Agonist-promoted trafficking of human bradykinin receptors: arrestin- and dynamin-independent sequestration of the B_2 receptor and bradykinin in HEK293 cells

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In this study, we analysed the agonist-promoted trafficking of human B_2 (B_2R) and B_1 (B_1R) bradykinin (BK) receptors using wild-type and green fluorescent protein (GFP)-tagged receptors in HEK293 cells. B_2R was sequestered to a major extent upon exposure to BK, as determined by the loss of cell-surface B_2R using radioligand binding and by imaging of B_2R–GFP using laser-scanning confocal fluorescence microscopy. Concurrent BK sequestration was revealed by the appearance of acid-resistant specific BK receptor binding. The same techniques showed that B_1R was sequestered to a considerably lesser extent upon binding of des-Arg^{10}-kallidin. B_2R sequestration was rapid (half-life ~ 5 min) and reached a steady-state level that was significantly lower than that of BK sequestration. B_2R sequestration was minimally inhibited by K44A dynamin ($22.4 \pm 3.7\,\%$), and was insensitive to arrestin-(319–418), which are dominant-negative mutants of dynamin I and β-arrestin respectively. Furthermore, the B_2R-mediated sequestration of BK was completely insensitive to both mutants, as was the association of BK with a caveolae-enriched fraction of the cells. On the other hand, agonist-promoted sequestration of the β_2-adrenergic receptor was dramatically inhibited by K44A dynamin ($81.2 \pm 16.3\,\%$) and by arrestin-(319–418) ($36.9 \pm 4.4\,\%$). Our results show that B_2R is sequestered to a significantly greater extent than is B_1R upon agonist treatment in HEK293 cells. Furthermore, B_2R appears to be recycled in the process of sequestering BK, and this process occurs in a dynamin- and β-arrestin-independent manner and, at least in part, involves caveolae.

Key words: caveolae, clathrin-coated pit, G-protein-coupled receptor, internalization, peptide.

INTRODUCTION

Many G-protein-coupled receptors (GPCRs) are sequestered in the cell upon agonist binding. The most well described pathway of GPCR sequestration involves clathrin-coated pits, and is closely linked to receptor desensitization [1,2]. Exemplified by the β_2-adrenergic receptor (β_2AR), this pathway involves the recruitment of a GPCR kinase to the receptor and phosphorylation of specific serine and threonine residues in the third or fourth intracellular domain of the receptor. Subsequent binding of β-arrestin to the receptor interferes with the interaction of the receptor with G-proteins. In addition, β-arrestin binds to clathrin, and thereby targets the receptor to clathrin-coated pits. Dominant-negative mutants of β-arrestin [3] and dynamin I [4], a GTPase necessary for the closure and pinching off of clathrin-coated pits, have been used to delineate this pathway for several other GPCRs [1,2,5].

The sequestration of some GPCRs is either insensitive to dominant-negative β-arrestin and dynamin mutants or inhibited by only one of them, suggesting mechanisms of sequestration that are distinct from that of the β_2AR, and possibly independent of clathrin-mediated endocytosis. For example, the AT_1 angiotensin [6] and secretin [7] receptors are sequestered independently of both β-arrestin and dynamin, whereas the sequestration of the m_1, m_3 and m_4 muscarinic receptors is β-arrestin-independent but dynamin-dependent [8,9]. On the other hand, the sequestration of the m_2 muscarinic receptor is independent of dynamin [9,10], whereas dependence on β-arrestin varies with the cell system [10,11].

Caveolae are clathrin-independent domains that compartmentalize and internalize certain molecules [12]. These domains have been implicated in the agonist-promoted sequestration of the B_2 bradykinin (BK) receptor (B_2R) [13,14], the cholecystokinin receptor [15], the m_2 muscarinic receptor [16] and the ET_A endothelin receptor [17], and their cognate G-protein α subunits [13]. A dominant-negative dynamin I mutant and antibodies against dynamin I inhibit the pinching off of caveolae and the formation of transport vesicles, as well as cholera toxin uptake [18,19]. This has led to the proposal that dynamin is also involved in certain aspects of caveolae function, even though the exact role of dynamin in this function is not known.

The B_2R and B_1 BK receptor (B_1R) subtypes are prototypical GPCRs [20,21] that mediate the actions of kinins, which are potent pro-inflammatory peptides of 8–10 amino acids in length [22–24]. Agonist stimulation of the B_1R leads to a sustained response that is subject to limited desensitization [25,26], and the agonist–receptor complex is sequestered to a very limited extent [27]. On the other hand, the B_2R response is transient and subject to extensive desensitization [25], and the agonist–B_2R complex is sequestered rapidly [28,29]. A major portion of the agonist–B_2R complexes sequestered in cells remains associated with the plasma membrane [28,29], where it appears to reside, at least in part, in caveolae [13,14]. However, very little is known about the mechanism of sequestration of these receptors. Furthermore, the

Abbreviations used: BK, bradykinin; β_2AR, β_2-adrenergic receptor; B_2R, B_2 bradykinin receptor; B_1R, B_1 bradykinin receptor; DMEM, Dulbecco's modified Eagle's medium; GPCR, G-protein-coupled receptor; GFP, green fluorescent protein; WT, wild type.

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putative role of dynamin, as well as the potential role of β-arrestin, in the trafficking of GPCRs proposed to utilize the caveolae pathway has not been evaluated.

In the present study, we used human wild type (WT) and green fluorescent protein (GFP)-tagged receptors and dominant-negative mutants of dynamin I and β-arrestin expressed in HEK293 cells to analyse the trafficking of B_2Rs and B_1Rs. Our results show that the B_2R is sequestered rapidly in these cells, whereas the B_1R is sequestered to a very limited extent. The B_2R appears to recycle in the process of sequestering BK, and the process is independent of both dynamin and β-arrestin.

MATERIALS AND METHODS

Materials

[2,3-*prolyl*-3,4-^{3}H]BK (90–114 Ci/mmol), des-Arg^{10}-[3,4-*prolyl*-3,4-^{3}H]kallidin (64–107 Ci/mmol), [^{3}H]CGP12177 (36 Ci/mmol) and *myo*-[^{3}H]inositol (10–20 Ci/mmol) were obtained from NEN Life Sciences (Boston, MA, U.S.A.). WT dynamin I and K44A dynamin cDNAs were obtained from M. G. Caron (Duke University Medical Center, Durham, NC, U.S.A.), and WT β-arrestin, arrestin-(319–418), and β_2AR–GFP cDNAs were obtained from J. L. Benovic (Thomas Jefferson University, Philadelphia, PA, U.S.A.). Monoclonal antibodies against caveolin were purchased from ICN (Costa Mesa, CA, U.S.A.), and monoclonal antibodies against dynamin I were from Transduction Laboratories (Lexington, KY, U.S.A.). BK and des-Arg^{10}-kallidin were from Bachem (Torrance, CA, U.S.A.). GFP cDNA, Dulbecco's modified Eagle's medium (DMEM), Leibovitz's L-15 medium, Hanks balanced salt solution and Optiprep were from Gibco BRL (Gaithersburg, MD, U.S.A.). Reagents for calcium phosphate transfections were purchased from 5 Prime → 3 Prime, Inc. (Boulder, CO, U.S.A.). Enzymes were obtained from Gibco BRL and New England Biolabs (Beverly, MA, U.S.A.). An immunodetection kit was obtained from Amersham Corp. Sera and all other peptides and chemicals were from Sigma (St. Louis, MO, U.S.A.).

Construction of receptor–GFP fusion proteins

The original human B_1R and B_2R cDNAs in vector pcDNA3 (Invitrogen) were obtained from J. F. Hess (Merck Research Laboratories, West Point, PA, U.S.A.), and vector pDTGFP was kindly provided by L. Frank Kolakowski (University of Texas Health Science Center). Upon receptor DNA insertion, pDTGFP creates, in-sequence, the hexa-His tag and the FLAG tag at the N-terminus immediately following the initial methionine in the receptor, and GFP at the C-terminus immediately before the stop codon. Primers were designed to amplify the B_2R, with an *Xba*I site at the N-terminus (underlined) and the initial methionine mutated to a valine (5′-CG TCT AGA CTG CTC AAT GTC ACC TTG CAA-3′) and an *Mlu*I site at the C-terminus (underlined) immediately before the stop codon (5′-CG TCA ACG CGT CTG TCT GCT CCC TGC CCA-3′). The PCR product was cut with *Xba*I and *Mlu*I and cloned into the pDTGFP vector. The B_1R was modified similarly, with an *Xba*I site at the N-terminus and the initial methionine mutated to a valine. In addition, an endogenous *Xba*I site was removed by changing one base in the B_1R (5′-CG TCT AGA CTG GCA TCA TCC TGG CCC CCC CTA GAG CTC CAA T-3′). The C-terminus of the B_1R was modified in the same way as for the B_2R (5′-CG TTA ACG CGT ATT CCG CCA GAA AAG TT-3′). The identities of the constructs were confirmed by cycle sequencing. Pure plasmid DNA for transfections into mammalian cells was isolated with the Qiagen Plasmid Maxi kit (Qiagen).

Cell culture and transfection

HEK293 cells were grown in DMEM supplemented with 10 % (v/v) heat-inactivated horse serum at 37 °C in 10 % CO_2. Transient transfection was performed using the calcium phosphate precipitation method in the presence of 0.2–12 μg of cDNA per 35-mm well in six-well plates, as described previously [30]. This protocol yielded a transfection efficiency of 70–80 %, as determined in parallel transfections with GFP-expressing cDNA. Cells were used 48 h after transfection. Stable transfections were made by transfecting cells at 60–80 % confluency with 3 μg of cDNA in a 35 mm dish using the calcium phosphate precipitation method. At 48 h after transfection, the cells were trypsinized and transferred to a T-75 flask. At 24 h after the transfer, the medium was exchanged and supplemented with 600 μg/ml G418. Following incubation for 2 weeks, the cells were grown in medium supplemented with 200 μg/ml G418.

Enrichment of caveolae

Enrichment of caveolae from cells was carried out using the procedure of Smart et al. [31], with a few modifications as described previously [13].

Cell membrane preparation

Cells were washed twice with ice-cold PBS and then pelleted by centrifugation at 2000 ***g*** for 10 min. The cells were then resuspended in buffer A (25 mM 2-{[2-(hydroxymethyl)ethyl]-amino}ethanesulphonic acid, pH 6.8, 0.5 mM EDTA, 0.2 mM $MgCl_2$ and 1 mM 1,10-phenanthroline) and homogenized using an Ultra-Turrax at 20500 rev./min for 10 s. Membranes were isolated by centrifugation at 45000 ***g*** for 30 min at 4 °C. The pellets were then resuspended in buffer B (buffer A including 0.1 % BSA and 140 μg/ml bacitracin).

Radioligand binding

Radioligand binding on cell membranes was carried out as described previously [30]. Briefly, membranes were incubated with various concentrations of either [^{3}H]BK or des-Arg^{10}-[^{3}H]kallidin in buffer B for 60–90 min at 25 °C. Non-specific binding was determined in the presence of 1 μM non-radioactive peptide. The assays were terminated by dilution with 4 ml of ice-cold PBS/0.3 % BSA and rapid vacuum filtration on Whatman GF/C filters soaked previously in 1 % poly(ethyleneimine). The trapped membranes were then washed with an additional 2 × 4 ml of ice-cold PBS/0.3 % BSA. The filters were counted for radioactivity in a Beckman LS5000TD scintillation counter.

Radioligand binding on intact cells with [^{3}H]BK and des-Arg^{10}-[^{3}H]kallidin was carried out as described previously [32], with a few modifications. Briefly, cells grown in 6-well dishes were washed with Leibovitz's L-15 medium, pH 7.4, containing 20 mM Hepes, 1 mM 1,10-phenanthroline, 0.1 % BSA and 140 μg/ml bacitracin, and then incubated with various concentrations of radioligand for 90 min at 4 °C. The assays were terminated by rinsing the cells with 2 × 3 ml of ice-cold PBS/0.3 % BSA, followed by solubilization in 1 % SDS/0.1 M NaOH/0.1 M Na_2CO_3. The lysates were then counted for radioactivity. Radioligand binding with [^{3}H]CGP12177 was done essentially as described above, except that cells were washed with PBS and

Table 1 Binding constants for BK and des-Arg^{10}-kallidin on human WT and GFP-tagged receptor constructs expressed in stably transfected HEK293 cells

The K_d values were obtained from saturation binding experiments using membrane preparations, and were calculated using the Radlig curve-fitting program. The values are presented as means ± S.E.M. of at least three experiments. UB, undetectable binding of the radioligand to the construct. Significance of differences: $^*P < 0.05$ compared with WT B_2R; $\dagger P < 0.02$ compared with WT B_1R.

	K_d (nM)	
Receptor	BK	Des-Arg^{10}-kallidin
WT B_2R	0.14 ± 0.02	UB
B_2R–GFP	0.36 ± 0.07*	UB
WT B_1R	UB	0.27 ± 0.11
B_1R–GFP	UB	3.18 ± 0.80†

incubated in the presence of [^{3}H]CGP12177 for 4 h at 4 °C. Non-specific binding was assayed in the presence of 10 μM alprenolol.

The agonist binding constants for B_2R, B_2R–GFP and B_1R presented in Table 1 were obtained from saturation binding experiments using either various concentrations of radiolabelled agonist or a single concentration of radiolabelled agonist and various concentrations of non-radiolabelled agonist; the constants were calculated using Radlig software (Biosoft). The constants generated from the two types of experiments agreed very well. The agonist binding constant for B_1R–GFP was generated using only the latter type of experiment, since it has a value (3.18 ± 0.80 nM) that made it difficult to reach saturation using only radiolabelled agonist. Furthermore, radioligand binding to the B_1R–GFP-expressing cells decreased with time in culture, making it difficult to assess the number of binding sites expressed on the cells. The specific binding of 2 nM des-Arg^{10}-[^{3}H]kallidin obtained immediately after establishing the cells was 5.4 fmol/10^6 cells.

Agonist sequestration and release

Agonist sequestration was monitored essentially as described by Munoz and Leeb-Lundberg [28]. Cells were incubated with a saturating concentration of ^{3}H-labelled agonist (1–3 nM) in buffer C (DMEM, pH 7.4, 1 mM 1,10-phenanthroline, 0.1 % BSA and 140 μg/ml bacitracin) for various times at 37 °C in the absence and presence of 1 μM non-radioactive agonist. B_1R–GFP-expressing cells were not analysed for this process, since it was technically difficult to use a saturating concentration of radiolabelled agonist for a receptor construct with such a high K_d value (see above). After washing the cells with ice-cold PBS/0.3 % BSA, they were incubated for 6 min with 0.05 M glycine, pH 3.0, at 4 °C. This incubation was followed by a 30 s wash with the same solution and then a final wash with PBS/0.3 % BSA. The cells were solubilized in 1 % SDS/0.1 M NaOH/0.1 M Na_2CO_3, and the lysates were counted for radioactivity. As described originally by Haigler et al. [33] and Ascoli [34], and subsequently by us for specific [^{3}H]BK binding [28], radioactivity associated with the cells following the above acid wash is considered intracellular and inaccessible to the extracellular environment and, thus, was taken as a measurement of the amount of agonist sequestration.

To determine the release of cell-associated agonist, cells were first labelled with a saturating concentration of ^{3}H-labelled agonist at 4 °C, as described above. The cells were then rinsed twice with 3–5 ml of ice-cold PBS/0.3 % BSA to remove free radioligand, and then incubated further for various times at 37 °C. At each time point, the medium was collected and counted for radioactivity. The fragmentation pattern of the released BK-derived radioactivity was determined by reverse-phase HPLC on a C18 μ-Bondapak column, as described previously [28].

To determine the association of BK with caveolae, cells were first incubated with a saturating concentration of [^{3}H]BK (1–3 nM) in buffer C for 15 min at 37 °C in the absence or presence of 1 μM non-radioactive BK. After washing the cells with ice-cold PBS/0.3 % BSA, the labelled cells were processed as described above under 'Enrichment of caveolae'.

Receptor sequestration

B_2R and B_1R sequestration was monitored as described by Munoz et al. [29]. Briefly, cells were pretreated with 1 μM agonist in buffer C for various times at 37 °C, and then subjected to an acid wash to remove any surface-bound agonist as described above. β_2AR–GFP sequestration was monitored as described by Kallal et al. [35]. Briefly, cells were incubated with 10 μM isoprenaline and 0.1 mM ascorbic acid in PBS for various times and then washed with PBS to remove the agonist. Receptors were then assayed as described above for radioligand binding. The amount of receptors lost upon agonist exposure was taken as a measurement of agonist-promoted receptor sequestration. B_1R–GFP-expressing cells were not analysed for this process, for the reasons indicated above.

Phosphoinositide hydrolysis

Phosphoinositide hydrolysis was assayed as described previously [32], with a few modifications. Briefly, cells were incubated in six-well dishes with 4 μCi/ml *myo*-[^{3}H]inositol in DMEM containing 1 % (v/v) heat-inactivated horse serum for 18 h at 37 °C in 10 % CO_2. Prior to experimentation, the cells were washed with 4 × 1 ml of Leibovitz's L-15 medium, pH 7.4, including 50 mM LiCl for 30 min. Following replacement with the same medium, the cells were incubated with agonist for 20 min at 37 °C. Inositol phosphates were then extracted and isolated using anion-exchange chromatography.

Immunoblotting

Proteins were separated by SDS/PAGE on 12 % (w/v) gels and transferred to nitrocellulose membranes, and immunoreactive bands were visualized with an immunodetection kit (Amersham Corp.) using peroxidase-labelled sheep anti-mouse antibodies, as described previously [13].

Fluorescence microscopy

Cells were seeded on round glass coverslips pretreated with 5 μg/ml fibronectin at 48 h prior to use. The coverslip was then placed in a Leiden holder in Leibovitz's L-15 medium, pH 7.4, containing 1 mM 1,10-phenanthroline, 0.1 % BSA and 0.1 % glucose. Agonists were added manually. The cells were viewed with a Nikon T-300 Inverted Confocal Laser Scanning Microscope using a 60× oil immersion objective lens. Data were collected with Bio-Rad MicroRadiance Plus software.

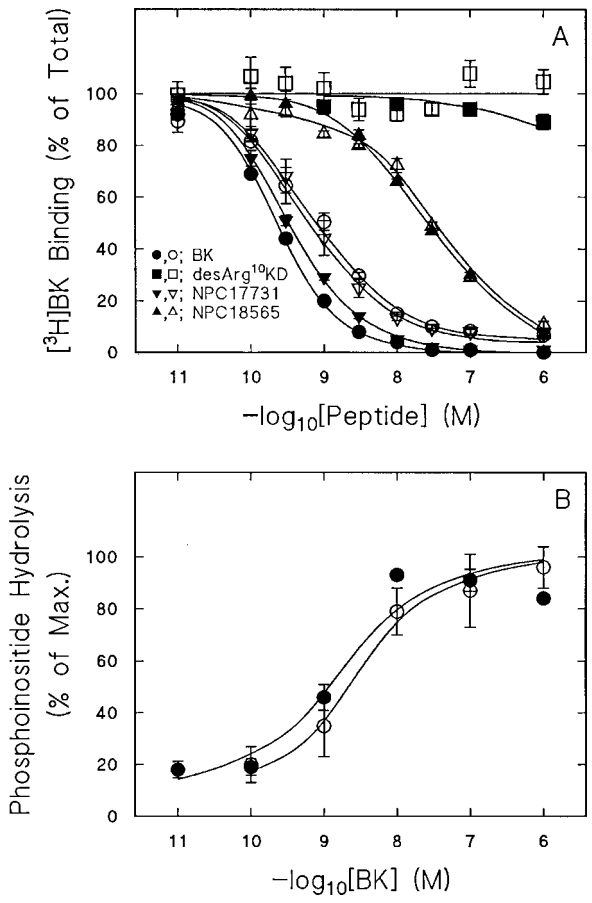

Figure 1 BK binding and BK-stimulated phosphoinositide hydrolysis in HEK293 cells expressing B_2R and B_2R–GFP

(**A**) Particulate preparations of stable B_2R cells (closed symbols) or stable B_2R–GFP cells (open symbols) were incubated with K_d concentrations of [^{3}H]BK in the absence or presence of increasing concentrations of BK (●, ○), des-Arg^{10}-kallidin (KD) (■, □), NPC17731 (▼, ▽) or NPC18565 (▲, △), and were assayed for radioligand binding as described in the Materials and methods section. The results are presented as % of maximum, where 100% represents [^{3}H]BK binding to B_2R in the absence of competitor. (**B**) Stable B_2R cells (○) or stable B_2R–GFP cells (●) labelled with *myo*-[^{3}H]inositol were incubated with increasing concentrations of BK and assayed for phosphoinositide hydrolysis as described in the Materials and methods section. The results are presented as % of maximum, where 100% is the response of B_2R to 1 μM BK (basal, 10954 ± 1261 d.p.m./well; BK, 63160 ± 1261 d.p.m./well). The results are means ± S.E.M. of three experiments, with each point assayed in duplicate. Some points have error bars that are smaller than the symbols.

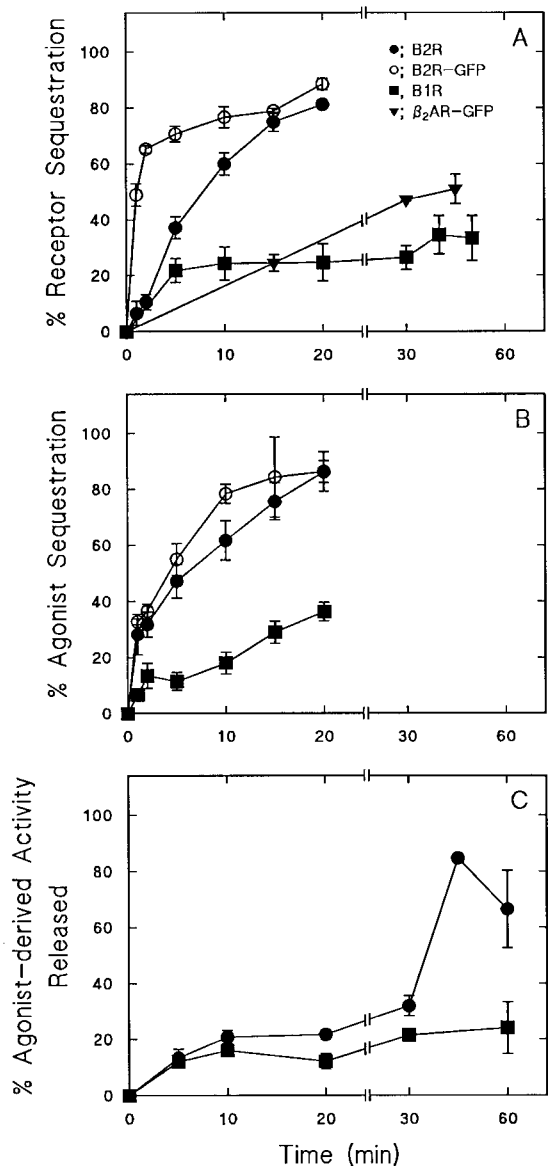

Figure 2 Time courses of receptor sequestration, agonist sequestration and agonist release in HEK293 cells expressing B_2R, B_2R–GFP, B_1R and β_2AR–GFP

(**A**) Cells stably expressing B_2R (●), B_2R–GFP (○) or B_1R (■), or transiently expressing β_2AR–GFP (▼), were assayed for receptor sequestration by measuring the decrease in specific [^{3}H]BK binding (B_2R and B_2R–GFP), des-Arg^{10}-[^{3}H]kallidin binding (B_1R) or [^{3}H]CGP12177 binding (β_2AR–GFP) upon stimulation with 1 μM BK, 1 μM des-Arg^{10}-kallidin or 10 μM isoprenaline respectively. (**B**) Agonist sequestration was determined as the appearance of acid-stable specific [^{3}H]BK binding (B_2R and B_2R–GFP) or des-Arg^{10}-[^{3}H]kallidin binding (B_1R). Symbols are as in (**A**). (**C**) Release of [^{3}H]BK-derived (B_2R) and des-Arg^{10}-[^{3}H]kallidin-derived (B_1R) activity from cells expressing the indicated constructs. Symbols are as in (**A**). Assays were performed as described in the Materials and methods section. The results are means ± S.E.M. of three experiments, with each point assayed in duplicate. Some points have error bars that are smaller than the symbols.

RESULTS

Pharmacological and functional properties of WT and GFP-tagged receptors

Human B_2Rs and B_1Rs were analysed in HEK293 cells expressing the WT receptors and receptors fused at the C-terminus to GFP. Agonist-promoted receptor trafficking was monitored by radioligand binding and by confocal laser-scanning microscopy of GFP fluorescence. WT B_2R and B_2R–GFP were detected using [^{3}H]BK, a B_2-selective agonist, and WT B_1R and B_1R–GFP were detected using des-Arg^{10}-[^{3}H]kallidin, a B_1-selective agonist. GFP fusion to B_2R did not significantly alter the agonist affinity (Figure 1A; Table 1) or the ligand-binding profile (Figure 1A) of the receptor, or receptor function as determined by agonist-stimulated phosphoinositide hydrolysis (Figure 1B). On the other hand, GFP fusion to B_1R significantly decreased the agonist affinity by about 10-fold (Table 1).

Receptor and agonist sequestration

Three methods were used to assess agonist-promoted receptor sequestration. Two methods, the loss of cell surface receptors

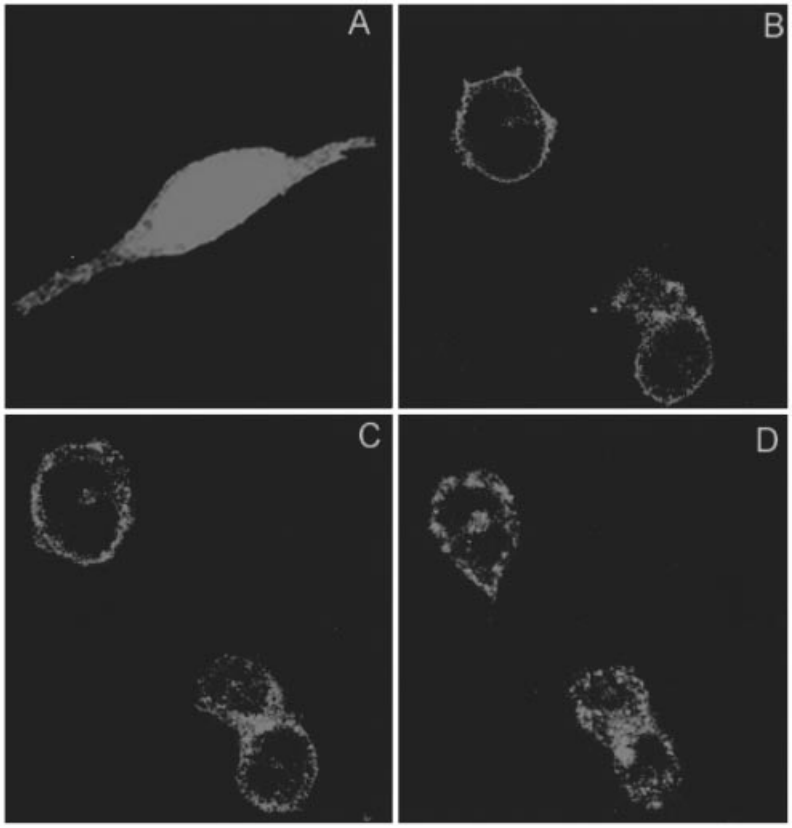

Figure 3 Fluorescence imaging of the agonist-stimulated redistribution of B_2R–GFP in live HEK293 cells

Cells transiently transfected with GFP (**A**) or stable B_2R–GFP cells (**B**–**D**) were grown on glass coverslips. The cells were then stimulated with 1 μM BK, and images were captured at 0 min (**B**), 3 min (**C**) and 15 min (**D**) by a Nikon T-300 confocal microscope and processed with Bio-Rad MicroRadiance Plus software, as described in the Materials and methods section.

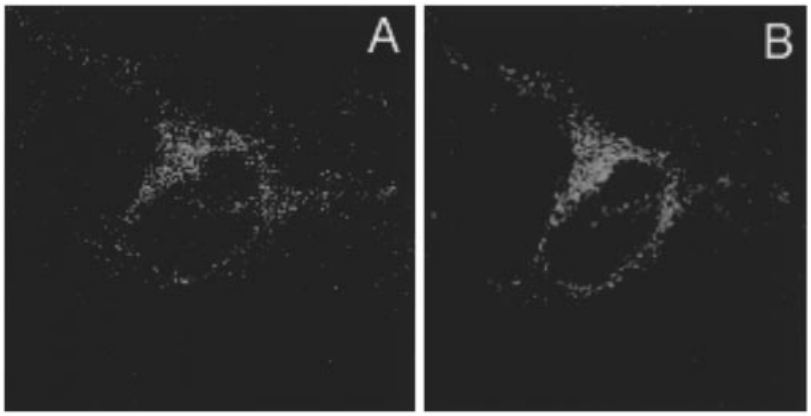

Figure 4 Fluorescence imaging of the agonist-stimulated redistribution of B_1R–GFP in live HEK293 cells

Stable B_1R–GFP cells were grown on glass coverslips. The cells were then stimulated with 1 μM des-Arg^{10}-kallidin and images were captured at 0 min (**A**) and 12 min (**B**) by a Nikon T-300 confocal microscope and processed with Bio-Rad MicroRadiance Plus software, as described in the Materials and methods section.

and confocal microscopy of GFP-tagged receptor fluorescence, monitored specifically the receptor, and one method, the appearance of acid-resistant specific agonist receptor binding, monitored the receptor-bound agonist.

As shown in Figure 2(A), treatment of HEK293 cells stably transfected with B_2R (termed 'stable B_2R cells' and expressing 23.8 ± 1.3 fmol of $B_2R/10^6$ cells) with 1 μM BK at 37 °C resulted in the time-dependent sequestration of the B_2R, as determined by the loss of receptors accessible on the cell surface. The response was rapid ($t_{1/2} \sim 5$ min) and appeared to involve a major portion of the receptors. B_2R sequestration was more or less paralleled by the sequestration of BK, as determined by the appearance of

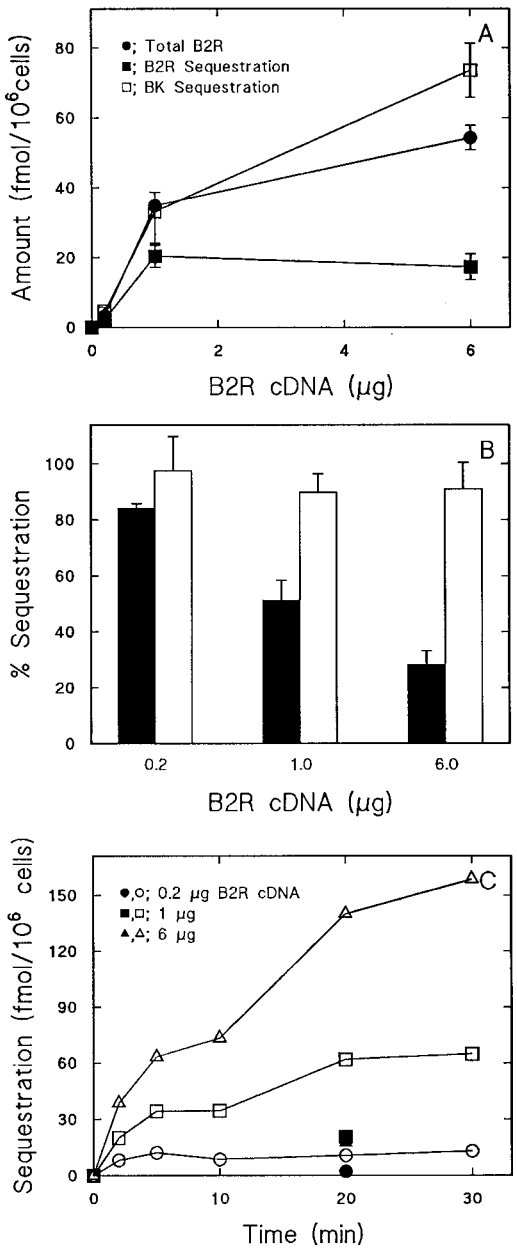

Figure 5 Effect of B_2R density on receptor and BK sequestration in HEK293 cells

HEK293 cells were transiently transfected with 0.2, 1 or 6 μg of receptor cDNA per 35 mm well in 6-well plates. (**A**) The total amount of B_2R expressed, B_2R sequestration and BK sequestration at 20 min were assayed under each condition, as described in the Materials and methods section. The results are means $\pm$ S.E.M. of three experiments, with each point assayed in duplicate. (**B**) The data presented in (**A**) were normalized for the total amount of receptor expressed under each condition, and the results are presented as % sequestration. Closed bars, B_2R sequestration; open bars, BK sequestration. (**C**) Time course of the amount of BK sequestration (open symbols) at the various B_2R levels, measured as described in the Materials and methods section. B_2R sequestration (closed symbols) at 20 min was also determined. The results are representative of at least three experiments, with each point assayed in duplicate.

acid-resistant specific BK binding (Figure 2B). Rapid sequestration of receptor and agonist also occurred in the stable B_2R–GFP cells, which expressed 1.2 ± 0.6 fmol of $B_2R/10^6$ cells. The relative amounts of receptor and agonist sequestration in the stable B_2R–GFP cells were very similar to those in stable B_2R cells. The kinetics of agonist sequestration in the two types of cells were also similar. On the other hand, receptor sequestration in the stable B_2R–GFP cells was significantly faster than that in the cells expressing B_2R.

The sequestration and cellular processing of BK was also apparent from the release of [^{3}H]BK-derived radioactivity from stable B_2R cells. In this case, cells were prelabelled with [^{3}H]BK at 4 °C, washed, and subsequently incubated at 37 °C to allow sequestration of the ligand. As shown in Figure 2(C), the release during the incubation at 37 °C was biphasic. The early phase was half-maximal at $\leqslant 5$ min, included a minor portion ($\sim 20\%$) of the activity originally bound to the receptors, and was presumed to represent rapidly dissociating cell-surface-bound agonist. The late phase appeared at about 30 min and included the major portion ($\sim 65\%$) of the receptor-bound activity, and the source of this activity was presumed to be intracellular, sequestered BK subsequently released from the cells. Furthermore, reverse-phase HPLC revealed that the released BK-derived radioactivity represented primarily intact BK (results not shown).

Confocal laser-scanning microscopy of live stable B_2R–GFP cells was also used to show agonist-promoted B_2R sequestration. As depicted in Figure 3(B), B_2R–GFP was located almost exclusively in the plasma membrane in naïve cells at 37 °C. This pattern is in sharp contrast with the localization of GFP, which is soluble and was located exclusively in the cytosol (Figure 3A). Treatment of B_2R–GFP-expressing cells with 1 μM BK for various times at 37 °C resulted in a time-dependent redistribution of the B_2R–GFP fluorescence (Figures 3C and 3D). The emergence of a punctate pattern of fluorescence in the plasma membrane was already obvious at 3 min of agonist treatment (Figure 3C), and this pattern had broadened at 15 min of treatment (Figure 3D).

Treatment of stable B_1R cells (which expressed 10.0 ± 0.1 fmol of $B_1R/10^6$ cells) with 1 μM des-Arg^{10}-kallidin caused a relatively rapid but very limited sequestration of B_1R, with only about 20% sequestered after 30 min (Figure 2A). The limited loss of B_1R was matched by an apparently stoichiometric sequestration of des-Arg^{10}-[^{3}H]kallidin (Figure 2B). The limited sequestration of B_1R and des-Arg^{10}-[^{3}H]kallidin was also reflected in the limited release of des-Arg^{10}-[^{3}H]kallidin-derived radioactivity from the cells. Indeed, only about 15% of the activity originally bound to the receptor was accounted for in the medium by 60 min (Figure 2C). Receptor and agonist sequestration in the B_1R–GFP-expressing cells was not assessed, primarily because of a decrease in the expression of the construct with time in culture and the relatively high K_d value of des-Arg^{10}-kallidin binding. Nevertheless, confocal laser-scanning microscopy images (Figures 4A and 4B) reveal that B_1R–GFP appeared to be located primarily in the plasma membrane in naïve cells at 37 °C. The impression of a cytoplasmic localization of this construct in the upper left corner of the cell is due to the uneven shape of the cell, which creates more than one level of image of the cell. The distribution of B_1R–GFP did not seem to change upon treatment with 1 μM des-Arg^{10}-kallidin for 12 min.

Effect of receptor density on BK and B_2R sequestration

In order to analyse further the relationship between BK and B_2R sequestration, HEK293 cells were transiently transfected with B_2R cDNA to express different receptor levels. As shown in Figure 5(A), increasing amounts of B_2R cDNA increased the total level of B_2R in the cells. At 0.2 μg of cDNA per 35-mm dish, which resulted in a receptor density of 3.3 ± 0.9 fmol of $B_2R/10^6$

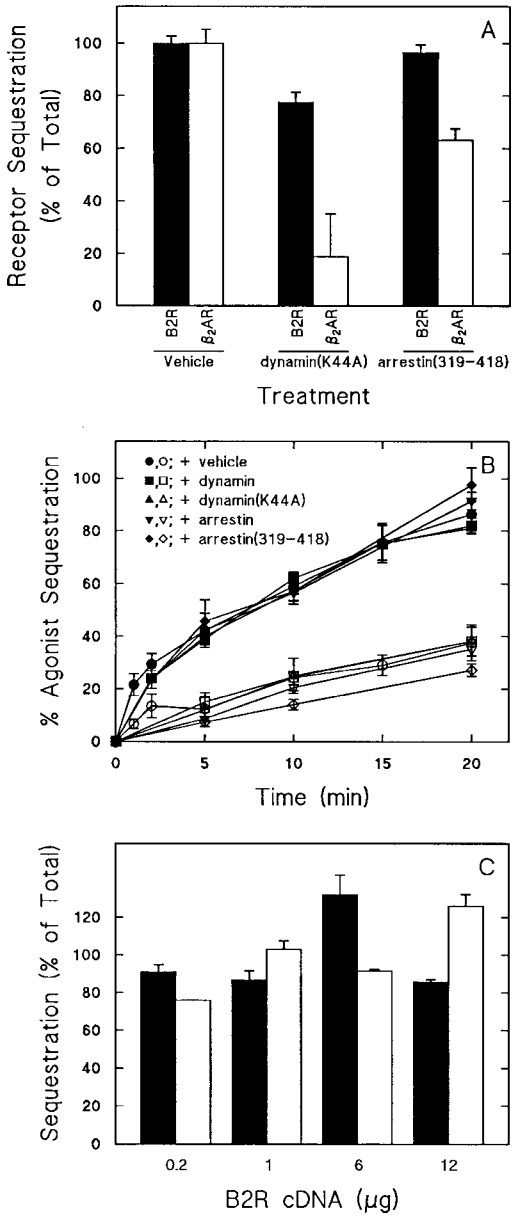

Figure 6 Effects of dominant-negative β-arrestin and dynamin on receptor and agonist sequestration in HEK293 cells

(**A**) Stable B_2R cells (closed bars) or HEK293 cells transiently transfected with β_2AR–GFP (open bars) were co-transfected with either arrestin-(319–418) or K44A dynamin. Sequestration of B_2R and β_2AR–GFP was then determined following stimulation with 1 μM BK for 20 min or with 10 μM isoprenaline for 45 min respectively, as described in the Materials and methods section. The results are presented as % of total, where 100% is the amount of receptor sequestered in the absence of dominant-negative proteins (Vehicle). (**B**) HEK293 cells stably expressing B_2R (filled symbols) or B_1R (open symbols) were co-transfected with WT or dominant-negative mutants of dynamin I or β-arrestin, and agonist sequestration was monitored as described in the Materials and methods section. (**C**) HEK293 cells were transiently co-transfected with 0.2–12 μg of WT B_2R and 3 μg of arrestin-(319–418) in 35-mm wells in six-well plates. The sequestration of agonist (closed bars) and receptor (open bars) was then monitored. The results are means $\pm$ S.E.M. of three experiments, with each point assayed in duplicate.

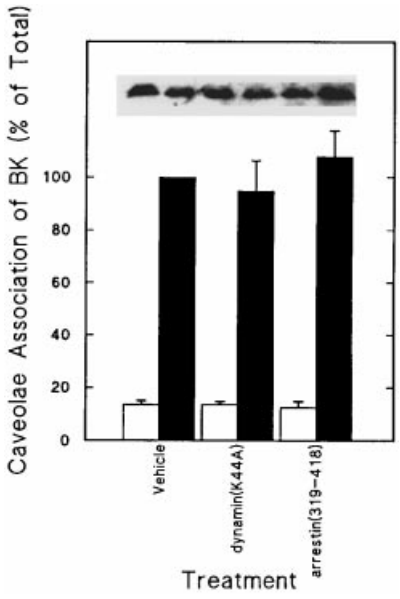

Figure 7 Effects of dominant-negative β-arrestin and dynamin on association of BK with caveolae

Stable B_2R cells were transfected with K44A dynamin or arrestin-(319–418). The cells were incubated with [^{3}H]BK for 1 min (open bars) or 20 min (closed bars). The cells were enriched for caveolae as described in the Materials and methods section, and then solubilized and counted for radioactivity. Results are presented as % of total, where 100% represents the amount of [^{3}H]BK associated with caveolae at 20 min in the absence of dominant-negative proteins (Vehicle). The results are means $\pm$ S.E.M. of three experiments, with each point assayed in duplicate. The inset shows the results under each condition when the caveolae fraction was immunoblotted with antibodies against caveolin-1, as described in the Materials and methods section.

cells, the amount of BK sequestered was almost identical with the amount of receptor sequestered (Figures 5A and 5B). This pattern was very similar to that observed in stable B_2R cells, which expressed 23.8 ± 1.3 fmol of $B_2R/10^6$ cells. As the density of receptors rose to 34.9 ± 3.8 and 54.3 ± 3.5 fmol/10^6 cells following transfection of 1 and 6 μg of cDNA respectively, the amount of BK sequestered also rose. On the other hand, the amount of receptor sequestered did not rise.

The apparently non-stoichiometric sequestration of BK and B_2R was analysed further by performing time courses of BK sequestration at the different receptor levels. Figure 5(C) shows that the amount, as well as the rate, of BK sequestration was directly dependent on the level of receptor expressed. Furthermore, the amount of BK sequestration was significantly higher than that of receptor sequestration. Considering that BK sequestration is receptor-mediated, the higher amount of sequestered BK relative to sequestered B_2R at given receptor densities suggests that the receptor recycles in the process of sequestering BK.

Effects of dominant-negative mutants of dynamin I and β-arrestin on B_2R and BK sequestration, and association of BK with caveolae

To dissect further the mechanism of B_2R and BK sequestration, we used dominant-negative mutants of dynamin I and β-arrestin. K44A dynamin, a dominant-negative mutant of dynamin I, caused a small decrease ($22.4 \pm 3.7\%$) in B_2R sequestration following BK treatment of stable B_2R cells for 20 min (Figure 6A). In contrast, arrestin-(319–418), a dominant-negative mutant of β-arrestin, had no significant effect on this process (Figure

6A). Virtually identical results were obtained in cells transiently co-transfected with B_2R and either K44A dynamin or arrestin-(319–418) (results not shown). Furthermore, BK sequestration was completely insensitive to both of these mutants (Figure 6B), and the lack of an effect of arrestin-(319–418) was completely independent of the receptor level, as determined in cells transiently co-transfected with B_2R (Figure 6C). Figure 6(B) shows that these dominant-negative mutants did not have any significant effect on the limited sequestration of des-Arg^{10}-kallidin in stable B_1R cells; again, the same results were obtained when the B_1R was transiently co-transfected with either of the two mutants (results not shown).

To ensure that the dominant-negative mutants were expressed and functional in HEK293 cells, their effects on the sequestration of a GFP-tagged β_2AR (β_2AR–GFP) were analysed in transiently transfected HEK293 cells. As described previously by Gagnon et al. [36], this receptor is sequestered through clathrin-coated pits in an arrestin- and dynamin-dependent manner. Figure 2(A) shows that, when cells were treated with 10 μM isoprenaline, approx. 50 % of the β_2AR–GFP was sequestered, with a $t_{1/2}$ of $\sim$ 15 min. K44A dynamin inhibited β_2AR–GFP sequestration by 81.2 $\pm$ 16.3 %, and arrestin-(319–418) inhibited sequestration by 36.9 $\pm$ 4.4 % (Figure 6A). Furthermore, immunoblotting revealed that dynamin immunoreactivity was increased in both the WT dynamin- and K44A dynamin-transfected cells (results not shown). Consequently, the dominant-negative mutants were expressed and functional in these cells. Thus BK-promoted B_2R sequestration and B_2R-mediated BK sequestration proceed through a dynamin- and β-arrestin-independent pathway(s) in HEK293 cells.

We and others have shown previously that BK and B_2R associate with caveolae [13,14]. To evaluate the dependency of this process on dynamin I and β-arrestin, we transfected stable B_2R cells with K44A dynamin or arrestin-(319–418) cDNA, exposed cells to [^{3}H]BK for 1 and 20 min, and then enriched for caveolae according to the procedure of Smart et al. [31] as described previously by us [13]. An immunoblot of caveolin-1, a marker protein for caveolae, was also performed on each enriched fraction to determine recovery. Figure 7 shows that neither K44A dynamin nor arrestin-(319–418) had any significant effect on the time-dependent association of BK with caveolae. Thus the B_2R-mediated association of BK with caveolae occurs in a dynamin- and β-arrestin-independent manner.

DISCUSSION

In the present study, we show that the major portion of human B_2Rs expressed in HEK293 cells is sequestered rapidly in response to agonist binding, whereas agonist binding to the human B_1R promotes a very limited amount of sequestration of this receptor. Furthermore, B_2R appears to recycle in the process of sequestering agonist, and the mechanism for this process is independent of dynamin I and β-arrestin and, at least in part, involves caveolae. The limited amount of B_1R sequestration is also independent of these proteins.

The present study is the first to directly image B_2R and B_1R in living cells. Receptor–GFP fusion did not interfere with the pharmacological, functional or regulatory properties of B_2R, whereas fusion of GFP with B_1R decreased agonist affinity slightly. Expression of both B_2R–GFP and B_1R–GFP appeared to be restricted to the plasma membrane in naïve cells. The pattern of expression of B_1R–GFP was not as clear as that of B_2R–GFP, in part due to the lower level of expression of the former construct. As expected, stimulation of B_1R–GFP with des-Arg^{10}-kallidin did not appear to change the cellular distribution pattern of this construct. On the other hand, BK treatment of B_2R–GFP resulted in the rapid formation of a punctate pattern of fluorescence in the cell membrane, and this pattern broadened as agonist exposure increased.

The agonist-promoted loss of accessible cell surface receptors is a common way of monitoring and quantifying GPCR sequestration. GPCRs that bind peptidic ligands provide the alternative, quicker method of monitoring sequestration by assaying the occurrence of acid-stable specific ligand binding. Indeed, this parameter is often used in lieu of the former, as in most cases it is assumed that agonist and receptor are sequestered as a complex. Whereas the agonist and receptor must initially exist as a complex, the relationship between the two during the subsequent steps in the sequestration process has not been rigorously analysed in most systems, and might not coincide.

Analysis of the sequestration of B_2R and BK in cells expressing different B_2R densities revealed that receptor sequestration reaches a steady-state level that is significantly lower than the level of BK sequestration. The amount and the rate of BK sequestration were directly dependent on the amount of B_2Rs in the cell, indicating that BK sequestration is B_2R-specific. Assuming that BK binds to B_2R in a 1:1 stoichiometry, the only explanation for the non-stoichiometric relationship of BK and B_2R sequestration is that, upon continuous BK exposure, the receptor is rapidly recycled in the process of sequestering BK. BK is released from the cell, but this does not occur to any significant extent until beyond 30 min.

Little is known about compartments for sorting of ligands and receptors in the plasma membrane. The resolution of B_2R–GFP fluorescence is clearly below that required to identify such a compartment directly. Receptor-mediated endocytosis through clathrin-coated vesicles is the best described mechanism for the intracellular sorting of ligands and receptors, and is utilized by some GPCRs [1,2]. To address the role of this pathway in the sequestration of BK and B_2R, we determined the sensitivity of these events to the dominant-negative dynamin I mutant, K44A dynamin. Dynamin I is a GTPase that is involved in the pinching off of clathrin-coated vesicles [4]. Dynamin I has also been implicated in the pinching off of caveolae [18,19]. Caveolae can exist in different shapes, from relatively flattened structures to clear invaginations to pinched-off vesicles [37], but the shape and behaviour of caveolae in their putative role of sequestering GPCRs is not known. Consequently, we also used a dominant-negative β-arrestin mutant, arrestin-(319–418) [3]. β-Arrestin is a protein that binds to GPCRs in a receptor-phosphorylation-sensitive manner during homologous desensitization and prevents G-protein coupling [1,2]. In addition, β-arrestin targets at least some GPCRs to clathrin-coated pits. The role of β-arrestin in caveolae targeting has not been evaluated, even though some GPCRs that bind β-arrestin are apparently processed through this compartment [5].

B_2R sequestration in the stable B_2R cells was inhibited by K44A dynamin to a very small extent, whereas BK sequestration was not perturbed by this mutant. Furthermore, the sequestration of neither component was perturbed by arrestin-(319–418), regardless of the B_2R density. In contrast, both mutants drastically inhibited the sequestration of β_2AR. Thus B_2R and BK do not appear to be sequestered through the clathrin-coated pit pathway in HEK293 cells. Furthermore, the limited degradation of the released BK-derived material provided further evidence against a typical receptor-mediated endocytic mechanism in this process. A previous study reported that receptor-mediated BK sequestration in CHO cells stably expressing the human B_2R was partially inhibited by arrestin-(319–418) [38]. Even though the

role of dynamin was not evaluated and no positive GPCR control for the clathrin-coated pit pathway was included in that study, it is possible that the dependency of BK trafficking on β-arrestin varies with the cell system. Interestingly, B_1R sequestration in HEK293 cells was also insensitive to the dominant-negative mutants of dynamin I and β-arrestin. Thus the limited amount of sequestration of this receptor may be proceeding through a pathway(s) similar to that for B_2R in these cells.

We have shown previously that BK associates with caveolae in a B_2R-mediated fashion in DDT_1 MF-2 cells [13]. Continuous exposure of the cells to BK yielded a steady-state level of BK in caveolae, whereas exposure to one round of receptor-bound BK resulted in a transient BK association. Thus BK apparently transits this compartment during its intracellular routing. A subsequent study reported the immunocytochemical localization of B_2R in caveolae-like structures following exposure to BK in A431 cells [14]. That a clathrin-independent compartment, such as caveolae, plays a significant role in BK sequestration in HEK293 cells was shown by the fact that neither total sequestered nor caveolae-associated BK was perturbed by either K44A dynamin or arrestin-(319–418). The small inhibition of total B_2R sequestration by K44A dynamin may indicate that a small portion of the receptor-containing caveolae pinch off during B_2R sequestration. Alternatively, caveolae and clathrin-coated pits may be positioned in sequence in the same sequestration pathway. In other words, following the redistribution and association of B_2R with caveolae, the receptors may subsequently associate with clathrin-coated pits. Evidence for this scenario in receptor trafficking comes from the recent observation that some growth factor receptors that are sequestered via clathrin-coated pits are first present in caveolae [39–41]. Furthermore, clathrin-coated vesicles and caveolae have been proposed to be sequestered in a common endosomal pool [42]. Some GPCRs, including for example the cholecystokinin type A receptor [15] and the β_2AR [43,44], have been identified in both clathrin-coated and -uncoated structures.

In conclusion, the non-stoichiometric relationship between B_2R sequestration and B_2R-mediated BK sequestration in HEK293 cells suggests that the B_2R is recycled rapidly in the process of sequestering BK. The fact that the sequestration of both B_2R and BK is virtually insensitive to K44A dynamin and arrestin-(319–418) indicates that these events proceed though a clathrin-independent pathway, and several recent lines of evidence support a major role for caveolae in this pathway.

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