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Stable expression of human kinin B_1 receptor in 293 cells: pharmacological and functional characterization

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1 We compared the binding properties of [^{3}H]-desArg^{10}-[Leu^9]-kallidin, a radiolabelled kinin B_1 receptor antagonist, to membranes from IMR-90 human embryonic fibroblasts and from 293 cells transiently or stably transfected with the human B_1 receptor.

2 The dissociation constant (K_D) of [^{3}H]-desArg^{10}-[Leu^9]-kallidin and the affinity of several kinin receptor agonists and antagonists were similar between the native and cloned receptor, either transiently or stably expressed in 293 cells. In IMR-90 cells, the rank order of potency was that expected for a kinin B_1 receptor.

3 The receptors transiently or stably expressed in 293 cells were fully functional with respect to their signalling properties. Phosphoinositide hydrolysis was increased in a concentration-dependent manner by the B_1 receptor agonist, desArg^{10}-kallidin. Functional coupling to the calcium pathway was also demonstrated for the native and stably expressed human B_1 receptor.

4 In conclusion, the established stable and functional 293 cell clone may provide an important tool for further analysis of the molecular mechanisms involved in binding, activation, and coupling of the kinin B_1 receptor.

Keywords: Kinin B_1 receptor; binding; phosphoinositosides cascade; calcium

Introduction

Kinins, such as bradykinin (BK) and kallidin (Lys-bradykinin, KD), are biologically active peptides derived from large precursors (kininogens) by the action of serine proteases, named kallikreins (Regoli & Barabé, 1980). Kinins are released in response to tissue injury and activate sensory pain fibres, contract smooth muscle, cause endothelium-dependent vasodilatation and induce plasma extravasation (Regoli & Barabé, 1980; Proud & Kaplan, 1988; Dray & Perkins, 1993).

Kinins mediate their effects through the activation of two types of G-protein coupled receptors, B_1 and B_2. These receptors have been cloned (Hess *et al*., 1992; Menke *et al*., 1994). Most of the physiological effects of kinins seem to be mediated by the activation of constitutive B_2 receptors, which bind BK and KD with high affinities. The enzymatic removal of the C-terminal arginine from BK and KD generates desArg^9-BK and desArg^{10}-KD, respectively, which are both agonists for the B_1 receptor. While the existence of a B_1 receptor subtype was established over 15 years ago (Regoli *et al*., 1977; Regoli & Barabé, 1980), detailed information about its tissue distribution, biological function and molecular properties are limited. Several isolated tissue preparations, such as rabbit arteries, exhibit the capacity to increase selectively their sensitivity to desArg-kinins in a time- and protein synthesis-dependent manner (Bouthillier *et al*., 1987; Deblois *et al*., 1988; Pruneau & Belichard, 1993). Interleukin-1β (IL-1β), lipopolysaccharide (LPS) or tissue injury cause an increase of kinin B_1 receptor expression both *in vitro* and *in vivo* (Bouthillier *et al*., 1987; Drapeau *et al*., 1991; Pruneau *et al*., 1994). Moreover, this up-regulation process has been observed in several models of persistant hyperalgesia in the rat (Perkins *et al*., 1993). Such a feature has led to the hypothesis that B_2 receptors play a significant role in the earlier stages of an inflammatory process, whereas B_1 receptors are more important for the maintenance of the chronic inflammatory response.

Recently, the cDNA encoding the human B_1 receptor was obtained by expression cloning in *Xenopus* oocytes and was transiently expressed in COS-7 cells (Menke *et al*., 1994). In the present study, we describe the cloning, functional expression and pharmacological characterization of the cloned receptor stably expressed in 293 cells, with a B_1 receptor antagonist radioligand, [^{3}H]-desArg^{10}-[Leu^9]-KD.

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Methods

Cell culture

Human embryonic lung fibroblasts (IMR-90) and 293 cells were from American Type Culture Collection (Rockville, MD). IMR-90 and 293 cells were grown in Dulbecco's modified Eagles Medium (DMEM) containing 4.5 g l^{-1} glucose, 1% glutamax (v/v), 1% non essential aminoacid (NEM) (v/v), 1 mM sodium pyruvate, 100 μg ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% foetal bovine serum.

PCR cloning

Total RNA was extracted from IMR-90 cells by use of Trizol (Life Technologies), according to the method of Chomczynski & Sacchi (1987) and poly(A)$^+$ RNA was subsequently prepared by oligo(dT) cellulose chromatography as described by Sambrook *et al*. (1989). cDNA was obtained with the Superscript II RT kit (Life Technologies).

A 1.1 kb cDNA encoding the human B_1 receptor was amplified by polymerase chain reaction (PCR) by use of oligonucleotides based on the sequence published by Menke *et al*. (1994). Oligonucleotides were designed to include the coding domain plus 6 basepairs of the 5′ untranslated region and to generate a BamHI restriction site at the 5′ end and a KpnI site at the 3′ end (sense: 5′-CGC GGA TCC CTG TGC ATG GCA TCA TCC TGG CCC CCT CTA GA-3′ and antisense: 5′-GCG GGT ACC CTT CAA TGC TGT TTT AAT TCC GCC AGA AAA G-3′). The 1.1 kb PCR product was digested with BamHI and KpnI and subcloned into pGEM7zf (+) (Promega, Madison, WI) and sequenced by the dideoxy method of Sanger *et al*. (1977). The recombinant plasmid was digested with XhoI and BamHI and the insert was subcloned into the mammalian expression vector pcDNA3 (Invitrogen, Leek, The Netherlands).

Transfection

For transient cell expression, 293 cells were seeded into 175 cm^2 dishes and left to adhere overnight. Ten micrograms of vector containing the cDNA fragment encoding the B_1 receptor were then transfected into the cells by means of the calcium phosphate precipitation method (Chen & Okayama, 1987). As a negative control, one dish of cells received a precipitate containing the native pcDNA3 vector. Precipitates were left on cells for 5 h and, after washing, the 293 cells were allowed to recover in a standard growth medium for 48 h before membrane preparation or functional studies.

For stable cell expression, the selection of transfectants was initiated 48 h post-transfection by addition of geneticin (500 $\mu g\ ml^{-1}$). Resistant colonies, visible two weeks after treatment, were lifted into 12-well plates (1 cell/well) and were expanded with a maintenance concentration of 500 $\mu g\ ml^{-1}$ geneticin before assaying for specific binding of [^{3}H]-desArg^{10}-[Leu^9]-KD (1 nM). Clone IIID6, which exhibited the highest binding capacity was selected for further radioligand binding and functional assays.

Membrane preparation

IMR-90 cells treated for 2.5 h with 2.5 ng ml^{-1} IL-1β or transfected 293 cells were rinsed twice with Ca^{2+}/Mg^{2+} free ice-cold PBS and scrapped from the dishes with a rubber policeman in 5 ml binding buffer (TES 25 mM, 1,10-phenanthroline 1 mM, bacitracin 140 $\mu g\ ml^{-1}$ and bovine serum albumin 0.1%) at pH 7.4. They were homogenized with a Polytron (Kinematica, Lucerne, Switzerland) and centrifuged at 40,000 $\times g$ for 20 min. The pellet was then resuspended in binding buffer and stored in liquid nitrogen.

Binding assay

Binding experiments were performed at room temperature with 20–40 μg membrane protein/assay in binding buffer. For saturation experiments, [^{3}H]-desArg^{10}-[Leu^9]-KD (0.1 to 5 nM) was incubated for 1 h in a final volume of 500 μl. The assay was terminated by filtration on Whatman GF/B filters presoaked for 2 h in polyethyleneimide 0.1% (w/v). Filters were rinsed three times with 5 ml ice-cold 50 mM TES and the radioactivity was determined by liquid scintillation counting in 5 ml Optima Gold (Packard, Rungis, France). Non-specific binding was determined in the presence of 10 μM desArg^{10}-[Leu^9]-KD. Competition binding experiments were carried out in the presence of 1 nM [^{3}H]-desArg^{10}-[Leu^9]-KD with various concentrations of kinin analogues. All assays were carried out in duplicate. Protein concentration was measured by the method of Bradford (1976).

Assay of phosphoinositide hydrolysis

Transiently transfected 293 cells were suspended in phosphoinositide (PI) buffer of the following composition (in mM): HEPES 20, NaCl 116, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 5, glucose 11, captopril 0.01 and bacitracin 140 $\mu g\ ml^{-1}$; pH 7.4. Cells were loaded with 1 $\mu Ci\ ml^{-1}$ *myo*-[^{3}H]-inositol, maintained for 1 h at 37°C and washed twice with PI buffer. Labelled cells were incubated in PI buffer containing 10 mM LiCl for 15 min at 37°C. Stimulation of PI hydrolysis was induced by desArg^{10}-KD for 30 min. The reaction was stopped by chloroform-methanol (2/1:v/v). Labelled inositol phosphates were separated by ion-exchange chromatography on Dowex AG1-X8 according to the modified method of Berridge *et al.* (1982).

Subconfluent stably transfected 293 cells maintained in 12 well plates were labelled with *myo*-[^{3}H]inositol, 1 μCi/well in serum-free 199 medium. After 24 h, cells were washed with PBS, and incubated 15 min at 37°C in PI buffer supplemented with 10 mM LiCl (500 μl/well). Stimulation of PI hydrolysis was induced by desArg^{10}-KD for 15 min. After removal of PI buffer, the reaction was stopped by addition of a 5% $HClO_4$ solution (500 μl/well) and wells were rinsed with 500 μl of 2.5% $HClO_4$. After 30 min on ice, 800 μl of cellular suspension were neutralized with 155 μl of 2 M K_2CO_3. Samples were then applied on anion exchange columns (Dowex AG1-X8) and the different inositol phosphates were separated as described elsewhere (Berridge *et al.*, 1982).

Measurement of intracellular calcium

IMR-90 cells or stably transfected 293 cells were seeded onto 10 × 25 mm glass cover slips and used after reaching confluence. After washing twice in Minimum Essential Medium (MEM), cover slips containing adherent monolayers were incubated for 30 min at 37°C in MEM supplemented with 5.5 μM Fura-2/AM and excess Fura-2/AM was removed by washing twice for 10 min in Hank's balanced salt solution (HBSS). Intracellular calcium levels were monitored in 3.5 ml HBSS at room temperature with a spectrofluorimeter (Perkin Elmer LS50-B, Buckinghamshire, UK). Cytosolic Ca^{2+} concentrations were calculated as described by Grynkiewicz *et al.* (1985), by use of the following equation:

$$[Ca^{2+}]_i = K_D \times (R - R_{min})/(R - R_{max}) \times Sf_2/Sb_2$$

where K_D is the dissociation constant of the Ca^{2+}/Fura-2 complex (224 nM), R is the experimentally determined 510 nm emission ratio at the two excitation wavelengths of 340 and 380 nm, respectively. R_{max}, the maximal ratio, is measured in the presence of 0.5% (v/v) Triton X-100, whereas R_{min}, the ratio given by Ca^{2+}-free-dye, is measured in HBSS supplemented with 25 mM EGTA. Sf_2/Sb_2 is the ratio of Fura-2 fluorescence values at 380 nm in Ca^{2+}-free and Ca^{2+}-saturated medium, respectively.

Analysis of data

Competition experiments and concentration-response curves for PI hydrolysis were analysed by use of GraphPADInPlot (GraphPAD Software, San Diego, CA). In order to evaluate the potency of the antagonists, we calculated a pK_B value and its s.e.mean by applying the following equation:

$$K_B = [B]/(slope - 1)$$

in which slope is that of the double-reciprocal plot of equieffective concentrations of agonist (A) in the absence (1/A) and in the presence (1/A′) of the antagonist (B) and [B] represents the antagonist concentration (Kenakin, 1993).

Statistical analysis was performed by use of Statview (Abacus Concept, Palo Alto, CA). A one-way analysis of variance followed by Student's t test was used to establish significant differences between basal levels, maximum responses, B_{max}, EC_{50}, K_i or K_D values. A P value less than 0.05 was considered to be statistically significant.

Drugs

[^{3}H]-desArg^{10}-[Leu^9]-KD (100–120 Ci $mmol^{-1}$) was obtained from NEN (Boston, MA) and *myo*-[^{3}H]-inositol (80–120 Ci $mmol^{-1}$) from Amersham (Les Ullis, France). Bradykinin and its analogues were from Bachem California (Basel, Switzerland). Hoe140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-bradykinin) was kindly provided by Pr J. Martinez (CNRS, URA 1845, Montpellier, France). DesArg^{10}-kallidin, desArg^{10}-[Leu^9]-kallidin and desArg^{10}-Hoe140 were from Neosystem (Strasbourg, France). All molecular biology and cell culture reagents were purchased from Life Technologies (Cergy-Pontoise, France). All other chemicals were supplied by Sigma Chemical Co. (St. Louis, MO).

Results

Molecular biology

The nucleotidic sequence analysis of the 1.1 kb amplified cDNA revealed a 1,059 base pair open reading frame encoding a 353 amino acid protein which was homologue of the sequence described by Menke *et al*. (1994), except for Arg which replaced Gly in position 146.

Binding experiments

IMR-90 cells and 293 cells transiently or stably transfected with a mammalian expression vector containing the cDNA for the human B_1 receptor exhibited a specific saturable binding for [^{3}H]-desArg^{10}-[Leu^9]-KD (Table 1). Scatchard analysis revealed the presence of a single high affinity site. No statistically significant differences were observed between the affinities of [^{3}H]-desArg^{10}-[Leu^9]-KD for the native human B_1 receptor of IMR-90 cells ($K_D = 1.02 \pm 0.19$ nM, $n = 6$) and for the cloned receptor either transiently ($K_D = 0.47 \pm 0.06$ nM, $n = 5$) or stably ($K_D = 0.63 \pm 0.16$ nM, $n = 12$) expressed in 293 cells.

Mock-transfected 293 cells expressed a very low level of specific binding which was less than 4% of the specific binding obtained in transiently transfected cells. Maximum binding

Table 1 The dissociation constant (K_D) and binding density (B_{max}) for [^{3}H]-desArg^{10}-[Leu^9]-KD binding in different cell membranes

	K_D (nM)	B_{max} (fmol mg^{-1} protein)
Native B_1 receptor		
IMR-90 cells	1.02 ± 0.19	183 ± 26
Cloned B_1 receptor		
293 cells transiently transfected	0.47 ± 0.06	3148 ± 961
293 cells stably transfected	0.63 ± 0.16	830 ± 460

Each value represents the mean ± s.e.mean from 5 to 12 experiments in duplicate.

Table 2 Binding affinities (K_i, nM) of various kinin derivatives at [^{3}H]-desArg^{10}-[Leu^9]-KD binding sites in human native and cloned B_1 receptors

	Native B_1 receptor	*Transiently expressed B_1 receptor*	*Stably expressed B_1 receptor*
Bradykinin (BK)	> 10,000	> 10,000	> 10,000
Kallidin (KD)	3.23 ± 0.84	2.54 ± 1.13	1.35 ± 0.21
DesArg^9-BK	1,250 ± 228	1,930 ± 329	1,370 ± 45
DesArg^{10}-KD	0.29 ± 0.07	0.12 ± 0.02	0.12 ± 0.02
DesArg^9-[Leu^8]-BK	367 ± 98	276 ± 39	382 ± 60
DesArg^{10}-[Leu^9]-KD	1.96 ± 0.43	0.58 ± 0.14	0.90 ± 0.18
Hoe140	1,000 ± 142	437 ± 32	1,270 ± 321
DesArg^{10}-Hoe140	68.5 ± 29.8	17.4 ± 2.8	24.5 ± 1.0

Each value represents the mean ± s.e.mean from 3 or 4 experiments in duplicate.

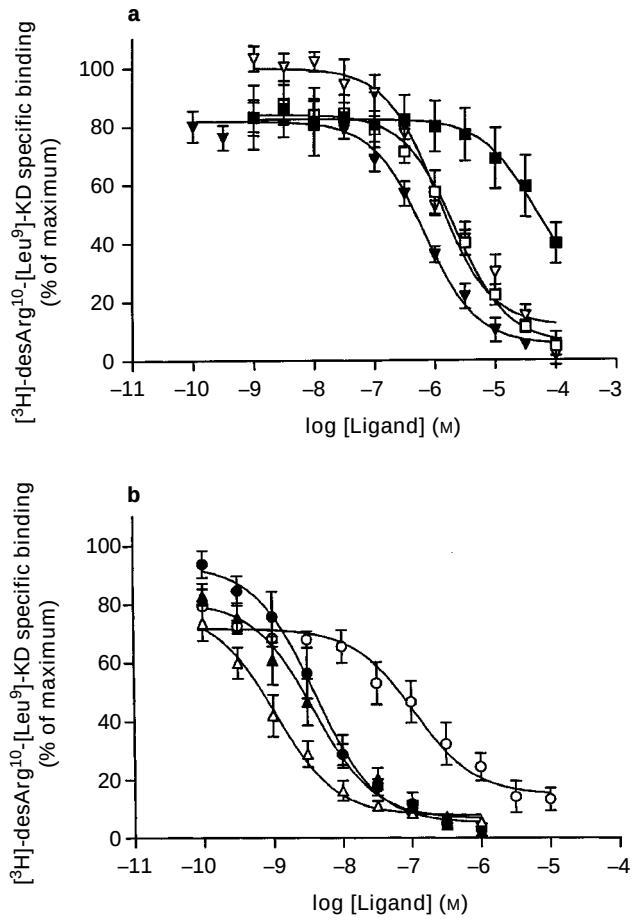

Figure 1 Displacement curves of [^{3}H]-desArg^{10}-[Leu^9]-KD to membranes of IMR-90 cells treated with IL-1β. (a) BK (■), desArg^9-BK (□), desArg^9-[Leu^8]-BK (▼), Hoe140 (▽); (b) KD (▲), desArg^{10}-KD (△), desArg^{10}-[Leu^9]-KD (●), desArg^{10}-Hoe140 (○). Values are means from 4–7 experiments in duplicate; vertical lines show s.e.mean.

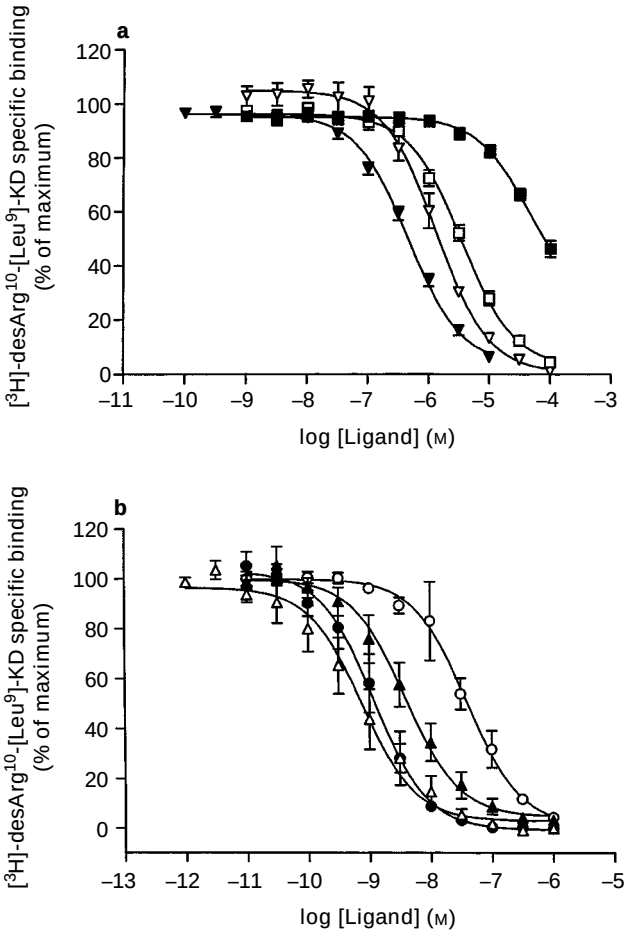

Figure 2 Displacement curves of [^{3}H]-desArg^{10}-[Leu^9]-KD to membranes of 293 cells transiently transfected with the human B_1 receptor. (a) BK (■), desArg^9-BK (□), desArg^9-[Leu^8]-BK (▼), Hoe140 (▽); (b) KD (▲), desArg^{10}-KD (△), desArg^{10}-[Leu^9]-KD (●), desArg^{10}-Hoe140 (○). Values are means from 4–7 experiments in duplicate; vertical lines show s.e.mean.

capacity was 4 times lower in the clone IIID6 than in transiently transfected 293 cells (Table 1). However, this clone showed a stable expression of the human B_1 receptor for at least 6 months of cell culture.

Pharmacological characterization of the human native kinin B_1 receptor in IMR-90 membranes was achieved by analysing the competition of [^{3}H]-desArg^{10}-[Leu^9]-KD with several kinin receptor agonists and antagonists (Figure 1). All curves were monophasic with a slope factor close to unity, suggesting a homogeneous population of binding sites. K_i values are given in Table 2. The rank order of potency was as follows: desArg^{10}-KD > desArg^{10}-[Leu^9]-KD > KD > desArg^9-[Leu^8]-BK > desArg^9-BK > > BK. The B_2 receptor antagonist, Hoe140, gave a K_i value of 1,000 ± 142 nM.

Similar binding affinity data were obtained with the human cloned B_1 receptor either transiently (Figure 2) or stably (Figure 3) expressed in 293 cells. K_i values for kinin receptor ligands are given in Table 2. The correlation coefficient of the affinities between the cloned and the native B_1 receptor was highly significant ($r = 0.98$ for transiently transfected 293 cells *vs* IMR-90 cells; $r = 0.97$ for stably transfected 293 cells *vs* IMR-90 cells; $r = 0.98$ for transiently transfected 293 cells *vs* stably transfected 293 cells).

Functional characterization

As kinin B_2 receptors are known to couple to phospholipase C activation (Yano *et al*., 1984), the B_1 receptor agonist, desArg^{10}-KD, was tested for its ability to induce phosphoinositide hydrolysis in transiently transfected 293 cells. DesArg^{10}-KD produced a concentration-dependent increase in the accumulation of inositol 1,4,5-monophosphate (IP_1) with an EC_{50} value of 0.15 ± 0.08 nM (Figure 4a). The maximum increase in IP_1 production was 4.8 ± 0.3 fold over basal values. Mock-transfected 293 cells did not respond to desArg^{10}-KD (1 μM). The phosphoinositide response induced by desArg^{10}-KD was inhibited by the selective B_1 receptor antagonist desArg^{10}-[Leu^9]-KD (100 nM) with a pK_B value of 9.42 ± 0.17. The maximal response to desArg^{10}-KD was not affected by desArg^{10}-[Leu^9]-KD.

The functional coupling of the human stably transfected kinin B_1 receptor in 293 cells was also demonstrated by phosphoinositide hydrolysis assay in clone IIID6 (Figure 4b). DesArg^{10}-KD produced a concentration-dependent increase in accumulation of IP_1, inositol 1,4,5-diphosphateIP_2 and inositol 1,4,5-trisphosphate IP_3 with EC_{50} values of 2.88 ± 0.59 nM, 1.42 ± 0.48 nM and 1.08 ± 0.32 nM, respectively ($n = 4-7$, data not shown). The maximum increase in IP_1 level over basal values was 3.0 ± 0.5 fold. In the presence of desArg^{10}-[Leu^9]-KD (100 nM), the concentration-response curve to desArg^{10}-KD was shifted to the right with EC_{50} values of 29.3 ± 13.9 nM ($P < 0.05$), 30.7 ± 11.1 nM ($P < 0.05$) and 3.81 ± 0.84 nM for IP_1, IP_2 and IP_3, respectively. In contrast to results obtained with transiently transfected 293 cells, the maximal response to desArg^{10}-KD was significantly reduced in the presence of desArg^{10}-[Leu^9]-KD (100 nM) in stably transfected 293 cells. The phosphoinositide response induced by desArg^{10}-KD was also inhibited by 100 μM Hoe 140 with a pK_B value of 4.66 ± 0.41.

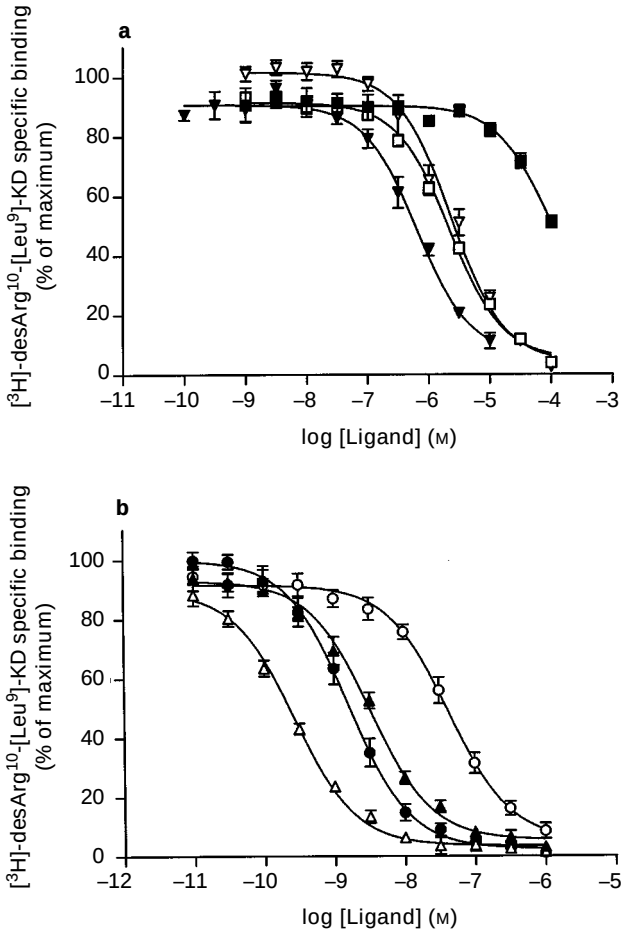

Figure 3 Displacement curves of [^{3}H]-desArg^{10}-[Leu^9]-KD to membranes of 293 cells stably transfected with the human B_1 receptor (clone IIID6). (a) BK (■), desArg^9-BK (□), desArg^9-[Leu^8]-BK (▼), Hoe140 (▽); (b) KD (▲), desArg^{10}-KD (△), desArg^{10}-[Leu^9]-KD (●), desArg^{10}-Hoe140 (○). Values are mean from 3–6 experiments in duplicate; vertical lines show s.e.mean.

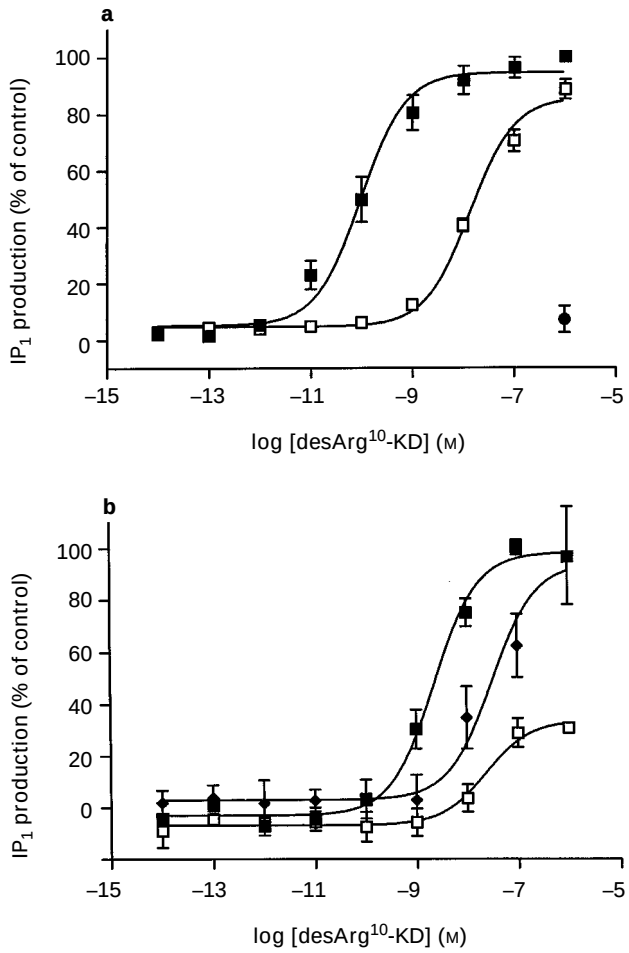

Figure 4 Concentration-response curve for [^{3}H]-inositol monophosphate accumulation in 293 cells transiently (a) or stably (b) tranfected with the human kinin B_1 receptor induced by desArg^{10}-KD in the absence (■) or presence (□) of desArg^{10}-[Leu^9]-KD (100 nM) or (◆) Hoe140 (100 μM). (●) Represent the effect of desArg^{10}-KD (1 μM) in mock-transfected 293 cells. Values are mean from 4–7 experiments in duplicate; vertical lines show s.e.mean.

To characterize further the human kinin B_1 receptor, measurements of intracellular calcium were performed in IMR-90 cells and stably transfected 293 cells. The basal level of $[Ca^{2+}]_i$ was lower ($P<0.05$) in transfected 293 cells (27.4 ± 6.0 nM, $n=5$) than in IMR-90 cells (51.3 ± 10.2 nM, $n=3$). The B_1 agonist, desArg^{10}-KD, increased intracellular calcium in IMR-90 cells (Figure 5a, b) and in stably transfected 293 cells (Figure 5c, d). Maximal $[Ca^{2+}]_i$ responses induced by desArg^{10}-KD (100 nM) were different between transfected 293 cells and IMR-90 cells (143 ± 52 nM *vs* 75 ± 4 nM, $P<0.05$). The kinetics of the $[Ca^{2+}]_i$ responses were similar and pretreatment with desArg^{10}-[Leu^9]-KD (100 nM) totally abolished desArg^{10}-KD-induced $[Ca^{2+}]_i$ responses in both cell lines.

Discussion

It has been previously shown that the human embryonic fibroblast cell line IMR-90 expressed the kinin B_1 receptor subtype (Goldstein & Wall, 1984). On another hand, IL-1β was shown to increase the number of B_1 receptors without affecting their affinity for desArg^{10}-KD (Galizzi *et al*., 1994; Levesque *et al*., 1995). The initial aim of this study was to characterize the kinin B_1 receptor described on IL-1β-treated IMR-90 cells, by use of a selective radiolabelled antagonist, [^{3}H]-desArg^{10}-[Leu^9]-KD. Interestingly, the kinin B_1 receptor expressed by IMR-90 cells exhibited a lower affinity for the des-arginine derivatives of bradykinin than for those of kallidin. Thus, the most potent natural ligand for the human B_1 receptor appears to be desArg^{10}-KD. In the present study, the pharmacological profile of the B_1 receptor was similar to that previously demonstrated with [^{3}H]-desArg^{10}-KD as a radioligand (Menke *et al*., 1994), except for Hoe140 which competed with [^{3}H]-desArg^{10}-[Leu^9]-KD binding giving a K_i value of $1{,}000\pm142$ nM. This effect was not dependent on the radioligand since two other groups have recently shown similar binding properties of Hoe140 to the native human B_1 receptor with [^{3}H]-desArg^{10}-KD as a radioligand. Burkard *et al*. (1996) obtained an IC_{50} value of 1,000 nM for Hoe140 in IMR-90 cells and Phagoo *et al*. (1996) a K_i value of 595 ± 119 nM in WI38 fibroblasts. In addition, Hoe140 has also been shown to antagonize contractions to desArg^9-BK with pA_2 values of 5.48 ± 0.15 in the human isolated umbilical vein (Gobeil *et al*., 1996) and 5.75 ± 0.11 in the human ileum (Zuzack *et al*., 1996), respectively. Furthermore, in bovine cultured aortic endothelial cells, production of cyclic GMP induced by desArg^9-BK was totally inhibited by Hoe140 (100 nM) (Wiemer & Wirth, 1992). In this study, the authors suggested heterogeneity

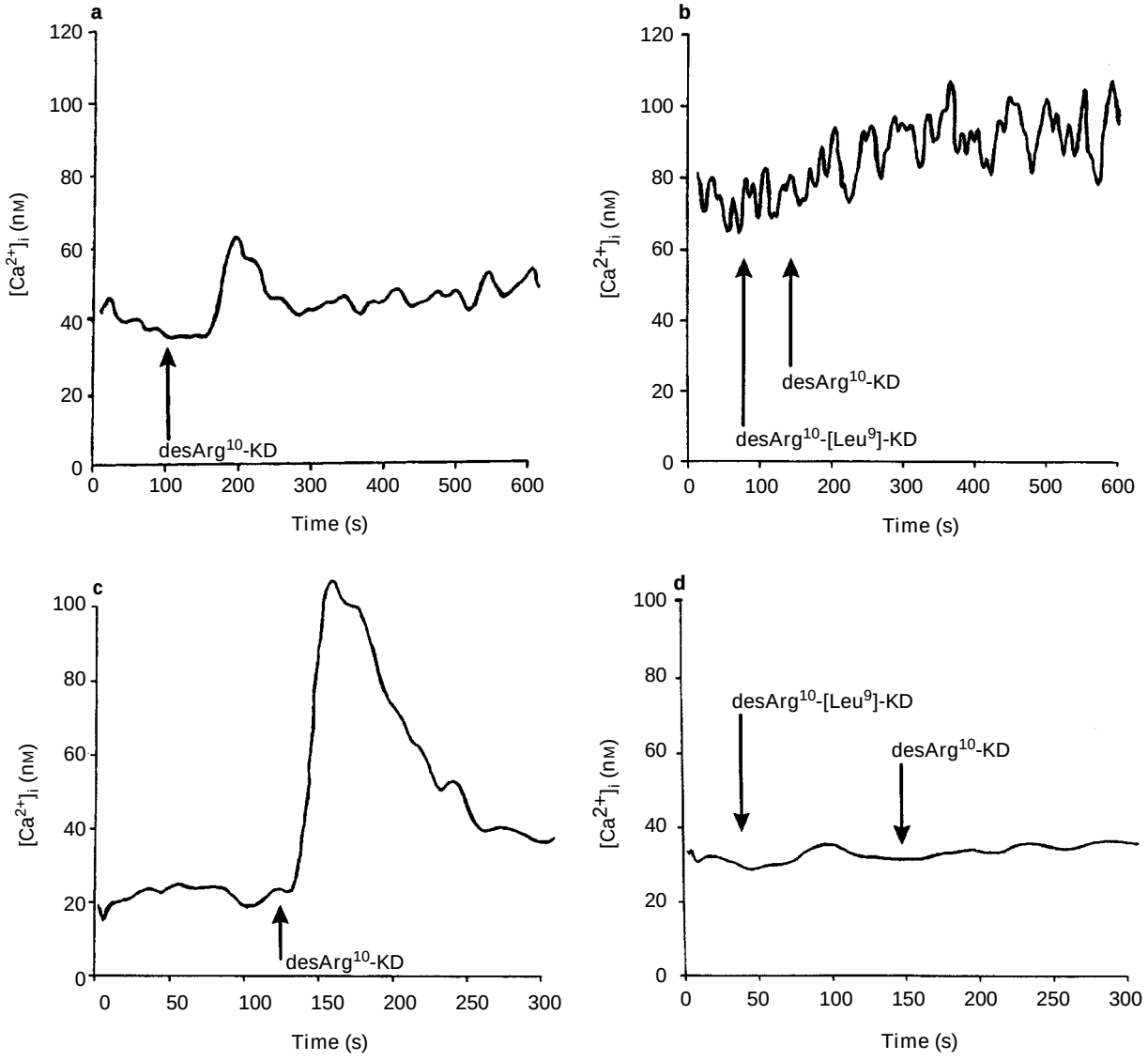

Figure 5 Original tracings showing the $[Ca^{2+}]_i$ response induced by desArg^{10}-KD (100 nM) in IMR-90 cells (a, b) and stably transfected 293 cells (c, d) before (a, c) and after (b, d) preincubation with desArg^{10}-[Leu^9]-KD (100 nM). Tracings are representative of at least 3 independent experiments.

amongst B_1 kinin receptors. Thus, it remains to be determined whether the moderate affinity of Hoe140 for the B_1 receptor is restricted to this molecule or is a general feature of peptide B_2 receptor antagonists described so far.

As previously found in rat mesangial cells and in bovine tracheal smooth muscle cells (Issandou & Darbon, 1991; Marsh & Hill, 1994), we demonstrated functional coupling to the calcium signalling in IMR-90 cells. DesArg^{10}-KD increased intracellular calcium concentration, a response which probably resulted from phospholipase C and phosphoinositosides cascade activation.

The second aim of this study was to compare the pharmacological profile of native and cloned human B_1 receptors. We found that the affinity of [^{3}H]-desArg^{10}-[Leu^9]-KD and the K_i values of various kinin receptor agonists and antagonists were similar to those found with the native receptor. The receptor expressed in 293 cells exhibited the pharmacological profile of a B_1 subtype (outlined in Table 2). These results suggest that there is no difference between the native and cloned human kinin B_1 receptor. The level of expression in transiently transfected 293 cells appears to be higher (B_{max} = 3,150 fmol mg^{-1} protein) than previously described in COS-7 cells (B_{max} = 100 fmol mg^{-1} protein) (Menke *et al*., 1994). Furthermore, we describe here the first stable expression of the human kinin B_1 receptor.

Recently, the B_1 receptor has been shown to activate the phospholipase C pathway in a primary culture of rabbit aortic smooth muscle cells, rabbit mesenteric artery smooth muscle cells and rat mesangial cells (Issandou & Darbon, 1991; Tropea *et al*., 1993; Schneck *et al*., 1994). Phosphoinositide hydrolysis assay and intracellular calcium measurement were used to characterize further the human B_1 receptor expressed in 293 cells. DesArg^{10}-KD increased intracellular calcium in stably transfected 293 cells, a response which was totally abolished by the B_1 receptor antagonist, desArg^{10}-[Leu^9]-KD. The maximal effect of desArg^{10}-KD at inducing the calcium response was larger in stably transfected 293 cells compared to IMR-90 cells. This difference might be related to the density of B_1 receptors, which was 4.5 fold lower in human fibroblasts, or to the size of the pool of releasable calcium in the two cell lines. In transiently and stably transfected 293 cells, desArg^{10}-KD increased IP_1 in a concentration-dependent manner and with a similar maximal response. This effect was receptor-dependent, since it was inhibited by the B_1 receptor antagonist desArg^{10}-[Leu^9]-KD. Surprisingly, the concentration of desArg^{10}-KD required for half-maximal stimulation was 15 fold higher in transiently transfected 293 cells than in clone IIID6 stably expressing the human kinin B_1 receptor. Furthermore, in contrast to results obtained with transiently transfected 293 cells, the maximal effect of desArg^{10}KD was reduced in the presence of desArg^{10}-[Leu^9]-KD in stably transfected 293 cells. Similar results were obtained when IP_1 accumulation was measured in cells in suspension or maintained in 12 well plates (data not shown). Although we have no adequate explanation, we suggest that these differences could be related to the number of receptors, which was 4-fold lower in the clone IIID6 than in transiently transfected 293 cells. Alternatively, it is possible that the maturation state of the cloned B_1 receptor might be different between transiently and stably transfected cells. In this respect, a recent study comparing transient and stable expression of the V_2 vasopressin receptor in 293 cells demonstrated the existence of a pool of immature receptors expressed in transiently transfected cells. In contrast, receptors extracted from stably transfected cells were composed mostly of mature receptor protein. The distinction was based on the size of proteins, their half-life and the degree of maturation of their sugar moieties (Innamorati *et al*., 1996). Moreover, a recent study showed that the extent of murine gastrin-releasing peptide receptor glycosylation affected the G-protein coupling and the receptor affinity (Kusui *et al*., 1994). Thus, it is possible that functional differences observed between transiently and stably expressed human kinin B_1 receptors might be dependent on the maturation state of the protein, although further studies are needed to investigate this point. While the K_i value of Hoe140 in stably transfected 293 cells was 1,270 nM, the phosphoinositide response induced by desArg^{10}-KD in these cells was inhibited by Hoe140 with a pK_B value of 4.66. A different potency of Hoe140 in the binding and the functional assays has also been found with the human kinin B_2 receptor by Burkard *et al*.(1996). These authors obtained a pIC_{50} value of 9.8 for Hoe140 on the human cloned B_2 receptor and a pA_2 value of 8.1 in human ileum. This unexpected difference is also seen with desArg^{10}-[Leu^9]-KD which exhibits a pK_B value of 7.96 in human ileum (Zuzack *et al*., 1996) and an IC_{50} value of 1.3 nM in IMR-90 cells (Menke *et al*., 1994).

In summary, the present study demonstrates that IMR-90 cells express high-affinity [^{3}H]-desArg^{10}-[Leu^9]-KD binding sites which display the classical pharmacological profile of a kinin B_1 receptor, with the exception of a moderate affinity for the B_2 receptor antagonist, Hoe140. [^{3}H]-desArg^{10}-[Leu^9]-KD binding and K_i values of various kinin ligands were similar between the native and the cloned B_1 receptor. The established stable and functional 293 cell clone might be an important tool for further analysis of the molecular mechanisms involved in binding, activation and coupling of the kinin B_1 receptor.

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