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RESEARCH PAPER

Effects of inactivation-resistant agonists on the signalling, desensitization and down-regulation of bradykinin B_2 receptors

Marie-Thérèse Bawolak[1]*, Sébastien Fortin[2]*, Johanne Bouthillier[1], Albert Adam[3], Lajos Gera[4], René C.-Gaudreault[2] and François Marceau[1]

[1]*Centre de recherche en rhumatologie et immunologie, Centre Hospitalier Universitaire de Québec and Department of Medicine, Université Laval, Québec, QC, Canada,* [2]*Biotechnology and Bioengineering Unit, Hôpital St-François d'Assise, Centre Hospitalier Universitaire de Québec, Québec, QC, Canada,* [3]*Faculty of Pharmacy, Université de Montréal, Montréal, QC, Canada, and* [4]*Department of Biochemistry, University of Colorado Denver, Aurora, CO, USA*

Background and purpose: A peptide bradykinin (BK) B_2 receptor agonist partially resistant to degradation, B-9972, down-regulates this receptor subtype. We have used another recently described non-peptide agonist, compound 47a, as a tool to study further the effects of metabolically more stable and thus persistent, agonists of the BK B_2 receptor on signalling, desensitization and down-regulation of this receptor.
Experimental approach and key results: Compound 47a was a partial agonist at the B_2 receptor in the human umbilical vein, where it shared with B-9972 a very slow relaxation on washout, and in HEK 293 cell lines expressing tagged forms [myc, green fluorescent protein (GFP)] of the rabbit B_2 receptor. Compound 47a desensitized the umbilical vein to BK. In the cellular systems, the inactivation-resistant agonists induced $[Ca^{2+}]_i$ transients as brief as those of BK but affected other functions with a longer duration than BK [12 h; receptor endocytosis, endosomal β-arrestin$_{1/2}$ translocation, protein kinase C-dependent extracellular signal-regulated kinases (ERK)1/2 phosphorylation and c-Fos expression]. The B_2 receptor–GFP was degraded in cells exposed to B-9972 or compound 47a for 12 h. The non-peptide B_2 receptor antagonist LF 16-0687 prevented all effects of compound 47a, which were also absent in cells lacking recombinant B_2 receptors.
Conclusion and implications: Inactivation-resistant agonists revealed a long-lasting assembly of the agonist–B_2 receptor–β-arrestin complexes in endosomal structures and induce 'biased signalling' (in terms of activation of ERK and c-Fos) as a function of time. Further, B-9972 and compound 47a, unlike BK, efficiently down-regulated BK B_2 receptors.
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Keywords: bradykinin; B_2 receptor; compound 47a; B-9972; LF 16-0687; β-arrestin; receptor endocytosis; receptor down-regulation; ERK1/2; c-Fos

Abbreviations: BK, bradykinin; cherryFP, cherry fluorescent protein; ERK1/2, extracellular signal-regulated kinases 1/2; GFP, green fluorescent protein

Introduction

Agonist stimulation of bradykinin (BK) B_2 receptors promotes the internalization of the hormone–receptor complex and functional desensitization, with Ser/Thr phosphorylation and dephosphorylation events that precede the internalization and recycling to the surface of the B_2 receptor respectively (Blaukat *et al.*, 1996; 1999; Faussner *et al.*, 1998; Pizard *et al.*, 1999). The internalized BK broken down into oligopeptides is documented (Munoz and Leeb-Lundberg, 1992). We modelled the agonist-induced endocytosis/re-expression cycle using a fluorescent rabbit B_2 receptor-green fluorescent protein conjugate (B_2 receptor–GFP) in a series of recent papers (Houle *et al.*, 2000; 2003; Bachvarov *et al.*, 2001; Marceau *et al.*, 2002; Houle and Marceau, 2003; Bawolak *et al.*, 2007; Morissette *et al.*, 2007) and showed that the receptor's recycling at the cell surface is essentially complete unless special procedures are applied: rapid heterologous down-regulation by extracellular proteases or slow homologous down-regulation by using a peptidase-resistant BK analogue, B-9972. The latter peptide, largely resistant to inactivation in

Correspondence: François Marceau, Centre Hospitalier Universitaire de Québec, Centre de Recherche en Rhumatologie et Immunologie, CHUQ, Pavillon CHUL, T1-49, 2705 Laurier Blvd., Québec (Québec), Canada G1V 4G2. E-mail: francois.marceau@crchul.ulaval.ca
*These authors contributed equally to this work.
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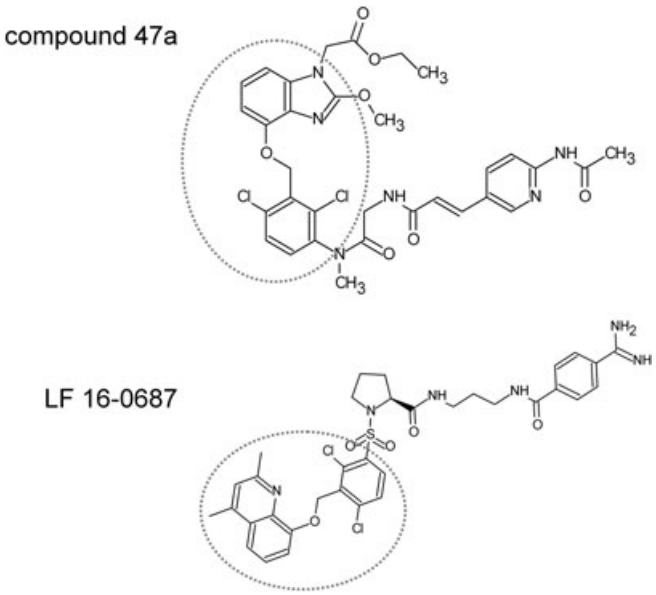

Figure 1 Structures of compound 47a, an agonist of the bradykinin B_2 receptor, and of LF 16-0687, a competitive antagonist of this receptor. Circled areas show some structural similarities between 47a and LF 16-0687.

the presence of receptor-expressing cells, also down-regulated a different myc-tagged rabbit B_2 receptor construct and desensitized cells for prolonged periods to functional responses elicited by BK (Bawolak *et al.*, 2007). β-Arrestin$_2$ colocalizes with the presumably phosphorylated B_2 receptor in endosomes in cells stimulated with BK for 15 min, and dissociation of the two molecules is necessary for the re-expression of the receptors at the cell surface (Simaan *et al.*, 2005).

The BK B_2 receptor has been the subject of recent medicinal chemistry efforts to develop non-peptide antagonists (Fortin and Marceau, 2006; Alexander *et al.*, 2008). A good example of a non-peptide B_2 receptor antagonist, optimized for use at the human form of the receptor is LF 16-0687 (anatibant) (Figure 1) (Pruneau *et al.*, 1999). Non-peptide partial agonists analogous to the novel B_2 receptor antagonists are also known (Aramori *et al.*, 1997). FR190997 is an early example of a high-affinity non-peptide B_2 receptor stimulant that has been studied in several laboratories and showed prolonged inflammatory and hypotensive effects (Ueno *et al.*, 1999; Majima *et al.*, 2000; Hayashi *et al.*, 2001) as well as a propensity for prolonged desensitization of the B_2 receptor in some systems (Cuthbert, 1999; Gobeil *et al.*, 1999). Further work to improve the intrinsic activity of non-peptide agonists led to compound 47a (Figure 1; Sawada *et al.*, 2004b), reportedly a full agonist at the human recombinant BK B_2 receptor. This compound has evident structural similarities to the antagonist LF 16-0687, notably in the 'scaffold 2' (nomenclature as in Fortin and Marceau, 2006) where the structural transition from an antagonist to an agonist resides (Figure 1, circled structures).

We have used the compound 47a along with B-9972 as potentially inactivation-resistant agonists to study further the cycling of BK B_2 receptors. The kinetics of arrestin association with the receptor, receptor down-regulation and signalling were particularly addressed.

Methods

Contractility assay

A local ethics committee approved the anonymous use of human umbilical cord segments obtained after elective Caesarean section deliveries. The isolated human umbilical vein provides an established contractile bioassay mediated by BK B_2 receptors, as described by Bawolak *et al.* (2007). The agonist effect of compound 47a on the human vein was studied for potency, antagonism by LF 16-0687 treatment and desensitizing effect on BK-induced contractions, as further described in *Results*.

Vectors and cell transfection, microscopy

The derivation of a HEK 293 cell line stably expressing B_2 receptor–GFP and its properties are described elsewhere (Houle *et al.*, 2000; Bachvarov *et al.*, 2001). In this construct, the coding sequence of enhanced GFP was fused at the C-terminus of the rabbit B_2 receptor. Non-transfected HEK 293 cells were used in control experiments. Another line of similar cells, HEK 293a, was obtained from Sigma-Aldrich (Mississauga, ON, Canada) and used for experiments dealing with transient vector expression. Vectors coding for various proteins were transiently transfected or co-transfected in HEK 293a cells: myc-tagged rabbit B_2 receptor (myc–B_2 receptor, described by Bawolak *et al.*, 2007; myc tag located at the extracellular N-terminus of the receptor sequence), β-arrestin$_2$–GFP fusion protein in pcDNA3 (gift of Dr Michel Bouvier, Université de Montréal; Bernier *et al.*, 2007) and β-arrestin$_1$–cherry fluorescent protein (cherryFP) (vector given by Dr J.-M. Beaulieu, Université Laval, Quebec City, Canada). HEK 293a cells, grown until 70% confluent, were transiently (24–48 h) transfected or co-transfected with the above-mentioned vectors with the use of the EX-Gen 500 transfection reagent (MBI Fermentas, Flamborough, Canada) as directed. The cells were further treated with B_2 receptor ligands and observed in epifluorescence microscopy. Some intact cells were counterstained with the nuclear dye Hoechst 33258 (final concentration 25 μM in culture medium, last 15 min of incubation at 37°C, Morissette *et al.*, 2008).

Calcium mobilization

To quantify the calcium mobilization induced or influenced by several BK receptor ligands, FURA-2 fluorometry (Molecular Probes, Invitrogen Detection Technologies, Carlsbad, CA, USA) was applied to the HEK 293 or to cells stably expressing B_2 receptor–GFP. FURA-2 fluorometry (Molecular Probes, Invitrogen Detection Technologies) was applied to HEK 293 cells maintained in serum-containing medium, detached using trypsin-EDTA, suspended in complete culture medium containing foetal bovine serum (to neutralize trypsin), counted, centrifuged 5 min at 600× *g* at room temperature and resuspended in Hank's balanced salt solution (1×, pH 7.4, prepared from 10× concentrate, Multicell Wisent, St Bruno, Canada) with 10 mM HEPES and 1.6 mM $CaCl_2$. At this point, FURA-2-AM was added to cell suspensions (final concentration 1 μM) and incubated 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 (FluoroLog-3; HORIBA Jobin-Yvon, Edison, NJ, USA; excitation 340 nm and emission 510 nm) in 2 mL suspension of cells loaded with FURA-2 (2.5×10^6 cells mL^{-1}). After the readings, the maximum mean fluorescence (F_{max}) was measured by adding ionomycin (5 μM) and the minimum mean fluorescence (F_{min}) by adding $MnCl_2$ (25 mM). Calcium mobilization concentrations were established with the following equation ($[Ca^{+2}] = 224((y - F_{min})/(F_{max} - y))$, where y represents the fluorescence reading from the sample, as described by Burelout *et al.* (2004).

[^{3}H]BK binding assay

HEK 293 cells stably expressing the B_2 receptor–GFP were used to determine the affinity of compound 47a using the competition of the binding of 3 nM [^{3}H]BK precisely as described earlier (Bawolak *et al.*, 2007). LF 16-0687 and BK were used in each assay for comparison. Binding data were fitted by non-linear regression to a one-site competition equation to derive IC_{50} values (Prism 4.0, GraphPad Software, San Diego, CA, USA).

Immunoblots

The agonist action of compound 47a on the extracellular signal-regulated kinase 1/2 (ERK1/2) MAP kinase phosphorylation assay was tested as described in HEK 293 cells stably expressing B_2 receptor–GFP or in HEK 293a cells expressing myc–B_2 receptors (Bawolak *et al.*, 2007). Induction of the expression of the transcription factor c-Fos is a response controlled by ERK1/2 activity in many cellular systems (Glauser and Schlegel, 2007) and this sequence of events can be produced by the activated BK B_2 receptor in some cells (Vidal *et al.*, 2005). Total HEK 293 cell extracts were immunoblotted to detect c-Fos expression using the K-25 rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:100; general methods as in Bachvarov *et al.* (2001). Immunoblots for B_2 receptor–GFP were performed as previously described (Bawolak *et al.*, 2007) using the monoclonal anti-GFP antibodies JL8 (Clontech, Palo Alto, CA, USA). This system can be exploited to demonstrate agonist-induced receptor degradation (see *Results*).

Data analysis

The EC_{50} values were obtained from averaged concentration–effect curves by interpolation from two points on each side of the half-maximal effect on a semi-logarithmic scale. The Schild plot parameters (pA_2, slope) and their respective SEM were then calculated from the experimental points by a computer program (PCS/Pharm; Bawolak and Marceau, 2007). Numerical values are reported as means ± SEM. Non-normally distributed groups of values were analysed using non-parametric analysis of variance (Kruskall–Wallis test) followed by Dunn's multiple comparison test. Normally distributed sets of values were compared using ANOVA followed by Dunnett's test to compare the effect of treatments to a common control.

Materials

LF 16-0687 (anatibant; XY2405; 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), a previously described non-peptide B_2 receptor antagonist (Pruneau *et al.*, 1999), was a gift from Laboratoires Fournier (Daix, France). B-9972 (D-Arg-[*trans*-4-hydroxyprolyl^3, α-(2-indanyl)glycyl^5, (3as, 7as)-octahydroindol-2-yl-carbonyl^7, α-(2-indanyl) glycyl^8]-BK) is a peptide B_2 receptor agonist that incorporates resistance to several peptidases (Taraseviciene-Stewart *et al.*, 2005; Bawolak *et al.*, 2007). BK, GF109203x and Hoechst 33258 were purchased from Sigma-Aldrich. PD 98059 was from Research Biochemicals International (Natick, MA, USA).

Synthesis of compound 47a

Compound 47a (ethyl {4-[(3-{[({(2E)-3-[6-(acetylamino)-3-pyridinyl]-2-propenoyl}amino)acetyl](methyl)amino}-2,6-dichlorobenzyl) oxy]-2-methoxy-1H-benzimidazol-1-yl} acetate) was synthesized according to Sawada *et al.* (2004b), with further reference to older reports from the same group (Abe *et al.*, 1998a,b; Sawada *et al.* 2004a). The molecular structure of compound 47a was confirmed by ^{1}H-NMR, ^{13}C-NMR and ESI-MS (Department of Chemistry, University of Montreal, Montreal, Canada).

Results

Pharmacological profile of compound 47a in an isolated vascular smooth muscle preparation

The human isolated umbilical vein is a contractile bioassay for the human B_2 receptor (Marceau *et al.*, 1994). Compound 47a stimulated the contraction of this preparation with an EC_{50} of 25.3 nM and a maximal effect of ~82% of that of BK (this was determined by adding 23.6 μM BK at the end of the cumulative concentration–effect curve in response to compound 47a) (Figure 2). This contraction was mediated by B_2 receptors because it was competitively antagonized by LF 16-0687. Schild regression from the contractility data showed a pA_2 of 8.46 ± 0.10 (slope –1.11 ± 0.07, Figure 2, right).

The umbilical vein can be repeatedly stimulated with BK with a stable contractile response (Marceau *et al.*, 1994). Using this contractile bioassay, a protocol was devised to test whether B_2 receptor agonists resistant to inactivation, the peptide B-9972 and compound 47a, desensitized the preparation to the effect of BK, or vice versa, by constructing two concentration–effect curves for different agonists at 3 and 6 h after mounting the tissue (Figure 3). When used first, BK did not desensitize the umbilical vein to the other agonists B-9972 or compound 47a (EC_{50} of 69.5 and 68.8 nM respectively; control values 40.3 and 53.4 nM respectively; Figure 3A and C). However, when applied first, compound 47a, but not B-9972, decreased the apparent potency of BK (EC_{50} of 672 and 36.8 nM respectively; control value 33.3–41.7 nM for BK; Figure 3B and D). In addition, the maximal effect of BK appeared to be depressed after initial exposure to compound 47a (Figure 3D). An additional parameter was

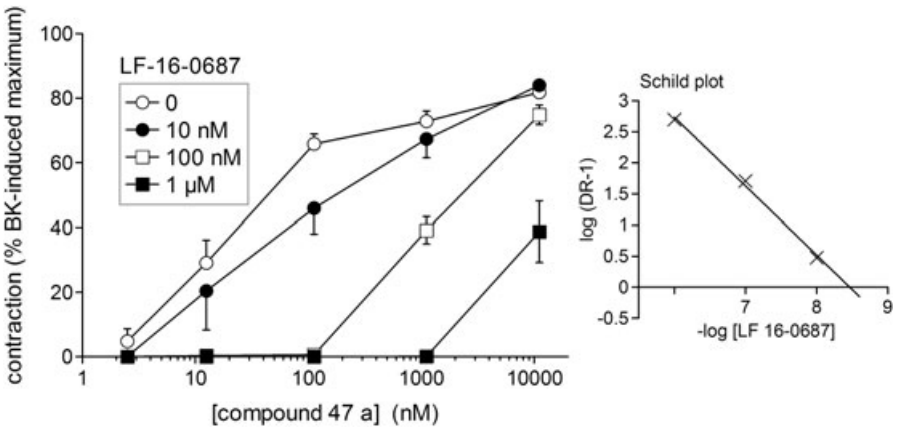

Figure 2 Antagonist effect of LF 16-0687 in a bioassay for the bradykinin (BK) B_2 receptor, the human umbilical vein stimulated with compound 47a. Values are the means ± SEM. (*n* = 6). Right: Schild plot analysis. See *Results* for details.

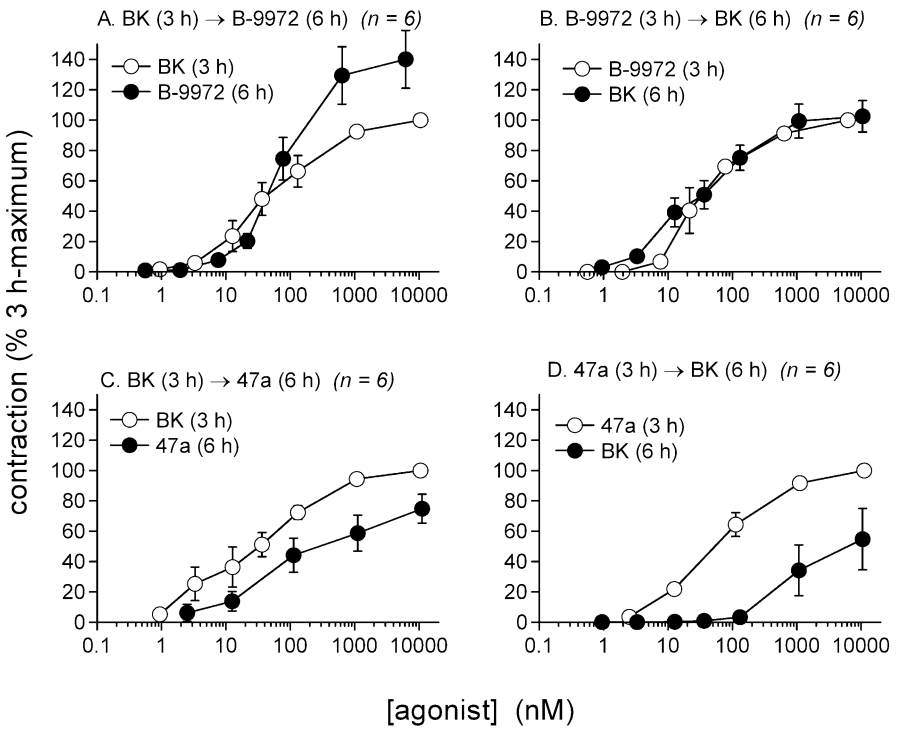

Figure 3 Sequential stimulation of the human umbilical vein preparation to demonstrate agent-specific desensitization. Full concentration–effects curves were constructed at times 3 and 6 h relative to the beginning of the *in vitro* incubation in the order indicated above each of the four panels. Values are the per cent of maximal stimulant-induced contraction recorded at 3 h.

derived from this series of experiments: the time for half-relaxation from maximal effect upon agonist washout for the three agonists in naive tissues (values derived from the first concentration–effect curves only). Tissues contracted with BK at the maximal cumulative concentration (10.5 μM) were half-relaxed in 6.7 ± 0.8 min (Figure 4A and B).The corresponding $t_{1/2}$ for B-9972 (6.2 μM) was higher (Bawolak *et al.*, 2007) and that of compound 47a (11.1 μM), even higher than the two other values (Figure 4A and B).

Affinity of compound 47a at the B_2 receptors

The B_2 receptor–GFP construction is essentially a tagged rabbit receptor and the results showed that the affinity of compound 47a was weaker than that of BK (calculated IC_{50} 168 nM, ~10-fold less potent than BK; Figure 5) in a [^{3}H]BK binding competition assay applied to HEK 293 cells stably expressing the receptor conjugate. In this cell line, LF 16-0687 was a pure and potent antagonist (IC_{50} 3.6 nM; Figure 5). The human MG-63 osteosarcoma cell line has been previously shown to respond robustly to BK via B_2 receptors and to express the corresponding radioligand binding sites (Wang *et al.*, 2001; Brechter and Lerner, 2002). We therefore used this cell line also to generate ligand competition curves for the binding of [^{3}H]BK and essentially confirmed that compound 47a was 10.2-fold less potent, and LF 16-0687 4.5-fold more potent than BK at the naturally expressed human B_2 receptor (data not shown).

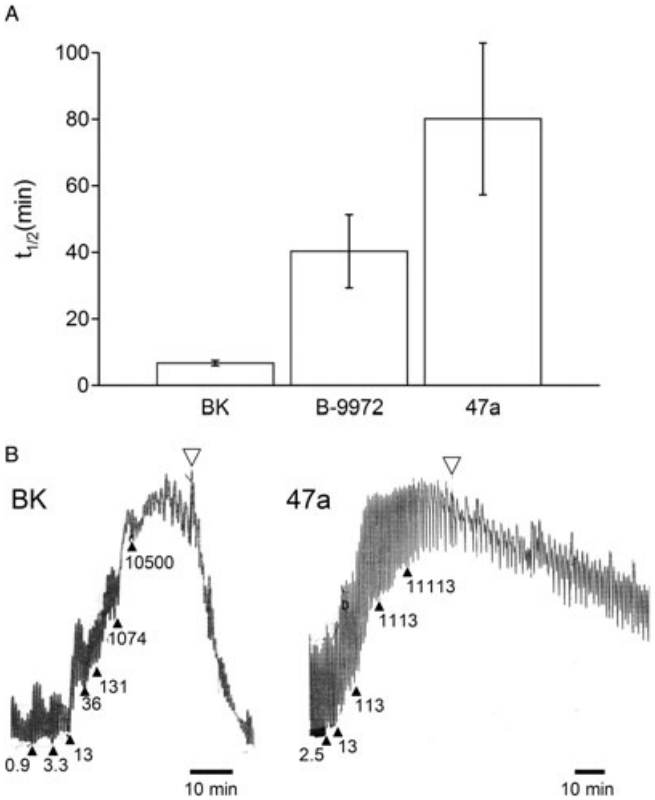

Figure 4 (A) Time for half-relaxation from the first maximal response for each bradykinin (BK) B_2 receptor stimulant after washout of the maximal concentration (same tissues as in Figure 3 for all three agonists). Kruskall–Wallis test indicated that the three groups were heterogeneous ($P < 0.001$). Further comparison with the values for BK using Dunn's multiple comparison test indicated statistically significant differences between BK and B-9972 ($P < 0.05$) and BK and compound 47a ($P < 0.001$). (B) Samples of cumulative concentration–effect curves for BK and compound 47a. Solid symbols represent drug application (nM cumulative concentrations indicated) and open symbols, the first of a series of tissue washouts.

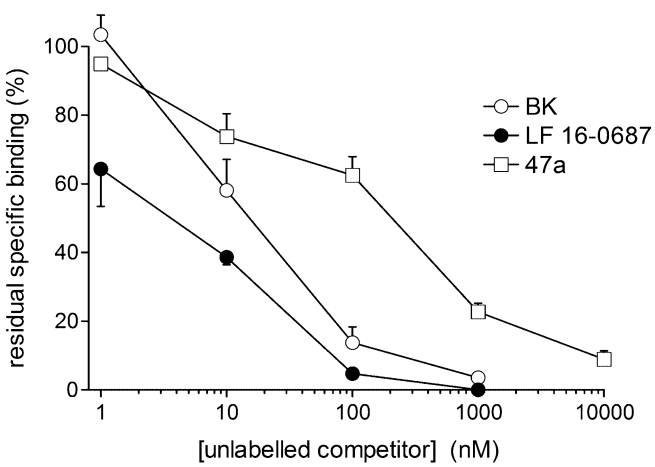

Figure 5 Displacement of [^{3}H]BK (3 nM) bound to HEK 293 cells stably expressing BK B_2 receptor–GFP by three unlabelled ligands. Values are the means ± SEM. (duplicate determinations, $n = 3$–4). Average specific binding for this concentration of radioligand (100%) was 128 ± 27 fmol per well and the non-specific binding averaged 3.3 ± 0.5% of total binding.

Effect of compound 47a and other agonists on HEK 293 cells expressing rabbit B_2 receptors

HEK 293 cells that stably express B_2 receptor–GFP are suitable for morphological studies of endocytosis and for a biochemical approach of agonist-induced down-regulation (Bachvarov *et al.*, 2001; Bawolak *et al.*, 2007). It was reasoned that a partial agonist activity of compound 47a at the rabbit B_2 receptor would trigger B_2 receptor–GFP endocytosis. Compound 47a induced a slow translocation of the membrane fluorescence, corresponding to the fluorophore-tagged receptor, to very fine cytosolic particles (Figure 6); full agonist peptides elicit a different endosomal morphology, the rapid formation of coarse aggregates (this is illustrated by the effect of BK and B-9972 in Figure 6). The effects of B-9972 on endocytosis persisted for 3 h after stimulation and a loss of the total fluorescent signal was manifested 12 h after stimulation; BK-induced B_2 receptor–GFP endocytosis was totally reversible in 3 h without agonist washout (Figure 6). When cells were exposed to agonists for 30 min, washed, then incubated for an additional 2.5 h period, the persistence of receptor-containing endosomal structures was observed in cells treated with B-9972, but not in those treated with BK. The results for compound 47a under these conditions were not as clear because receptor endocytosis is modest in 30 min. Compound 47a-induced translocation of the receptor fluorescence was not reversible over 12 h, with a progressive loss of the membrane localization and a decreased total signal, suggesting partial down-regulation at the protein level, the construction being expressed under the control of a viral promoter independent from kinin receptor signalling. The morphological changes induced by compound 47a were prevented by co-treatment with LF 16-0687 (Figure 6).

A quantitative evaluation of calcium transients was based on FURA-2 in HEK 293 cells expressing recombinant B_2 receptor–GFP (Figure 7). Non-transfected HEK 293 cells showed small and brief responses to B_2 receptor agonists (data not shown). In cells that stably expressed the receptor fusion protein, BK at the maximal concentration of 100 nM induced a fast Ca^{+2} mobilization followed by a slower fall in Ca^{+2} signal. B-9972 (100 nM) and a higher concentration of compound 47a (1 μM) essentially elicited similar responses. The effect of all three stimuli declined towards baseline in about 2.5 min (Figure 7).

HEK 293 cells stably expressing B_2 receptor–GFP can detect the stimulant effect of peptide partial agonists at the rabbit B_2 receptor with specific endpoints such as ERK1/2 MAP kinase phosphorylation (Morissette *et al.*, 2007). Using this assay, compound 47a (1 μM) stimulated phospho-ERK formation in 10 min, but less intensely than BK (10 nM) (Figure 8A). The effect of compound 47a was dependent on B_2 receptor–GFP because it was absent in untransfected cells and was blocked by LF 16-0687 pretreatment (Figure 8A). In this assay, longer incubation times with agonists revealed that B-9972 and 47a were still active after 12 h, although their effect was declining (Figure 8B), which may be explained in part by receptor down-regulation (see below). By comparison, the effect of BK was barely detectable after 3 h of stimulation and not at all after 12 h. Similar findings were made in HEK 293a cells transiently expressing the myc–B_2 receptor construct (effects of agonists dependent on the presence of recombinant

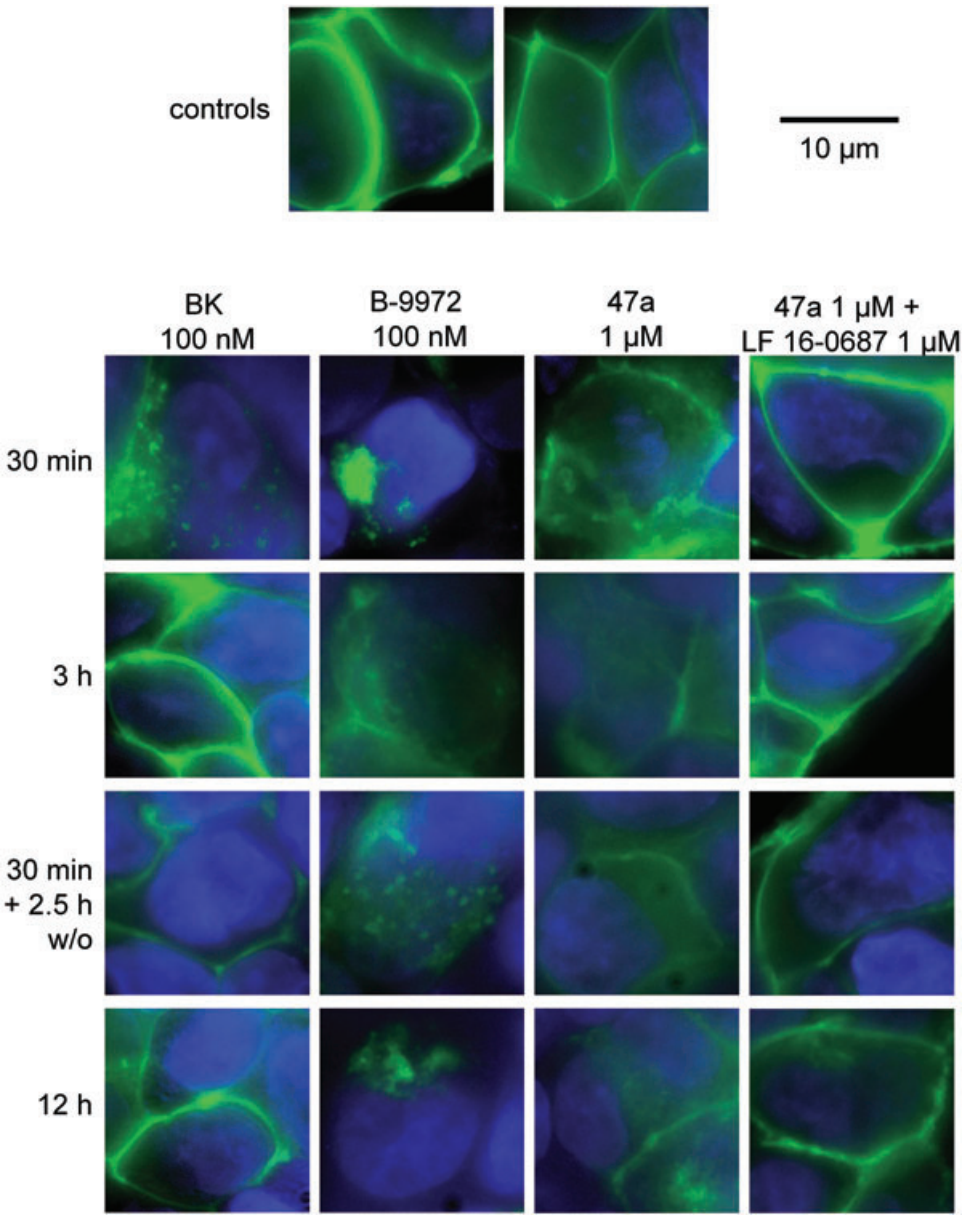

Figure 6 Epifluorescence microscopy studies of live HEK 293 cells stably expressing bradykinin (BK) B_2 receptor–GFP and stimulated for 30 min, 3 h or 12 h with B_2 receptor ligands at the indicated concentrations. In the third row, ligands were applied for 30 min and then washed out for 2.5 h. Control cells generally exhibit sharply defined plasma membrane-associated green fluorescence. Blue fluorescence: nuclear counterstain with Hoechst 33258. Original magnification 1000×.

receptors, those of B-9972 and 47a persisting over 12 h; Figure 8C), with the exception of the BK signal at 3 h, which was as strong as that of B-9972.

In these experiments, cells treated with an agonist for 12 h were maintained in serum-containing medium to maintain viability while keeping an acceptably low ERK1/2 phosphorylation background. The effect of agonists on B_2 receptor–GFP-mediated ERK1/2 phosphorylation at both time points 10 min or 3 h were prevented by the protein kinase C (PKC) inhibitor GF109203x, regardless of the agonist used (Figure 8D). An inhibitor of the tyrosine kinase domain of the epidermal growth factor receptor, AG 1478 (1 µM), did not reduce the phosphorylation of ERK1/2 induced by BK or B-9972 (10 min or 3 h, data not shown). HEK 293 cells stably expressing B_2 receptor–GFP did not contain c-Fos; however, a

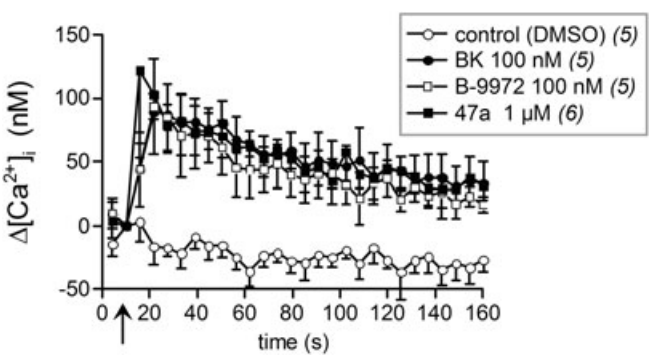

Figure 7 Calcium mobilization in HEK 293 cells stably expressing bradykinin (BK) B_2 receptor–GFP. The time course of effects elicited by acute application of B_2 receptor ligands (at 10 s, arrows) is shown. Values are means ± SEM. The number of replicates is indicated between parentheses.

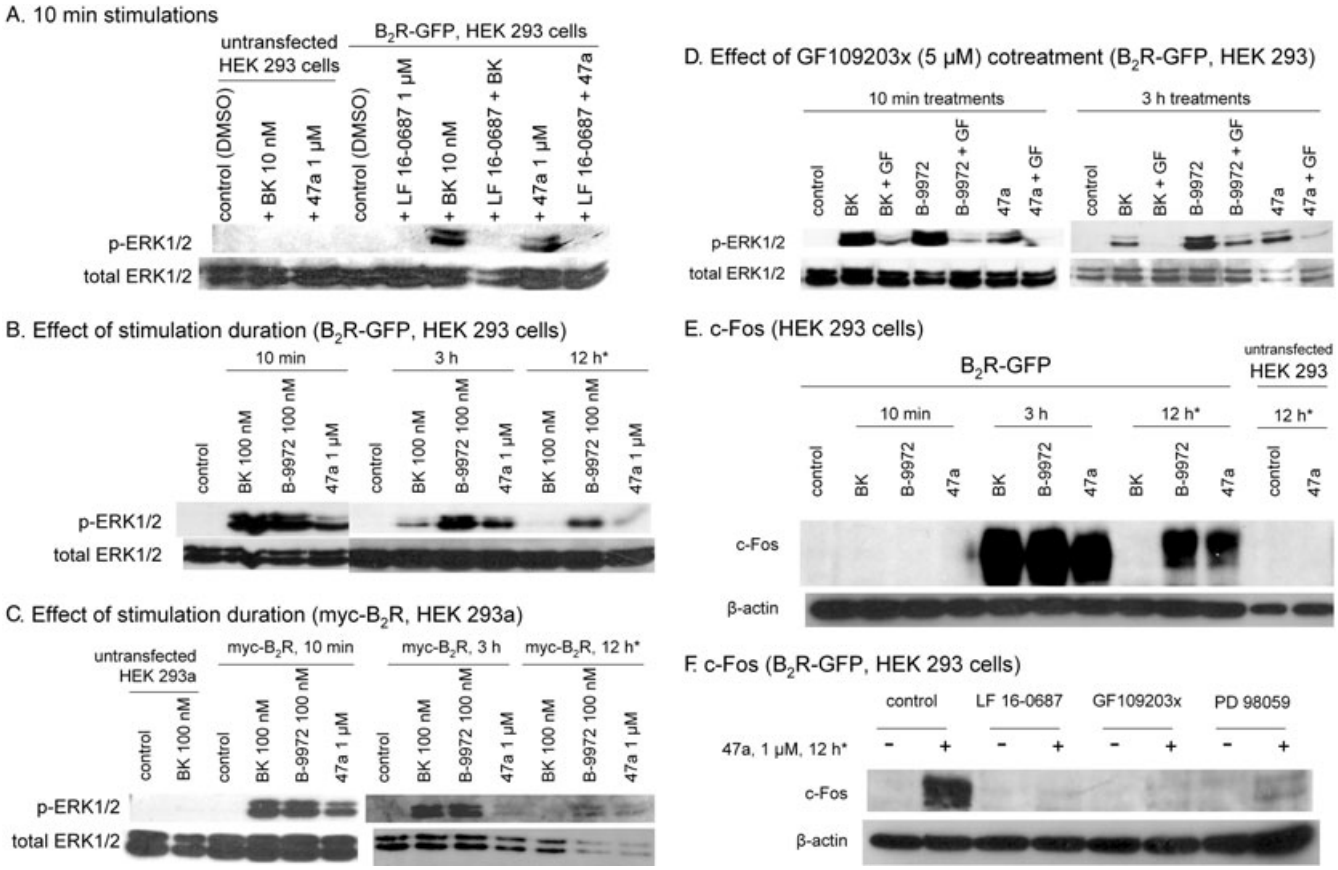

Figure 8 Differential signalling responses induced by agonists with different resistance to inactivation in HEK 293 cells stably expressing bradykinin (BK) B_2 receptor–GFP (A, B, D, E, F) or HEK 293a cells transiently expressing myc–BK B_2 receptor (C). (A) Immunoblots for phospho-ERK1/2 and total ERK1/2 in response to BK or compound 47a (10 min treatments). B_2 receptor agonist-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 compound 47a (1 µM 10 min) on untransfected cells and by the antagonist effect of LF 16-0687 (1 µM; 10 min) on the activation of ERK1/2 induced by BK or compound 47a in cells stably expressing B_2 receptor–GFP. Total ERK1/2 is shown to compare loading in tracks. Representative results of two replicates. (B) Effect of duration of stimulation with agonists on activation of ERK1/2. Cells incubated for 10 min or 3 h were maintained in low serum medium, but not those stimulated for 12 h (*regular medium). (C) Effect of myc–B_2 receptor transient expression, agonists and time on ERK1/2 phosphorylation. Presentation as in (A, B). (D) Effect of the protein kinase C inhibitor GF109203x on kinin-induced ERK1/2 phosphorylation. (E) Induction of c-Fos expression in HEK 293 cells stably expressing B_2 receptor–GFP or in untransfected cells treated as indicated with one of the three agonists for various time periods; representative result of two experiments. (F) Effect of inhibitory drugs on c-Fos induction by compound 47a (1 µM, 12 h). Drug concentrations: LF 16-0687, 1 µM; GF109203x, 5 µM; PD98089, 25 µM.

slow induction of c-Fos expression was observed following treatment with B_2 receptor agonists (Figure 8E). Specifically, no signal was present at 10 min, but BK and B-9972 produced a robust signal at 3 h and the partial agonist 47a, a weaker one. However, at 12 h of stimulation, the signal induced by 47a and B-9972 was still present while that of BK virtually disappeared. At 12 h, the apparent molecular weight of c-Fos was increased. c-Fos induction by 47a (at 12 h) was absent in HEK 293 cells that did not express the B_2 receptor–GFP (Figure 8E) and, in the presence of B_2 receptor–GFP, was inhibited by the B_2 receptor antagonist LF 16-0687, the PKC inhibitor GF109203x or the MEK1 inhibitor PD98059 (Figure 8F).

β-Arrestin$_2$ is a molecular partner of the B_2 receptor during endocytosis (Simaan *et al.*, 2005). The GFP-conjugated form of β-arrestin$_2$ is essentially cytosolic in resting, transiently transfected HEK 293a cells (Figure 9). The fluorescent protein was translocated to intracytosolic particles 10–30 min following BK stimulation, but only if co-expressed with the non-fluorescent myc–B_2 receptor of rabbit origin (Figure 9). The cells returned to their baseline appearance at times 3 h or 12 h post stimulation. However, treatment with B-9972 or compound 47a (at 100 nM and 1 µM respectively) induced a prolonged translocation of β-arrestin$_2$–GFP to cytosolic granules (3 or 12 h duration, Figure 9). Further morphological investigations of the cellular distribution of β-arrestins are summarized in Figure 10. β-Arrestin$_1$ (cherryFP conjugate) was also translocated to cytosolic granules in HEK 293 cells expressing B_2 receptor–GFP and treated for 12 h with one of the two persistent agonists. The red fluorescent signal was co-localized in some, but not all intracellular structures where the green fluorescence of GFP was seen. A 12 h treatment with BK had no effect on the distribution of β-arrestin$_1$–cherryFP, relative to control cells.

The down-regulation of B_2 receptor–GFP is conveniently detected by the decrease of the B_2 receptor–GFP ~101 kDa band and the appearance of GFP-sized metabolite(s) (~27 kDa) in immunoblots of total HEK 293 cells (anti-GFP monoclonal antibodies), because GFP is a stable globulin in

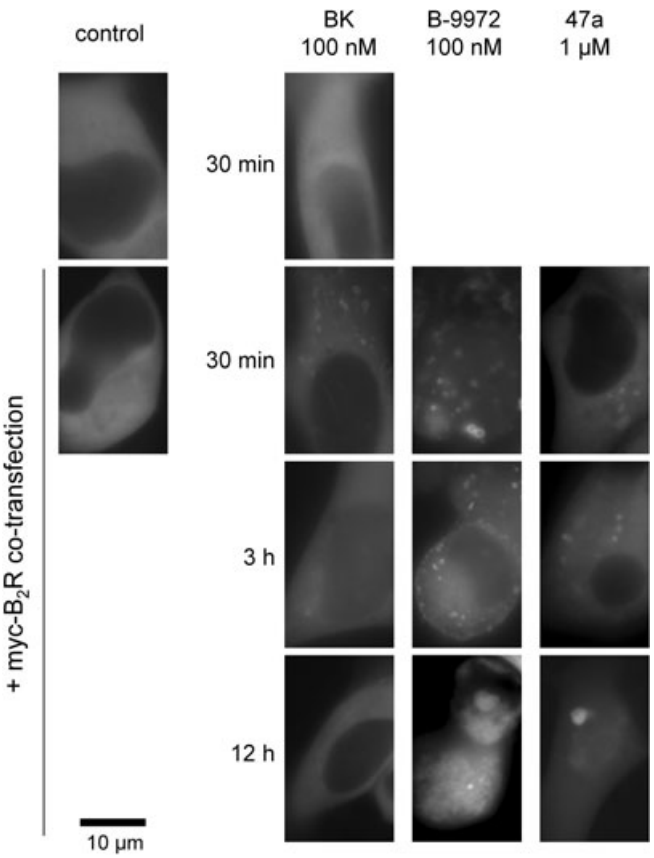

Figure 9 Epifluorescence microscopy studies of HEK 293a cells transiently expressing β-arrestin$_2$–GFP and, optionally, myc–B_2 receptor and stimulated for 10 min to 12 h with B_2 receptor ligands at the indicated concentrations. Control cells exhibit diffuse cytosolic fluorescence. Original magnification 1000×.

mammalian cells (Bawolak *et al.*, 2007) and should remain in the cytosol upon degradation of the fusion protein. A 12 h treatment with B-9972 has previously been shown to slowly but extensively degrade B_2 receptor–GFP (Bawolak *et al.*, 2007), BK being inactive in this respect. These findings were replicated in the present study (100 nM of each, Figure 11). Compound 47a (1 μM) behaved as a persistent agonist, promoting receptor degradation less than that induced by B-9972, but clearly above background. Further, compound 47a did not antagonize the effect of B-9972. LF 16-0687 co-treatment inhibited the down-regulation of B_2 receptor–GFP induced by compound 47a (Figure 11).

Discussion

The synthesis of a non-peptide agonist for peptide hormone receptors is an intriguing goal that has also been achieved for the angiotensin II AT_1 receptor (with L-162,313; Perlman *et al.*, 1995) and the vasopressin V_2 receptor (with OPC-51803; Nakamura *et al.*, 2004). As for the BK B_2 receptor, a previous study has shown that FR190007 produced 70% of BK-induced maximal contraction in the umbilical vein and 30% of that recorded in the rabbit jugular vein (Rizzi *et al.*, 1999). Thus, despite the previous claim that compound 47a is a full BK receptor agonist at the recombinant B_2 receptor (Sawada *et al.*, 2004b), compound 47a does not possess a clearly greater intrinsic activity, especially at the rabbit B_2 receptor where it is a partial agonist in cell-based assays (ERK1/2 phosphorylation, c-Fos induction, B_2 receptor–GFP down-regulation). The effect of compound 47a is mediated by a classical BK B_2 receptor in the umbilical vein, as the Schild plot parameters of LF 16-0687 measured against BK (pA_2 8.27 ± 0.19, slope –1.04; Bawolak and Marceau, 2007) are very similar to those measured against compound 47a (Figure 2). Although possessing lower receptor affinity than B-9972 (Bawolak *et al.*, 2007), compound 47a exhibited a particularly slow relaxation on washout of the agonist (Figure 4), consistent with the previously suggested use of the relaxation velocity to evaluate resistance to inactivation, regardless of receptor affinity (Bawolak *et al.*, 2007). Further, compound 47a desensitized the umbilical vein to BK during 3 h, an effect not shared by the partially inactivation-resistant peptide B-9972 (Figure 3). Thus, the results of the contractility assays were compatible with a prolonged signalling and desensitization of the B_2 receptor after stimulation with compound 47a. Detailed molecular mechanisms of the desensitization process, which may or not include down-regulation, cannot be derived from the contractility assay.

The cellular system exploited to characterize degradation-resistant B_2 receptor agonists expressed the tagged constructs, B_2 receptor–GFP or myc–B_2 receptor. The B_2 receptor–GFP has the same affinity as the rabbit wild type B_2 receptor for [^{3}H]BK (Bachvarov *et al.*, 2001) and activates relevant signalling pathways (phospholipase A_2, calcium, ERK1/2 phosphorylation) (Houle *et al.*, 2000; Morissette *et al.*, 2007). The radioligand affinity at myc–B_2 receptor is also identical to that of the wild type (Bawolak *et al.*, 2007). The fact that ERK1/2 phosphorylation was activated with rather similar time courses in the present experiments (Figure 8B and C) suggests that myc–B_2R and B_2R–GFP are functionally comparable. It has been argued that C-terminal GFP conjugation can slow down GPCR cycling in general (McLean and Milligan, 2000) and perhaps increase the proportion of B_2 receptors recycled, over that of those degraded following stimulation with BK (Kalatskaya *et al.*, 2006). However, immunoblotting showed that both constructs were degraded after a 12 h treatment with B-9972, not by a 3 h treatment, with comparable down-regulation at the receptor protein level over a long period despite the different location of the tag sequences in the receptor constructs (Bawolak *et al.*, 2007).

The cellular behaviour of the non-peptide compound 47a is similar in many respects to that of the peptide B-9972: the very persistent endocytosis and receptor down-regulation have been described previously for B-9972 (Bawolak *et al.*, 2007). A 6–8 min half-life for BK was previously estimated in the same experimental system, using an enzyme immunoassay (Bachvarov *et al.*, 2001); therefore, the reversibility of BK-induced effects can be attributed to agonist breakdown and receptor re-expression at the cell surface. All three agonists elicited similar temporal profiles of calcium transients (Figure 7) that were mediated by recombinant B_2 receptor–GFP and possibly a small endogenous population of B_2 receptors in this cell type (Kramarenko *et al.*, 2009). LF 16-0687, which has no effect on calcium mobilization, prevented the

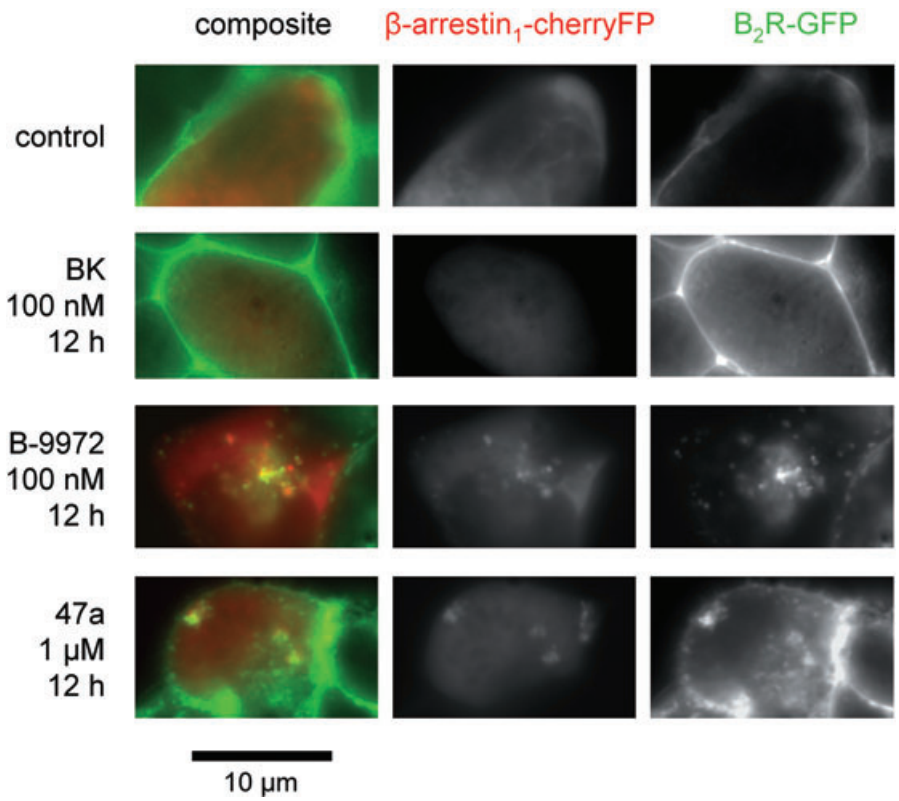

Figure 10 Translocation of β-arrestin$_1$–cherryFP induced by 12 h treatment with bradykinin (BK) B_2 receptor agonists (concentrations as in Figure 9), and colocalization with B_2 receptor–GFP in HEK 293 cells. Original magnification 1000×.

effect of BK in a previous report based on the same transfectant cells (Morissette *et al.*, 2007). Novel aspects of the present study include the persistence of B_2 receptor–GFP in endosomal structures following B-9972 treatment followed by 2.5 h agonist washout, the very long lasting translocation of β-arrestins$_{1/2}$ to these structures and prolonged MEK/ERK and c-Fos signalling under stimulation with either persistent agonist. These findings suggest, on one hand, the persistence of the agonist–receptor–arrestin complex in endosomes and, on the other, a 'biased' agonist activity of the persistent agonists of the B_2 receptor that induced an unbalanced recruitment of signalling pathways as a function of time, relative to BK (especially the persistence of the ERK1/2 and c-Fos pathways over calcium, Figures 7 and 8). Differential effects on c-Fos expression are particularly interesting findings, as this response integrates the effect of ERK1/2 stimulation through several successive mechanisms as a function of time (transcriptional stimulation, inhibition of the proteasomal clearance of the protein, multiple phosphorylation events, compatible with an apparent increase of molecular weight (Figure 8E; Glauser and Schlegel, 2007). The ERK1/2-dependent pathway of activation of c-Fos has been previously observed in human keratinocytes stimulated with a B_2 receptor agonist (Vidal *et al.*, 2005) and was also seen here in HEK 293 cells (Figure 12).

Protein kinase C-dependent MEK1/ERK MAP kinase signalling is well known to be activated by another type of receptor, mainly coupled to protein $G\alpha_q$, the angiotensin AT_1 receptor (Violin and Lefkowitz, 2007; DeWire *et al.*, 2008), and a postulated β-arrestin-mediated component in this pathway was recognized by its resistance to a pharmacological PKC inhibitor. In the present study, the effect of all three B_2 receptor agonists were profoundly inhibited by the PKC inhibitor GF109203x (Figure 8C), apparently ruling out MAP kinase signalling via an arrestin in this system, although the two types of β-arrestin were translocated to endosomes for extended periods of time under the effect of agonists resistant to degradation. Whether the 'biased agonist' status attributed to an angiotensin II analogue with preferred β-arrestin$_2$-mediated signalling, [Sar^1, Ile^4, Ile^8]angiotensin II (Violin and Lefkowitz, 2007), can be attributed to unforeseen resistance to inactivation conferred by the N-terminal sarcosine residue is a possibility as the AT_1 receptor, related to the B_2 receptor, is also subject to a cycle of phosphorylation, endocytosis and cell surface re-expression.

The present data about the persistence of β-arrestin translocation to endosomal granules induced by B-9972 or compound 47a resolve the conflict in the classification of the BK B_2 receptor regarding the short and membrane-located (type A) or long and intracellular (type B) classes, in terms of the β-arrestin association with receptors (Hamdan *et al.*, 2007). When assessed using an inactivation-resistant agonist, such as B-9972 which persistently translocates B_2 receptor–GFP to endosomal structures, even when washed out from cells (Figure 6), the B_2 receptor unequivocally falls into the B class, consistent with its persistent association with β-arrestin in endosomes and propensity for agonist-induced receptor down-regulation. Furthermore, class A receptors exhibit a

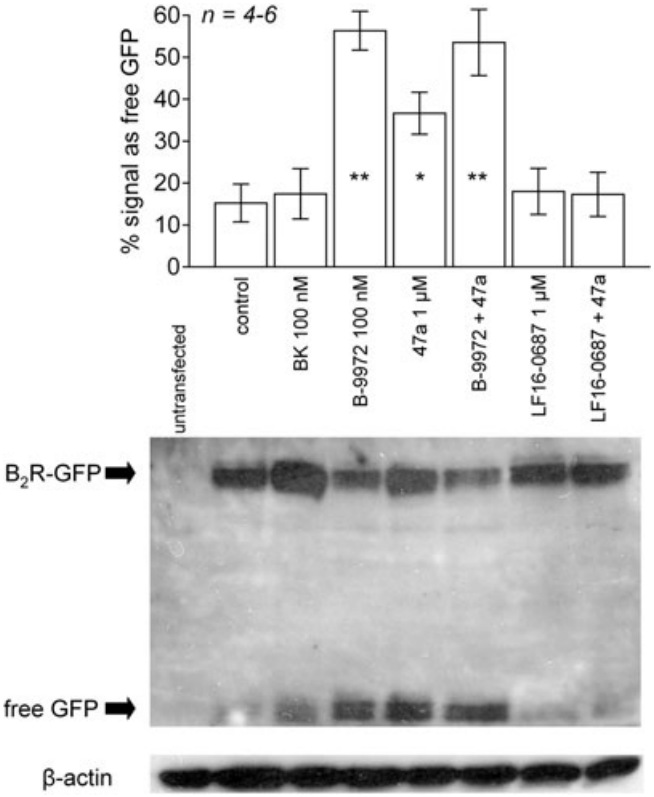

Figure 11 Effect of agonists on B_2 receptor–GFP expressed in HEK 293 cells: immunoblot of total cell extracts based on anti-GFP antibodies. The cells were submitted to the indicated treatments for 12 h in the usual culture medium with heat-inactivated foetal bovine serum before extraction. Upper graph: Average densitometry values of immunoblots for four to six experiments with ligand treatments as shown. Lower figure: representative experiments also showing the prevention of stimulant-induced B_2 receptor–GFP degradation by LF 16-0687 co-treatment. ANOVA indicated that the groups of values were different ($P < 0.001$). Comparison with control GFP/B_2 receptor–GFP density ratio (Dunnett's test): $*P < 0.05$; $**P < 0.01$.

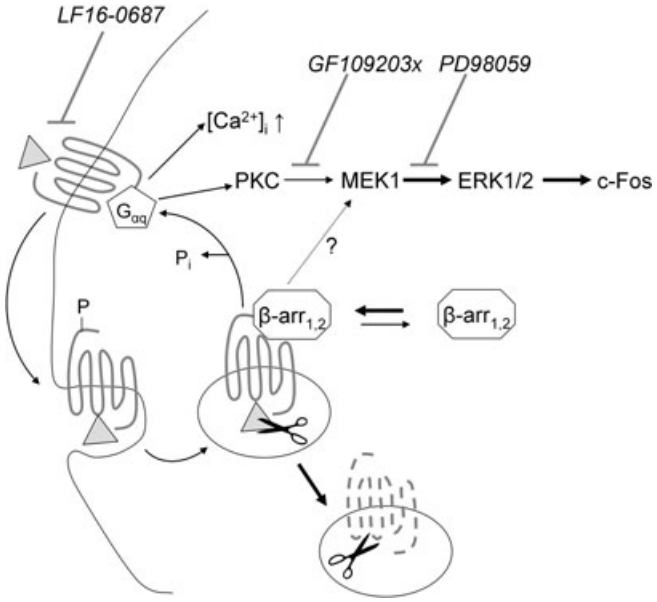

Figure 12 Schematic representation of the mechanisms of bradykinin B_2 receptor-mediated responses to agonists in HEK 293 cells. The dotted arrow represents a pathway that has been proposed in other systems but is not supported in the present one. Thick arrows represent pathways that are favoured by agonists resistant to inactivation in long-term experiments.

high affinity for β-arrestin$_2$, not β-arrestin$_1$, whereas class B receptors bind to the two types of β-arrestin with comparable high affinities (Oakley *et al.*, 2000). The present data showing the translocation of both types of arrestins to endocytic structures also support the classification of the B_2 receptor as a class B, G protein coupled receptor.

Inactivation-resistant agonists revealed the long-lasting assembly of the agonist–B_2 receptor–β-arrestin complexes. These agonists also induced 'biased signalling' as a function of time, because the activation of ERK and of c-Fos persisted for much longer than that induced by BK. Further, B-9972 and compound 47a, unlike the endogenous agonist BK, efficiently down-regulated BK B_2 receptors.

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Conflict of interest

The authors state no conflict of interest.

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