British Journal of Pharmacology (2002) 135, 462–468
www.nature.com/bjp

Pharmacological and functional characterization of the guinea-pig B_2 bradykinin receptor stably expressed in CHO-K1 cell line

*,[1]C. Robert, [1]D. Pruneau & [1]J.-L. Paquet

[1]Groupe de Pharmacologie des Récepteurs, Centre de Recherche, Laboratoires Fournier, 50 rue de Dijon, 21121-Daix, France

1 In the present study, pharmacological properties of a bradykinin B_2 receptor amplified either from guinea-pig ileum or lung and homologous to the previously reported sequence except two amino-acid changes $L^{124}\rightarrow P$ and $N^{227}\rightarrow Y$ in the receptor protein were characterized.

2 Tritiated bradykinin ([^{3}H]-BK) specifically bound to the cloned guinea-pig B_2 bradykinin receptor stably expressed in Chinese hamster ovary cells (CHO-K1) with a K_D value of 0.29 ± 0.07 nM. In competition experiments, bradykinin (BK) affinity constant value was 0.21 ± 0.05 nM while the two specific kinin B_1 ligands, des-Arg^9-bradykinin (DBK) and des-Arg^9-Leu^8-bradykinin (DLBK) were unable to compete with [^{3}H]-BK. As the specific peptide antagonist D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (HOE140), (E)-3-(6-acetamido-3-pyridil)-N-[-N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl]phenyl]-N-methylaminocarbonylmethyl]acrylamide (FR173657) and 1-[[3-[2,4-dimethylquinolin-8-yl)oxymethyl]-2,4-dichloro-phenyl]sulfonyl]-2(S)-[[4-[4-(aminoiminomethyl)-phenylcarbonyl]piperazin-1-yl]carbonyl]pyrrolidine (LF16-0335C) exhibited a high affinity for this receptor with K_i values of 7.34 ± 2.45 nM and 8.54 ± 1.55 nM respectively.

3 BK and kallidin (KD) increased inositol phosphates (IPs) levels with EC_{50} values of 0.44 ± 0.12 nM and 6.88 ± 0.28 nM, respectively. Neither DLBK nor DBK (0.01 nM to 10 μM) stimulated or inhibited IPs turnover and as expected HOE140 did not raise IPs production. HOE140 (0.1 μM) and LF 16-0335c (1 μM) right shifted the BK response curve with pK_B values of 9.2 ± 0.4 and 8.4 ± 0.3, respectively.

4 The results indicate that this cloned guinea-pig receptor displayed typical pharmacological properties of a bradykinin B_2 receptor and support the existence of a single B_2 receptor in this species.

British Journal of Pharmacology (2002) **135,** 462–468

Keywords: Bradykinin; guinea-pig; B_2 receptor; cDNA; sequence analysis; mutation; binding; phosphoinositides; CHO-K1

Abbreviations: BK, bradykinin; cDNA, complementary deoxyribonucleic acid; CHO, chinese hamster ovary cells; DBK, des-Arg^9-bradykinin; DKD, des-Arg^{10}-kallidin; DLBK, des-Arg^9-[Leu^8]bradykinin; GPCRs, G-protein coupled receptors; ICL2, second intracellular loop; ICL3, third intracellular loop; IPs, inositol phosphates; KD, kallidin; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT–PCR, reverse transcription polymerase chain reaction; TES, N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; TM3, third transmembrane domain

Introduction

The hydrolysis of high and low molecular weight kininogens by tissue and plasma kallikreins releases endogenous kinins including the decapeptide kallidin (KD) and the nonapeptide bradykinin (BK) (Bhoola *et al*., 1992). Carboxypeptidases further cleave kinins into active metabolites including des-Arg^9-BK (DBK) and des-Arg^{10}-KD (DKD) (Regoli & Barabe, 1980). Based on pharmacological and molecular studies, kinin receptors have been classified into two subtypes, named B_1 and B_2. Kinin B_2 receptors are constitutively expressed in numerous tissues and display a high binding affinity for BK and KD but not for DBK and DKD (Proud & Kaplan, 1988: Hess *et al*., 1992). In contrast, the B_1 kinin receptor (Menke *et al*., 1994) is *de novo* expressed (Marceau, 1997), selectively activated by DBK and DKD whilst it does not recognize BK (Deblois *et al*., 1988: Austin *et al*., 1997). These two receptors are members of the super family of G-protein coupled receptors (GPCRs) bearing seven transmembrane domains. Among the different second messenger pathways coupled to kinin B_2 or B_1 receptors, a well characterized transduction signaling is the activation of phospholipase C which results in inositol phosphates (IPs) generation and a subsequent increase of intracellular Ca^{2+} (Austin *et al*., 1997; Ransom *et al*., 1992; Bastian *et al*., 1997).

Intensive research for B_2 receptor antagonists led first to the discovery of peptide derivatives including D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (HOE140) which was a potent and selective B_2 receptor antagonist displaying only a weak affinity for the B_1 receptor (Regoli *et al*., 1990; Rhaleb *et al*., 1992; Trifilieff *et al*., 1992). Phosphonium,[[4-2[[2[[bis(cyclohexylamino)-methylene]amino]-3-(2-naphtalenyl) 1-oxopropyl]amino]-phenyl]-methyl]tributyl, chloride, monohydro-chloride (WIN64338) which was the first non-peptide B_2 receptor antagonist had a moderate affinity for the human receptor and also lacked selectivity (Stewart, 1995). More recently, (E)-3-(6-acetamido-3-pyridil)-N-[-N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl]phenyl]-N-methylaminocarbonylmethy-

*Author for correspondence; E-mail: c.robert@fournier.fr

l]acrylamide (FR173657) was described as a potent and selective B_2 receptor antagonist (Aramori *et al*., 1997) introducing a novel generation of non-peptide antagonist which now includes 1-[[3-[2,4-dimethylquinolin-8-yl)oxymethyl]-2,4-dichloro-phenyl]sulfonyl]-2(S)-[[4-[4-(aminoiminomethyl) - phenylcarbonyl]piperazin - 1 - yl]carbonyl]pyrrolidine (LF16-0335C) (Pruneau *et al*., 1998; 1999).

Cloning of the cDNAs encoding for bradykinin receptor subtypes and their functional expression in cultured cells provide a useful system to study the pharmacological profile of a given molecule (Hess *et al*., 1994). This is important since the binding affinity and functional antagonist property of a drug for the B_2 receptor may extensively vary from one species to another (Paquet *et al*., 1999). Until now, the kinin B_2 receptor has been cloned from the rat, mouse, rabbit and human (Hess *et al*., 1992; 1994; McEachern *et al*., 1991; Bachvarov *et al*., 1995), and more recently, from the guinea-pig (Farmer *et al*., 1998). In this latter study, the kinin B_1 receptor agonist, DBK as well as the kinin B_1 receptor antagonist des-Arg^9-[Leu^8]-BK (DLBK) elicited a Ca^{2+} efflux in CHO cells transfected with the guinea-pig lung bradykinin B_2 receptor cDNA despite DBK did not bind to the receptor. Furthermore, the kinin B_2 receptor antagonist, HOE140 behaved as a fairly potent agonist on this cloned receptor as revealed by a concentration-dependent increase of the Ca^{2+} efflux. These atypical pharmacological properties led us to further study this guinea-pig kinin receptor. In this respect, we cloned the cDNA encoding for the kinin B_2 receptor from both ileum and lung parenchyma and we subsequently characterized this receptor expressed in CHO-K1 cells using binding and functional assays.

Methods

Cell culture

Chinese Hamster Ovary cells (CHO-K1, CCL-61) were purchased from American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown to 80% confluence in nutrient mixture F-12 (HAM) supplemented with 10% Fetal Bovine Serum and containing 2 mM non essential amino acid (NEM), 1 mM sodium pyruvate, 2 mM Glutamax, 10^5 UI l^{-1} penicillin, 100 mg l^{-1} streptomycin.

PCR cloning of the bradykinin receptor from both lung and ileum of the guinea-pig

Total RNA was extracted from lung parenchyma and ileum fragments using RNeasy™ protocol (Qiagen, Courtaboeuf, France). Subsequently, mRNA was reverse-transcribed using Expand Reverse transcriptase (Boehringer, Mannheim, Germany). Based on the published cDNA receptor sequence (Farmer *et al*., 1998), a 49 mers 5′ oligonucleotide was designed to include an EcoRI restriction site and an improved consensus translation initiation site sequence adjacent to the initiation codon (Kozak, 1987). The 3′ oligonucleotide was 34 mers long and included a *Xho*I restriction site flanking downstream the stop codon.

The expected 1.1 kb cDNA was amplified by PCR using Red Goldstar polymerase (Eurogentec, Serain, Belgium). After digestion by the two aforementioned restriction endonucleases, the PCR amplified and purified fragment was ligated into the mammalian expression vector pcDNA3.0 (Invitrogen, Leek, The Netherlands) and in a bicistronic vector derivative of pcDNA3.0 (pIRES) generated in our laboratory. Briefly, in this latter vector, an Internal Ribosomal Entry Site of the encephalomyocarditis virus was added upstream of the Open Reading Frame of the neomycin resistance selection coding region (Rees *et al*., 1996). This construct allowed both the gene of interest and the resistance gene to be transcribed from a single bicistronic mRNA. The inserted fragment was subsequently sequenced using a SequiTherm™ Excell II Long-Read™ DNA Sequencing Kit (Tebu, Le Perray-en-Yvelines, France) and a DNA sequencer LI-COR 4000 (Science Tec, Les Ulis, France).

Stable expression of the guinea-pig receptor

Transfection of *Pvu*I linearized pcDNA3.0 and pIRES constructs was achieved in CHO-K1 using Superfect reagent (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. Forty-eight hours after recovering in complete medium, selection was initiated by the addition of 750 μg ml^{-1} neomycin (G418). One hundred and forty-four individual resistant clones were isolated by dilution plating and screened for their ability to bind [^{3}H]-bradykinin. After a 1 month selection period, G418 (750 μg ml^{-1}) was removed and clones were assessed. Clone XA2, which exhibited a high binding capacity was selected for further radioligand binding and functional assays.

[^{3}H]bradykinin binding experiments

Membranes of scrapped XA2 cells were prepared in TES (25 mM) binding buffer according the previously described method (Paquet *et al*., 1999). Saturation isotherms and competition binding experiments were performed as mentioned by Paquet *et al*. (1999) with increasing concentrations of [^{3}H]-BK from 0.05 to 2 nM. In order to determine inhibitory binding constants values (K_i) of various ligands, competition binding experiments were performed at room temperature by a 90 min incubation of membranes with [^{3}H]-bradykinin concentrations close to the K_D value and 11 increasing concentrations of competitor ligands.

Phosphoinositides hydrolysis assay

Cells were seeded at 2.5×10^5 ml^{-1} in 12 well-plates. Twenty-four hours later, the medium was replaced by 0.5 ml of serum free complete 199 medium containing 1 mCi l^{-1} [^{3}H]-*myo*-inositol for a 24 h period. The labeled cell sheet was washed twice with a phosphate buffer solution and incubated for 15 min at 37°C, 5% CO_2 in 0.5 ml of a phosphate buffer (pH 7.4) composed as follows: (mM) NaCl 136, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, HEPES 5, glucose 11. Ten mM LiCl was added for another 15 min period. Cells were subsequently incubated with a range of concentrations of bradykinin receptor ligands (BK, KD, DLBK, DBK, HOE140, LF16-0335C, NPC567, WIN64338) for 20 min.

In another set of experiments, HOE140 (0.1 μM) or LF16-0335C (1 μM) were added 20 min prior to incubation with increasing concentrations of BK. The reaction was stopped

by removal of the reaction medium and the addition of ice cold 5% perchlorid acid (0.5 ml) containing 50 mg l^{-1} phytic acid. After 30 min on ice, the solution was entirely sampled and the wells were rinsed with 0.5 ml of chilled 2.5% perchlorid acid. The 1 ml combined mixture was neutralized with 0.155 ml K_2CO_3 (2 M) and 0.025 ml HEPES (1 M), pH 7.4. Total inositol phosphate components (IPs) were assessed by applying 0.8 ml samples on anion exchange columns (Dowex AG1-X8) as described elsewhere (Berridge *et al*., 1982).

Drugs

BK, KD, D-Arg^0-[Hyp^3-D-Phe^7]-bradykinin (NPC567), D-Arg^0-[Hyp^3-D-Phe^7-Leu^8]-bradykinin, DBK and DLBK were from Bachem AG (Bubendorf, Switzerland). HOE140 was from Neosystem (Strasbourg, France). FR173657, WIN64338 and LF16-0335C were synthesized at Laboratoires Fournier. [^{3}H]-bradykinin (114.0 Ci mmol^{-1}) and [^{3}H] *myo*-inositol (22.2 Ci mmol^{-1}) were obtained from New England Nuclear (Les Ulis, France).

All cell culture reagents were purchased from Life Technologies (Cergy-Pontoise, France) except fetal bovine serum which was provided by Hyclone Laboratories Inc. (Logan, UT, U.S.A.).

Data analysis

Binding competition data and concentration-response curves for phosphoinositide hydrolysis were analysed using GraphPADInPlot (GraphPAD Software, San Diego, CA, U.S.A.). The maximal binding of [^{3}H]-BK at the equilibrium (B_{max}) and the equilibrium dissociation constant (K_D) were derived from saturation curves fitted with one site ligand binding model.

Values of inhibitory binding constants (K_i) were obtained from the Cheng-Prusoff's equation (Cheng & Prusoff, 1973):

$$K_i = [IC_{50}]/(1 + [L]/K_D)$$

where L and K_D are the concentration and equilibrium dissociation constant of the radioligand, respectively, and IC_{50} is the concentration of competing ligand reducing specific binding by 50%.

Results

Cloning

Based on the published sequence of the guinea-pig bradykinin receptor (Farmer *et al*., 1998), we amplified a 1.1 kb fragment containing the full open reading frame of the protein from lung parenchyma and ileum. The nucleotides sequence analysis of the guinea-pig parenchyma amplified cDNA was performed on three different clones and revealed that the 1116 base pairs Open Reading Frame encoded for a 372 amino-acid protein which was homologous to the previously reported sequence (Farmer *et al*., 1998) except for two bases, cytosine in position 371 and thymine in position 679 that were identified instead of thymine and adenine, respectively (Figure 1). The two corresponding codons led to a translation of the nucleic acid sequence into P^{124} and Y^{227} instead of L and N (Figure 1). The same modifications were found in the nucleic sequence of the cDNA encoding for the B_2 receptor of the guinea-pig ileum.

A

```
acc No AJ003243 355 ATCTGCTTCCTGATGCTGGTGAGCATTGACCGCTACCTGGCC 396
                119  I  C  F  L  M  L  V  S  I  D  R  Y  L  A  132

amplified cDNA  355 ATCTGCTTCCTGATGCCGGTGAGCATTGACCGCTACCTGGCC 396
                119  I  C  F  L  M  P  V  S  I  D  R  Y  L  A  132
```

B

```
acc No AJ003243  667 CAGGTGCTTCGCAACAACGAGATG 690
                 223  Q  V  L  R  N  N  E  M  230

amplified cDNA   667 CAGGTGCTTCGCTACAACGAGATG 690
                 223  Q  V  L  R  Y  N  E  M  230
```

Figure 1 Alignment of nucleotidic and corresponding protein translations of GenBank deposited cDNA sequence (accession No. AJ003243) and of the present amplified cDNA sequence from the guinea-pig B_2 receptor third transmembrane domain (A) and third intracellular loop (B). The mutations are indicated by underlined bold characters.

Binding experiments

In order to determine the influences of these two mutations, preliminary studies were performed using Cos-7 cells (CRL-1651, American Type Culture Collection, Rockville, MD, U.S.A.) transiently transfected with the cDNA encoding for the ileum B_2 receptor. Saturation binding experiments revealed that cell membranes bound specifically (80%) [^{3}H]-BK with a high affinity (K_D value of 0.29 ± 0.07 nM, $n = 6$) and exhibited a single saturable site (B_{max} value of 589 ± 171 fmoles mg^{-1}). Moreover BK, WIN64338 and HOE140 competed with [^{3}H]-BK giving K_i values of 0.39 ± 0.04 nM, 206.2 ± 48.3 nM and 0.19 ± 0.04 nM, respectively whereas both DBK and DLBK did not (K_i > 10,000 nM). Since nucleotide sequences of the cDNAs originating from two different tissues were the same, subsequent experiments focused on the characterization of the clone XA2 (pIRES construct) stably expressing the lung B_2 receptor in CHO-K1 cells.

XA2 cell membranes preparations exhibited a specific (93%) high affinity single and saturable binding site giving a K_D value of 0.09 ± 0.02 nM ($n = 4$) and a maximal binding capacity of $1{,}492 \pm 174$ fmoles mg^{-1} protein. Competition binding experiments by several kinin receptor ligands were performed and the corresponding K_i are reported in Table 1. BK bound to the cloned receptor leading to a K_i value of 0.21 ± 0.05 nM while the two specific kinin B_1 receptor ligands, DBK and DLBK were unable to compete with [^{3}H]-BK (K_i > 10,000 nM). The B_2 receptor peptide antagonist, HOE140, had a better affinity than NPC567, a non-selective B_1 and B_2 receptor ligand. Among the three non peptide B_2 antagonist compounds, WIN64338 exhibited the lowest affinity (211.20 ± 70.70 nM) whilst FR173657 and LF16-0335C, two novel selective non peptide B_2 receptor ligands, displayed a comparable affinity (Table 1).

Functional characterization

Activation of phospholipase C occurs when BK binds to the B_2 receptor leading to the hydrolysis of phosphoinositides

Table 1 Affinities of kinin ligands for the cloned guinea-pig bradykinin B_2 receptor stably expressed in CHO-K1 cells (clone XA2)

Ligands	K*i* (nM)
Bradykinin	0.21±0.05
Kallidin	0.03±0.02
des-Arg^9-bradykinin	>10000
des-Arg^9-[Leu^8]bradykinin	>10000
NPC 567	14.20±2.70
HOE140	0.22±0.18
D-Arg^0-[Hyp^3-D-Phe^7-Leu^8]-BK	13.10±2.70
WIN64338	211.20±70.80
FR173657	7.34±2.45
LF16-0335C	8.54±1.55

Reported *Ki* values are means±s.e.mean of four different [^{3}H] BK binding competition experiments.

and generation of inositol triphosphates (IPs) which induces the release of intracellular Ca^{2+} (Austin *et al*., 1997). BK induced a marked concentration-dependent increase of IPs accumulation in XA2 cells with an EC_{50} value of 0.44±0.12 nM as shown in Figure 2, demonstrating the functional coupling of the stably transfected B_2 guinea-pig receptor. The maximum response was reached at the concentration of 1 μM of BK so that data were normalized and expressed as percentage of this maximal BK response. Using this functional assay, the capacity of several B_1 and B_2 receptors ligands to stimulate the IPs turnover was assessed. Similarly to BK, KD induced a concentration-dependent increase of IPs with an EC_{50} value of 6.88±0.28 nM. Neither DLBK nor DBK stimulated or inhibited IPs production in a range of concentrations of 0.01 nM to 10 μM (Figure 2). HOE140 (from 0.01 nM to 10 μM) did not stimulate IPs production in XA2 cells. A modest 20% decrease of basal IPs content occurred with WIN64338 or LF16-0335C (Figure 3). NPC567 slightly decreased by 25% IPs formation from 0.1 nM to 1 nM whilst at higher concentrations it produced a 20% increase of IPs with an EC_{50} value of 9.2 nM. HOE140 (0.1 μM) shifted to the right the concentration-response curve to BK (Figure 4) and depressed the maximal response by 40%. The pK_B value was 9.2±0.4 according to Kenakin's method of calculation (Kenakin, 1993). LF16-0335C (1 μM) inhibited IPs production induced by BK with a pK_B value of 8.4±0.3 (Figure 4).

Discussion

Based on the GenBank deposited sequence (accession No. AJ003243), we have amplified cDNAs encoding for the B_2 bradykinin receptor from guinea-pig lung parenchyma and ileum. The analysis of the two cDNA amplified sequences were identical suggesting that a single B_2 receptor is expressed in both tissues thus confirming previous findings based on pharmacological responses (Pruneau *et al*., 1995). Additionally, cDNAs encoding for the ileum and the lung B_2 receptor contained two mutations when compared to the original published sequence (Farmer *et al*., 1998). These two nucleic acid differences induced modifications of the corresponding codons resulting in P^{124} and Y^{227} instead of L and N, respectively.

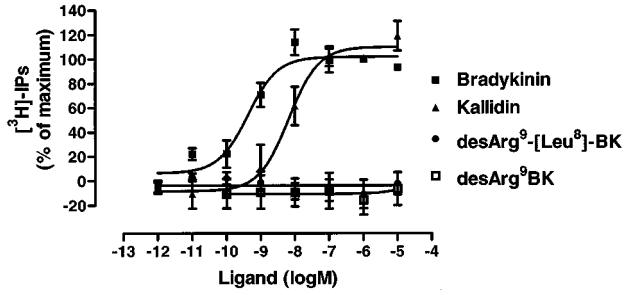

Figure 2 Concentration-response curves of total [^{3}H]-inositol phosphate accumulation induced by kinins. Each point is the mean± s.e.mean of at least three different experiments. The maximal response to 1 μM BK was 100% whilst 0% represented the basal condition without any stimulation.

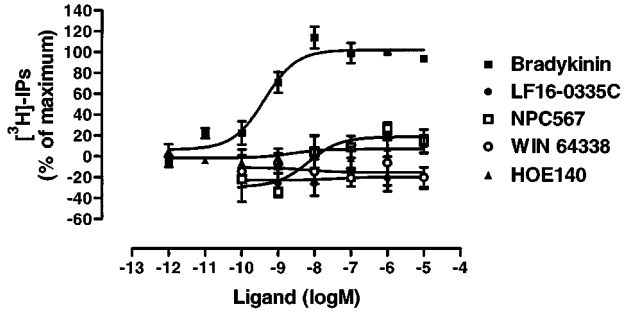

Figure 3 Concentration-response curves of total [^{3}H]-inositol phosphate accumulation induced by kinin B_2 receptor ligands. Each point is the mean±s.e.mean of at least three different experiments. The maximal response to 1 μM BK was 100% whilst 0% represented the basal condition without any stimulation.

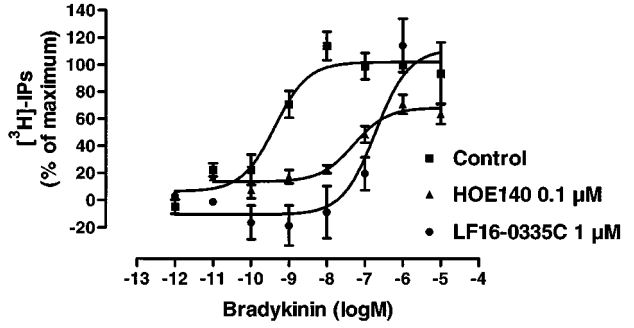

Figure 4 Effect of HOE140 (0.1 μM) and LF16-0335C (1 μM) on the total [^{3}H]-inositol phosphate production induced by increasing concentrations of BK. Control curve was represented by the concentration-response curve of BK on IPs production. Each point is the mean±s.e.mean of at least three different experiments. The maximal response to 1 μM BK was 100% whilst 0% represented the basal condition without any stimulation.

Neither an alternative splicing, nor RNA editing events were probably involved in these mutations. Alternative splicing may occur depending on the cell type, the stage of cell development, the chemical an physical environment resulted in the synthesis of different functional elongated or truncated proteins (Lewin, 1994). On the other hand, RNA editing in mammalian species modify C to U, U to C, A to I,

G to A or U to A (Brennicke *et al*., 1999) and this biological process cannot explain the two mutations. Finally, a misincorporation of nucleotides was also unlikely as reverse transcriptions that have been performed from ileum mRNA and from lung mRNA purifications led to amplify two identical cDNAs.

Whether these changes of amino-acid within the guinea-pig bradykinin B_2 receptor protein sequence may alter the pharmacological properties was primarily investigated by transfecting transiently the cDNA amplified from the ileum in Cos-7 cells. Subsequent binding studies revealed that the protein encoded by this cDNA exhibited a high affinity for [^{3}H]-bradykinin and competition experiments suggested that the pharmacological profile was typical of a bradykinin B_2 receptor. Then, the cDNAs amplified from the lung was stably transfected in CHO-K1 cells for further ligand binding and functional characterization. One clone, named XA2, expressing a moderate amount of receptors was used to determine the affinity of several well-known kinin ligands. In accordance with a previous study (Regoli & Barabe, 1980), BK and KD but not DBK and DLBK, had a high affinity for the guinea-pig bradykinin B_2 receptor. Moreover, HOE140 potently bound to this receptor as previously described with guinea-pig ileum membrane preparations (Regoli *et al*., 1990; Rhaleb *et al*., 1992; Trifilieff *et al*., 1992; Hock *et al*., 1991) and the affinity of other tested kinin receptor ligands for the cloned receptor was within the same range than for the native ileum receptor (Hock *et al*., 1991; Asano *et al*., 1997). These results demonstrate that the protein encoded by the amplified cDNA from guinea-pig displayed the binding features expected for a bradykinin B_2 receptor. Although the two amino-acid modifications did not affect the binding profile of the cloned receptor we investigated their role in the functional property of the receptor. Indeed, the sequence published by Farmer *et al*. (1998) encoded for a receptor exhibiting important functional differences in respect to the native guinea-pig bradykinin B_2 receptor such as a vigorous stimulation of Ca^{2+} signal in response to HOE140 and DBK (Farmer *et al*., 1998). In clone XA2 cells, BK stimulated IPs formation in a concentration-dependent manner whilst the B_2 receptor antagonists, HOE140, WIN64338 and LF16-0335C, failed to activate IPs hydrolysis. NPC 567 showed a weak agonist property in agreement with previous data (Félétou *et al*., 1994). Interestingly, we observed approximately a 20% reduction of basal IPs production with LF16-0335C, NPC567 (from 0.1 to 1 nM) and to a lesser extent with WIN64338. This may reflect an inverse agonist property of these compounds as previously described for LF 16-0335C in the cloned human B_2 receptor (Marie *et al*., 1999). However, this would clearly need further investigation.

Both HOE140 and LF16-0335C, a new potent and selective non-peptide B_2 receptor antagonist (Pruneau *et al*., 1998), inhibited BK-stimulated IPs turnover with similar pK_B values than those previously reported using isolated guinea-pig ileum (Rhaleb *et al*., 1992; Hock *et al*., 1991). As expected, specific B_1 receptor ligands, DBK and DLBK failed to increase IPs production.

The cDNA sequence that encoded for the guinea-pig B_2 receptor as previously described (Farmer *et al*., 1998) has been constructed using site directed mutagenesis technique and the CHO-K1 clone 2–3 stably expressing this receptor was subsequently assessed in our experimental conditions. [^{3}H]-BK bound specifically to the receptor ($96.3 \pm 0.7\%$) with a B_{max} value of 1420 ± 365.8 fmol mg^{-1} protein and a single KD value of 0.54 ± 0.09 nM. In competition binding experiments, the natural B_2 receptor agonist ligand BK competes with [^{3}H]-BK giving a K_i value of 0.04 ± 0.02 nM. The specific peptide B_2 receptor antagonist HOE140 and the specific non peptide B_2 receptor antagonist WIN64338 competitively displaced [^{3}H]-BK leading K_i values of 0.050 ± 0.001 nM and 67.8 ± 16.6 nM respectively, whilst the natural specific B_1 receptor agonist DBK was unable to compete with [^{3}H]-BK ($K_i > 10{,}000$ nM). In clone 2–3 functional assay, BK induced a concentration-dependent increase of IPs production with an EC_{50} value of 12.5 ± 3.9 nM. DBK stimulated IPs production with an EC_{50} of 643.8 ± 323.3 nM despite the B_1 agonist ligand DBK failed to compete with [^{3}H]-BK in binding experiments. The B_2 antagonist ligands HOE140 and WIN64338 raised IPs production by 35% from 0.1 nM to 10 μM.

These aforementioned results which are consistent with those from Farmer *et al*. (1998) showed that binding and functional properties of this receptor were not similar to that observed in clone XA2. They were also not typical of a classical guinea-pig type 2 bradykinin receptor, since the B_2 antagonists HOE140 and WIN64338 and the B_1 agonist DBK behaved as agonists of this receptor.

Therefore, we conclude that the receptor we have cloned fulfils the classical features of a bradykinin B_2 receptor and displays a pharmacological profile quite similar to the native guinea-pig bradykinin B_2 receptor.

The two amino-acid modifications between the published sequence and the present sequence appear to affect markedly the function of the receptor. The first mismatch Leucine 124 in Proline is located 4 amino-acids upstream of the G-protein coupled receptor consensus DRY sequence which is involved in the signal transduction of the B_2 receptor (Prado *et al*., 1997; 1998). Amino acid residues at the boundary between the third transmembrane domain (TM3) and the second intracellular loop (ICL2) of GPCRs have been implicated in preserving high affinity agonist binding while reducing activation of the alpha2A-adrenergic receptors (Wang *et al*., 1991). The second difference consisting in an Asparagine instead of a Tyrosine is located at position 227 of the third intracellular loop (ICL3) of the receptor which is important in coupling the β-Adrenergic receptor to G proteins (Wong *et al*., 1990). The study of the beta 3-adrenergic receptor ICL3 highlighted the critical role of this region for coupling to G proteins (Guan *et al*., 1995). In addition, Frandberg *et al*. (1998) reported that a point mutation in the ICL3 of human melanocortin 1 receptor reduced its functional response although the ligand binding affinity was not affected.

We conclude that the sequence of the guinea-pig B_2 bradykinin receptor was identical between the lung and the ileum further supporting the existence of a single bradykinin B_2 receptor subtype in this species (Pruneau *et al*., 1995). In addition, it does not favor the presence of a B_3 receptor subtype as previously postulated (Farmer *et al*., 1998; Farmer & Desiato, 1994). We also propose that the two amino-acid residue differences found in TM3 and ICL3 as compared to the first reported cDNA sequence play a role in the receptor activation. Further point-directed mutagenesis studies of these two amino-acids would be needed to further elucidate their importance in guinea-pig B_2 receptor activation.

References

ARAMORI, I., ZENKOH, J., MORIKAWA, N., O'DONNELL, N., ASANO, M., NAKAMURA, K., IWAMI, M., KOJO, H. & NOTSU, Y. (1997). Novel subtype-selective nonpeptide bradykinin receptor antagonists FR167344 and FR173657. *Mol. Pharmacol.,* **51,** 171 – 176.

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.

AUSTIN, C.E., FAUSSNER, A., ROBINSON, H.E., CHAKRAVARTY, S., KYLE, D.J., BATHON, J.M. & PROUD, D. (1997). Stable expression of the human kinin B_1 receptor in Chinese hamster ovary cells. Characterization of ligand binding and effector pathways. *J. Biol. Chem.,* **272,** 11420 – 11425.

BACHVAROV, D.R., SAINT-JACQUES, E., LARRIVEE, J.F., LEVESQUE, L., RIOUX, F., DRAPEAU, G. & MARCEAU, F. (1995). Cloning and pharmacological characterization of the rabbit B_2 receptor. *J. Pharmacol. Exp. Ther. U.S.A.,* **275,** 1623 – 1630.

BASTIAN, S., LOILLIER, B., PAQUET, J.L. & PRUNEAU, D. (1997). Stable expression of human kinin B1 receptor in 293 cells: pharmacological and functional characterization. *Br. J. Pharmacol.,* **122,** 393 – 399.

BERRIDGE, M.J., DOWNES, C.P. & HANLEY, M.R. (1982). Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.,* **206,** 587 – 595.

BHOOLA, K.D., FIGUEROA, C.D. & WORTHY, K. (1992). Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol. Rev.,* **44,** 1 – 80.

BRENNICKE, A., MARCHFELDER, A. & BINDER, S. (1999). RNA editing. *FEMS Microbiol. Rev.,* **23,** 297 – 316.

CHENG, Y. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (IC50) of an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099 – 3108.

DEBLOIS, D., BOUTHILLIER, J. & MARCEAU, F. (1988). Effect of glucocorticoids, monokines and growth factors on the spontaneously developing responses of the rabbit isolated aorta to des-Arg^9-bradykinin. *Br. J. Pharmacol.,* **93,** 969 – 977.

FARMER, S.G. & DESIATO, M.A. (1994). Effects of a novel nonpeptide bradykinin B_2 receptor antagonist on intestinal and airway smooth muscle: further evidence for the tracheal B_3 receptor. *Br. J. Pharmacol.,* **112,** 461 – 464.

FARMER, S.G., POWELL, S.J., WILKINS, D.E. & GRAHAM, A. (1998). Cloning, sequencing and functional expression of a guinea pig lung bradykinin B_2 receptor. *Eur. J. Pharmacol.,* **346,** 291 – 298.

FÉLÉTOU, M., GERMAIN, M., THURIEAU, C., FAUCHÈRE, J.L. & CANET, E. (1994). Agonist and antagonistic properties of the bradykinin B_2 receptor antagonist, Hoe 140, in isolated blood vessels from different species. *Br. J. Pharmacol.,* **112,** 683 – 689.

FRANDBERG, P.A., DOUFEXIS, M., KAPAS, S. & CHHAJLANI, V. (1998). Human pigmentation phenotype: a point mutation generates nonfunctional MSH receptor. *Biochem. Biophys. Res. Commun.,* **245,** 490 – 492.

GUAN, X.M., AMEND, A. & STRADER, C.D. (1995). Determination of structural domains for G protein coupling and ligand binding in beta 3-adrenergic receptor. *Mol. Pharmacol.,* **48,** 492 – 498.

HESS, J.F., BORKOWSKY, J.A., MACNEIL, T., STONESIFER, G.Y., FRAHER, J., STRADER, C.D. & RANSOM, R.W. (1994). Differential pharmacology of cloned human and mouse B_2 bradykinin receptors. *Mol. Pharmacol.,* **45,** 1 – 8.

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). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.,* **102,** 769 – 773.

KENAKIN, T. (1993). Allotopic, noncompetitive, and irreversible antagonism. In *Pharmacologic Analysis of Drug-receptor Interaction*. ed. Kenakin, T., pp. 323 – 343. New York: Raven Press.

KOZAK, M. (1987). An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.,* **15,** 8125 – 8148.

LEWIN, B. (1994). The apparatus for nuclear splicing. In *Gene V*. pp. 911 – 939. New York: Oxford University Press.

MARCEAU, F. (1997). Kinin B_1 receptor induction and inflammation. In *The Kinin System*. ed. Farmer, S.G., pp. 143 – 156. London: Accademic Press.

MARIE, J., KOCH, C., PRUNEAU, D., PAQUET, J.L., GROBLEWSKI, T., LARGUIER, R., LOMBARD, C., DESLAURIERS, B., MAIGRET, B. & BONNAFOUS, J.C. (1999). Constitutive activation of the human bradykinin B_2 receptor induced by mutations in transmembrane helices III and VI. *Mol. Pharmacol.,* **1,** 92 – 101.

MCEACHERN, A.E., SHELTON, E.R., BHAKTA, S., OBERNOLTE, R., BACH, C., ZUPPAN, P., FUJISAKI, J., ALDRICH, R.W. & JARNAGIN, K. (1991). Expression cloning of a rat B_2 bradykinin receptor. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 7724 – 7728.

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. Biol. 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.

PRADO, G.N., MIERKE, D.F., PELLIGRINI, M., TAYLOR, L. & POLGAR, P. (1998). Motif mutation of bradykinin B_2 receptor second intracellular loop and proximal C terminus is critical for signal transduction, internalization, and resensitization. *J. Biol. Chem.,* **273,** 33548 – 33555.

PRADO, G.N., TAYLOR, L. & POLGAR, P. (1997). Effects of intracellular tyrosine residue mutation and carboxyl terminus truncation on signal transduction and internalization of the rat bradykinin B_2 receptor. *J. Biol. Chem.,* **272,** 14638 – 14642.

PROUD, D. & KAPLAN, A.P. (1988). Kinin formation: mechanisms and role in inflammatory disorders. *Annu. Rev. Immunol.,* **6,** 49 – 83.

PRUNEAU, D., LUCCARINI, J.M., DEFRENE, E., PAQUET, J.L. & BELICHARD, P. (1995). Pharmacological evidence for a single bradykinin B_2 receptor in the guinea-pig. *Br. J. Pharmacol.,* **116,** 2106 – 2112.

PRUNEAU, D., LUCCARINI, J.M., FOUCHET, C., DEFRENE, E., FRANCK, R.M., LOILLIER, B., DUCLOS, H., ROBERT, C., CREMERS, B., BÉLICHARD, P. & PAQUET, J.L. (1998). LF 16.0335, a novel potent and selective nonpeptide antagonist of the human bradykinin B_2 receptor. *Br. J. Pharmacol.,* **125,** 365 – 372.

PRUNEAU, D., LUCCARINI, J.M., FOUCHET, C., DEFRENE, E., FRANCK, R.M., LOILLIER, B., DUCLOS, H., ROBERT, C., CREMERS, B., BELICHARD, P. & PAQUET, J.L. (1999). *In vitro* and *in vivo* effects of the new nonpeptide bradykinin B_2 receptor antagonist; LF 16-0335C, on guinea-pig and rat kinin receptors. *Fundam. Clin. Pharmacol.,* **13,** 75 – 83.

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.

REES, S., COOTE, J., STABLES, J., GOODSON, S., HARRIS, S. & LEE, M.J. (1996). Bicistronic vector for the creation of stable mammalian cell lines that predisposes all antibiotic-resistant cells to express recombinant protein. *Biotechniques,* **20,** 102 – 110.

REGOLI, D. & BARABE, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.,* **32,** 1 – 46.

REGOLI, D., RHALEB, N.E., DION, S. & DRAPEAU, G. (1990). New selective bradykinin receptor antagonists and bradykinin B_2 receptor characterization. *Trends Pharmacol. Sci.,* **11,** 156 – 161.

RHALEB, N.E., ROUISSI, N., JUKIC, D., REGOLI, D., HENKE, S., BREIPHOL, G. & KNOLLE, J. (1992). Pharmacological characterization of a new highly potent B_2 receptor antagonist (HOE 140: D-Arg-[Hyp3,Thi5,D-Tic7,Oic8]bradykinin). *Eur. J. Pharmacol.,* **210,** 115 – 120.

STEWART, J.M. (1995). Bradykinin antagonists: development and applications. *Biopolymers,* **37,** 143–155.

TRIFILIEFF, A., DASILVA, A., LANDRY, Y. & GIES, J.P. (1992). Effect of Hoe 140, a new B_2 noncompetitive antagonist, on guinea pig tracheal bradykinin receptors. *J. Pharmacol. Exp. Ther.,* **263,** 1377–1382.

WANG, C.D., BUCK, M.A. & FRASER, C.M. (1991). Site-directed mutagenesis of alpha 2A-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol. Pharmacol.,* **40,** 168–179.

WONG, S.K., PARKER, E.M. & ROSS, E.M. (1990). Chimeric muscarinic cholinergic: beta-adrenergic receptors that activate Gs in response to muscarinic agonists. *J. Biol. Chem.,* **265,** 6219–6224.

(Received August 13, 2001
Accepted November 8, 2001)