

RESEARCH PAPER

Antagonist, partial agonist and antiproliferative actions of B-9870 (CU201) as a function of the expression and density of the bradykinin B₁ and B₂ receptors

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Background and purpose: A bradykinin (BK) B₂ receptor (B₂R) antagonist, B-9870 (CU201), has been proposed to behave as a 'biased agonist' at B₂Rs and to exert anti-neoplastic effects. It was unclear whether these effects were determined by the activation of B₂Rs by the drug. B-9870 was evaluated for antagonism or stimulation of several responses mediated by the rabbit B₂R or B₁ receptor (B₁R); its anti-proliferative activity was also characterized.

Experimental approach and key results: B-9870 was an insurmountable B₂R antagonist in the rabbit jugular vein contractility assay, but a partial agonist in HEK 293 cells expressing the rabbit B₂R or a green fluorescent protein (GFP) conjugate of the latter (ERK1/2 phosphorylation, [Ca²⁺]_i, [³H]-arachidonate release, endocytosis). The agonist-like effects of B-9870 were inhibited by the B₂R antagonist LF 16.0687 and absent in untransfected cells. In addition, B-9870 was a surmountable antagonist of the rabbit B₁R in the aorta contractility assay, and blocked Lys-des-Arg⁹-BK-induced ERK1/2 phosphorylation in HEK 293 cells expressing a fluorescent B₁R conjugate. B-9870 inhibited the growth of MDA-MB-231 cells. The latter effect was not influenced by B₁R or B₂R antagonists and was not apoptotic. MDA-MB-231 cells expressed a small population of B₂Rs but no B₁Rs; they responded to BK (small calcium transients) and B-9870 behaved as an antagonist.

Conclusion and implications: B-9870 is a dual B₁R and B₂R antagonist with confirmed stimulating effects at the B₂R in high expression systems only. Its cell type-specific anti-proliferative effect occurs at a high concentration, independently from kinin receptors and apoptosis.

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Abbreviations: B₁R, B₁ receptor; B₂R, B₂ receptor; BK, bradykinin; ERK1/2, extracellular signal regulated kinases 1/2; GFP, green fluorescent protein; Hyp, 4-hydroxyproline; Igl, α (2-indanyl)glycine; Oic, L-(3aS,7aS)-octahydroindol-2-yl-carbonyl; PARP, poly(ADP-ribose)polymerase I; PLA₂, phospholipase A₂; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; YFP, yellow fluorescent protein

Introduction

Bradykinin (BK)-related peptides (the kinins) are blood-derived peptides that are vasodilator, hypotensive, natriuretic and pro-inflammatory (Leeb-Lundberg *et al.*, 2005). The role of the preformed B₂ receptors (B₂Rs) and the inducible

B₁ receptors (B₁Rs) is prominent in the mediation of kinin effects in cell types such as the endothelial and smooth muscle cells, the afferent nerve terminals, the renal epithelium and others. B₁Rs and B₂Rs are G protein-coupled receptors primarily linked to phospholipase C activation, mainly via G_q proteins (De Weerd and Leeb-Lundberg, 1997; Leeb-Lundberg *et al.*, 2005). Following agonist stimulation, B₂Rs are redistributed to caveolae and endocytic vesicles that are not clathrin-coated (De Weerd and Leeb-Lundberg, 1997; Haasemann *et al.*, 1998). Ser/Thr phosphorylation and dephosphorylation events precede the internalization and recycling to the surface of the B₂R, respectively (Blaukat *et al.*, 1996; Pizard *et al.*, 1999). A fluorescent rabbit B₂R-

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green fluorescent protein conjugate (B₂R-GFP) is confined to a recycling endosome compartment, and it is completely recycled in agonist-stimulated HEK 293 cells (Bachvarov *et al.*, 2001).

'Biased agonists' have been defined as receptor ligands causing the activation of a subset of the signal transduction pathways activated by the reference agonist (MacKinnon *et al.*, 2001; Chan *et al.*, 2002a). This concept may differ from the definition of a partial agonist, a drug that causes a fraction of the maximal activation of all signalling pathways of the receptor. Synthetic drugs consisting of dimers of peptide B₂R antagonists, linked at their N-terminus by the suberimidyl moiety (Stewart *et al.*, 1997), are proposed to behave as biased agonists of the B₂R. The most active drug in this respect, B-9870 (also called CU201), blocked the proliferation of tumour-derived cell lines, activated c-Jun kinase and caspase-3 while antagonizing BK-induced calcium signalling (Chan *et al.*, 2002a). The monomer structure of B-9870, D-Arg-[Hyp³, Igl⁵, D-Igl⁷, Oic⁸]BK (B-9430) was essentially devoid of the biased agonist properties of the dimer, but was a potent B₂R antagonist and, in previous investigations, a significant antagonist at the related B₁R as well (Stewart *et al.*, 1996; Chan *et al.*, 2002a). B-9870 inhibits tumour cell growth both *in vitro* and *in vivo* (Stewart *et al.*, 2005). The pharmacological effects of B-9870 on tumour cells have been postulated to be mediated by endogenously expressed B₂Rs, although this has not been directly evaluated (Chan *et al.*, 2002a, b).

The major aim of the present work was to verify whether the antiproliferative effect of B-9870 is a generalized cellular response mediated by kinin receptors. To support this line of investigation, the pharmacological profile of B-9870 has been initially established at the natural or recombinant rabbit receptors of both B₁ and B₂ subtypes. Both rabbit kinin receptors exhibit a high degree of pharmacological convergence with the human orthologues (Leeb-Lundberg *et al.*, 2005). The characterization of the pharmacological profile included testing of B₂R stimulation by B-9870 and related constrained peptide antagonists. B-9870 actions have been compared with the possible similar effects of B-10346 (the first pass metabolite of B-9870 in which imide groups are hydrolysed into amides at both ends of the divalent linker; JM Stewart, L Gera and DC Chan, unpublished results), of Hoe 140 and of a dimer of the latter peptide, B-9872 (also composed of two monomers linked at their N-terminus by the suberimidyl moiety; Gera *et al.*, 1996). Hoe 140 (icatibant, D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK) has been successfully used as a B₂R antagonist in many systems (Leeb-Lundberg *et al.*, 2005), but may exert some atypical effects reminiscent of the agonist, depending on the experimental system and the species. Hoe 140 activates the mitogen-activated kinases ERK1/2 and cell growth in some human tumour cell lines (Drube and Liebmann, 2000); a slow form of receptor endocytosis correlated with an insurmountable type of antagonism was observed at the rabbit B₂R for Hoe 140 and the related drug NPC 17731 (D-Arg-[Hyp³, D-HypE(transpropyl)⁷, Oic⁸]-BK) (Houle *et al.*, 2000), consistent with the finding that significant phospholipase C activation is elicited by both peptides in cells that express the recombinant human B₂R (Fathy *et al.*, 1999).

Further, the expression and stimulation of the kinin B₁R has been implicated in proliferation, migration and invasiveness of prostate cancer PC3 cells (Taub *et al.*, 2003). Thus, we have verified whether B-9870 is a B₁R ligand and of which functional type it belongs to. Finally, a tumour-derived cell line reportedly responsive to B-9870 has been evaluated for kinin receptor expression and the possible mediation of the antiproliferative effect by kinin receptors.

Methods

Cells

The derivation of HEK 293 cell lines stably expressing B₂R-GFP or B₁R-YFP and their properties are described elsewhere (Houle *et al.*, 2000; Bachvarov *et al.*, 2001; Sabourin *et al.*, 2002). The conjugation with a fluorescent protein has no detectable effect on the pharmacological profile of either receptor, and allows the visual monitoring of constant receptor expression in a simple manner. Non-transfected HEK 293 cells were used in some control experiments. Confocal microscopy for B₂R-GFP was applied to the live cells as described previously (Houle *et al.*, 2000; Bachvarov *et al.*, 2001), and supplemented with epifluorescence microscopy. An arachidonic acid release assay was performed, as described elsewhere, to evaluate the activation of the phospholipase A₂ (PLA₂) following a stimulation with ligands in HEK 293 cells stably expressing B₂R-GFP (24-well plates, 20 min release period at 37°C) (Houle *et al.*, 2000).

The effect of B-9870 on the proliferation of HEK 293 cells stably expressing B₂R-GFP or not was tested in the following manner: 5×10^4 cells were seeded in 35 mm Petri dishes in the usual serum-containing culture medium, optionally supplemented with B-9870 (applied 24 h after seeding). After 4 days of culture (3 days of drug treatment), the cells were detached using trypsin-ethylenediaminetetraacetic acid (EDTA) and manually counted using a haemocytometer. A similar procedure to this protocol has been applied to evaluate the effect of several kinin receptor ligands on the proliferation of the human breast cancer cell line MDA-MB-231 (originally obtained from American Type Culture Collection); 5×10^4 cells were seeded in 35 mm dishes containing 2 ml of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Burlington, ON, Canada) supplemented with 5% fetal bovine serum (FBS). After a 24-h incubation period, the medium was changed and the drugs were introduced for an additional 48 h incubation period. At 72 h post-seeding, the cells were detached with trypsin-EDTA and counted.

Binding assays

The binding of 2 nM [³H]-BK (Perkin Elmer Life Sciences; 90 Ci mmol⁻¹) or 1 nM [³H]-Lys-des-Arg⁹-BK (Perkin Elmer Life Sciences, Shelton, CT, USA; 69–80 Ci mmol⁻¹) to adherent intact HEK 293 cells stably expressing B₂R-GFP or B₁R-YFP (24-well plates), respectively, was evaluated as described previously (Houle *et al.*, 2000; Sabourin *et al.*, 2002). The assays were applied to construct binding competition curves for a series of unlabelled antagonists. The same radioligands were used in binding experiments involving adherent, intact MDA-MB-231 cells grown in 24-well plates to monitor

endogenous kinin receptor expression (protocols for each radioligand identical to those applied to HEK 293 cells; saturation curve in the 0.25–7 nM concentration range for [³H]-BK).

Immunoblots

For the analysis of B₂R-GFP in total cell extracts, immunoblots were generally performed as described previously (based on an anti-GFP monoclonal from BD Biosciences Clontech, Palo Alto, CA, USA, Marceau *et al.*, 2002). For the detection of phospho-ERK1/2 and total ERK1/2, cells maintained in reduced serum (0.5% FBS) were stimulated with BK-related and other drugs for definite periods of time; total cell extracts were prepared and submitted to electrophoresis (Marceau *et al.*, 2002). Transferred proteins were revealed using two types of antibodies for each sample: phospho-ERK1/2 (monoclonal, New England Biolabs, Pickering, ON, Canada, dilution 1/1000) and total ERK1/2 (to show comparable loading; polyclonal, New England Biolabs, dilution 1/1000). Staining was revealed using the appropriate peroxidase-conjugated secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Poly(ADP-ribose)polymerase I (PARP, 116 kDa) and cleaved PARP (89 kDa) immunoblots were applied as described by Morissette *et al.* (2004b). Briefly, total cell extracts (attached and detached cells) of MDA-MB-231 cells seeded in 25 cm² flask, maintained in 5% serum for 24 h and treated for another 48 h, were migrated and blotted with anti-PARP antibodies (polyclonal, Cell Signalling Technologies, Inc., Pickering, ON, Canada; 1/1000). Actinomycin D 0.4 μM was used as a positive control treatment that induces PARP cleavage and apoptosis.

Contractility assays

A local ethics committee approved procedures based on rabbits and the specific pathogen-free animals (Charles River) were cared for in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care). Rabbit jugular vein contractile responses to BK (mediated by B₂Rs) or histamine (mediated by H₁ receptors) were measured as generally described by Houle *et al.* (2000). Specifically, cumulative concentration–effect curves were established in each tissue at 1.5 h for BK and 3.5 h for histamine (in order to present all contraction results as a per cent of the maximal histamine-induced effect, a standard independent from BK receptors). In some tissues, B₂R ligands tested as antagonists were applied 30 min before BK. Kinins contract rabbit aortic rings via B₁Rs, and the effect of different concentrations of B-9870 on the cumulative concentration–effect curves of the B₁R agonist des-Arg⁹-BK were determined and analysed as described previously (Morissette *et al.*, 2004a).

Calcium transient imaging

HEK 293 cells (35 mm dishes) were co-transfected with a pcDNA3-based vector coding for the wild-type rabbit B₂R (Bachvarov *et al.*, 1995) and the pHcRed1-N1 vector (Clontech) coding for a red fluorescent protein (used to assess the efficacy of the transient transfection and identify

transfected cells) using the Ex-Gen 500 transfection reagent (MBI Fermentas Inc., Flamborough, Canada) as directed by the manufacturer. Forty-eight hours later, cells were transferred in serum-free α-minimal essential medium (MEM) containing Fluo-3-AM (Molecular Probes, Eugene, OR, USA; 10 μM), bovine serum albumin (BSA) (0.1%), Pluronic F-127 (Sigma-Aldrich, St Louis, MO, USA; 0.01%) and dimethylsulphoxide (DMSO) (0.1%). The cells were incubated for 20 min at room temperature, then thoroughly rinsed with serum-free α-MEM and further incubated in this medium for 1 h at 37°C. Cells were immediately observed at × 400 using a fluorescence microscope (blue excitation, green emission) equipped with a heated chamber (37°C) and containing a controlled atmosphere (humid air, 5% CO₂). B₂R ligands were added and the green fluorescence of Fluo-3 bound to calcium was observed as a function of time using time lapse photography (electronic camera); at the end of the recordings, the calcium ionophore A23187 (5 μM) was added to the cell dishes as a positive control. Fluo-3 imaging has been applied to cells expressing the wild-type receptor because the emission spectrum of this fluorophore overlaps with that of GFP (present in the B₂R-GFP construction).

Calcium mobilization

To quantify the calcium mobilization induced or influenced by several BK receptor ligands, Fura-2 fluorometry (Molecular Probes, Invitrogen Detection Technologies) was applied to the non-transfected HEK 293, stably transfected B₂R-GFP HEK 293 or MDA-MB-231 cells. The cells were detached using trypsin–EDTA, suspended in complete culture medium containing FBS (to neutralize the trypsin), counted, centrifuged for 5 min at 1100 r.p.m. at room temperature and resuspended in Hank's balanced salt solution (HBSS) (1 ×, pH 7.4, prepared from 10 × concentrate, Multicell Wisent, St Bruno, Canada) with 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) and 1.6 mM CaCl₂. At this point, Fura-2-AM was added to cell suspensions (final concentration 1 μM), and they were incubated for 30 min in a 37°C bath with agitation. After the incubation, cells were centrifuged and rinsed twice. Calcium mobilization was read with a thermostated (37°C) spectrofluorimeter (SLM8000C; excitation 340 nm and emission 510 nm) in 2 ml suspension of cells loaded with Fura-2 (2.5 × 10⁶ cells ml⁻¹ for the two HEK 293 cell lines, 5 × 10⁶ for MDA-MB-231) with optional drug pretreatments 15 min before stimulations. After the readings, the maximum mean fluorescence (*F*_{max}) was measured by adding 15 μl of 10% Triton X-100 and the minimum mean fluorescence (*F*_{min}) with further addition of 15 μl of NaOH 1 N and 100 μl of ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) 100 mM. Calcium mobilization concentrations were established with the following equation ($[Ca^{2+}] = 224((\gamma - F_{min}) / (F_{max} - \gamma))$), where γ represents the fluorescence reading from the sample, as described by Burelout *et al.* (2004).

Drugs

BK was purchased from Bachem (Plymouth Meeting, PA, USA). The non-peptide LF 16.0687 (1-[2,4-dichloro-3-[(2,4-

dimethylquinolin-8-yl)oxy)methyl]phenyl)sulphonyl]-N-[3-[[4-(aminoiminomethyl)-phenyl]carbonylamino]propyl]-2(S)-pyrrolidinecarboxamide, mesylate salt) and the peptide Hoe 140 (icatibant), antagonists of BK at the rabbit and human B₂R (Pruneau *et al.*, 1999; Houle *et al.*, 2000), were gifts from Laboratoires Fournier (Daix, France). Compound 11 (2-[(2R)-1-[(3,4-dichlorophenyl)sulphonyl]-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]-N-[2-[4-(4,5-dihydro-1H-imidazol-2-yl)-phenyl]ethyl]acetamide), a powerful non-peptide antagonist at the rabbit and human B₁R (Su *et al.*, 2003; Morissette *et al.*, 2004a), was a gift from Dr DJ Pettibone Merck Research Labs (West Point, PA, USA). The other kinin receptor antagonists B-9870, B-10346 (suberyl-(D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-Digl-Oic-Arg)₂TFA; the N-terminal amide analogue of B-9870) and B-9872 were synthesized using general methods described elsewhere (Gera *et al.*, 1996; Stewart *et al.*, 2002). PD 98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) was purchased from Research Biochemicals International (Natick, MA, USA). The other drugs were purchased from Sigma-Aldrich (St Louis, MO, USA).

Data and statistical analyses

Data points and values are presented as means \pm s.e. of *n* determinations. Where appropriate, experimental groups were compared by use of Student's *t*-test or analysis of variance (ANOVA) followed by Dunnett's test (GraphPad Instat, version 3, San Diego, CA, USA). *P* < 0.05 was considered to be statistically significant.

Results

Effect of B-9870 and related compounds at the recombinant rabbit B₂R

We tested whether a set of five compounds that are nominally B₂R antagonists could displace the specific binding of [³H]-BK (2 nM) from HEK 293 cells stably expressing B₂R-GFP (Figure 1). It can be seen that the non-peptide antagonist LF 16.0687, the peptide antagonist Hoe 140, its dimer B-9872, the anti-tumoral ligand B-9870 and its metabolite B-10346 possess comparable affinities for the rabbit B₂R, with IC₅₀ values in the range 50–75 nM.

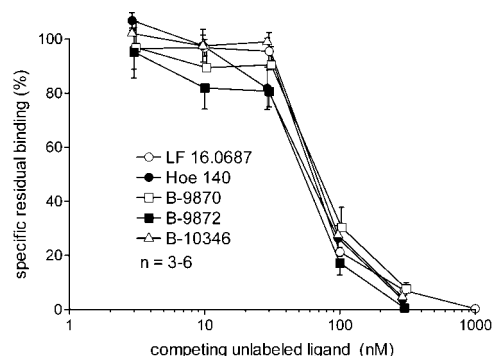


Figure 1 Competitive inhibition of [³H]-BK (2 nM) binding to HEK 293 cells stably expressing B₂R-GFP by a panel of cold drugs. Values are the means \pm s.e.m. (vertical lines) of 3–6 experiments composed of duplicate determinations. Average specific binding without competitor (100%) was 116 ± 20 fmol/well.

A phosphorylation assay of the mitogen-activated protein (MAP) kinases ERK1/2 was applied to HEK 293 cells stably expressing B₂R-GFP in search of agonist activity (Figure 2a). Cells were stimulated with drugs at concentrations known to occupy the receptors. The stimulant effect of the reference agonist, BK, is shown. Other peptides exerted stimulative actions in the following decreasing order of effect amplitude: the proposed biased agonist B-9870 (Figure 2a and c), Hoe-140 (Figure 2c), its dimer B-9872 (data not shown) and B-10346 (10 min treatments, Figure 2b). The non-peptide antagonist LF 16.0687 had no agonist effect, but could antagonize both BK- and B-9870-induced ERK1/2 activation

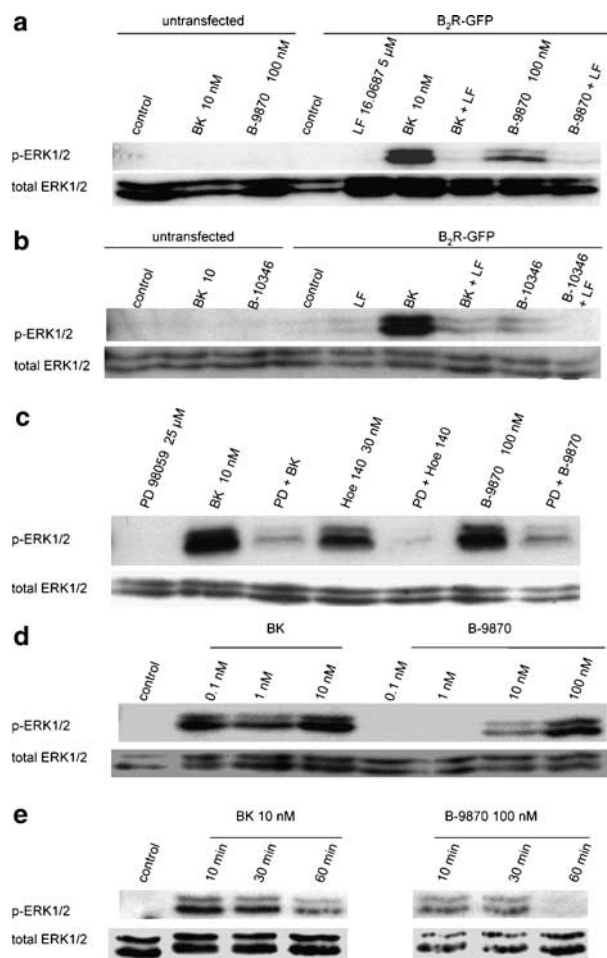


Figure 2 Immunoblots for phospho-ERK1/2 and total ERK1/2 in HEK 293 cells stably expressing B₂R-GFP in response to BK, B-9870, its metabolite B-10346, Hoe-140 or its dimer B-9872 (10 min treatments). (a) B₂R ligand-induced ERK1/2 phosphorylation in HEK 293 cells is receptor-dependent, as shown by the lack of effect of BK (10 nM; 10 min) or B-9870 (100 nM; 10 min) on untransfected cells and by the antagonist effect of LF 16.0687 (5 μ M; 30 min) on the activation of ERK1/2 induced by BK or B-9870 in cells stably expressing B₂R-GFP. Total ERK1/2 is shown to compare loading in tracks. (b) Effect of B-10346, the B-9870 metabolite, on the phosphorylation of ERK1/2. Presentation as in (a). (c) PD 98059 (25 μ M, 30 min), the inhibitor of the upstream MAP kinase MEK-1, prevents the effect of all B₂R-GFP peptide ligands (last 10 min) on ERK1/2 phosphorylation in HEK 293 cells. (d) Concentration-effect relationship for BK and B-9870 (10 min). (e) Time-effect relationship for BK and B-9870 (the control track was common to both stimulants). In all panels, the number of replicates was 2–3.

(Figure 2a) as well as the smaller effects of B-10346 (Figure 2b), Hoe 140 or B-9872 (data not shown). Non-transfected HEK 293 cells were unresponsive to BK, B-9870 or B-10346, further showing that the stimulant effect of these peptides is receptor-mediated (Figure 2a and b). The pharmacological inhibitor of the upstream MAP kinase MEK-1, PD 98059 (Dudley *et al.*, 1995), profoundly inhibited the effect of BK, B-9870, Hoe 140 (Figure 2c) and also B-9872 (data not shown). In the ERK phosphorylation assay, B-9870 exhibited an active threshold concentration of 10 nM (higher than that of BK, Figure 2d), and the effect of B-9870 was generally lower in amplitude and not more durable than that of BK (Figure 2e), indicating that the desensitization of B₂R-mediated MAP kinase signalling applies to both agents.

PLA₂ activation is a documented response to BK in HEK 293 cells stably expressing B₂R-GFP (Houle *et al.*, 2000). As found previously, Hoe 140 (0.1–1000 nM) did not stimulate PLA₂ over baseline in these cells (Figure 3a); it was shown elsewhere that this drug antagonizes BK in this assay (Houle *et al.*, 2000). B-9870 (10–1000 nM) clearly stimulated arachidonate release with a maximal effect inferior to that of BK, whereas the Hoe 140 dimer B-9872 was inactive in this respect. The non-peptide antagonist LF 16.0687 (1 μ M) had no direct effect on arachidonate release by cells, but could antagonize the stimulant effects of BK (1 nM) and B-9870 (30 nM) (Figure 3b). BK and B-9870 failed to release [³H]-arachidonate from untransfected HEK 293 cells (Figure 3c).

As the MEK-1-ERK1/2 signalling pathway activation seems to be somewhat correlated to the ability to stimulate arachidonate release in the series of compounds, we have verified whether the latter effect is dependent on MAP kinase signalling. The MEK-1 inhibitor PD 98059 had a slight, nonsignificant stimulative effect in the PLA₂ assay, but did not prevent arachidonate release by BK or B-9870 (Figure 3b).

In confocal microscopy studies of live HEK 293 cells stably expressing B₂R-GFP, cells were stimulated for 30 min with B₂R ligands (Figure 4). Control cells generally exhibit sharply defined plasma membrane-associated fluorescence. The agonist BK promotes the endocytosis of fluorescent receptors in the form of vesicles of various sizes. The biased agonist B-9870 produces fluorescent receptor internalization in the form of very fine particles with a slow kinetic, in a manner similar to B-9872 (see Figure 4) or Hoe 140 (Figure 4a, Houle *et al.*, 2000). None of these ligands promoted B₂R-GFP degradation, as judged with immunoblot of total cell extracts based on anti-GFP antibodies (data not shown; positive control: extracellular trypsin produces GFP-sized fragments of the fusion protein in 10 min; Houle *et al.*, 2003). Cell treatment with PD 98059 did not prevent peptide ligand-induced translocation (data not shown). LF 16.0687 does not promote B₂R-GFP endocytosis (Houle *et al.*, 2000). The non-peptide antagonist LF 16.0687 had no direct effect on the cellular fluorescence distribution, but decreased the specific dislocation pattern of the membrane fluorescence induced by BK or B-9870 (Figure 4b).

HEK 293 cells expressing the wild-type rabbit B₂R responded by a transient increase of calcium concentration in response to BK (10 nM, Figure 5, Fluo-3 fluorescence); this applied to some cells only, consistent with the fractional effect of transient transfections. The response, detected by the fluorescence of Fluo-3 bound to calcium, was maximal in

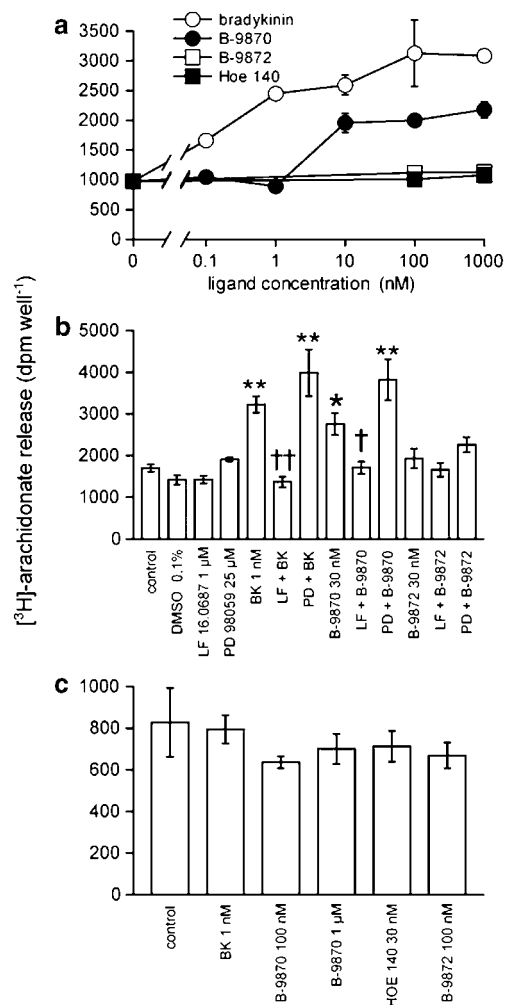


Figure 3 PLA₂ assay applied to HEK 293 cells stably expressing B₂R-GFP (a, b) or untransfected cells (c). (a) Concentration-effect relationship for PLA₂ activation by some peptide B₂R ligands. Values are the means \pm s.e.m. (vertical lines) of 4–5 determinations. (b) Effect of the MEK-1 inhibitor PD 98059 and of the B₂R antagonist LF 16.0687 on arachidonate release induced by peptide antagonists/biased agonists. The effect of the DMSO vehicle for PD 98059 is also shown. Values are means \pm s.e.m. (vertical lines) of 5–6 determinations. Inhibitory drugs were applied 30 min before stimuli. Analysis of variance (ANOVA) indicated that the groups were heterogeneous ($P < 0.001$). Dunnett's test for comparison with the control group: * $P < 0.05$; ** $P < 0.01$. Comparison of the inhibitor-treated groups with the appropriate stimulant-treated group, t -test: $^{\dagger}P < 0.01$; $^{\dagger\dagger}P < 0.01$. (c) Lack of effect of BK, B-9870 or other ligands on untransfected HEK 293 cells ($n = 4$).

30 s and decreased afterwards. B-9870 (30 or 100 nM) was consistently observed to induce a small response in some cells and this response was delayed in some cases (Figure 5). This response appeared to be receptor-dependent, as it was not recorded in cells that did not exhibit the red fluorescence caused by co-transfection of the B₂R with the HcRed1-coding vector (data not shown; each microscopic field contains numerous untransfected cells that can be taken as controls).

A more quantitative evaluation of calcium transients was based on Fura-2 in HEK 293 cells stably expressing B₂R-GFP (Figure 6). This approach confirmed that BK (10 or 100 nM) induced a fast Ca²⁺ mobilization followed by decreasing signal (Figure 6a). B-9870 (30 or 100 nM) produced smaller

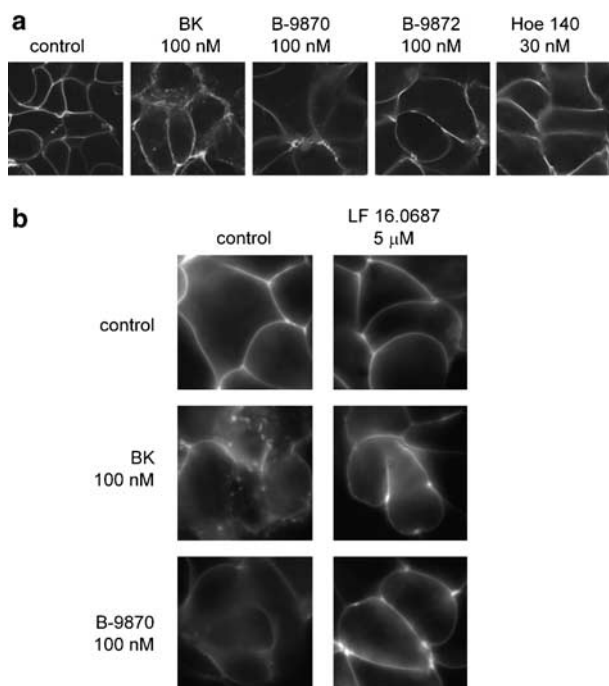


Figure 4 (a) Confocal microscopy studies of live HEK 293 cells stably expressing B₂R-GFP and stimulated for 30 min with B₂R ligands at the indicated concentrations. Control cells generally exhibited sharply defined plasma membrane-associated fluorescence. All photomicrographs represent square fields with 40 μm sides. Typical results of at least two independent days of observation in multiple microscopic fields. (b) LF 16.087 pretreatment (10 min, 5 μM) decreased the dislocation of the membrane fluorescence induced by BK or B-9870 (30 min treatment). Epifluorescence imaging, original magnification × 1000.

and protracted responses with no clear dependence on the concentration (Figure 6a and b). LF 16.0687, which had no effect on calcium mobilization, prevented the effects of BK (10 nM) or B-9870 (100 nM) (Figure 6b). Non-transfected HEK 293 cells failed to respond to B-9870 and exhibited a small response to BK (100 nM only) that was inhibited by either B-9870 or LF 16.0687.

Effect of constrained peptides on naturally expressed rabbit B₂R

Both B-9870 and the Hoe 140 dimer, B-9872, behaved as insurmountable antagonists of BK in the rabbit jugular vein contractility assay (a tissue that expresses the wild type B₂R) (Figure 7). The peptide analogues had no agonist contractile effects. This behaviour is similar to that of Hoe 140 in the same preparation (Houle *et al.*, 2000).

Effect of B-9870 at the rabbit B₁R

Reminiscent of the effect of the monomer B-9430 (Stewart *et al.*, 1996), the dimer B-9870 also is a surmountable B₁R antagonist in the aortic contractility assay (Figure 8a). It antagonized, with a pA₂ value of 7.40 (Schild plot; Figure 8b, regression slope −0.88), the rabbit aorta contraction induced by the B₁R agonist des-Arg⁹-BK. B-9870, although at a higher concentration than compound 11, displaced Lys-des-Arg⁹-BK binding to HEK 293 cells stably expressing B₁R-YFP (Figure 8c). Finally, in contrast with the B₂R-GFP system, only the specific agonist Lys-des-Arg⁹-BK (10 nM) activated the phosphorylation of ERK1/2 in HEK 293 stably expressing B₁R-YFP (Figure 8d). This phosphorylation was receptor-dependent as the highly selective non-peptide B₁R antagonist compound 11 could prevent it; no activation was

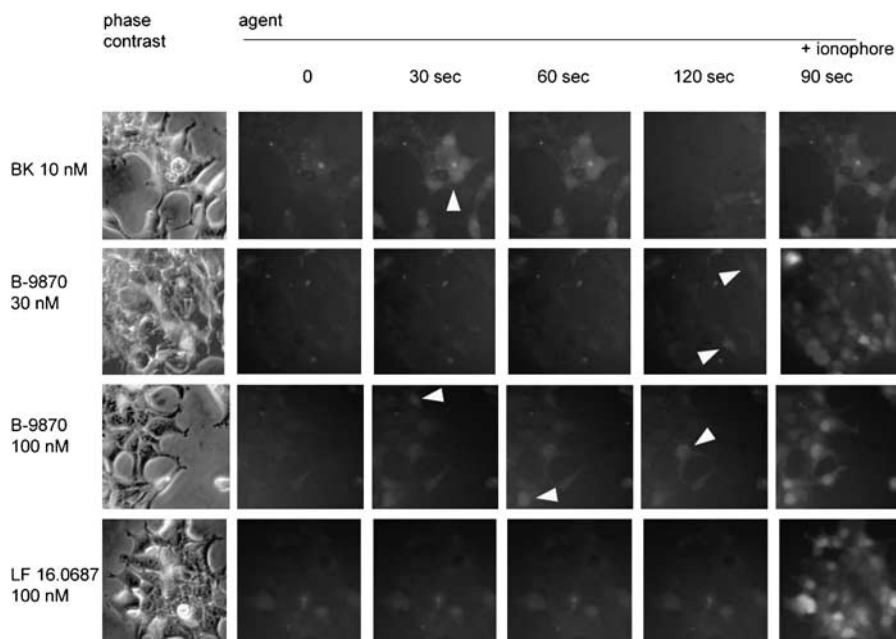


Figure 5 Imaging of Fluo-3 fluorescence in HEK 293 cells transiently expressing rabbit B₂R_s and stimulated with the indicated drugs. At the end of each sequence of time lapse photography, the cells were stimulated with the calcium ionophore (5 μM) in order to provide a positive control. Arrowheads indicate some of the responsive cells. Cells were not responsive to saline, the peptide vehicle (data not shown). Results are representative of several replicates performed over three separate days.

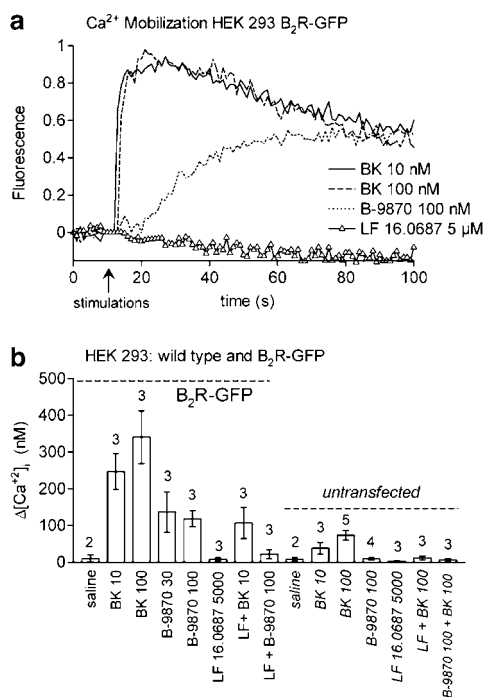


Figure 6 Calcium mobilization in untransfected HEK 293 cells or in cells stably expressing B_2R -GFP at high density. (a) Representative tracings of the Fura-2 fluorescence to show the time course of effects elicited by acute application of B_2R ligands (conventionally at time 10 s). (b) Peak intracellular calcium measurements observed in cells as a function of drug treatment or co-treatments. Drug concentrations are indicated in nM, and that of LF 16.0687 was always 5 μM (5000 nM). Values are means \pm s.e.m. (vertical lines). The number of replicates is indicated close to each column.

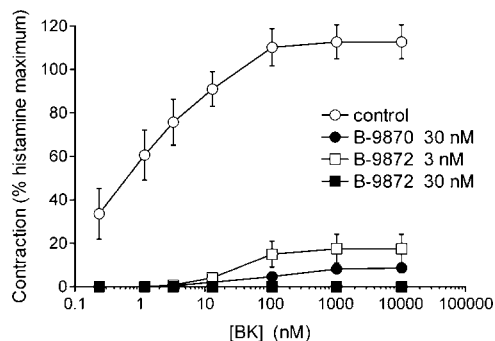


Figure 7 Effect of B-9870 and B-9872 on cumulative concentration-effect curve for BK in the rabbit jugular vein contractility assay. The peptide analogues had no direct contractile effects. Replicate numbers: control (4), B-9870 (5), B-9872 3 nM (5), B-9872 30 nM (2). Vertical lines show mean \pm s.e.m.

observed in the untransfected cells in response to any of the B_1R ligands. Thus, B-9870 possesses a fair affinity for the B_1R without a stimulative effect on ERK1/2 phosphorylation or on aortic contractility, suggesting that B-9870 behaved as a competitive antagonist on this receptor.

Effect of B-9870 on cell proliferation

After 4 days of culture, cell wells seeded with 5×10^4 HEK 293 cells stably expressing B_2R -GFP contained $3.79 \pm 0.51 \times 10^5$

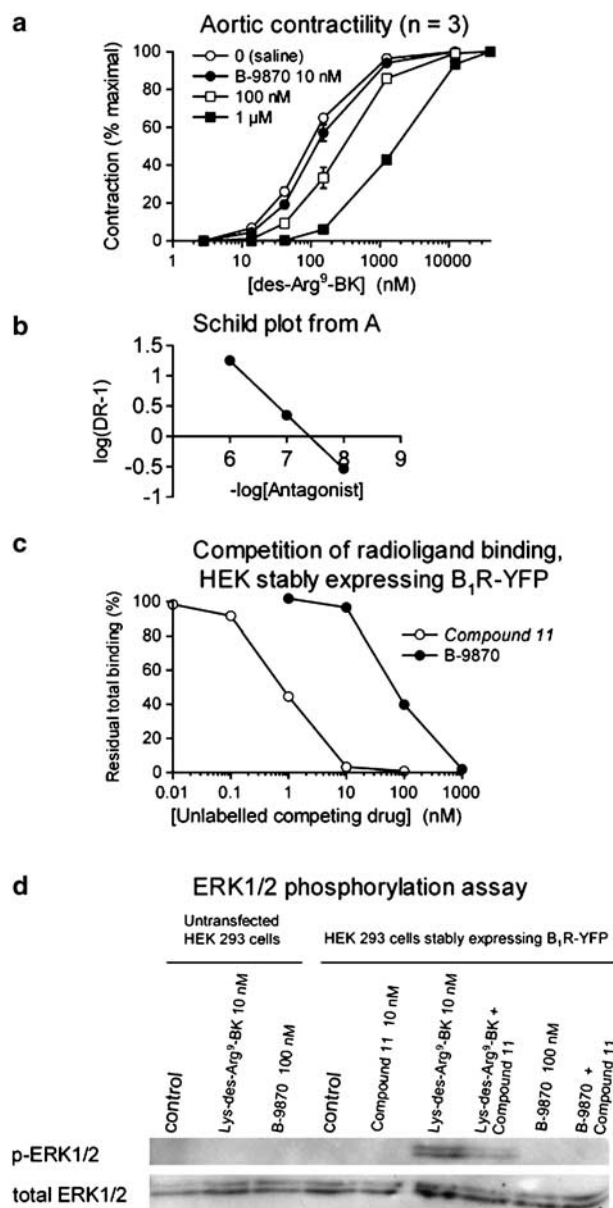


Figure 8 (a) Effect of B-9870 on rabbit isolated aorta contractions induced by the B_1R agonist des-Arg⁹-BK (values are means \pm s.e.m. of triplicate determinations). (b) Schild plot analysis based on the averaged data in (a). (c) Displacement of [³H]-Lys-des-Arg⁹-BK (1 nM) binding to HEK 293 cells stably expressing B_1R -YFP by B-9870 or compound 11 (a highly selective non-peptide B_1R antagonist). Values are the means of duplicate determinations. The average specific binding without competitor (100%) was 139 fmol/well. (d) Immunoblots for total or phosphorylated ERK1/2 in untransfected HEK 293 and HEK 293 cells stably expressing B_1R -YFP and maintained for 24 h in reduced serum (0.5% FBS). Compound 11 applied 30 min before extraction, and the agonist 10 min.

cells (controls, $n = 4$). Culture in the presence of LF 16.0687 (1 μM) or B-9870 (100 nM) did not significantly change the extent of proliferation, although a nonsignificant stimulative trend was observed for the latter drug (final cell counts of $5.05 \pm 0.50 \times 10^5$ and $5.36 \pm 0.78 \times 10^5$ cells, respectively; $n = 4$). Higher concentrations of B-9870 (1 or 5 μM) had no consistent effect on the proliferation of HEK 293 cells

expressing B₂R-GFP; all these observations also applied to untransfected HEK 293 cells (data not shown).

MDA-MB-231 is one of the five breast cancer cell lines found by Chan *et al.* (2002a) to respond to B-9870 (CU201) by growth inhibition. In our model, a rather modest 30.6% inhibition of MDA-MB-231 proliferation was observed after 48 h of B-9870 treatment (5 μ M, Figure 9). No significant inhibition was found at 1 μ M. The N-terminal amide analogue and first pass metabolite of B-9870, B-10346 was less potent as it exerted no statistically significant effect on cell proliferation (Figure 9a). B-9870 has been proposed to exert its antiproliferative effects via a B₂R biased agonist mechanism. Thus, we used the non-peptide antagonist LF

16.0687 (1 μ M) to verify whether peptide ligands stimulate endogenous B₂R to mediate proliferation inhibition. LF 16.0687 has a \sim 10-fold greater affinity for the human B₂R, relative to the rabbit orthologue (Pruneau *et al.*, 1999; Houle *et al.*, 2000). The inhibitory effect of B-9870 was not reversed by cell co-treatment with LF 16.0687 (Figure 9a). The highly selective non-peptide B₁R antagonist, compound 11 (1 μ M, Figure 9a), or this drug combined with LF 16.0687 (data not shown), also failed to reverse the inhibitory effect of B-9870 on proliferation. The non-peptide antagonists, alone (Figure 9a) or combined (not shown), failed to influence carcinoma cell proliferation. To further ascertain that the competitive antagonist LF 16.0687 was used optimally in the proliferation experiments against a high concentration of B-9870, experiments were repeated with a different ratio of the drugs (10 and 5 μ M, respectively; Figure 9b). The results again failed to show an inhibition of the antiproliferative effect of the peptide by the non-peptide agent.

Pre-apoptotic signalling has been shown to be activated by B-9870 in at least some responsive tumour-derived cell lines (Chan *et al.*, 2002a). PARP is cleaved during apoptotic events. Actinomycin D (0.4 μ M, 48 h treatment) strongly inhibited MDA-MB-231 proliferation (Figure 9a), and induced the typical apoptotic PARP cleavage (89 kDa; Figure 9c). However, no PARP cleavage was observed upon B-9870 or B-10346 treatment (48 h; Figure 9c).

The MDA-MB-231 cell line failed to exhibit specific binding sites for [³H]-Lys-des-Arg⁹-BK (1 nM, Figure 10a), but expressed some specific sites for [³H]-BK (Figure 10b). The specific binding was smaller than the nonspecific binding and low in absolute abundance in this system; applying a single site model was not attempted owing to the uncertainty of the specific binding at low ligand concentrations. Nevertheless, MDA-MB-231 cells exhibit a small calcium mobilization in response to BK (100 nM, Figure 11). This response is not mimicked by B-9870 (100 nM or 1 μ M), but is prevented by pretreatment with this peptide or with LF 16.0687 (Figure 11b).

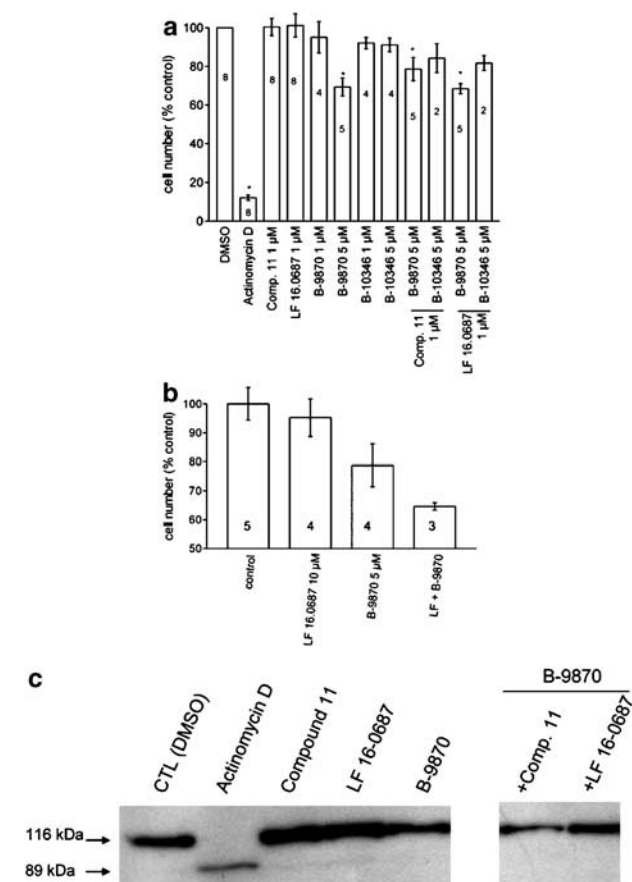


Figure 9 (a) Effects of bradykinin receptor ligands on the proliferation of breast carcinoma MDA-MB-231 cells. Petri dishes were seeded with 5×10^4 cells at time 0; at time 24 h the media were changed and drug treatments were introduced for an additional 48 h period; cells were trypsinized and counted at time 72 h. Values are the means \pm s.e.m. (vertical lines) of the indicated number of replicates indicated in each column and are expressed as the percentage of cells relative to controls established for each day of the experiments (100% in absolute cell counts = $156\,000 \pm 12\,000$). ANOVA $P < 0.001$; Dunnett's test relative to controls: $*P < 0.01$. Adding the non-peptide antagonists LF 16.0687 or compound 11 to the peptide ligands B-9870 or B-10346 did not produce effects that were significantly different from those of the peptide ligands alone. (b) Effect of a different ratio of LF 16.0687 (10 μ M) and B-9870 (5 μ M) on the proliferation of MDA-MB-231 cells. (c) Immunoblot of PARP and cleaved PARP detected in the MDA-MB-231 extracts. Cells were seeded, stimulated and harvested (attached and detached cells) at time 0, 24 and 72 h, respectively. Drug concentrations as in (a). Representative results of two experiments.

Discussion

In the present experiments, the peptide B-9870 has been observed to behave either as a potent and insurmountable BK antagonist in the rabbit jugular vein contractility assay and in the calcium mobilization assay in MDA-MB-231 cells or as a partial agonist in other assays (ERK1/2 phosphorylation, PLA₂ activity, calcium transients and receptor endocytosis in HEK 293 cells expressing high densities of recombinant rabbit B₂R). These results extend the number of tested end points relative to a previous study (Chan *et al.*, 2002a). The stimulative effects of B-9870 were strictly receptor-dependent, as shown by the lack of drug effect on untransfected cells or the antagonism exerted on the peptide drug effect on MAP kinase and calcium or PLA₂ signalling by the non-peptide drug LF 16.0687. The lack of agonist effect in jugular vein contractility studies (Figure 7) and in calcium assays in MDA-MB-231 cells (a system with low receptor density) seems, at first sight, to conflict with the results obtained in HEK 293 cells expressing recombinant rabbit

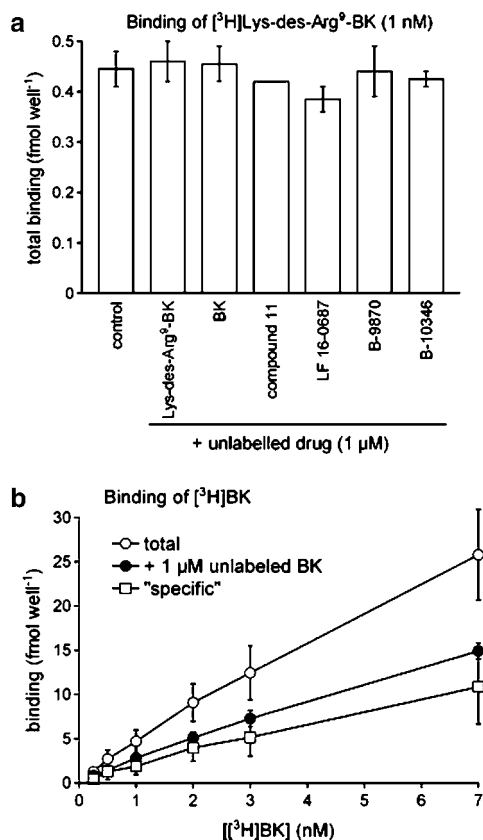


Figure 10 Surface expression of receptors for kinins in MDA-MB-231 cells. (a) Total binding of a saturating concentration (1 nM) of [³H]-Lys-des-Arg⁹-BK to the tumour-derived cells. The unchanged amount of bound ligand in the presence of a large excess of agonist or antagonist drugs is consistent with the absence of B₁Rs. (b) Total, nonspecific and specific binding of [³H]BK to cells. In both (a) and (b), values are means \pm s.e.m. (vertical lines) of two experiments composed of duplicate determinations.

receptors, where B-9870 exerts agonist-like effects, but consistent with the previously reported antagonist effect of B-9870 on BK-induced calcium signalling in a tumour-derived cell line (Chan *et al.*, 2002a). However, ligands with low efficacy are more likely to behave as pure antagonists if a pharmacological effect requires the stimulation of a very large proportion of the receptors on each cell (i.e., if there are no spare receptors; Leslie, 1987; Kenakin, 1987; Mathieu *et al.*, 2005). As the recombinant receptors (either the wild-type or the GFP conjugate) are expressed at a high density (Bachvarov *et al.*, 1995, 2001), these systems are more likely to reveal the agonist-like actions of a low efficacy partial agonist. Untransfected HEK 293 cells unexpectedly exhibited a small calcium response to BK, but no response in the ERK1/2 phosphorylation or PLA₂ assays. B-9870 behaved as an antagonist of the small calcium response, consistent with a small population of B₂Rs in untransfected HEK 293 cells. The biased agonist activity of B-9870 for a subset of signalling pathways activated by BK, as previously defined (see Introduction), is not necessary to account for the present data.

Although widely exploited as a B₂ receptor antagonist, Hoe 140 may be a stimulator at the rabbit B₂R (see Introduction).

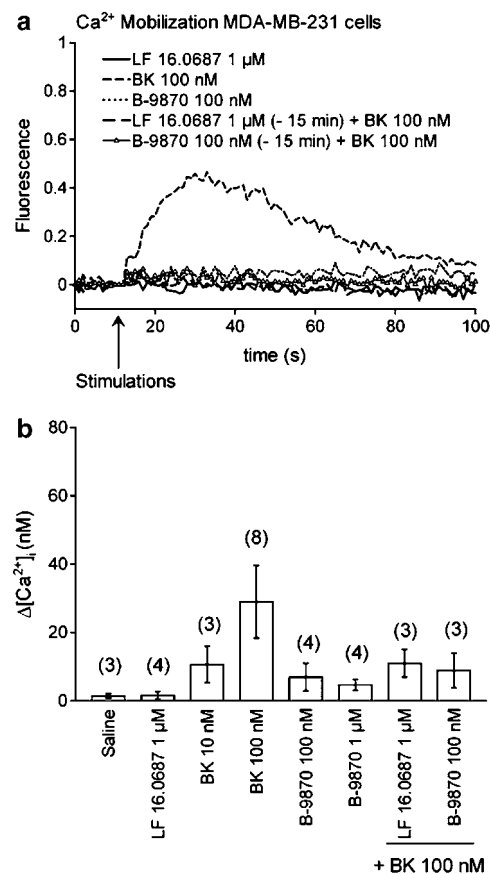


Figure 11 Calcium mobilization in MDA-MB-231 cells exposed to B₂R agonists or antagonists. (a) Representative tracings of the Fura-2 fluorescence to show the time course of effects elicited by acute application of B₂R ligands (conventionally at time 10 s). (b) Peak intracellular calcium measurements observed in cells as a function of drug treatment or co-treatments. Values are means \pm s.e.m. (vertical lines). The number of replicates is indicated close to each column.

This type of effect may be analogous to the partial agonist actions of B-9870, but with an even lower efficacy (limited to a minor activation of endocytosis and ERK1/2 kinase activation). The same contention applies to the Hoe 140 dimer, B-9872 and to the first pass metabolite and amide analogue of B-9870, B-10346 (ERK1/2 phosphorylation Figure 2b). Hoe 140 has a large stimulative effect at the sheep B₂R (Félétou *et al.*, 1994) and is a full agonist at the ornithokinine receptor, a bird homologue of the mammalian B₂R (Schroeder *et al.*, 1997). The efficacy in this series is not related to affinity as these three synthetic peptides and B-10346, as well as the pure antagonist LF 16.0687, exhibit comparable affinities at B₂R-GFP (Figure 1). Thus, a specific structure-activity relationship must exist for the partial agonist activity among the structurally constrained peptide B₂R antagonists. Another property of the constrained peptide antagonists is the high resistance to metabolic breakdown; by comparison, BK is destroyed rapidly following endocytosis of the receptor-agonist complex (Munoz and Leeb-Lundberg, 1992) and also in serum-containing culture media (Bachvarov *et al.*, 2001). Thus, even a low efficacy agonist activity may be amplified over time, as observed with the slow but very persistent endocytosis of

B₂R-GFP induced by Hoe 140 (Houle *et al.*, 2000). Resistance to catabolism may explain why B-9870 was more persistent than BK as a function of time to mobilize Ca²⁺ (Figure 5 and 6a).

B-9870 (CU201) has been shown to exert antitumour cell line proliferation activity and to have a significant anti-neoplastic action when used alone or in combination with other drugs in animal models (Stewart *et al.*, 2005). The effect of B-9870 on tumour-derived cell lines (including MDA-MB-231 Figure 9a; Chan *et al.*, 2002a) has not been observed on HEK 293 cells, whether they expressed B₂R-GFP or not (proliferation assay over 4 days). In addition, B-9870-induced proliferation inhibition observed in MDA-MB-231 cells was not blocked by the non-peptide B₂R antagonist LF 16.0687 (Figure 9a and b). The latter non-peptide drug has no direct effect on carcinoma cell growth *in vitro*. Although the stimulation of kinin B₁R has also been implicated in proliferation, migration and invasion of cancer cells (Taub *et al.*, 2003), and despite the fact that B-9870 possesses a fair affinity for this receptor as an antagonist (rabbit aortic contractility, radioligand binding competition and ERK1/2 phosphorylation in HEK 293 stably expressing B₁R-YFP; Figure 8), its inhibitory action on growth was not mediated by B₁Rs, as evidenced by the lack of effect of compound 11 co-treatment (Figure 9a) and the absence of specific binding for [³H]-Lys-des-Arg⁹-BK (Figure 10a). Although MDA-MB-231 cells possess a small functional B₂R population, the concentrations of B-9870 needed to inhibit proliferation in this or other studies ($\geq 5 \mu\text{M}$) were much higher than those that efficiently inhibited either BK receptor subtype in the present pharmacological study. Cell type-dependent, kinin receptor-independent pathway(s) must exist for the anti-proliferative effect of B-9870, which does not apply to HEK 293 cells and may vary from one tumour cell type to another. In contrast with the lung cancer line used in most signalling experiments described by Chan *et al.* (2002a), inhibition of MDA-MB-231 cell growth was not apoptotic (lack of PARP cleavage, Figure 9c; small sub-G₁ peaks unaltered by B-9870 in ploidy experiments, data not shown) and did not involve JNK activation (lack of phospho-c-jun in cells treated with either B-9870 or BK, data not shown).

Arginine-rich peptides possessing six or more Arg or D-Arg residues are effective at infusing into the cytosol of several cell types (Tung and Weissleder, 2003). The dimer B-9870 has six of these residues (four Arg and two D-Arg). Two such Arg-rich sequences of physiological significance are found in the TAT domain from the transcription activating factor of HIV and 'penetratin' sequence from the *Drosophila* homeo-domain protein *Antennapedia* (Melikov and Chernomordik, 2005). Arg-rich peptide conjugates with a 'cargo', for example, a cytotoxic drug, have been designed as cell permeant agents with a cytosolic site of action. It is not clear whether B-9870 has structural features that explain its cell type-specific effect on proliferation and the induction of apoptosis observed in specific cell types (Chan *et al.*, 2002a). The linker region of the dimer may be critical for an effect of B-9870 on the cell cycle, because its metabolite that differs only in this region, B-10346, is not active at depressing MDA-MB-231 cell proliferation (Figure 9a). Thus, B-9870 may not be a pro-drug of a more active cytotoxic agent.

B-9870 is a dual rabbit B₁R and B₂R antagonist with confirmed significant stimulative effects at the latter receptor subtype only in systems where B₂Rs are overexpressed; the compound may be a partial agonist of the B₂R with low efficacy and be highly resistant to metabolic breakdown. In any event, the antiproliferative effect on the carcinoma cell line studied occurred at a relatively high concentration range and was not dependent on kinin receptors and apoptosis. The contention that cell type-specific, non-receptor-mediated effects are mediated by the direct entry of this peptide, rich in Arg or D-Arg residues, into specific cells remains an intriguing possibility.

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Conflict of interest

The authors state no conflict of interest.

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