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Human vascular kinin receptors of the B_2 type characterized by radioligand binding

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1 The human umbilical vein responds to bradykinin (BK) with contractions that are mediated by B_2 receptors. In the present study, the corresponding vascular smooth muscle B_2 binding sites have been investigated.

2 [^{3}H]-BK, a full agonist labelled ligand, was used to demonstrate a single binding site giving a K_d value of 0.51 ± 0.02 nM and a B_{max} of 24 ± 1 fmol mg^{-1} protein. Scatchard plots were linear ($r = 0.98$) in the 0.05–5 nM range of concentrations. Non-specific binding was found to be 30% of total binding.

3 Competition binding curves gave the following order of potency for various B_2 receptor agonists: BK-[Hyp^3]-BK $\geqslant$ Lys-BK $>>$ [Aib^7]-BK $>>>$ [desArg^9]-BK, which is typical of B_2 receptors. There was no binding to B_1 receptors since the selective B_1 receptor ligand, Lys-[desArg^9]BK was inactive up to 10 μM ($n = 4$).

4 Characterization of the binding site with antagonists, performed with three chemically distinct series of peptide and non-peptide compounds, revealed a high affinity of Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK) (K_i 0.17 nM; $n = 4$) which was more potent that FR 173657 ([(E)-3-(6-acetamido-3-pyridyl)-N-[N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl]phenyl]-N-methylaminocarbonylmethyl] acrylamide]) (K_i 1.94 nM; $n = 4$), D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK (K_i 256 nM; $n = 4$) and Win 64338 (phosphonium, [[4[[2[[bis(cyclohexylamino)methylene]amino]-3-(2-naphthalenyl)-1-oxopropyl]amino]phenyl]methyl]tributyl, chloride, monohydrochloride) (K_i 1,450 nM; $n = 4$).

5 The present study describes and characterises B_2 receptor binding sites in the vascular smooth muscle of the human umbilical vein. The binding assay appears to be suitable for studying new agonists or antagonists designed to activate or block the B_2 receptor class that mediate the majority of the physiopathological effects of kinins in man.

Keywords: Kinins; B_2 receptor; [^{3}H]-bradykinin; receptor binding; vascular smooth muscle; human umbilical vein

Introduction

Bradykinin (BK) and kallidin (Lys-BK) are potent vasoactive peptides that can be released in the vessel walls (Regoli & Barabé, 1980) to induce vasodilatation, increase of vascular permeability (Marceau, 1995) and venoconstriction (Gaudreau *et al*., 1981). The main biological effects of kinins are mediated by B_2 receptors which are widespread and can be found in endothelia, smooth muscles (Regoli & Barabé, 1980) and sensory nerve endings (Steranka *et al*., 1988). B_2 receptors have been studied with functional experiments (Regoli & Barabé, 1980) as well as with binding assays (Innis *et al*., 1981; Manning *et al*., 1986) and, more recently, the receptor genes have been cloned and receptors expressed in appropriate systems for characterization (McEachern *et al*., 1991; Eggerickx *et al*., 1992; Hess *et al*., 1992; 1994; McIntyre *et al*., 1993; Park *et al*., 1994). Binding assays on B_2 receptor have been performed in a variety of tissues from animals and man by use of [^{3}H]-BK or iodinated derivatives of BK (see review by Hall, 1992; and Hall & Morton, 1997). As for human vascular tissues, to our knowledge all studies have been performed on cultured cells (Goldstein & Wall, 1984; Hess *et al*., 1992; 1994; Bathon *et al*., 1992; Burch *et al*., 1992; Sawutz *et al*., 1994).

The main purposes of the present study were: (1) to set up a binding assay using an intact human vessels where the B_2 receptor functional site has already been characterized (Gobeil *et al*., 1996a); (2) to use [^{3}H]-BK as a ligand since it is the naturally occurring ligand with full biological activity that has been used in the majority of such B_2 receptor binding studies (Hall & Morton, 1997) and (3) to characterize the human B_2 binding site with a variety of agonists and antagonists appropriately selected for their pharmacological features, in order to enable a comparison of the human B_2 receptor with those of other species. These data are compared with, and discussed in relation to, information in the literature on B_2 receptors obtained from isolated tissue bioassay studies, radioligand binding studies and molecular biological studies of the human B_2 receptor.

Methods

Human umbilical vein membrane preparation

Experiments on human tissues were performed under conditions and with experimental protocols reviewed and approved by the Ethics Committee of the Medical School of the University of Ferrara, Ferrara, Italy; written informed consent was obtained from each parturient woman.

Human umbilical cords ($n = 50$) from 31 ± 0.7 years old women were collected after spontaneous delivery at term and used either immediately or after storage in Krebs solution at 4°C for no longer than 12 h. In the laboratory, the middle segment of the cord, 10–15 cm. in length, was cut and the vein was dissected free of surrounding tissues and mechanically denuded of endothelium. The tissues were used immediately for obtaining membrane preparations or stored at −80°C for up to 25–30 days. Tissues were homogenized in ice-cold buffer (50 mM sodium trimethylamino-ethanesulphonate (TES) and 1 mM 1,10-phenantroline, pH 6.8) with a Polytron homogenizer (Kinematica, Gmbh, Luzern, Switzerland). The homogenate was centrifuged ($1,000 \times g$, 20 min, 4°C) to remove cellular debris and the supernatant was centrifuged

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(100,000 × g, 60 min, 4°C) to obtain a membrane pellet. Then, the pellet was resuspended in ice-cold assay buffer of the following composition: 50 mM TES, 1 mM 1,10-phenantroline, 140 μg ml^{-1} bacitracin, 1 mM dithiothreitol, 1 μM captopril and 0.1% bovine serum albumin (BSA), at pH 6.8. A concentration of 160–200 μg protein/100 μl was used for binding experiments. The protein concentration was determined according to a Bio-Rad method (Bradford, 1976) using bovine albumin as reference standard.

[^{3}H]-bradykinin binding assay

The specific binding of [^{3}H]-BK to plasma membranes of human umbilical veins was assayed in a total volume of 250 μl containing the assay buffer (see composition above), according to the method previously described by Manning *et al.* (1986), with minor modifications. In saturation experiments, umbilical vein membranes were incubated with 8 to 10 different concentrations of [^{3}H]-BK ranging from 0.05 to 5 nM. In inhibition experiments, carried out to determine the IC_{50} values of several compounds, 0.5 nM [^{3}H]-BK was incubated with 100 μl of membrane preparations in the presence of 8 to 10 different concentrations of each agonist (BK, Lys-BK, [Hyp^3]-BK, [Aib^7]-BK, [desArg^9]-BK, Lys-[desArg^9]-BK) or antagonist (D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK, Win 64338, Hoe 140, FR 173657, [Leu^8,desArg^9]-BK, Lys-[Leu^8,desArg^9]-BK, R-715, [desArg^{10}]-Hoe 140). Inhibitory binding constants, (K_i values) were calculated from the IC_{50} values according to the Cheng and Prusoff equation (Cheng & Prusoff, 1973), $K_i = IC_{50}/(1 + [C^*]/K_d^*)$, where [C*] is the concentration of the radioligand and K_d* is the dissociation constant. Non-specific binding was defined as the binding obtained in the presence of 1 μM unlabelled BK. Incubation time was 60 min at room temperature. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fibre filters previously treated with 0.1% polyethyleneimine for at least 45 min in a Brandel cell harvester. The incubation mixture was diluted with 3 ml of ice-cold buffer (50 mM Tris-HCl), and then rapidly filtered under vacuum and the filter was washed three times with 3 ml of buffer. The filter-bound radioactivity was counted in a Beckman LS-1800 Spectrometer (efficiency 55%).

Data analysis

All data are expressed as mean ± s.e. mean of n experiments; for K_i values the 95% confidence limits are shown. A weighed non linear least-squares curve fitting programme LIGAND (Munson & Rodbard, 1980) was used for computer analysis of saturation and inhibition experiments.

The pharmacological terminology adopted in this paper is in line with the recent IUPHAR recommendations (Jenkinson *et al.*, 1995; Vanhoutte *et al.*, 1996).

Materials

Peptide receptor agonists and antagonists were prepared by solid-phase synthesis and purified by high pressure chromatography, as described by Drapeau and Regoli (1988). Abbreviations of non-natural residues used for the peptide synthesis are as follows: Aib: 2-aminoisobutyric acid; Hyp: trans-4-hydroxy-L-proline; Thi: β-(2-tienyl)-L-alanine; D-Tic: D-(1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid); Oic: L-(3aS, aS)-octahydro-indol-2-carboxylic acid. Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK) (Hock *et al.*, 1991) was made available by Hoechst AG (Frankfurt, Germany). Win 64338 (phosphonium, [[4[[2[[*bis*(cyclohexylamino)methylene]amino]-3-(2-naphthalenyl)-1-oxopropyl]amino]phenyl]methyl]tributyl, chloride, monohydrochloride) (Salvino *et al.*, 1993) was supplied by Sterling Winthrop (Collegeville, PA, U.S.A). FR 173657 ([(E)-3-(6-acetamido-3-pyridyl)-N-[N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl]phenyl]-N-methylamino-carbonylmethyl]acrylamide]) (Asano *et al.*, 1996) was a gift of Fujisawa (Osaka, Japan). Captopril ([2S]-1-[3-mercapto-2-methyl-propionyl]-L-proline) was purchased from Squibb (Canada). [^{3}H]-BK (specific activity 76 Ci mmol^{-1}) was from Amersham (U.K.) and Aquassure was from NEN Research Products (Boston, Mass., U.S.A.). All other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Concentrated solutions (1 mM) of peptides and other agents were made in bidistilled water and stored at × 20°C. The stock solution of FR 173657 was made in dimethyl sulphoxide (DMSO) such that the final bath concentration did not exceed 0.1%.

Results

[^{3}H]-BK binding

Preliminary experiments were performed either on plasma membranes obtained from fresh tissues or from tissues stored at −80°C for 20–30 days in order to find out if we could pool together several umbilical cords to prepare large fractions of

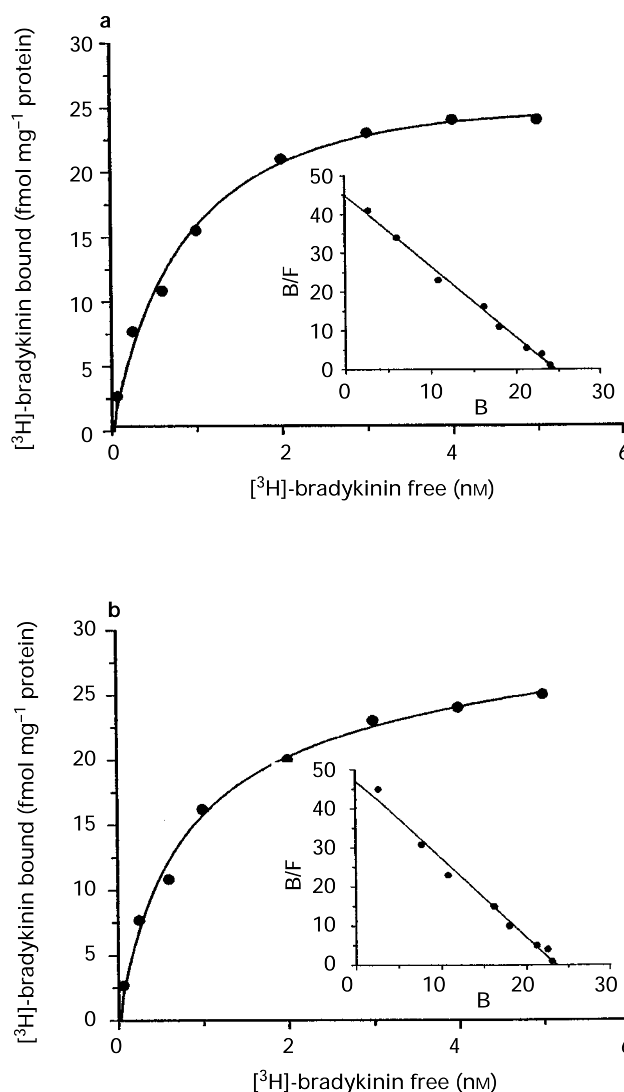

Figure 1 Typical saturation curves of [^{3}H]-bradykinin binding to umbilical vein kinin B_2 receptors in both fresh (a) and frozen (b) tissues. Experiments were performed as described in Methods. In the inset the Scatchard plot of corresponding data is shown. K_d values (nM) were 0.51 ± 0.02 and 0.53 ± 0.03 in fresh and frozen tissues, respectively, and corresponding B_{max} values (fmol mg^{-1} protein) were 24.00 ± 1.14 and 22.75 ± 1.30. Values are the mean ± s.e.mean of four separate experiments performed in triplicate. Non-specific binding was determined in the presence of 1 μM BK.

vascular smooth muscle plasma membranes. The presence and binding capacity of B_2 receptors in the two experimental conditions was evaluated by measuring the saturation curve of [^{3}H]-BK. Results obtained in four separate experiments, performed in triplicate, are presented in Figure 1a and b The figure shows typical saturation curves of [^{3}H]-BK binding to B_2 receptors and the corresponding Scatchard plots (inset). These data indicate the presence of a single class of binding sites with K_d value of 0.51 ± 0.02 nM and B_{max} value of 24.00 ± 1.14 fmol mg^{-1} of protein in fresh tissues identical to those obtained in tissues stored for 20–30 days at -80°C ($K_d = 0.53 \pm 0.03$ nM; $B_{max} = 22.75 \pm 1.30$ fmol mg^{-1} of protein). All the experiments presented below were performed on tissues that had been stored at -80°C.

Displacement studies

The pharmacological characterisation of the binding site was made by using several bradykinin related peptides that, in numerous studies have been shown to act as agonists of either the B_1 or the B_2 kinin receptors. Figure 2 shows that BK, Lys-BK, [Hyp^3]-BK [Aib^7]-BK compete with the binding of [^{3}H]-BK. The concentration curves are parallel to that of BK, the reference and most potent compound, suggesting that all compounds bind to the same site, but differ in their affinity. The order of potency of agonists in the [^{3}H]-BK binding displacements assay is the following: BK = [Hyp^3]-BK = Lys-BK > > [Aib^7] - BK > > > [desArg^9] - BK = Lys - [desArg^9] - BK BK, Lys-BK and [Hyp^3]-BK were the most potent compounds, their affinity being in the low nanomolar range, while the selective B_1 receptor agonists displayed low affinity values (> 10 μM). Similar experiments were performed with antagonists which have been shown to inhibit B_2 receptor mediated responses, namely D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK, Hoe 140, Win 64338, FR 173657, or the B_1 receptor ([Leu^8,desArg^9]-BK, Lys-[Leu^8,desArg^9]-BK, R-715 (Ac-Lys[D-βNal^7,Ile^8,desArg^9]-BK). Results illustrated in Figure 3 indicate that these compounds differ markedly in their affinities, but the displacements of the concentration-response curves are parallel and extend over a range of 3 log units. The order of potency of antagonists in [^{3}H]-BK binding displacement assays was: Hoe 140 > FR 173657 > > D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK > Win 64338 = [desArg^{10}]-Hoe 140 > [Leu^8,desArg^9]-BK > Lys-[Leu^8,desArg^9]-BK = R 715. Hoe 140 and FR 173657 displayed a high affinity, K_i values being in the nanomolar range. In contrast, typical B_1 receptor antagonists were the weakest compounds with K_i values in the micromolar range or less.

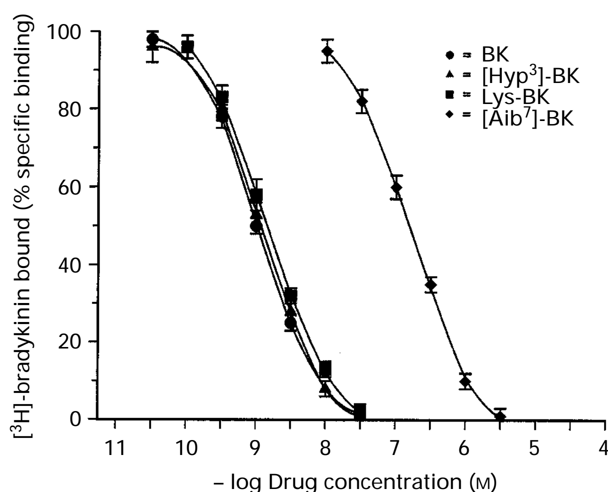

Figure 2 Inhibition curves of specific [^{3}H]-bradykinin (0.5 nM) binding to human umbilical vein membranes by kinin receptor agonists. Mean values of four experiments done in triplicate are shown; vertical lines show s.e.mean. Non-specific binding was determined in the presence of 1 μM BK.

Hill coefficients of all compounds studied were not significantly different from unity ($P > 0.05$; data not shown).

The curves shown in Figures 2 and 3 were used to calculate the K_i values (nM), which are presented in Table 1 where it can be seen that BK, Lys-BK and [Hyp^3]-BK are equipotent, and [Aib^7]-BK is 200 fold weaker, whereas the B_1 agonists are unable to displace the binding of [^{3}H]-BK. Among the antagonits, Hoe 140 was the most potent, around 10 fold more than FR 173657 and more than 1000 fold more active than D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK. Win 64338 was very weak, more than 10,000 fold less potent than Hoe 140 and the B_1 receptor antagonists were all inactive except [desArg^{10}]-Hoe 140.

Angiotensin II and 5-hydroxytryptamine (5-HT) did not displace the ligand when used at 10 μM ($P > 0.05$; data not shown).

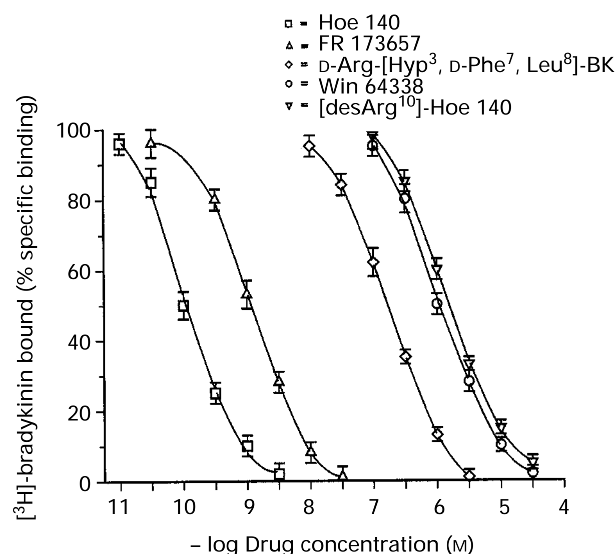

Figure 3 Inhibition curves of specific [^{3}H]-bradykinin (0.5 nM) binding to human umbilical vein membranes by kinin receptor antagonists. Mean values of four experiments done in triplicate are shown; vertical lines show s.e.mean. Non-specific binding was determined in the presence of 1 μM BK.

Table 1 Inhibition of [^{3}H]-BK binding to human umbilical vein membrane B_2 receptors by kinin receptor agonists and antagonists

	K_i (nM)
Agonists	
BK	1.22 (0.84–1.77)
Lys-BK	1.59 (1.26–2.01)
[Hyp^3]-BK	1.24 (0.8–1.92)
[Aib^7]-BK	250 (171–365)
[desArg^9]-BK	> 10,000
Lys-[desArg^9]-BK	> 10,000
Antagonists	
D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK	256 (172–381)
Win 64338	1,450 (1,070–1,970)
Hoe 140	0.17 (0.04–0.67)
FR 173657	1.94 (1.10–3.43)
[Leu^8,desArg^9]-BK	> 10,000
Lys-[Leu^8,desArg^9]-BK	> 10,000
R-715	> 10,000
[desArg^{10}]-Hoe 140	1,510 (990–2,300)
Angiotensin II	> 10,000
5-HT	> 10,000

The data are mean of four experiments done in triplicate; for K_i values the 95% confidence limits are shown.

Discussion

The major finding of the present study is that [^{3}H]-BK, a radioactive derivative of BK with full biological activity, appears to be suitable for labelling the kinin B_2 receptors of the human umbilical vein. In the human umbilical vein, the B_2 receptor is a single binding site of fairly high affinity (BK; K_i 1.23 nM). The binding site is present in the smooth muscle fibres, since the endothelium was systematically removed from all tissues used to prepare the plasma membranes. To our knowledge this is the first time that vascular smooth muscle B_2 receptors have been demonstrated in a binding assay. Similar binding has been shown to be present in several human cell types in culture, including foetal lung fibroblasts (Goldstein & Wall, 1984), RAW 2647 macrophages (Burch *et al*., 1992), synovial cells (Bathon *et al*., 1992) and different isolated tissues (see the review articles by Hall, 1992, and Hall and Morton, 1997). In the latter studies, the affinity measured in human native B_2 receptors varied from 1 to 4 nM, consistent with the present data.

When compared with the binding assay most frequently used for the kinin B_2 receptor, namely the guinea pig ileum, (initially described by Innis *et al*., (1981) and further characterized later by Manning *et al*. (1986), Tousignant *et al*. (1991) and other workers (Ransom *et al*., 1992; Tousignant *et al*., 1992)), the present assay offers the advantage of a single site, in contrast to the ileum, in which very high (K_i 13 pM) and a lower (K_i 910 pM) affinity sites have been found (Manning *et al*., 1986), Similarly two sites, in the epithelium and the smooth muscle have been demonstrated by Trifilieff *et al*. (1992) in the guinea-pig trachea. Another interesting feature of the present assay is the good match between the antagonist affinity values found in the present study and those determined in functional assays, which is in contrast with that observed in the guinea-pig ileum, where 20–30 fold differences of affinity and potency have been observed between the binding and the biological assay (Ransom *et al*., 1992; see also Hall, 1992; Burch *et al*., 1993; Kyle & Burch, 1993).

A close relationship between the data obtained with a series of bradykinin related peptides both agonists and antagonists (see Table 1) indicates that the human B_2 receptor is similar to the rabbit receptor, because of its high affinity for [Hyp^3]-BK, and low affinity for [Aib^7]-BK: this differs from the guinea-pig ileum, where [Aib^7]-BK is more active than [Hyp^3]-BK (Regoli *et al*., 1993). The same conclusion can be drawn from the data obtained with antagonists: in fact, Win 64338, which is almost inactive in rabbit tissues (Gobeil & Regoli, 1994; Regoli *et al*., 1993), shows very little affinity, if any, for human B_2 receptors, while it antagonizes guinea-pig B_2 receptors with a fairly high affinity (pA_2 7.8) (Gobeil & Regoli, 1994). Data obtained in guinea-pig tissues cannot be easily explained by the presence of two different B_2 receptor subtypes or of B_3 receptors in this species, as suggested by Farmer *et al*. (1989), because two different studies (Trifilieff *et al*., 1992; Pruneau *et al*., 1995) have demonstrated that B_2 receptors of the guinea-pig ileum (Pruneau *et al*., 1995) or the guinea-pig trachea (Trifilieff *et al*., 1992) are pharmacologically identical. It is therefore suggested that binding assays performed in membrane preparations of guinea-pig tissues, especially the ileum, may be not suitable for a correct evaluation of new B_2 receptor agonists and antagonists to be used in man.

Table 2 Relative potencies of kinin receptor agonists and antagonists on human B_2 receptor

	Native UV strips bioassay[a]	*Native UV membranes receptor binding (this study)*
Agonists		
BK	100	100
Lys-BK	159	77
[Hyp^3]-BK	159	98
[Aib^7]-BK	0.63	0.49
[desArg^9]-BK	<0.05	<0.01
Lys-[desArg^9]-BK	<0.05	<0.01
Antagonists		
D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK	0.08	0.47
Win 64338	<0.05	0.08
Hoe 140	63	615
FR 173657	40[b]	61
[Leu^8,desArg^9]-BK	<0.05	<0.01
Lys-[Leu^8,desArg^9]-BK	<0.05	<0.01
R-715	<0.05[c]	<0.01
[desArg^{10}]-Hoe 140	<0.05[c]	0.08

UV: umbilical vein. References: [a]Gobeil *et al*. (1996a); [b]Rizzi *et al*. (1997); [c]Gobeil *et al*. (1996b). The data are expressed relative to BK (BK = 100).

The B_2 receptor binding assay in the human umbilical vein is also well documented with respect to antagonists. From a comparison of three B_2 receptor antagonists that belong to the first (D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK), the second (Hoe 140) and third (FR 173657) generation, it emerges that Hoe 140 is by far the most potent antagonist, 10 fold more potent than FR 173657 (in agreement with data obtained in other studies (Aramori *et al*., 1997)), 1,500 more potent than D-Arg-[Hyp^3,D-Phe^7,Leu^8]-BK and 8,500 more potent than Win 64338.

Data obtained on a large number of compounds (agonists and antagonists) in binding (present results) and biological assays (Gobeil *et al*., 1996a) carried out in the same human vessel are now available to compare the binding with the corresponding functional sites. Such a comparison is presented in Table 2. The correspondence between the two sets of data is very good indeed, both for agonists and for antagonists. Such a good correlation strongly supports our claim that binding or functional assays performed on the human umbilical vein can be efficiently used for the identification and characterization of new products directed to activate or block the human B_2 receptor.

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