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Bradykinin Antagonists in Human Systems: Correlation between Receptor Binding, Calcium Signalling in Isolated Cells, and Functional Activity in Isolated Ileum*

*Maciej Wieczorek,†‡ Anna Pilyavskaya,† Michael Burkard,† John S. Zuzack,† Steven W. Jones,†§ Mary D. Francis,†‖ Virginia E. Beckey,† Sherman E. Ross,† Val S. Goodfellow,† Timothy D. Fitzpatrick,†¶ Manoj V. Marathe,† Albert Gyorkos,† Lyle W. Spruce,† William M. Selig,† John M. Stewart,** Lajos Gera** and Eric T. Whalley†*

†CORTECH, INC., DENVER, CO 80221, U.S.A.; AND **DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF COLORADO SCHOOL OF MEDICINE, DENVER, CO 80262, U.S.A.

ABSTRACT. The determination of the relationship between ligand affinity and bioactivity is important for the understanding of receptor function in biological systems and for drug development. Several physiological and pathophysiological functions of bradykinin (BK) are mediated via the B_2 receptor. In this study, we have examined the relationship between B_2 receptor (soluble and membrane-bound) binding of BK peptidic antagonists, inhibition of calcium signalling at a cellular level, and *in vitro* inhibition of ileum contraction. Only human systems were employed in the experiments. Good correlations between the studied activities of BK antagonists were observed for a variety of different peptidic structures. The correlation coefficients (r) were in the range of 0.905 to 0.955. In addition, we analyzed the effect of the C-terminal Arg^9 removal from BK and its analogs on B_2 receptor binding. The ratios of binding constants (K_i^{+Arg}/K_i^{-Arg}) for the Arg^9 containing compounds and the corresponding des-Arg^9 analogs varied from about 10 to 250,000. These ratios strongly depend on the chemical structures of the compounds. The highest ratios were observed for two natural agonist pairs, BK/des-Arg^9-BK and Lys^0-BK/des-Arg^9-Lys^0-BK. BIOCHEM PHARMACOL **54**;2:283–291, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. bradykinin antagonists; B_2 receptor; calcium flux; human ileum

BK†† is a vasoactive nonapeptide that is formed locally from kininogen precursors following inflammation or tissue damage. BK causes vasodilation and increases vascular permeability, resulting in edema. It also produces contraction of bronchiolar, gastrointestinal, and uterine smooth muscles [1, 2]. The actions of BK and its metabolite, des-Arg^9-BK, are mediated through at least two different types of receptors named B_2 and B_1, respectively [1–3]. Since the determination of the BK sequence in 1960, a large number of antagonists have been synthesized and tested *in vivo* and *in vitro*, mostly in animal systems [4–7]. However, in recent years new biochemical methods have been developed that allow one to evaluate BK and its antagonists in human cell systems [8–12]. Present availability of many human cell lines and recombinant receptors [13–15] greatly facilitates signal transduction and binding studies on human BK receptors. It is well known that calcium release from intracellular stores is an important event in signalling, triggered by peptide hormone receptors. It has been shown that the B_2 receptor is also coupled to calcium release in many cell types including smooth muscle cells, fibroblasts, and synovial, endothelial, and neuronal cells [12, 16–19]. A good correlation between antagonist binding and inhibition of receptor bioactivity is expected and is observed frequently [20]. However, this kind of structure-to-function relationship is often very complex and cannot be extrapolated easily from one receptor system to another for a variety of reasons. These may include different receptor subclasses or receptor states, events con-

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‡ Corresponding author: Dr. Maciej Wieczorek, Cortech, Inc., 6850 North Broadway, Denver, CO 80221. Tel. (303) 650-1200; FAX (303) 650-1220; E-mail: mwieczorek@crtq.com

§ Present address: Sepracor, Marlborough, MA 01752.

‖ Present address: Cadus Pharmaceutical Corp., Lakewood, CO 80214.

¶ Present address: Synthetech, Inc., Albany, OR 97321.

†† *Abbreviations:* B_2, bradykinin$_2$ receptor; BK, bradykinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; EMEM, Eagle's Minimum Essential Medium; FBS, fetal bovine serum; mR, membrane-bound receptor; PCR, polymerase chain reaction; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; pX, −Log(X); sR, soluble receptor; and TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid.

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trolled by kinetics rather than equilibrium, and possible interactions with some other receptors or membrane proteins that may not be detected by binding assays.

This paper describes studies on the correlation between binding using soluble and membrane-bound human B_2 receptor, calcium flux in human fibroblasts, and pA_2 values in human ileum. We have used only human receptor systems to limit problems associated with interspecies variability. Further, only peptide-based compounds, including several novel BK antagonists, were employed in these studies. In addition, we have analyzed the effect of removal of Arg^9 from the agonists and antagonists on B_2 receptor binding.

MATERIALS AND METHODS

Materials

Bradykinin, kallidin, digitonin, BSA, TES, HEPES, PIPES, CHAPS, glucose, 1,10-phenanthroline, and EGTA were purchased from the Sigma Chemical Co. (St. Louis, MO). Bacitracin was obtained from Calbiochem (San Diego, CA). [^{3}H]BK was purchased from DuPont-NEN (Boston, MA). Fura-2AM and Pluronic F-127 were obtained from Molecular Probes Inc. (Eugene, OR). EMEM, 200 mM L-glutamine, and sodium pyruvate were from BioWhittaker (Walkersville, MD), and penicillin-streptomycin and F-12 Ham's medium were from Gibco BRL (Gaithersburg, MD). FBS was from Irvine Scientific (Santa Ana, CA) or Hyclone (Logan, UT). Human lung diploid fibroblasts WI-38 were purchased from the American Type Culture Collection (Rockville, MD) at 14 passages and used in the Ca^{2+}-flux assay from passage 16 to 28. BK antagonists coded with a "B" prefix were synthesized at the University of Colorado; all other antagonists were synthesized at Cortech.

Isolation and Expression of the Human B_2 Receptor Gene

RNA was isolated from human lung fibroblasts (CCD-16 LU obtained from the ATCC) using the method of Chirgwin *et al.* [21]. The RNA was transcribed into cDNA using MMLV reverse transcriptase with the primer GACTCG AGTCGACATCGATTTTTTTTTTTTTTT according to the procedure of Maniatis *et al.* [22]. The human B_2 receptor cDNA was amplified selectively using nested PCR. The first round PCR utilized the two primers CTCCGAG GAGGGGTGGG and CCTGAAAAGCAACTGTCCC and Taq DNA polymerase (Promega, Madison, WI). Twenty-five rounds of PCR were done under the following experimental conditions: 94°, 1 min for denaturation, 50°, 1 min for annealing, followed by 72° and 3 min for extension. Excess primers were removed with a Centricon-30 micro concentrator (Amicon Inc., Beverly, MA). A portion of this first round reaction was used as a template in a second round of PCR with the following primers GCAAGCTTCGTGAGGACTCCGTGCCC and CGC TCTAGACAAATTCACAGCCC. The number of rounds of PCR and the conditions were the same as those used for the first round. The DNA obtained after the second round was digested with the restriction enzymes *Hin*dIII and *Xba*I using standard methodology. Cesium chloride-purified pRc/CMV (Invitrogen, San Diego, CA) was also digested with *Hin*dIII and *Xba*I, using standard methodology. The products of the two digests were resolved on a 1% low melt agarose gel. The human B_2 receptor DNA (approximately 1.1 kb) and the pRc/CMV DNA (approximately 5.5 kb) were excised from the gel. The gel slices containing these DNAs were heated at 65°, and aliquots were combined in a reaction containing T4 DNA ligase. The reaction was incubated overnight at 15°. An aliquot of this reaction was used to transform frozen competent *Escherichia coli* DH5α cells (Gibco BRL). Transformants containing the human B_2 receptor DNA were selected on LB+amp plates. One of the transformants was chosen, and the sequence of the human B_2 receptor DNA insert was determined using the Sequenase enzyme (United States Biochemical, Cleveland, OH) according to the manufacturer's instructions. This sequence was compared to the sequence of Hess *et al.* [13]. Two nucleotide misincorporations were detected, and those that altered the amino acid sequence of the receptor were corrected by site-directed mutagenesis [23]. Cesium chloride purified human B_2 receptor-pRc/CMV plasmid was transfected into CHO-K1 (ATCC) cells using the Lipofectamine reagent (Gibco BRL). Transfectants were selected with the antibiotic G418 and screened (see details below) for [^{3}H]BK (Dupont NEN) binding. One clone, S34f, was chosen based upon binding levels, binding kinetics, and inhibition patterns as the clone to be used for all human B_2 receptor binding assays. This clone also displayed detectable calcium flux elicited by BK (see "Calcium Flux Assay" below).

Binding to Membrane-Bound B_2 Receptor

Preparation of CHO-K1 cell membranes containing human B_2 receptor and the binding assay were performed as described previously [24]. Briefly, human clone membrane suspensions were incubated with 0.3 nM [^{3}H]BK with or without test compounds in 25 mM TES, 2 μM 1,10-phenanthroline, 2 μM captopril, 0.014% bacitracin, 0.1% BSA, pH 6.8, at room temperature for 45 min. The membranes were harvested by quick filtration in a Tomtec harvester with ice-cold wash Tris buffer (pH 7.5) onto Wallac printed glass fiber "B" filtermats presoaked with polyethylenimine. Membrane-bound radioactive material, retained by the filtermats, was counted in a Wallac 1450 microbeta counter. Nonspecific binding was determined by the addition of 1000-fold excess of nonradioactive BK over [^{3}H]BK. The K_d value for BK was 0.72 ± 0.23 nM.

Binding to Solubilized B_2 Receptor

For the preparation of soluble receptors, the membranes of transfected CHO-K1 cells were thawed to 4° and centri-

fuged in an Eppendorf centrifuge (15,000 g) at 4° for 2–4 min. Pellets were reconstituted with 5 parts of 20 mM PIPES, 1 mM 1,10-phenanthroline, 0.1 mM bacitracin, 0.25% CHAPS [25], pH 6.8, mixed for 10 min at 4° and centrifuged for 30 min in a Beckman L7 Ultracentrifuge (96,075 g), and the supernatant was stored on ice until used. The solubilized receptor solutions were incubated at 4° for 6 hr in the presence of 5 nM [^{3}H]BK with or without test compounds in the PIPES buffer described above containing 0.125% CHAPS. The B_2 receptor concentration was less than 0.25 nM. After incubation, the receptor–[^{3}H]BK complex was separated from free [^{3}H]BK on an analytical Sephadex G-50 column at 4°. Radioactivity of the complex was measured using Wallac Hi-Load scintillation mixture (LKB) in a Wallac 1450 microbeta counter. The K_d value for BK was 1.5 ± 0.2 nM. Nonspecific binding was determined as described above.

Calcium Flux Assay

WI-38 fibroblasts were cultured in EMEM with Earle's BBS (BioWhittaker) containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 5% penicillin-streptomycin at 37° in an atmosphere of 7% CO_2–93% air. Cells were kept for 1.5 hr before experiments in modified EMEM containing 0.1% BSA (EMEM–BSA). At confluence, cells were harvested with cold (4°) 0.025% trypsin/EDTA solution (Clonetics, San Diego, CA) for 1 min, washed twice with EMEM–BSA, and loaded with 5 μM Fura-2AM containing 0.05% Pluronic F-127 [26] during a 30-min incubation at 37°, washed again with EMEM–BSA and twice with Krebs buffer, pH 7.4 (120 mM NaCl, 475 mM KCl, 1.1 mM $CaCl_2$, 1.44 mM $MgSO_4$, 1 mM KH_2PO_4, 25 mM HEPES, 5 mM $NaHCO_3$, 11 mM glucose, and 0.1 mM EGTA). Cells were kept on ice before experiments; however, all experiments were performed at room temperature. Different concentrations of antagonists were preincubated with 2×10^6 cells for 5 min, and BK was used at a concentration of 20 nM, which elicited approximately 80% of the maximal BK response (without antagonist). The EC_{50} value for BK was 7.5 ± 1.2 nM. The fluorescence measurements (corrected for cell autofluorescence) were performed using a Perkin–Elmer LC-50B Luminescence Spectrometer with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm [26, 27]. Maximum and minimum fluorescence ratio signals were measured using digitonin and EGTA at final concentrations of 75 and 50 μM, respectively.

Measurements of Antagonist Activity in Human Ileum

Whole human ileum was delivered on ice in Urrocolins buffer from the International Institute for the Advancement of Medicine (Exton, PA) approximately 24–36 hr after death. Donors were of either sex between the ages of 19 and 60. The mucosa layer was removed, and the tissue was cut into longitudinal strips approximately 5 by 20 mm. These strips were equilibrated for 1 hr in 8 mL siliconized tissue baths under 2 g resting tension at 37° in gassed (95% O_2 and 5% CO_2) Kreb's solution (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 11.1 mM glucose). Tissues were primed initially with 1 μM BK followed by a 45-min wash period. Antagonist potencies were measured using BK as an agonist. Cumulative concentration–response curves were repeated in the same tissue prior to and following a 15-min exposure to one of the antagonists. Data were calculated as percent of the maximum contraction in the control curve. Generally, three concentrations of antagonists were used. Shifts in the BK EC_{50} were used to calculate pA_2 (negative log of the molar concentration of antagonist that causes a 2-fold increase in the EC_{50}) values obtained against BK for each antagonist, according to the method of Arunlakshana and Schild [28]. Compound B9668 displayed a biphasic response with a minor phase and a major phase; only pA_2 for the major phase was used for the correlation studies. For compounds B9066 and B9812, only pK_b values were measured, where $pK_b = -\text{Log}([\text{antagonist}]/[x - 1])$ and x is the dose ratio defined as IC_{50}[antagonist + agonist]/IC_{50}[agonist].

In Vitro Proteolysis

Proteolytic stability studies of peptidic compounds were performed as described by Goodfellow *et al.* [24].

Data Analysis

Analysis of the receptor binding data was performed using a standard single-site competition model. The IC_{50} or Log(IC_{50}) values were calculated by a nonlinear least squares regression analysis program (GraFit, Erithacus Software). K_i constants were calculated from the equation $IC_{50} = K_i(1 + ([^3\text{H}]\text{BK})/K_d)$. In the binding experiments, the intra-assay standard errors for Log (IC_{50}) were in the range of 0.05 to 0.2 and in most cases below 0.1. The inter-assay standard deviations for pK_i or pIC_{50} were in the range of 0.05 to 0.3 except in the case of B9696 and B9698 with the membrane-bound receptor, where the inter-assay standard deviations were 0.62 and 0.55, respectively. In the case of the calcium flux assay, the IC_{50} or Log (IC_{50}) values were obtained using a four-parameter fitting routine as described by De Lean *et al.* [29]. The intra-assay and inter-assay variabilities of pIC_{50} for the calcium flux experiments were in the range of 0.01 to 0.2. All of the assays were performed one to three times depending on the experimental error evaluation during computer calculations. All of the experiments with human ileum were conducted using 5–15 ileum strips from 2 to 6 donors. The pA_2 or pK_b values were measured for each strip. The overall standard deviations (including different donors) of pA_2 and pK_b were in the range of 0.1 to 0.3 except for CP0678 with a standard deviation of 0.5.

TABLE 1. Amino acid sequences of BK antagonists

Compound	AA^0	AA^1	AA^2	AA^3	AA^4	AA^5	AA^6	AA^7	AA^8	AA^9
BK		Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg
KD	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg
B9066	DArg	Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg	
B9340	DArg	Arg	Pro	Hyp	Gly	Thi	Ser	DIgl	Oic	Arg
B9430	DArg	Arg	Pro	Hyp	Gly	Igl	Ser	DIgl	Oic	Arg
B9512		Arg	Pro	Hyp	Gly	Thi	Ser	DIgl	Oic	Arg
B9598		Arg	Pro	Hyp	Gly	Igl	Ser	DIgl	Oic	Arg
B9668	Gun-DArg	Arg	Pro	Hyp	Gly	Thi	Ser	DIgl	Oic	Arg
B9694	DArg	Arg	Pro	Pro	Gly	Igl	Ser	DIgl	Oic	Arg
B9696		Gun-Arg	Pro	Hyp	Gly	Igl	Ser	DIgl	Oic	Arg
B9698	Gun-DArg	Arg	Pro	Hyp	Gly	Igl	Ser	DIgl	Oic	Arg
B9812	DArg	Arg	Pro	Hyp	Gly	Igl	Ser	DIgl	Oic	
B10220	DArg	Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg	Arg
CP0088*	DArg	Arg	Pro	Hyp	Gly	Phe	Ser	DPhe	Leu	Arg
CP0298†	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Leu	
CP0597	DArg	Arg	Pro	Hyp	Gly	Thi	Ser	DTic	NChg	Arg
CP0678	daA	Arg	Pro	Hyp	Gly	Thi	Ser	DTic	NChg	Arg
CP0716	DArg	Arg	Pro	Hyp	Gly	Phe	Ser	DTic	NChg	Arg
CP0750	NK_1A‡-DArg	Arg	Pro	Hyp	Gly	Thi	Ser	DTic	NChg	Arg
CP0840	DHM-DLys	Arg	Pro	Hyp	Gly	Thi	Ser	DTic	NChg	Arg
CP0858	daA	Arg	Pro	Hyp	Gly	Phe	Ser	DTic	NChg	Arg
CP0885	DArg	Arg	Pro	Hyp	Gly	Phe	Cys	DTic	NChg	Arg
Hoe140§	DArg	Arg	Pro	Hyp	Gly	Thi	Ser	DTic	Oic	Arg
DRHoe140	DArg	Arg	Pro	Hyp	Gly	Thi	Ser	DTic	Oic	
NPC431‖		Arg	Pro	Pro	Gly	Thi	Ser	DPhe	Thi	Arg
NPC567‖	DArg	Arg	Pro	Hyp	Gly	Phe	Ser	DPhe	Phe	Arg
DRNPC567	DArg	Arg	Pro	Hyp	Gly	Phe	Ser	DPhe	Phe	

Abbreviations not defined previously: AA, amino acid residue; Cpg, α-cyclopentylglycine; DRHoe140, des-Arg^9-Hoe140; DRNPC567, des-Arg^9-NPC567; DTic, D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; daA, des-amino-Arginine; DHM, dihydromorphine; DHM-DLys, (5α,6α)-4,5-epoxy-1,7-methylmorphinan-6-ol-3-oxy-lauroyl-N-ε-DLys; Gun, guanidyl; Hyp, *trans*-4-hydroxyproline; Igl, α-2-indanylglycine; KD, kallidin (Lys^0-BK); NChg, *N*-cyclohexylglycine; NK_1A, neurokinin receptor antagonist; NK_1A-DArg, (*N*-methyl-indol-3-yl)carbonyl-(*N*-hexanoyl-*N*-α-DArg)-glycyl-*N*-methyl-*N*-phenylmethyl-3-(2-naphthyl)-L-alaninamide; Oic, (3aS,7aS)-octahydroindole-2-carboxylic acid; and Thi, α-2-thienylalanine.

* Regoli *et al.* [31].

† Regoli and Barabé [1].

‡ Modified neurokinin antagonist (NK_1A) [32], see also Abbreviations.

§ Hock *et al.* [33].

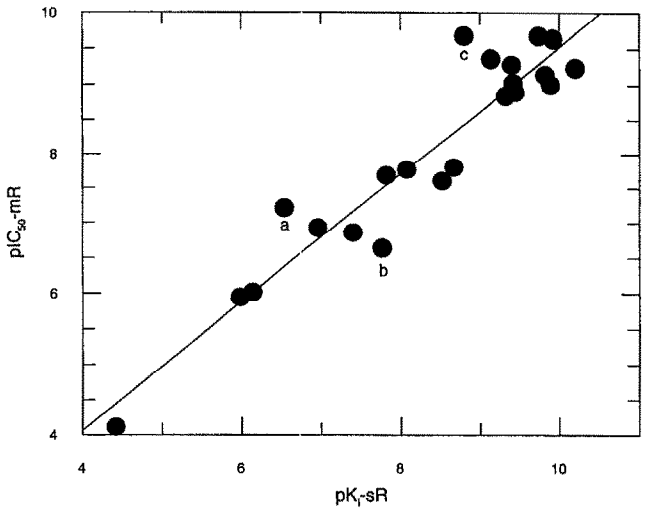
‖ Stewart and Vavrek [5], Hall [2].

RESULTS AND DISCUSSION

The determination of the relationship between ligand affinity and bioactivity is very important for understanding receptor function in biological systems and for drug development. Theoretically, a good correlation is expected and frequently observed between antagonist binding and inhibition of receptor bioactivity, because an antagonist only needs to block the receptor [20]. Weaker correlations are expected and observed in the case of agonists where the molecular events involve not only binding but also induction of a unique receptor conformation necessary for signal transduction [20]. However, even in the case of antagonists, significant deviations are also possible due to the following reasons: (1) different receptor subclasses or receptor states, (2) events controlled by kinetics rather than equilibrium, or (3) possible interactions with some other receptors or membrane proteins that may not be detected by the binding assays. Recent studies on the B_2 receptor gene in mouse indicate that a single gene appears to be responsible for conferring responsiveness to BK in this species [30]. Kinetic problems may be especially important in the case of very potent antagonists where very diluted compounds can react slowly with receptors despite their high k_{on} constants. In this situation, the duration of *in vitro* biological assays may be too short (because of the experimental design or technical limitations) to reach equilibrium. Ligand adsorption on plastic or glass surfaces may cause additional problems, especially with solutions without detergent or other agents used to eliminate surface adsorption.

For our studies we used only human systems and examined peptide-based BK analogs with diverse chemical structures (Table 1). Included in this list were two compounds with large molecular weight N-terminal substituents (CP0840 and CP0750 which contain dihydromorphine and a neurokinin A antagonist, respectively) and five compounds devoid of the C-terminal Arg residue. Several compounds are novel BK antagonists.

We selected two binding assays that employed detergent (CHAPS)-solubilized and membrane-bound recombinant human B_2 receptors (see Materials and Methods) to measure the receptor binding activity ($pK_i = -\text{Log}K_i$ or $pIC_{50} = -\text{Log}IC_{50}$) of these compounds. The rank order of



Compound	pK_i-sR	pIC_{50}-mR
CP0298	4.42	4.12
B9066	5.98	5.94
DRNPC567	6.14	6.01
B10220	6.54	7.21
NPC431	6.96	6.92
DRHoe140	7.40	6.85
CP0088	7.77	6.63
B9812	7.82	7.68
CP0678	8.08	7.76
CP0750	8.52	7.60
NPC567	8.67	7.80
B9340	8.80	9.67
CP0885	9.14	9.36
B9696	9.32	8.84
CP0716	9.40	9.28
B9598	9.42	9.02
CP0840	9.44	8.89
Hoe140	9.74	9.67
B9694	9.82	9.14
B9668	9.89	9.00
B9430	9.92	9.63
B9698	10.20	9.23

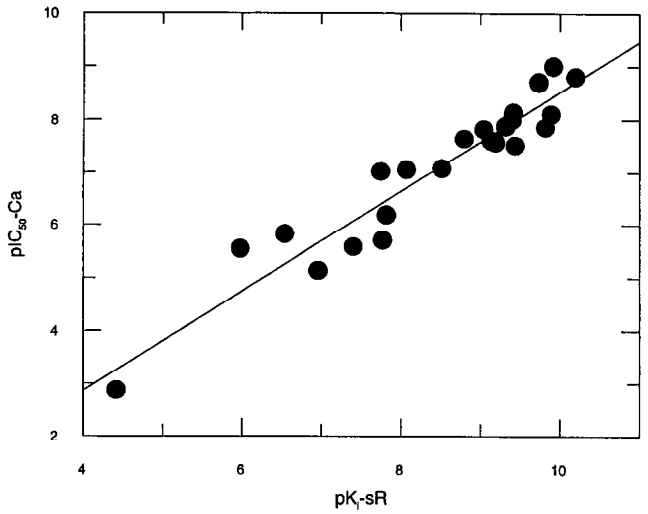
Correlation coefficient (r) = 0.9456; slope = 0.91.

FIG. 1. Correlation between antagonist binding affinities for membrane-bound and soluble receptors. Labels a, b, and c indicate compounds B10220, CP0088, and B9340, respectively.

potencies for some ligands was found to be in good agreement for both forms of the B_2 receptor [25]. However, the conformational state of the receptor bound to biomembrane fragments can still be different than the receptor state in detergent micelles; therefore, the correlation between the two forms of receptor is not necessarily predictable for all the ligands. The overall correlation between pK_i-sR and pIC_{50}-mR was reasonably good with a correlation coefficient (r) of 0.946; however, noticeable deviations were observed for B10220, CP0088, and especially B9340 (Fig. 1). The reason for this discrepancy is unknown. The chemical structures (Table 1), do not show distinct differences of these three compounds when compared with the other compounds. Proteolytic instability of these compounds is unlikely to be a problem, especially in the presence of proteolytic inhibitors. CP0088 showed a negative deviation (with respect to the calculated correlation line), but this compound was more resistant to *in vitro* proteolysis than BK (unpublished data; see also Materials and Methods). Further studies are necessary to clarify this issue.

Interestingly, a better correlation was observed between BK-elicited inhibition of calcium flux (pIC_{50}-Ca) in fibroblast and soluble receptor binding (pK_i-sR) with a correlation coefficient (r) of 0.955 (Fig. 2). Calcium is an important second messenger, but it only indicates a biochemical response to BK at the cellular level; therefore, we also studied the effects of the BK antagonists on BK-elicited human ileum contractions. Correlations between the pA_2 values in human ileum and soluble receptor binding, and pA_2 and inhibition of calcium flux are presented in Figs. 3 and 4, respectively. Only a small number of antagonists were tested in human ileum due to limited availability of this tissue and compounds. The correlation coefficients were 0.905 and 0.939, respectively. The deviations observed in Fig. 3 may be experimental rather than related to chemical structure (especially taking into account potential variability associated with different diseases of human donors and initial storage conditions of ileum samples). A relatively high negative deviation with B9812 most likely is not associated with proteolysis because this compound showed excellent *in vitro* stability in the presence of proteolytic enzymes (unpublished data). Also, the "binding paradox" [2], which addresses a discrepancy between absolute affinities determined from binding studies and those determined from functional pharmacological studies, does not seem to provide an adequate explanation for the present data. This discrepancy may be attributable to the differing ionic composition of assay buffers; however, the literature data [2] show that the rank order of antagonist affinities determined in binding studies correlates well with relative affinities obtained in functional studies.

The overall results (especially the correlation coefficients) described in this paper indicate that the binding constants of BK peptidic antagonists with the soluble or membrane-bound human B_2 receptors predict biological potency with good probability. However, one should be aware of kinetic limitations in functional assays. The addition of the calcium flux assay is also very useful for such predictions (Fig. 4) and necessary to differentiate agonists from antagonists. Some data showed relatively high variability; in two cases pK_b rather than pA_2 values were measured for human ileum, and several binding and calcium flux experiments were performed once (see "Data Analysis" in Materials and Methods), although using



Compound	pK_i-sR	pIC_{50}-Ca
CP0298	4.42	2.87
B9066	5.98	5.54
B10220	6.54	5.82
NPC431	6.96	5.13
DRHoe140	7.40	5.58
CP0858	7.75	7.01
CP0088	7.77	5.70
B9812	7.82	6.18
CP0678	8.08	7.05
CP0750	8.52	7.07
B9340	8.80	7.64
CP0597	9.05	7.82
CP0885	9.14	7.60
B9512	9.19	7.57
B9696	9.32	7.88
CP0716	9.40	8.00
B9598	9.42	8.13
CP0840	9.44	7.51
Hoe140	9.74	8.70
B9694	9.82	7.85
B9668	9.89	8.10
B9430	9.92	9.00
B9698	10.20	8.80

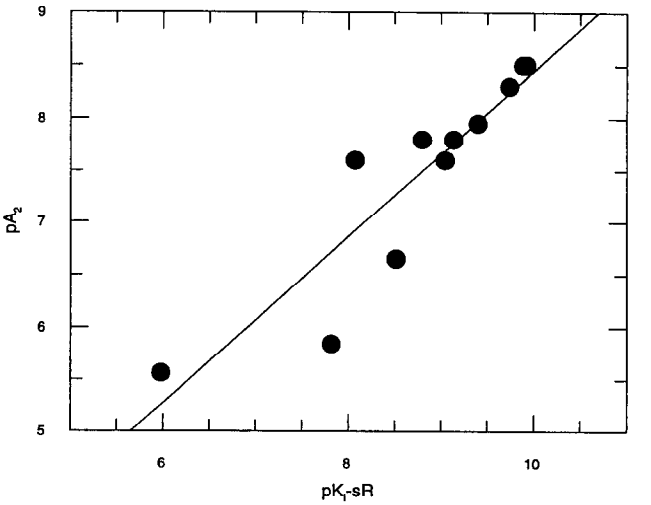
Correlation coefficient (r) = 0.9553; slope = 0.94.

FIG. 2. Correlation between inhibition of calcium flux and antagonist binding to soluble B_2 receptor.

enough data points to obtain statistically meaningful results.

It should be noted that two novel B_2 antagonists, B9430 and B9698 (Table 1, Figs. 1–3), showed comparable or better potency than the reference standard Hoe140 [33]. Compound B9430 also displayed very good stability [34]. B9698 (the α-guanido analog of B9430) was not tested for proteolytic stability; however, its resistance to proteolysis is expected to be very similar to that of B9430. A selective B_1 antagonist, CP0298 [1] (Table 1), was relatively inactive in these systems as expected (Figs. 1 and 2), and it did not undergo any significant proteolysis under the conditions of the calcium flux assay.

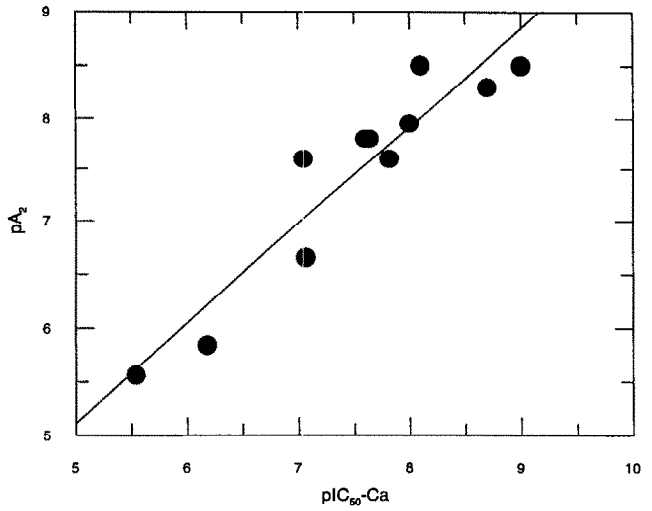
In addition, we studied the effect of removal of the C-terminal Arg^9 residue from BK analogs on B_2 receptor binding. Four pairs of the antagonists differed by the presence or absence of this amino acid residue. To be thorough, BK, des-Arg^9-BK, Lys^0-BK (KD), and des-Arg^9-Lys^0-BK were also included in this analysis. In each case, the removal of the C-terminal Arg^9 residue lowered the receptor binding potency (pK_i or pIC_{50}) of the ligands as expected [9, 25, 35]. The results for these compounds are



Compound	pK_i-sR	pA_2
B9066	5.98	5.56
B9812	7.82	5.84
CP0678	8.08	7.60
CP0750	8.52	6.65
B9340	8.80	7.80
CP0597	9.05	7.60
CP0885	9.14	7.80
CP0716	9.40	7.95
Hoe140	9.74	8.30
B9668	9.89	8.50
B9430	9.92	8.50

Correlation coefficient (r) = 0.9054; slope = 0.79.

FIG. 3. Correlation between pA_2 values in ileum and antagonist binding to soluble B_2 receptor.



Compound	pIC_{50}-Ca	pA_2
B9066	5.54	5.56
B9812	6.18	5.84
CP0678	7.05	7.60
CP0750	7.07	6.65
CP0885	7.60	7.80
B9340	7.64	7.80
CP0597	7.82	7.60
CP0716	8.00	7.95
B9668	8.10	8.50
Hoe140	8.70	8.30
B9430	9.00	8.50

Correlation coefficient (r) = 0.9395; slope = 0.93.

FIG. 4. Correlation between pA_2 values in ileum and inhibition of calcium flux in fibroblast.

presented in Table 2. The changes of the pK_i ($\Delta pK_i = p_{K_i}^{+Arg} - p_{K_i}^{-Arg}$) or pIC_{50} ($\Delta pIC_{50} = p_{IC_{50}}^{+Arg} - p_{IC_{50}}^{-Arg}$) values were very different, and the spread was from 0.6 to 5.4, which corresponds to the range of 10–250,000 for the ratios of the binding constants (K_i^{+Arg}/K_i^{-Arg}). Thus, the energetic contribution of Arg^9 in the binding is not additive, i.e. it strongly depends on the preceding chemical structure of the peptidic ligand. The largest differences (on the order of 5.3 to 5.4 and 4.0 to 4.3) were observed for the two agonist pairs, BK/des-Arg^9-BK and Lys^0-BK/des-Arg^9-Lys^0-BK, respectively. A similar drop of potency for these des-Arg^9 agonists has been reported before [9, 25, 35]. The lack of additivity may be partially associated with the loss of the Ser^6(O)-Arg^9(NH) hydrogen bond in the C-terminal β-turn [35, 36]. The absence of this hydrogen bond in the receptor binding pocket may create greater conformational flexibility (an unfavorable entropic factor in binding) of agonists like des-Arg^9-BK than that of more constrained antagonists like des-Arg^9-Hoe140 [8]. Another possible interpretation is that the mode of ligand binding to the B_2 receptor, and consequently the energy contribution of Arg^9 in the studied compounds, is different. Recent literature data suggest that some agonists and antagonists do not bind to identical sites on the receptor [35, 37]. More BK analogs, which would differ by single amino acid substitutions, need to be tested to define more precisely these structure-to-function relationships.

In conclusion, a significant correlation between BK B_2 receptor binding, inhibition of BK-elicited calcium flux, and inhibition of ileum contraction was observed for a variety of peptidic bradykinin antagonists in human *in vitro* systems. Our results strengthen earlier literature suggestions

TABLE 2. Effect of Arg^9 removal from peptidic ligands on B_2 receptor binding

Compound	pK_i-sR*	pIC_{50}-mR†	ΔpK_i	ΔpIC_{50}‡
BK	8.8	8.9		
			5.4	5.4
Des-Arg^9-BK	3.4	3.6		
KD	8.9	9.0		
			4.0	4.2
Des-Arg^9-KD	4.9	4.7		
NPC567	8.7	8.2		
			2.5	1.8
Des-Arg^9-NPC567	6.1	6.0		
Hoe140	9.7	9.8		
			2.3	2.8
Des-Arg^9-Hoe140	7.4	6.8		
B9430	9.9	9.8		
			2.1	1.9
Des-Arg^9-B9430	7.8	7.7		
B10220	6.6	7.2		
			0.6	1.3
Des-Arg^9-B10220	6.0	5.9		

* pK_i-sR, $-Log(K_i)$ with soluble receptor (see "Data Analysis" in Materials and Methods).
† pIC_{50}-mR, $-Log(IC_{50})$ with membrane-bound receptor.
‡ Similar ΔpIC_{50} values of 5.2 and 2.65 have been reported recently for BK and Hoe140, respectively [35].

that B_2 human receptor binding assays combined with calcium flux cell assays are very useful analytical techniques for identification of potent and selective bradykinin B_2 human receptor antagonists.

Additional comparisons of Arg^9 containing compounds and the corresponding des-Arg^9 analogs demonstrated that the removal of the C-terminal Arg residue from the former compounds produced very different effects on human B_2 receptor binding. These effects strongly depend on the chemical structure of the BK analogs. The largest changes in receptor binding constants were observed for two natural agonist pairs, BK/des-Arg^9-BK and Lys^0-BK/des-Arg^9-Lys^0-BK (kallidin).

References

1. Regoli D and Barabé J, Pharmacology of bradykinin and related kinins. *Pharmacol Rev* **32:** 1–46, 1980.
2. Hall JM, Bradykinin receptors: Pharmacological properties and biological roles. *Pharmacol Ther* **56:** 131–190, 1992.
3. Farmer SG and Burch RM, Biochemical and molecular pharmacology of kinin receptors. *Annu Rev Pharmacol Toxicol* **32:** 511–536, 1992.
4. Vavrek RJ and Stewart JM, Competitive antagonists of bradykinin. *Peptides* **6:** 161–164, 1985.
5. Stewart JM and Vavrek RJ, Chemistry of peptide B2 bradykinin antagonists. In: *Bradykinin Antagonists* (Ed. Burch RM), pp. 51–96. Marcel Dekker, New York, 1991.
6. Bathon JM and Proud D, Bradykinin antagonists. *Annu Rev Pharmacol Toxicol* **31:** 129–162, 1991.
7. Stewart JM, Gera L, Hanson W, Zuzack JS, Burkard M, McCullough R and Whalley ET, A new generation of bradykinin antagonists. *Immunopharmacology* **33:** 51–60, 1996.
8. Burch RM, Kyle DJ and Stormann TM, *Molecular Biology Intelligence Unit. Molecular Biology and Pharmacology of Bradykinin Receptors*. R.G. Landes Co., Austin, 1993.
9. Sawutz DG, Faunce DM, Houck WT and Haycock D, Characterization of bradykinin B_2 receptors on human IMR-90 lung fibroblasts: Stimulation of $^{45}Ca^{2+}$ efflux by D-Phe^7 substituted bradykinin analogues. *Eur J Pharmacol (Mol Pharmacol)* **227:** 309–315, 1992.
10. Sawutz DG, Salvino JM, Dolle RE, Casiano F, Ward S, Houck WT, Faunce DM, Douty BD, Baizman E, Awad MA, Marceau F and Seoane P, The nonpeptide WIN 64338 is a bradykinin B_2 receptor antagonist. *Proc Natl Acad Sci USA* **91:** 4693–4697, 1994.
11. Byron KL, Babnigg G and Villereal ML, Bradykinin-induced Ca^{2+} entry, release, and refilling of intracellular Ca^{2+} stores. *J Biol Chem* **267:** 108–118, 1992.
12. McAllister BS, Leeb-Lundberg MF, Javors MA and Olson MS, Bradykinin receptors and signal transduction pathways in human fibroblasts: Integral role for extracellular calcium. *Arch Biochem Biophys* **304:** 294–301, 1993.
13. Hess JF, Borkowski JA, Young GS, Strader CD and Ransom RW, Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. *Biochem Biophys Res Commun* **184:** 260–268, 1992.
14. Eggerickx D, Raspe E, Bertrand D, Vassart G and Parmentier M, Molecular cloning, functional expression and pharmacological characterization of a human bradykinin B2 receptor gene. *Biochem Biophys Res Commun* **187:** 1306–1313, 1992.
15. Powell SJ, Slynn G, Thomas C, Hopkins B, Briggs I and Graham A, Human bradykinin B2 receptor: Nucleotide sequence analysis and assignment to chromosome 14. *Genomics* **15:** 435–438, 1993.
16. Murray RK and Kotlikoff MI, Receptor-activated calcium influx in human airway smooth muscle cells. *J Physiol (Lond)* **435:** 123–144, 1991.
17. Bathon JM, Croghan JC, MacGlashan DW Jr and Proud D, Bradykinin is a potent and relatively selective stimulus for cytosolic calcium elevation in human synovial cells. *J Immunol* **153:** 2600–2608, 1994.
18. Willars GB and Nahorski SR, Heterologous desensitization of both phosphoinositide and Ca^{2+} signaling in SH-SY5Y neuroblastoma cells: A role for intracellular Ca^{2+} store depletion? *Mol Pharmacol* **47:** 509–516, 1995.
19. Wang Y, Shin WS, Kawaguchi H, Inukai M, Kato M, Sakamoto A, Uehara Y, Miyamoto M, Shimamoto N, Korenaga R, Ando J and Toyo-oka T, Contribution of sustained Ca^{2+} elevation for nitric oxide production in endothelial cells and subsequent modulation of Ca^{2+} transient in vascular smooth muscle cells in coculture. *J Biol Chem* **271:** 5647–5655, 1996.
20. Hulme EC and Birdsall NJM, Strategy and tactics in receptor-binding studies. In: *Receptor–Ligand Interactions. A Practical Approach* (Ed. Hulme EC), pp. 63–174. Oxford University Press, Oxford, 1992.
21. Chirgwin JM, Przybyla AE, MacDonald RJ and Rutter WJ, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18:** 5294–5299, 1979.
22. Maniatis T, Fritsch EF and Sambrook J, *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
23. Kunkel T, Roberts J and Zakour R, Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* **154:** 367–382, 1987.
24. Goodfellow VS, Marathe MV, Kuhlman KG, Fitzpatrick D, Cuadrado D, Hanson W, Zuzack JS, Ross SE, Wieczorek M, Burkard M and Whalley ET, Bradykinin receptor antagonists containing N-substituted amino acids: *In vitro* and *in vivo* B_2 and B_1 receptor antagonist activity. *J Med Chem* **39:** 1472–1484, 1996.
25. Faußner A, Heinz-Erian P, Klier C and Roscher AA, Solubilization and characterization of B_2 bradykinin receptors from cultured human fibroblasts. *J Biol Chem* **266:** 9442–9446, 1991.
26. Hallett MB, Dormer RL and Campbell AK, Cytoplasmic free calcium: Measurement and manipulation in living cells. In: *Peptide Hormone Action. A Practical Approach* (Eds. Siddle K and Hutton JC), pp. 115–150. Oxford University Press, New York, 1990.
27. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450, 1985.
28. Arunlakshana O and Schild HO, Some quantitative uses of drug antagonists. *Br J Pharmacol* **14:** 48–58, 1959.
29. De Lean A, Munson PJ and Rodbard D, Simultaneous analysis of families of sigmoidal curves: Application to bioassay, radioligand assay, and physiological dose–response curves. *Am J Physiol* **235:** E97–E102, 1978.
30. Borkowski JA, Ransom RW, Seabrook GR, Trumbauer M, Chen H, Hill RG, Strader CD and Hess JF, Targeted disruption of a B_2 bradykinin receptor gene in mice eliminates bradykinin action in smooth muscle and neurons. *J Biol Chem* **270:** 13706–13710, 1995.
31. Regoli D, Rhaleb NE, Dion S and Drapeau G, New selective bradykinin antagonists and bradykinin B_2 receptor characterization. *Trends Pharmacol Sci* **11:** 156–161, 1990.
32. Fujii T, Murai M, Morimoto H, Maeda Y, Yamaoka M, Hagiwara D, Miyake H, Ikari N and Matsuo M, Pharmaco-

logical profile of a high affinity dipeptide NK_1 receptor antagonist, FK888. *Br J Pharmacol* **107:** 785–789, 1992.
33. Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G, Henke St, Breipohl G, König W, Knolle J and Schölkens BA, Hoe 140 a new potent and long acting bradykinin-antagonist: *In vitro* studies. *Br J Pharmacol* **102:** 769–773, 1991.
34. Hanson WL, McCullough RG, Selig WM, Wieczorek M, Ross S, Whalley ET, Stewart JM and Gera L, *In vivo* pharmacological profile of novel, potent, stable BK antagonists at B1 and B2 receptors. *Immunopharmacology* **33:** 191–193, 1996.
35. Jarnagin K, Bhakta S, Zuppan P, Yee C, Ho T, Phan T, Tahilramani R, Pease JHB, Miller A and Freedman R, Mutations in the B_2 bradykinin receptor reveal a different pattern of contacts for peptidic agonists and peptidic antagonists. *J Biol Chem* **271:** 28277–28286, 1996.
36. Guba W, Haessner R, Breipohl R, Henke S, Knolle J, Santagada V and Kessler H, Combined approach of NMR and molecular dynamics within a biphasic membrane mimetic: Conformation and orientation of the bradykinin antagonist Hoe 140. *J Am Chem Soc* **116:** 7532–7540, 1994.
37. Alla SA, Quitterer U, Grigoriev S, Maidhof A, Haasemann M, Jarnagin K and Müller-Esterl W, Extracellular domains of the bradykinin B2 receptor involved in ligand binding and agonist sensing defined by anti-peptide antibodies. *J Biol Chem* **271:** 1748–1755, 1996.