LF16-0687 structure as raised by Marie et al. (2001).

Previous models of binding site of bradykinin at the rat bradykinin B_2 receptor, suggested that the N-terminal amino and guanidino group of Arg^1 interacts directly with the Asp receptor residues (Kyle, 1995). Experimental evidence supported this hypothesis by means of mutant receptors (Novotny et al., 1994; Nardone and Hogan, 1994), but did not indicate a similar interaction for peptide antagonist ligands. It has to be pointed out that in these studies homologous radioligand binding experiments might have biased or hindered the actual affinity of peptide antagonist ligands (Hjorth et al., 1996; Costa-Neto et al., 2000). Other studies performed by means of reagent cross-linking or anti-idiotypic antibodies, but not including the double mutation of the acidic resides, suggested a different positioning for peptide antagonists N-terminal side as compared with bradykinin (Abd Alla et al., 1996; Herzig and Leeb-Lundberg, 1995; Herzig et al., 1996).

We previously presented data describing the decrease in antagonist potency and binding affinity, both at human and guinea pig bradykinin B_2 receptors, when the DArg residues in position 1 or Arg in position 2 in the sequence of Icatibant, were substituted with Ala (Quartara et al., 2000). [Ala^1]-Icatibant and [Ala^2]-Icatibant, although showing a similar potency in antagonism of bradykinin or FR190997 induced IP production in cells expressing the wild-type receptor, were 3- and 63-fold less potent than Icatibant, respectively, thus confirming a role for the guanidine group in the high affinity interaction with the receptor. On the other hand, the fact that Icatibant potency is more (50-fold) impaired by the D266A/D284A mutation than that of [Ala^1]-Icatibant and [Ala^2]-Icatibant (10–20-fold), suggests that the presence of the two positive charges in the N-terminal sequence of the antagonist unfavours the antagonist-mutant receptor complex.

In conclusion, the present study supports evidence for a different interaction of peptide and non-peptide ligands at the human bradykinin B_2 receptor, and reappraise the role of basic charges present in the N-terminal sequence of peptide antagonists for an ionic interaction with D266 and D284 residues in the human bradykinin B_2 receptor.

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