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Selective labelling of bradykinin receptor subtypes in WI38 human lung fibroblasts

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1 Binding of the B_1 bradykinin receptor radioligand, [^{3}H]-des-Arg^{10}-kallidin (-KD) and the B_2 receptor radioligand [^{3}H]-bradykinin (-BK) was investigated in membranes prepared from WI38 human foetal lung fibroblasts.

2 One-site analysis of the saturation data for [^{3}H]-des-Arg^{10}-KD gave an equilibrium dissociation constant (K_D) value of 0.51 ± 0.12 nM and a maximum receptor density (B_{max}) of 260 ± 49 fmol mg^{-1} of protein. [^{3}H]-des-Arg^{10}-KD binding was displaced by ligands in the order: des-Arg^{10}-KD > KD > > des-Arg^9[Leu^8]-BK > des-Arg^9-BK > Hoe 140 > > BK, implying that it was binding selectively to B_1 receptors.

3 One-site analysis of the binding of [^{3}H]-BK to WI38 membranes indicated that it had a K_D value of 0.25 ± 0.06 nM and a B_{max} of 753 ± 98 fmol mg^{-1} of protein. The potencies for displacement of [^{3}H]-BK binding were: Hoe 140 > > BK = KD > > > des-Arg^{10}-KD = des-Arg^9[Leu^8]-BK = des-Arg^9-BK, which was consistent with binding to B_2 receptors.

4 This is the first characterization of [^{3}H]-des-Arg^{10}-KD binding to include both kinetic and equilibrium data, and demonstrates that [^{3}H]-des-Arg^{10}-KD has a high affinity for human B_1 bradykinin receptors and is sufficiently selective to be used as a radioligand for B_1 receptors in human cells or tissues expressing an excess of B_2 BK receptors.

Keywords: Bradykinin; B_1 and B_2 receptors; [^{3}H]-des-Arg^{10}-kallidin; [^{3}H]-bradykinin; binding; WI38 fibroblasts

Introduction

Bradykinin (BK) and kallidin (KD, Lys-BK) are released during tissue injury and inflammation as a result of activation of tissue and plasma kallikreins which cleave high and low molecular weight kininogen precursors. They play a key role in a number of pathophysiological processes (Hall, 1992) including pain and inflammation (Burch & Kyle, 1992; Farmer & Burch, 1992; Dray & Perkins, 1993).

Two types of BK receptors have been cloned: B_2 receptors (McEachern *et al.*, 1991; Eggerickx *et al.*, 1992; Hess *et al.*, 1992; McIntyre *et al.*, 1993) and B_1 receptors (Menke *et al.*, 1994; MacNeil *et al.*, 1995). Both belong to the superfamily of G-protein-coupled receptors with seven membrane spanning regions. B_2 receptors are widely distributed and appear to be responsible for many of the actions of BK (Sterenka *et al.*, 1988; Dray *et al.*, 1992). BK and KD are B_2 selective agonists and there are numerous sequence-related B_2 receptor antagonists (Regoli *et al.*, 1992). One of the most potent and selective of these is D-Arg [Hyp^3, Thi^5, D-Tic^7, Oic^8]BK (Hoe 140) (Hock *et al.*, 1991).

In contrast to B_2 receptors, B_1 receptors are not usually expressed on normal tissues and have been studied mainly in cytokine-activated or traumatized smooth muscle preparations, mainly from rabbit vascular tissues (e.g. Marceau *et al.*, 1980; Regoli *et al.*, 1981; Bouthillier *et al.*, 1987; Deblois & Marceau, 1987; Pruneau & Belichard, 1993). B_1 receptors are also expressed constitutively in several cell lines including WI38 human foetal lung fibroblasts (Crecelius *et al.*, 1986; Phillips *et al.*, 1992; Webb *et al.*, 1994). There is evidence that B_1 receptors are involved in persistent inflammatory hyperalgesia (Farmer *et al.*, 1991; Perkins & Kelly, 1993; Perkins *et al.*, 1993; Davis & Perkins, 1994) and other pathological conditions such as septic shock and hypotension (Hall, 1992). The carboxypeptidase metabolites of BK and KD, des-Arg^9-BK and des-Arg^{10}-KD respectively, are selective agonists at the B_1 receptor and have little or no activity at B_2 receptors. Des-Arg^9[Leu^8]-BK (Regoli *et al.*, 1977) and des-Arg^9-D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK (des-Arg^{10}-Hoe 140) (Wirth *et al.*, 1992) are selective antagonists at the B_1 receptor.

Detailed radioligand binding studies of B_1 receptors were not possible until [^{3}H]-des-Arg^{10}-KD was synthesized and demonstrated to exhibit a high affinity for B_1 receptors in mouse macrophages (Burch *et al.*, 1992). It has been used in smooth muscle cells (Schneck *et al.*, 1994) and Cos-7 cells expressing the cloned human or rabbit B_1 receptor (Menke *et al.*, 1994; MacNeil *et al.*, 1995). In the present study we have characterized this radioligand in WI38 human fibroblasts which express both B_1 and B_2 receptors. The results indicate that [^{3}H]-des-Arg^{10}-KD is sufficiently selective for B_1 receptors to be used in cells that also express a relatively large number of B_2 receptors.

Methods

Preparation of membranes from cultures of WI38 cells

WI38 fibroblasts (ICN Biomedicals Ltd., Oxon, UK) were used up to passage number 35 and were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% foetal bovine serum (Myoclone Plus, FBS), 100 iu ml^{-1} each of penicillin and streptomycin, 2 mM L-glutamine and 1% (v/v) of non-essential amino acids. Cells were maintained in a humidified atmosphere at 37°C with 5% CO_2. The cells were subcultured every 3-4 days at a ratio of 1:2 or 1:3 using 0.25% trypsin/1 mM ethylenediaminetetraacetate (EDTA) to detach them.

Membrane preparation

WI38 cells were harvested by incubating at 37°C with EDTA solution (1 mM EDTA, 10 mM *N*-[2-hydroxy-ethyl]piperazine-*N'*-[2-ethanesulphonic acid] (HEPES), in Hanks Buffered Salt Solution (HBSS), pH 7.4) containing a cocktail of peptidase inhibitors (1 mM 1,10 phenanthroline, 1 mM ethylene glycol *bis* (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid

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(EGTA), 1 μM each of captopril, leupeptin, soyabean trypsin inhibitor, DL-2-mercaptomethyl-3-guanidoethylthiopropanoic acid (Plummer's inhibitor, MERGETPA); 3.3 μM chymostatin and 0.1 mM phenylmethyl-sulphonyl fluoride (PMSF) for 5 min. All subsequent procedures were performed at 4°C. Cells were washed in 10 mM N-tris[hydroxymethyl]methyl-2-aminoethane-sulphonic acid (TES) buffer, pH 7.4 containing the peptidase inhibitor cocktail and homogenized with a Kinematica polytron homogeniser, set at 10 000 r.p.m. for 30 s. The homogenate was centrifuged at 40 000 *g* for 20 min. The pellet was washed twice, with intermediate rehomogenisation, then resuspended in TES buffer, pH 7.4, with the cocktail of peptidase inhibitors. The pellet from two 175 cm^2 flasks of cells was resuspended in 1 ml of buffer (giving approximately 2 mg membrane protein ml^{-1}). The membranes were frozen immediately in 1 ml aliquots on dry ice, then stored at −70°C until use. The protein concentration was determined by the method of Bradford (1976) using a Bio-Rad kit. Immediately prior to use, frozen membrane aliquots were thawed in binding buffer (see below) and mixed to give a homogeneous membrane suspension.

Bradykinin receptor binding assays

[^{3}H]-des-Arg^{10}-KD binding Binding assays were performed at 4°C in triplicate in micronic polypropylene tubes (1.2 ml) in a final volume of 1 ml. For association studies, 1 nM [^{3}H]-des-Arg^{10}-KD was incubated with WI38 membranes (usually 40–80 μg protein ml^{-1}) in binding buffer that was comprised of 10 mM TES buffer pH 7.4, 0.14 gl^{-1} bacitracin, 1 mM 1,10 phenanthroline and 1 gl^{-1} bovine serum albumin (BSA). Non-specific binding was defined as the amount of labelled ligand bound in the presence of 1 μM des-Arg^{10}-KD. The reaction was initiated by the addition of 750 μl of membrane suspension.

For dissociation studies, [^{3}H]-des-Arg^{10}-KD (1 nM) binding was allowed to reach equilibrium (60 min). The [^{3}H]-des-Arg^{10}-KD that remained bound to the membranes after the addition of 1 μM unlabelled des-Arg^{10}-KD was measured at the times indicated.

For saturation studies, WI38 membranes were incubated for 60 min in the presence of a range of concentrations of [^{3}H]-des-Arg^{10}-KD (initially over the range 0.03–8 nM, then over the range 0.03–40 nM; 4 experiments for each range) under the same conditions as described for the kinetic studies. In competition studies, competing ligands were added to the reaction mixture at the beginning of the incubation. Data were expressed as fractions of the specific binding in the absence of competing ligand. All incubations were terminated by rapid filtration through GF/B glass fibre filtermats that had been pre-soaked for at least 2 h in 6 gl^{-1} polyethylenimine (PEI), using a Brandel 48 cell harvester. The tubes and filters were washed 5 times with 1 ml aliquots of 50 mM Tris buffer (pH 7.4, 4°C). Filters were soaked in Ready Micro scintillation fluid for at least 4 h before counting.

[^{3}H]-BK binding Experiments were performed as described above, with substitution of [^{3}H]-BK for [^{3}H]-des-Arg^{10}-KD, with the following modifications. The incubation was initiated with the addition of approximately 30 μg ml^{-1} of WI38 membranes and non-specific binding was measured in the presence of 1 μM unlabelled BK.

Analysis of binding data Kinetic experiments were analysed using the kinetic programme in RADLIG (Elsevier Biosoft, Cambridge, UK). Saturation and competition data were processed by the method of Munson & Rodbard (1980) initially using the EBDA programme in RADLIG (McPherson, 1983) and then using LIGAND (National Institute of Health, Bethesda, USA). The maximum binding site density (B_{max}), equilibrium dissociation constant (K_D) and equilibrium inhibition constants (K_I) were determined with LIGAND. Where indicated, parameter values are given as means with approximate standard errors which were calculated by LIGAND as the percentage coefficient of variation of the affinity constant. All other values are given as mean ± s.e.means. To evaluate the goodness of fit, the *F*-ratio was used to test significance of both single and multiple site binding models from the residual mean squares of each analysis.

Materials

[^{3}H]-BK (specific activity 65 Ci $mmol^{-1}$) was obtained from Amersham International plc (Amersham UK, Bucks) and, at a higher specific activity (75–100 Ci $mmol^{-1}$), from Zeneca Cambridge Research Biochemicals (Cheshire, UK). [^{3}H]-des-Arg^{10}-KD (specific activity 107–110 Ci $mmol^{-1}$) was provided by Du Pont NEN (Hertfordshire, U.K.). BK and other peptides were obtained either from Sandoz Basle or Peninsula Laboratories Europe Ltd. (Merseyside). The following materials were obtained from the sources indicated: bacitracin, EDTA, EGTA, PMSF, captopril, TES, HEPES, BSA, 1,10-phenanthroline and PEI (Sigma Chemical Co Ltd., Poole, Dorset); leupeptin and soyabean trypsin inhibitor (Boehringer Corporation (London) Ltd., East Sussex); MERGETPA (Plummer's inhibitor) (Calbiochem/Novabiochem, Nottinghamshire); chymostatin (Peptide Products Ltd., Wiltshire); Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd., Hertfordshire); GF/B glass fibre filtermats (Semat Technical Ltd., Hertfordshire) HBSS and other culture reagents (Gibco Ltd., Scotland).

Results

Binding characteristics of [^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK in WI38 membranes

The specific binding of both [^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK was directly proportional to the membrane concentration (Figure 1). In order to achieve a useful level of specific binding, membrane concentrations of 40–200 μg ml^{-1} and 30–60 μg ml^{-1} were used in subsequent binding experiments with [^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK respectively (the amount of protein per tube used was adjusted according to membrane batch). At these protein concentrations, the specific binding represented approximately 60–70% of the total binding for [^{3}H]-des-Arg^{10}-KD and 80–90% of the total binding for [^{3}H]-

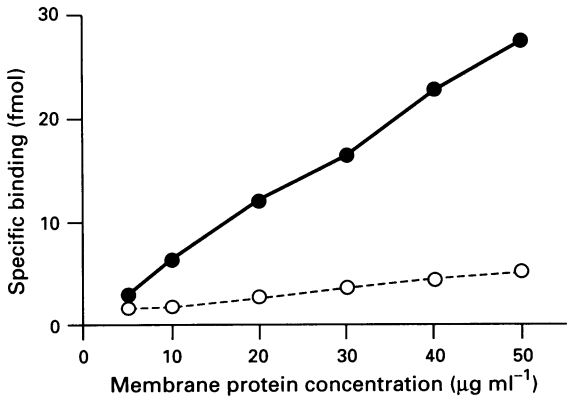

Figure 1 Effect of protein concentration on specific [^{3}H]-des-Arg^{10}-Kallidin (-KD) and [^{3}H]-bradykinin (-BK) binding in WI38 membranes. Specific [^{3}H]-des-Arg^{10}-KD (○) and [^{3}H]-BK (●) binding was plotted against membrane protein concentration. Binding was measured in the presence of 1 nM radioligand and incubations were carried out at 4°C for 60 min. The data points represent the means of triplicate determinations from 1 out of at least 3 similar experiments. In similar experiments (data not shown) specific binding was proportional to membrane protein concentrations tested up to 200 μg ml^{-1} for [^{3}H]-des-Arg^{10}-KD and up to 60 μg ml^{-1} for [^{3}H]-BK.

BK. Approximately 3% of the total radioactivity added bound to the membranes for each radioligand.

Kinetic analysis of [^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK binding to WI38 membranes

The association-time course for [^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK binding to fibroblast membranes at 4°C was rapid, both reaching equilibrium within 40 min and remaining stable for at least 50 min thereafter (Figure 2). An incubation time of 60 min was used for both radioligands in all subsequent experiments. After reaching equilibrium almost all of the specifically bound [^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK dissociated within 90 min of the addition of unlabelled ligand. The [^{3}H]-des-Arg^{10}-KD dissociation data could be fitted to a monophasic curve with a dissociation rate constant (k_{-1}) of 0.019 ± 0.003 min^{-1}. The [^{3}H]-des-Arg^{10}-KD association curve could be fitted to a pseudo first-order rate equation. The 'observed' association rate constant (k_{ob}; 0.131 ± 0.009 min^{-1}) was used to derive the actual association rate constant (k_1) using the equation, $k_1 = (k_{ob} - k_{-1})/[L]$, where [L] is the radioligand concentration. k_1 was calculated as 1.1E 8M^{-1} min^{-1}. The one-site kinetically determined K_D, derived from the ratio of k_{-1}/k_1 was 0.18 nM. Association and dissociation data for [^{3}H]-BK binding could also be fitted to monophasic curves, with a calculated k_{-1} value of 1.79E 8M^{-1} min^{-1} ($k_{ob} = 0.23 \pm 0.019$ min^{-1}) and a k_{-1} value of 0.014 ± 0.002 min^{-1}. The kinetically derived K_D for [^{3}H]-BK was 0.078 nM.

Equilibrium binding studies in WI38 membranes

Specific binding was a saturable function of radioligand concentration for both [^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK (Figure 3). The K_D and B_{max} values, calculated from a one-site model for the saturation isotherms spanning the concentration-range 0.03–40 nM for both [^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK, are summarised in Table 1. The WI38 membranes had significantly less B_1 than B_2 BK receptors. Also, in contrast to [^{3}H]-BK binding, there was some variability in the level of [^{3}H]-des-Arg^{10}-KD binding between membrane batches. This was manifested as a spread of data points at concentrations of [^{3}H]-des-Arg^{10}-KD above 5 nM (data not shown). LIGAND analysis of individual saturation curves indicated that the B_{max} ranged from 100 to 530 fmol mg^{-1} of protein.

Although a two-site binding analysis for [^{3}H]-des-Arg^{10}-KD (using data from 8 experiments) implied that a two-site fit was a significant improvement over a one-site binding model ($F = 13.8$, $P < 0.0001$), the low affinity site was very poorly defined (see Table 1 legend for values). The [^{3}H]-BK data could also be represented by a two-site model ($F = 6.5$, $P = 0.003$), but once again the low affinity site was very poorly defined.

Effects of B_1 and B_2 ligands on [^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK binding to WI38 membranes

The pharmacology of the binding sites for [^{3}H]-BK and [^{3}H]-des-Arg^{10}-KD in WI38 membranes was examined by in-

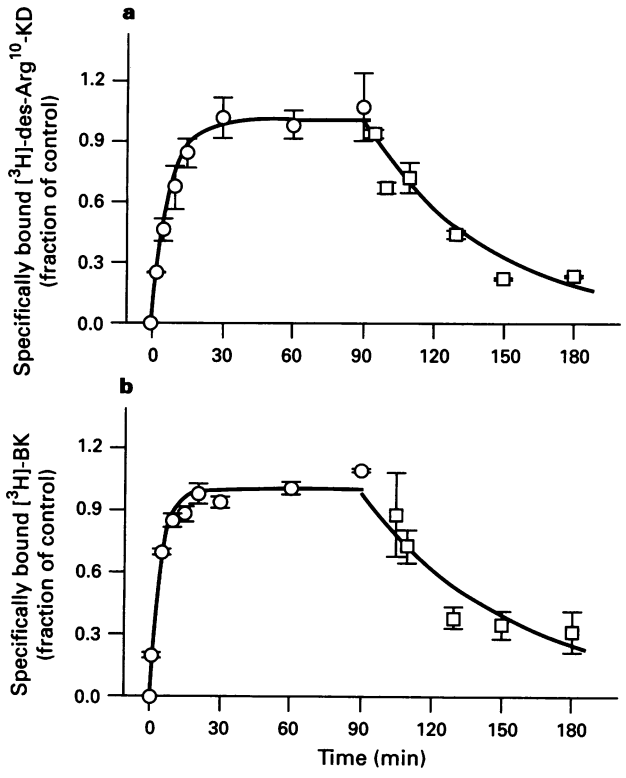

Figure 2 Association and dissociation time-courses for [^{3}H]-des-Arg^{10}-kallidin (-KD) and [^{3}H]-bradykinin (-BK) in WI38 membranes. Association (○) and dissociation (□) time-courses for the specific binding of (a) [^{3}H]-des-Arg^{10}-KD and (b) [^{3}H]-BK in WI38 membranes were plotted against incubation time at 4°C using approximately 80 μg ml^{-1} of membrane protein for [^{3}H]-des-Arg^{10}-KD experiments and 30 μg ml^{-1} of membrane protein for [^{3}H]-BK experiments. For dissociation curves, 1 μM unlabelled ligand was added to samples after equilibrium. The data points represent the mean($\pm$s.e.mean) of triplicate determinations from 3 experiments for both association and dissociation for each radioligand. One-site curve fits were performed by the kinetic programme in RADLIG.

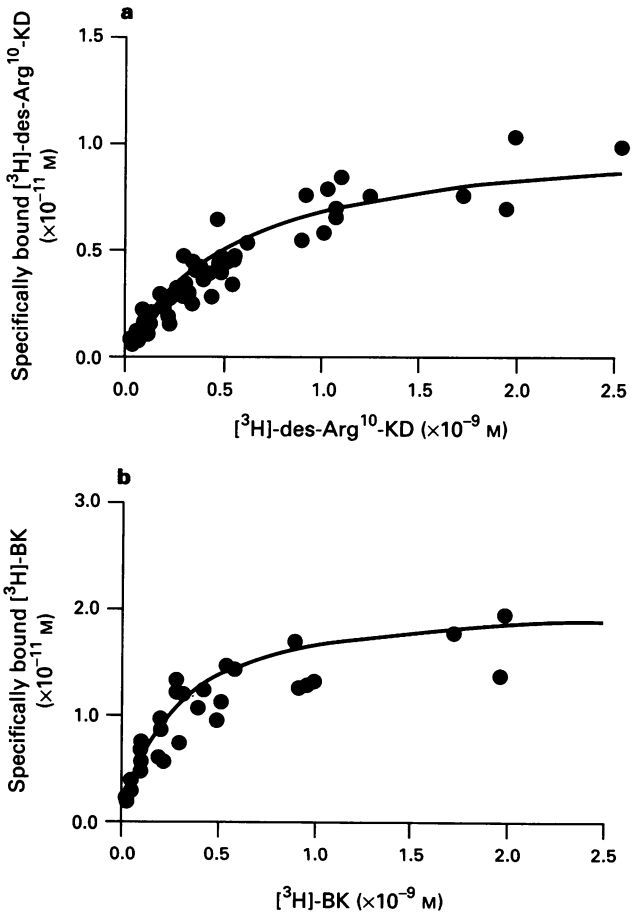

Figure 3 Saturation isotherms for [^{3}H]-des-Arg^{10}-kallidin (-KD) and [^{3}H]-bradykinin (-BK) binding in WI38 membranes. The saturation isotherm for [^{3}H]-des-Arg^{10}-KD (a) was obtained from 8 independent experiments (8 membrane preparations). The saturation isotherm for [^{3}H]-BK (b) binding was obtained from 5 independent experiments (5 membrane preparations). The data points represent the mean of triplicate determinations. Curve fitting of all values from a one-site model was fitted to all the data simultaneously by LIGAND.

Table 1 Saturation analysis of [^{3}H]-bradykinin (-BK) and [^{3}H]-des-Arg^{10}-kallidin (-KD) binding to WI38 membranes

	K_D (nM)	B_{max} (fmol mg^{-1})
[^{3}H]-des-Arg^{10}-KD	0.51±0.12*	260±49
[^{3}H]-BK	0.25±0.06**	753±98

The equilibrium dissociation constant (K_D) and maximum number of binding sites (B_{max}) (mean±approximate standard error calculated by LIGAND from at least 5 experiments performed in triplicate) were estimated by non-linear curve fitting of all values from a one-site binding model fitted to all the data sets simultaneously. *A two-site fit was a significant improvement (high affinity site: K_D value 45±32 pM, B_{max} 36±16 fmol mg^{-1}; low affinity site: K_D value 4.7±2.4 nM, B_{max} 988±385 fmol mg^{-1}; $F=25$, $P=0.00001$), but the low affinity site was poorly defined. **Although a two-site fit was a significant improvement ($F=7.0$, $P=0.002$), the lower affinity site was poorly defined (high affinity site: K_D value 0.13±0.05 nM, B_{max} value 144±52 fmol mg^{-1}; low affinity site: K_D value 5–20 nM, B_{max} value 1500–3500 fmol mg^{-1}).

Table 2 Inhibition of [^{3}H]-bradykinin (-BK) and [^{3}H]-des-Arg^{10}-kallidin (-KD) binding by a selection of B_1 and B_2 receptor agonists and antagonists

	Subtype selectivity	*[^{3}H]-des-Arg^{10}-KD* K_I (nM)	*[^{3}H]-BK* K_I (nM)
Agonists:			
des-Arg^{10}-KD	B_1	2.3±0.3	>3000
des-Arg^9-BK	B_1	285±63	>10000
BK	B_2	>10000	0.15±0.04
KD (Lys-BK)	B_2	9.3±2.7	0.12±0.02
Antagonists:			
des-Arg^9[Leu^8]-BK	B_1	231±37	>3000
des-Arg^{10}Hoe 140	B_1	6.9±3.6	80±18
Hoe 140	B_2	595±119	0.021±0.004

BK, bradykinin; des-Arg^{10}Hoe 140, des-Arg^9-D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK; Hoe 140, D-Arg [Hyp^3, Thi^5, D-Tic^7, Oic^8]BK; KD, kallidin; K_I, equilibrium inhibition constant. The K_I values were calculated from a one-site binding model applied to inhibition data using LIGAND and are the means±approximate s.e. (calculated by LIGAND) from at least 3 experiments.

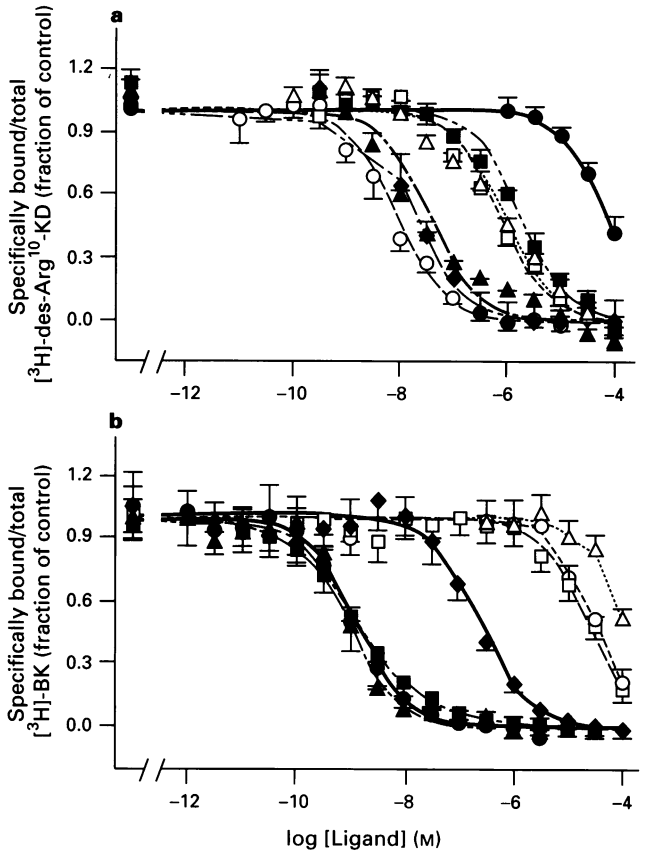

Figure 4 Inhibition of [^{3}H]-des-Arg^{10}-kallidin (-KD) and [^{3}H]-bradykinin (-BK) binding to WI38 membranes by a range of B_1 and B_2-selective ligands. (a) Specific [^{3}H]-des-Arg^{10}-KD binding and (b) specific [^{3}H]-BK binding were plotted against increasing concentrations of BK (●), KD (▲), D-Arg [Hyp^3, Thi^5, D-Tic^7, Oic^8]BK (Hoe 140) (■), des-Arg^{10}-KD (○), des-Arg^9-BK (△), des-Arg^9[Leu^8]-BK (□) and des-Arg^9-D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK (des-Arg^{10}-Hoe 140) (◆). [^{3}H]-des-Arg^{10}-KD (1 nM) and WI38 membranes (40–200 μg ml^{-1}) or [^{3}H]-BK (1 nM) and WI38 membranes (30–60 μg) were incubated as described in the Methods section in the presence of increasing concentrations of displacer. The data shown represent the mean of at least three separate determinations in triplicate. Curves were fitted by the simultaneous analysis of all data sets to a one-site binding model by LIGAND.

vestigating the ability of a number of kinin agonists and antagonists to inhibit binding. A radioligand concentration of 1 nM was used in all experiments and the K_I values that were obtained, using a one-site binding model, are summarized in Table 2. The inhibition curves for each radioligand are shown in Figure 4. Very low concentrations of the selective B_2 receptor agonist BK and of the B_2 selective antagonist, Hoe 140, displaced the binding of [^{3}H]-BK. Much higher concentrations of these ligands were required to displace the binding of [^{3}H]-des-Arg^{10}-KD. Conversely, the B_1-selective agonists, des-Arg^{10}-KD and des-Arg^9-BK and the antagonist, des-Arg^9[Leu^8]-BK were more potent in displacing [^{3}H]-des-Arg^{10}-KD than [^{3}H]-BK. Although KD was a fairly potent displacer of [^{3}H]-des-Arg^{10}-KD binding, it was almost 80 fold more potent against [^{3}H]-BK. When the activities of des-Arg^{10}-Hoe 140 and des-Arg^9[Leu^8]-BK were compared in the [^{3}H]-des-Arg^{10}-KD binding assay, des-Arg^{10}-Hoe 140 was approximately 12 times more potent than des-Arg^9[Leu^8]-BK.

Discussion

The existence of B_1 and B_2 BK receptors in WI38 human fibroblasts was noted when responses to B_1 and B_2 agonists were detected following the expression of mRNA from WI38 cells in *Xenopus* oocytes (Phillips *et al.*, 1992; Webb *et al.*, 1994). In the present study the selectivity of the B_1 radioligand [^{3}H]-des-Arg^{10}-KD has been assessed in parallel with the highly selective B_2 radioligand [^{3}H]-BK, in membranes from these fibroblasts. Previous attempts at characterizing B_1 receptors in binding studies has been limited due to the lack of a suitable radioligand. Bascands and colleagues (1993) used [^{3}H]-des-Arg^9-BK to label B_1 receptors on rat mesangial cells. However, it had a very low affinity, which prevented a rigorous examination of the binding sites and it was not even possible to identify specific binding sites in rabbit aorta, a tissue known to possess B_1 receptors, with [^{3}H]-des-Arg^9-BK (Barabé *et al.*, 1982). [^{3}H]-BK was used to try to label both B_1 and B_2 binding sites in endothelial cells (Sung *et al.*, 1988). A low affinity site was identified and postulated to represent binding to B_1 receptors, but the specific binding to this site could be partially displaced only with high concentrations of B_1 selective ligands and could also be displaced by similar concentrations of ligands (dopamine and ATP) for unrelated receptors. The availability of [^{3}H]-des-Arg^{10}-KD allowed B_1 receptors to be successfully identified in mouse macrophage cells (Burch & Kyle, 1992), rabbit muscle cells (Schneck *et al.*, 1994) and cells transfected with human or rabbit B_1 receptor cDNA (Menke *et al.*, 1994;

MacNeil *et al.*, 1995). In the present study in WI38 cells, [^{3}H]-des-Arg^{10}-KD, used at its K_D concentration, was highly selective for B_1 bradykinin receptors and did not appear to label the B_2 sites which are present in excess in these membranes.

[^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK bradykinin binding sites in WI38 membranes

The K_D values for [^{3}H]-des-Arg^{10}-KD determined kinetically (0.18 nM) and from saturation data (0.51 ± 0.12 nM) were considered to be similar, based on the data ranges. We have also performed kinetic experiments which indicate that the k_{ob} varies with radioligand concentration (data not shown). The K_D values for [^{3}H]-des-Arg^{10}-KD binding in WI38 membranes were close to the value reported in IMR90 human lung fibroblasts (0.5 nM; Menke *et al.*, 1994). They were also comparable to the value originally obtained in RAW 264 mouse macrophages (2.4 nM; Burch *et al.*, 1992). The ratio of B_1 to B_2 receptor density was, on average, 1:3 in WI38 membranes, but the B_{max} value for the B_1 receptors varied by as much as a factor of five between batches. We have obtained evidence that pretreatment with IL-1β can increase the B_{max} for [^{3}H]-des-Arg^{10}-KD by at least 2 fold (data not shown) and other factors, such as the time in culture, may also regulate B_1 receptor expression. This possibility is under investigation.

In contrast to B_1 receptors, there was less variation in B_2 receptor density between membrane batches. [^{3}H]-BK labelled high affinity binding sites in WI38 membranes with K_D values that were considered to be similar whether determined from kinetic (0.078 nM) or saturation (0.25 ± 0.06 nM) experiments based on the range of experimental data. Additional kinetic experiments have indicated that the k_{ob} varies with radioligand concentration (data not shown). The K_D values for [^{3}H]-BK binding in WI38 membranes are close to the value of 0.08 ± 0.04 nM recently reported by McIntyre and colleagues (1993) in which cDNA encoding the B_2 receptor from WI38 cells was expressed in Cos-7 cells and to the value reported for the cloned human B_2 receptor expressed in intact Chinese hamster ovary (CHO) cells (K_D value 0.21 ± 0.02 nM; Hess *et al.*, 1993).

Selectivity of [^{3}H]-des-Arg^{10}-KD and [^{3}H]-BK for B_1 and B_2 receptors in WI38 membranes

Competition binding data, using [^{3}H]-des-Arg^{10}-KD as the radioligand, yielded a rank order of affinity for B_1 and B_2 agonists of: des-Arg^{10}-KD > KD > > des-Arg^9-BK > > > BK. This is similar to the order of potency in IMR90 cells expressing B_1 receptors (Menke *et al.*, 1994) and in B_1 rabbit bioassays (Rhaleb *et al.*, 1990; Regoli *et al.*, 1990; 1992). The selective B_1 antagonist, des-Arg^9[Leu^8]-BK, displaced [^{3}H]-des-Arg^{10}-KD binding with a calculated K_I of 231 ± 37 nM but was inactive at 10 μM against [^{3}H]-BK. Correspondingly, the B_2 antagonist, Hoe 140 was almost 30,000 fold less effective at displacing [^{3}H]-des-Arg^{10}-KD than [^{3}H]-BK. Des-Arg^{10}-Hoe 140 has been reported to have a 10 fold higher affinity than des-Arg^9[Leu^8]-BK in B_1 bioassays (Wirth *et al.*, 1992). In the WI38 membranes it exhibited a > 30 fold higher affinity than des-Arg^9[Leu^8]-BK at the B_1 site (K_I value 6.9 ± 3.6 nM versus 231 ± 37 nM) which is in agreement with the order of potency in IMR90 fibroblasts (Menke *et al.*, 1994). It was, however, also effective in displacing [^{3}H]-BK binding, with a K_I value of 80 ± 18 nM. This suggests that des-Arg^{10}-Hoe 140 is a less selective ligand than des-Arg^{10}-KD for the B_1 site and is also less selective than is Hoe 140 for the B_2 site.

Although the pharmacology of the B_1 receptor in the WI38 membranes was similar to that in IMR90 human fibroblasts (Menke *et al.*, 1994), it was different from data reported for the rabbit B_1 receptor in the aorta (Schneck *et al.*, 1994) and in mesenteric artery muscle cells (Galizzi *et al.*, 1994) and for cloned rabbit B_1 receptors expressed in Cos-7 cells (MacNeil *et al.*, 1995). For example, at the human B_1 receptor, KD was 9 times more potent than des-Arg^9-BK in displacing [^{3}H]-des-Arg^{10}-KD binding. In contrast, KD and des-Arg^9-BK are equipotent at the rabbit B_1 receptor (Schneck *et al.*, 1994; Gallizi *et al.*, 1994; MacNeil *et al.*, 1995). At the human B_1 receptor, des-Arg^{10}-Hoe 140 was more potent than des-Arg^9[Leu^8]-BK but these ligands are also equipotent at the rabbit receptor. In addition, des-Arg^9[Leu^8]-BK is strikingly more potent at the rabbit B_1 receptor than at the human receptor (e.g. K_I value: 33 nM (Schneck *et al.*, 1994) versus 231 nM). The mouse B_1 receptor appears to be different from both the human and the rabbit receptor. In RAW 264 mouse macrophages, des-Arg^{10}-KD and des-Arg^9-BK are equipotent in displacing [^{3}H]-des-Arg^{10}-KD (Burch *et al.*, 1992). In contrast, des-Arg^{10}-KD is the most potent ligand at the human and rabbit B_1 receptors, with des-Arg^9-BK being less active in the rabbit and much less potent in the human. These discrepancies between human, mouse and rabbit imply that species differences exist between B_1 receptors.

The ability of a number of ligands to displace [^{3}H]-BK was also examined. The rank order of potency of the agonists tested; BK = KD > > > des-Arg^{10}-KD = des-Arg^9-BK, was similar to data reported elsewhere and confirmed the selectivity of des-Arg^{10}-KD. As expected, Hoe 140 (Hock *et al.*, 1991) displaced [^{3}H]-BK binding whereas the B_1 selective antagonist, des-Arg^9[Leu^8]-BK was ineffective at 10 μM.

LIGAND analysis of [^{3}H]-des-Arg^{10}-KD saturation data over the range 0.03 – 6 nM indicated that only a one-site model was possible. However, when the radioligand concentration-range was increased to 40 nM to permit any low affinity binding to be characterized (4 experiments), the data could be fitted to a two-site model (Table 1), giving a high affinity picomolar site (K_D value 45 ± 32 pM) and a very poorly defined lower affinity, nanomolar site (K_D value 4.7 ± 2.4 nM). It is likely that this apparent second site was the result of the increased scatter of specific binding data points that occurred as the specific bound:total bound ratio decreased at radioligand concentrations above 5 nM. However, an alternative explanation could be that conformational changes that occur following agonist binding to this G-protein-coupled receptor result in two affinity states (e.g. see Hess *et al.*, 1992; Leeb-Lundberg *et al.*, 1994). Additional experiments are currently underway to examine the effects of GTP analogues on [^{3}H]-des-Arg^{10}-KD binding. The saturation data for [^{3}H]-BK binding could also be fitted to a two-site binding model by LIGAND (see Table 2 legend). Again, the low affinity site was poorly defined and is also likely to have resulted from scatter of data at high radioligand concentrations. Competition binding experiments performed with an [^{3}H]-des-Arg^{10}-KD concentration of 1 nM produced a rank order of potency of ligands characteristic of a classical B_1 BK receptor (Hall, 1992), indicating that [^{3}H]-des-Arg^{10}-KD was unlikely to be binding to B_2 receptors. Similarly, the potencies for ligands displacing 1 nM [^{3}H]-BK was consistent with binding to B_2 and not B_1 receptors.

In conclusion, this is the first characterization of [^{3}H]-des-Arg^{10}-KD binding to include both kinetic and equilibrium data and indicates that [^{3}H]-des-Arg^{10}-KD is a radioligand with a high affinity for human B_1 bradykinin receptors and is sufficiently selective to be used in human cells in the presence of an abundance of B_2 bradykinin receptors.

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