Inhibitory effects of cytoskeleton disrupting drugs and GDP-locked Rab mutants on bradykinin B_2 receptor cycling

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Abstract

The bradykinin (BK) B_2 receptor (B_2R) is G protein coupled and phosphorylated upon agonist stimulation; its endocytosis and recycling are documented. We assessed the effect of drugs that affect the cytoskeleton on B_2R cycling. These drugs were targeted to tubulin (paclitaxel, or the novel combretastatin A-4 mimetic 3,4,5-trimethoxyphenyl-4-(2-oxoimidazolidin-1-yl)benzenesulfonate [IMZ-602]) and actin (cytochalasin D). Tubulin ligands did not alter agonist-induced receptor endocytosis, as shown using antibodies reactive with myc-tagged B_2Rs (microscopy, cytofluorometry), but rather reduced the progression of the ligand-receptor-β-arrestin complex from the cell periphery to the interior. The 3 fluorescent probes of this complex (B_2R-green fluorescent protein [B_2R-GFP], the fluorescent agonist fluorescein-5-thiocarbamoyl-D-Arg-[Hyp^3, Igl^5, Oic^7, Igl^8]-BK and β-$arrestin_2$-GFP) were condensed in punctuate structures that remained close to the cell surface in the presence of IMZ-602. Cytochalasin D selectively inhibited the recycling of endocytosed B_2R-GFP (B_2R-GFP imaging, [^{3}H]BK binding). Dominant negative (GDP-locked)-Rab5 and -Rab11 reproduced the effects of inhibitors of tubulin and actin, respectively, on the cycling of B_2R-GFP. GDP-locked-Rab4 also inhibited B_2R-GFP recycling to the cell surface. Consistent with the displacement of cargo along specific cytoskeletal elements, Rab5-associated progression of the endocytosed BK B_2R follows microtubules toward their (-)end, while its recycling progresses along actin fibers to the cell surface. However, tubulin ligands do not suppress the tested desensitization or resensitization mechanisms of the B_2R.

Keywords: arrestin; bradykinin; bradykinin B_2 receptors; microtubules; actin; Rab proteins.

1. Introduction

Bradykinin (BK) is a blood-derived nonapeptide that is a prominent cardiovascular regulator and also functions as a mediator of inflammation [1]. The BK B_2 receptor (B_2R) is a G protein-coupled receptor (GPCR) possessing a domain in its C-terminal tail that is phosphorylated by GPCR kinases upon agonist stimulation. Thus, the B_2R combines with either type of non-visual β-arrestins and the ligand-B_2R-β-arrestin complex gets translocated into endosomes [1, 2]. The fusion protein B_2R-green fluorescent protein (B_2R-GFP), β-arrestins conjugated to fluorescent proteins and BK sequences that were extended with fluorophores were instrumental in modeling the BK-induced endocytosis, but also its recycling to the cell surface and the persistence of the ternary agonist-receptor-β-arrestin complexes in intracellular structures [3-5] (and literature cited herein). Fluorescent BK analogs are rapidly transported by the B_2R into Rab5- and early endosome autoantigen 1 (EEA1)-positive early endosomes and are later sorted in Rab7-positive late endosomes [3, 4]. However, the BK-stimulated B_2Rs are essentially completely recycled to the cell surface as a function of time, unless the agonist has an altered structure that confers resistance to proteolytic breakdown. In this case, the length of the B_2R internalization and the duration of signaling are remarkably prolonged, and the secondary down-regulation of the receptors becomes prominent [2, 5].

Rab proteins are a family of small GTPases that organize vesicular trafficking in the cells by interacting with multiple molecular partners [6]. Rab5 and EEA1 are believed to be required for the transition of endosomes from the cell surface to the depth of the cytosolic space by traveling along the microtubules toward their minus end (that opposed to the cell surface) [6]. Since the agonist-stimulated B_2R is colocalized with Rab5 and EEA1, its inward movement is predicted to proceed

along microtubules. On the other hand, receptors sorted for recycling could travel along with Rab11 (as shown for the cannabinoid CB_2 receptor and β_2-adrenoceptors) [7, 8] along the actin cytoskeleton [6]. Thus, the rapid recycling of the interleukin-8 receptors CXCR1 and CXCR2 and that of β_2-adrenoceptors are inhibited by cytochalasin D, an actin filament-disrupting drug [8, 9]. Rab4 has also a role in the recycling of internalized cargo to the cell surface (transferrin receptor; β_2-adrenoceptor) [10, 11]. Whether this general model applies to the BK B_2R has been tested by exploiting, along with cytochalasin D, ligands of microtubules that act in complementary manner: paclitaxel (Taxol), that stabilizes microtubules, and a novel combretastatin A-4 mimetic that depolymerizes microtubules. The latter compound, IMZ-602, is a high potency representative of a family of drugs selectively targeted at the colchicine-binding site of tubulin [12]. Cytoskeleton-binding agents have been used mainly along visualization tools at specific times during BK-induced receptor cycling to isolate their effects on the endocytosis, progression and recycling.

2. Materials and methods

2.1. Drugs

The synthesis and properties of the tubulin-binding drug 3,4,5-trimethoxyphenyl-4-(2-oxoimidazolidin-1-yl)benzenesulfonate have been reported by Fortin et al. [12]. This compound (**45** in the original publication) will be designated by the code IMZ-602. This drug inhibited the growth of tumor-derived cell lines with IC_{50} values below 10 nM and morphologically disrupted the tubulin cytoskeleton at 4 nM (immunofluorescence of β-tubulin in M21 cells) [12]. FTC-B-9972 (fluorescein-5-thiocarbamoyl-D-Arg-[Hyp^3,Igl^5,Oic^7,Igl^8]-BK) is a previously characterized fluorescent B_2R agonist that is resistant to endosomal breakdown [4]. Paclitaxel and cytochalasin D were purchased from Sigma-Aldrich (St. Louis, MO) and BK, from Bachem (Torrance, CA). [^{3}H]BK was from Perkin Elmer Life Sciences (90 Ci/mmol).

2.2. Cell culture, transfection and microscopy

HEK 293a cells, obtained, maintained and transfected as described [3], were used for transient expression of recombinant proteins. A HEK 293 cell line stably expressing B_2R-GFP [13] was used as such for microscopic observations or immunoblotting of proteins in cell extracts; occasionally these cells were transiently transfected with an additional expression vector. DNA expression vectors coding for a number of proteins were exploited: myc-tagged rabbit B_2R (myc-B_2R), β-arrestin$_2$–GFP fusion protein in pcDNA3 (gift of Dr. Michel Bouvier, Université de Montréal) [15], C_1-Cherry fluorescent protein, dominant negative Rab5-GDP-locked-Cherry (S34N mutant of Genbank sequence M35520), Rab4-GDP-locked-Cherry (S22N mutant of Genbank sequence X56389) and Rab11-GDP-locked-Cherry (S25N mutant of Genbank sequence X56388) (graciously

given by Dr. Michel J. Tremblay, Centre de recherche du CHUQ, Québec, Canada). The 3 latter vectors had been prepared by recloning the insert from the GFP-conjugated constructions into the appropriate vector.

Cells were generally observed in epifluorescence microscopy at a 1000× magnification, at which the focal depth is limited in these relatively thick cells, and photographed using an Olympus BX51 microscope coupled to a CoolSnap HQ digital camera (filters for GFP and fluorescein: excitation 460-500 nm, emission 510-560 nm; for Cherry fluorescent protein: excitation 525-555 nm, emission 600-660). The objective lens was generally the 100× oil UPlanApo (Olympus). Most experiments were based on BK stimulation of different durations to monitor either the endocytosis of the B_2Rs (30 min) or their recycling (3 h), based on previous time course findings [2, 14] (up to 10 min of observation at room temperature may have been added to these treatment durations before capturing images, as in previous studies). B_2Rs possessing an N-terminal myc tag support the construction of immune complexes at the surface of intact cells and transport anti-myc antibodies into endosomes when stimulated with BK [16]. myc-B_2R was used in experiments designed to assess whether anti-tubulin drugs inhibit the agonist-induced internalization of the ligand-receptor complex. After drug treatment and BK stimulation, adherent and intact cells were incubated for 15 min at room temperature with anti-myc monoclonal antibodies (clone 4A6) conjugated with AlexaFluor-488 (Millipore, dilution $1{:}1000 \approx 3.3$ nM), then rinsed × 3 with phosphate buffered saline and observed for the exclusion of the antibody labeling (microscopy as described above or cytofluorometry in cells detached with the protease-free Cell Dissociation Buffer (Invitrogen): BD SORP LSR II cell analyzer, BD Biociences, Franklin Lakes, NJ; green fluorescence results analyzed using the BD FACS DIVA software).

2.3. Radioligand binding assay

To corroborate the effect of cytochalasin D on BK-induced cycling of the cell surface B_2Rs, a binding assay to recombinant B_2R-GFP stably expressed in HEK 293 cells was performed as described [2] using a [^{3}H]BK concentration (3 nM) superior to the radioligand K_D (2 nM). The cells had been pretreated with unlabeled BK with or without cytochalasin D prior to the binding assay.

2.4. Data analysis

The subcellular distribution of elements of the agonist-B_2R-β-arrestin complex was assigned to one of the two following categories: either peripheral (labeling at the plasma membrane or close to it, within vesicles possibly attached or juxtaposed to the plasma membrane) or non-peripheral, the latter being the typical B_2R distribution 30 min after agonist stimulation. These qualitative groupings, illustrated in Fig. 1, allowed the use of χ^2 statistics (comparison of frequencies) to determine drug effect on subcellular distributions of labeled molecules in large records of microphotographs. Other numerical values are reported as means ± SEM. ANOVA followed by the Tukey-Kramer multiple comparison test were used to analyze radioligand binding data.

3. Results

3.1. Microscopic study of the endocytosis process

HEK 293 cells that stably express B_2R-GFP exhibit a sharply defined fluorescence associated with the plasma membrane in more than 80% of evaluated cells (Fig. 2). This proportion was not significantly modified by cell pretreatment with the tubulin ligands of two different types, the stabilizing agent paclitaxel (1 µM) or the depolymerizing drug IMZ-602 (100 nM). However, the actin filament disrupting drug cytochalasin D (7.9 µM) slightly decreased the proportion of cells evaluated as possessing a predominantly peripheral fluorescence, defined as that located near the cell surface (whether or not coincident with the plasma membrane). Cell treatment with the agonist BK translocated the receptor-associated fluorescence to more or less defined intracellular structures, often very small and not well resolved, that were often located well within the cells, thus greatly reducing the proportion of cells possessing a peripheral fluorescence. This was not changed by co-treatment with cytochalasin D, but the intracellular translocation of condensed and relatively large structures containing B_2R-GFP from the cell surface was inhibited by either paclitaxel or IMZ-602 (Fig. 2), thus significantly increasing the proportion of cells exhibiting peripheral fluorescence relatively to cells treated with BK alone. On the basis of these results, the integrity of the tubulin cytoskeleton seems to be necessary for the intracellular progression of the structures containing agonist-stimulated B_2Rs.

Fluorescent arrestins constructions in cells that co-express B_2Rs rapidly condense in intracellular endosomes rather than at the plasma membrane (a so-called type B pattern) [17]. HEK 293a cells that transiently co-expressed the fusion protein β-arrestin$_2$-GFP and the non-fluorescent construction myc-B_2R were stimulated with BK, reproducing the known condensation of cytosolic fluorescence

into polymorphic endosomal structures located anywhere in the cytosol (thus few cells exhibited a peripheral distribution of endosomal structures, Fig. 3). However, pretreatment with IMZ-602 limited the migration of structures containing condensed β-arrestin$_2$-GFP from the cell surface, thus significantly increasing the proportion of cells exhibiting a peripheral distribution of fluorescent organelles that were still polymorphic, but often quite large (Fig. 3). The third element of the internalized complex organized around the B_2R is the agonist ligand. FTC-B-9972, a recently described fluorescent B_2R agonist resistant to endosomal breakdown [4], was exploited in HEK 293a cells that transiently expressed myc-B_2R (Fig. 4). Cells stimulated with FTC-B-9972 contained more or less defined fluorescent endosomal structures that could be very small and look like a cytosolic "haze" (not unlike the appearance of cells treated with fluorescent transferrin [18]) and the endosomal distribution was rarely predominantly peripheral (Fig. 4). However, in cells pretreated with IMZ-602, the peripheral distribution of fluorescent organelles was again sharply increased. Therefore, the intracellular translocation of all elements of the ligand-receptor-β-arrestin triad is inhibited by tubulin depolymerization.

An N-terminally myc-tagged B_2R (myc-B_2R) can be labeled in intact cells using anti-myc monoclonal antibodies and the receptor stimulated with BK transports these antibodies into endosomal structures [16]. A variant of this system was exploited to determine whether the pharmacologic ligands of tubulin block the endocytosis of the B_2R by using an AlexaFluor488-conjugated anti-myc monoclonal antibody (clone 4A6) to probe receptor presence at the surface of intact cells after agonist stimulation. Resting myc-B_2Rs are detected by the fluorescent antibody at the cell surface whether or not they had been pretreated with paclitaxel or IMZ-602 (Fig. 5, microscopy corroborated with cytofluorometry). BK treatment (100 nM, 30 min, 37°C, followed by 15 min incubation with the antibody at room temperature) importantly reduced the cell surface

labeling, implying that cell surface receptors were massively subjected to endocytosis into a compartment not accessible to extracellular antibodies. Pretreatment with either tubulin ligand did not inhibit BK-induced endocytosis of myc-B_2R, based on the equally weak cell labeling post-stimulation (Fig. 5). Control experiments based on non-transfected cells showed a low intensity of surface fluorescence that was not influenced by BK treatment and that includes cell autofluorescence and non-specific binding of the anti-myc antibodies (Fig. 5). The disruption of the tubulin cytoskeleton does not inhibit agonist-induced B_2R endocytosis, on the basis of this series of experiments.

3.2. Effect of cytochalasin D on the recycling process

The recycling of B_2R-GFP is conveniently assessed 3 h after stimulation with BK, a fragile peptide that is rapidly cleared by different pathways in both the serum-containing culture medium and in endosomes [5] (Fig. 6, top row of microphotographs). Previous studies on the B_2R-GFP model have shown that the radioactivity and the immunoreactive receptor are nearly back to background in the HEK 293 cell fraction that contains endosomes 3 h after stimulation with 10 nM [^{3}H]BK, whereas shorter incubation periods (0.25-1 h) were associated with variable quantities of internalized receptor and ligand [18]. Thus, the majority of cells exhibit a predominantly peripheral fluorescence, and after 3 h of BK stimulation, most are back to this type of distribution (with a slight but significant difference). The drugs that affect the cytoskeleton were applied in other cells 30 min after BK, to allow the full endocytosis of the receptor construction and isolate effects on recycling. These drugs alone had no effect or a mild significant effect (IMZ-602, cytochalasin D) on the fluorescence distribution of cells that were not stimulated with BK. Only cytochalasin D retarded the clearance of cytosolic endosomal structures, when tested in this manner (Fig. 6; quantified as a very low frequency of cells with predominantly peripheral fluorescence). The effect of cytochalasin D co-

treatment on B_2R-GFP recycling was corroborated using a [^{3}H]BK binding assay to measure the cell surface receptors (Fig. 7). A 3-h pretreatment with unlabeled BK (100 nM, in culture medium) did not reduce the expression of cell surface receptors, consistent with morphological recycling of the receptors and with the short half-life (<10 min) of the peptide in the serum-containing culture medium [18]. In cells co-treated with cytochalasin D 30 min after BK (a period allowed for agonist-induced internalization), the binding site recovery in 3 h was not complete. Both experimental approaches (morphologic, radioligand binding) support that functional actin inhibition inhibits at least in part the recycling of endocytosed B_2Rs to the plasma membrane.

3.3. GDP-locked Rab proteins reproduce the effects of drugs active on the cytoskeleton on the cycling of B_2R-GFP

HEK 293 cells that stably expressed B_2R-GFP were further transiently transfected with one of four vectors coding for red fluorescent proteins: Cherry or Cherry-conjugated dominant negative (GDP-locked) Rab5, Rab4 or Rab11 (Fig. 8). Only cells exhibiting the two types of fluorescence, green and red, were considered in the evaluation of the proportion of cells with peripheral distribution of the receptor. While the cycling of B_2R-GFP was not altered by co-expression of the Cherry fluorescent protein, Rab5-GDP-locked-Cherry significantly abated the early (30 min) translocation of B_2R-GFP from the periphery to the cell center (Fig. 8). Three h post-BK stimulation, fluorescent receptors returned to a smooth distribution at the level of the plasma membrane. In contrast, either Rab11-GDP-locked-Cherry or Rab4-GDP-locked-Cherry reproduced the effect of cytochalasin D, selectively preventing the recycling of receptors to the cell surface, as observed 3 h after cell stimulation with BK. Thus, the effect of dominant negative Rab5 reproduced that of tubulin ligands on B_2R cycling, inhibiting the progression of the cargo from the plasma membrane but not its

recycling, while the 2 other GDP-locked Rab constructions did not affect the internalization, but inhibited selectively B_2R recycling.

4. Discussion

We investigated the role of cytoskeletal elements on the cycling of the BK B_2Rs using mainly pharmacological and morphological approaches. Internalized B_2R-GFP is colocalized with transferrin, but not low-density lipoprotein, in HEK 293 cells stimulated with BK [18], suggesting that this model receptor molecule is confined to recycling endosomes under these conditions. Further, expression of GDP-locked Rab5 reportedly inhibited transferrin endocytosis [19] and GTP-locked Rab5, on the contrary, induced the formation of giant cell vesicles where transferrin or a fluorescent BK analog accumulate [3, 19]. Previous studies of intact cells that took advantage of fluorophore-labeled molecules have established the endosomal colocalization of each pair of molecules in the agonist-B_2R-β-arrestin triad [2-4]. Consistent with current models of Rab GTPase-mediated trafficking of vesicles [6], we hypothesized that the Rab5-mediated progression of the BK-B_2R-β-arrestin complex into the cytosol to the perinuclear region where endosomes are found would proceed toward the (-) end of microtubules.

The tubulin ligands, either depolymerizing (IMZ-602) or stabilizing (paclitaxel), exerted a similar effect that can be characterized as the inhibition of the progression of the ternary complex from the cell surface, but not necessarily the inhibition of endocytosis itself since endosomal structures containing any of the 3 members of the triad (the ligand, the receptor or the arrestin) were observed just below the plasma membrane surface (Figs. 2-4). An antibody-based approach confirmed that IMZ-602 or paclitaxel pretreatment did not prevent BK-induced clearing of most cell surface myc-B_2Rs (Fig. 5). The alternate tubulin ligand nocodazole failed to inhibit the interleukin-8-induced endocytosis of CXCR1 and -2 in another study [9], consistent with current findings, that therefore limit the role of microtubules in GPCR cycling to intracellular progression. Overexpression of the

dominant negative (GDP-locked) version of Rab 5 fully reproduced the effect of tubulin ligands on B_2R-GFP cycling (Fig. 8), in line with the model outlined above. In a previous study, the morphological effect of GDP-locked Rab5 on activated β_2-adrenoceptors was similar to that observed with B_2R-GFP: receptor-containing vesicles were said to remain in either close juxtaposition or attached to plasma membrane [11]. However, the receptors remained accessible to extracellular antibodies in that study, which may be a subtle differential effect of dominant negative Rab5 *vs.* that of tubulin ligands.

The recycling of endocytosed cannabinoid CB_2 receptors or β_2-adrenoceptors has been shown to be dependent on Rab11 [7, 8]. GDP-locked Rab11 also prevents the recycling of vasopressin V_2, somatostatin 3 and CXCR2 [20-22]. The actin cytoskeleton is currently considered as the substrate for this form of vesicular translocation [6]. Thus, cytochalasin D inhibited the recycling of agonist-stimulated CXCR1 and -2 and of α_{1B}-adrenoceptors [9, 23]. The striking effect of cytochalasin D as an inhibitor of B_2R-GFP recycling 3 h post-agonist stimulation indicates that the B_2R conforms to this model, based on microscopy and radioligand binding (Figs. 5, 7). The slightly increased cytosolic distribution of B_2R-GFP under the effect of cytochalasin D applied alone (significant in Figs. 2, 6) is an unexpected effect of the actin inhibitor that may result from the interference with a small intensity of agonist-independent cycling of the receptors. Overexpression of the dominant negative Rab11 reproduced the morphological effect of cytochalasin D on B_2R-GFP cycling, as did Rab4-GDP-locked-Cherry (Fig. 8). The activated mutant form Rab4-GTP-locked Cherry colocalized with B_2R-GFP in endosomal structures of BK-stimulated cells (30 min, data not shown), supporting that the Rab5 to Rab4 labeling transition of the endosomes containing transferrin receptors may apply to the B_2R [10]. Dominant negative Rab4 reportedly inhibited the resensitization of the dephosphorylated β_2-adrenoceptors by blocking receptor recycling to the cell surface [11].

Cytoskeletal elements that support Rab4-mediated cargo translocation are not defined as well as those related to Rab11 or Rab5.

Occasional discrepancies were observed in exploited models of B_2R endocytosis/recycling. Firstly, BK-induced internalization of the transiently expressed myc-B_2R is apparently more complete than that of stably expressed B_2R-GFP, based on the residual fluorescence apparently coincident with plasma membranes in BK-treated cells, whether or not co-treated with tubulin ligands (Fig. 2). This discrepancy is probably related to the ~6-fold higher expression of B_2R-GFP relative to that of myc-B_2R, as measured with [^{3}H]BK binding [14]; thus the whole population of B_2R-GFP may not be mobilized by the agonist. Secondly, cytochalasin D apparently reduced less the recycling of B_2R-GFP when measured with [^{3}H]BK (-44%, Fig. 7) than with the frequency of peripheral fluorescent labeling (-62%, Fig. 2). The morphological category may be oversensitive to the cytosolic retention of a fraction of fluorescent endosomes, thus providing a somewhat amplified assessment.

Tubulin ligands do not inhibit BK-induced B_2R internalization (Fig. 5) or association of the B_2R to β-arrestin (Fig. 3). Further, B_2R recycling to the cell surface is not altered by these drugs (Fig. 6). Therefore, no major effect of tubulin inhibitors on B_2R signaling is predicted as desensitization and resentitization mechanisms remain largely intact in cells treated with IMZ-602 or paclitaxel. This has been verified in 2 systems: BK-induced c-Fos expression in HEK 293 cells that express B_2R-GFP over 1-3 h and the BK-induced contractility of the freshly isolated human umbilical vein (methods as in [2, 14], data not shown). These systems are not affected, or only slightly potentiated by tubulin ligands (slight potentiation of BK EC_{50} values in the second case) and are concerned with a high- and low-density of cell surface B_2Rs, and slow *vs.* rapid kinetics, respectively. Whether tubulin-binding drugs would inhibit the down-regulation (degradation) of B_2Rs induced by

degradation-resistant agonists over 12 h [2, 5] is not readily testable because the employed tubulin ligands, developed as anti-cancer agents [12], are cytotoxic. Thus, the present study was limited to a pre-apoptotic time window. Further, the depolymerizing agent IMZ-602 and the microtubule-stabilizing drug paclitaxel exerted similar effects on all accounts when both were tested in parallel, a finding supporting that dynamic microtubule remodeling is necessary for cargo progression mediated by Rab5.

In summary, consistent with the displacement of cargo along specific cytoskeletal elements, Rab5-associated progression of the endocytosed BK B_2Rs follows microtubule toward their (-) end, while its Rab11-mediated recycling may progress along actin fibers to the cell surface (see Fig. 9, schematic representation). Based on the effect of the dominant negative constructions, Rab4 also seems to exert a role in the recycling. While similar approaches have been applied to other GPCRs, they have never been applied before to the BK B_2R. However, tubulin ligands do not suppress the tested desensitization or resensitization mechanisms of the B_2R.

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References

[1] Leeb-Lundberg LMF, Marceau F, Müller-Esterl W, Pettibone DJ, Zuraw BL. International Union of Pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. Pharmacol Rev 2005; 57: 27-77.

[2] Bawolak MT, Fortin S, Bouthillier J, Adam A, Gera L, C-Gaudreault R, Marceau F. Effects of inactivation-resistant agonists on the signalling, desensitization and down-regulation of bradykinin B_2 receptors. Br J Pharmacol 2009; 158: 1375-86.

[3] Gera L, Bawolak MT, Roy C, Lodge R, Marceau F. Design of fluorescent bradykinin analogs: application to imaging of B_2 receptor-mediated agonist endocytosis and trafficking and of angiotensin converting enzyme. J Pharmacol Exp Ther 2011; 337: 33-41.

[4] Gera L, Roy C, Bawolak MT, Charest-Morin X, Marceau F. N-terminal extended conjugates of the agonists and antagonists of both bradykinin receptor subtypes: structure-activity relationship, cell imaging using ligands conjugated with fluorophores and prospect for functionally active cargoes. Peptides 2012; 34: 433-46.

[5] Bawolak MT, Roy C, Gera L, Marceau F. Prolonged signalling and trafficking of the bradykinin B_2 receptor stimulated with the amphibian peptide maximakinin: insight into the endosomal inactivation of kinins. Pharmacol Res 2012; 65: 247-53.

[6] Grosshans BL, Ortiz D, Novick P. Rabs and their effectors: achieving specificity in membrane traffic. Proc Natl Acad Sci USA 2006; 103: 11821-7.

[7] Grimsey NL, Goodfellow CE, Dragunow M, Glass M. Cannabinoid receptor 2 undergoes Rab5-mediated internalization and recycles via a Rab11-dependent pathway. Biochim Biophys Acta 2011; 1813: 1554-60.

[8] Millman EE, Zhang H, Zhang H, Godines V, Bean AJ, Knoll BJ, Moore RH. Rapid recycling of β_2-adrenergic receptors is dependent on the actin cytoskeleton and myosin Vb. Traffic 2008; 9: 1958-71.

[9] Zaslaver A, Feniger-Barish R, Ben-Baruch A. Actin filaments are involved in the regulation of trafficking of two closely related chemokine receptors, CXCR1 and CXCR2. J Immunol 2001; 166: 1272-84.

[10] Sönnichsen B, De Renzis S, Nielsen E, Rietdorf J, Zerial M. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol 2000; 149: 901-14.

[11] Seachrist JL, Anborgh PH, Ferguson SSG. β2-adrenergic receptor internalization, endosomal sorting, and plasma membrane recycling are regulated by Rab GTPases. J Biol Chem 2000; 275: 27221–8.

[12] Fortin S, Wei L, Moreau E, Lacroix J, Côté MF, Petitclerc E, Kotra LP, C-Gaudreault R. Design, synthesis, biological evaluation, and structure-activity relationships of substituted phenyl 4-(2-oxoimidazolidin-1-yl)benzenesulfonates as new tubulin inhibitors mimicking combretastatin A-4. J Med Chem 2011; 54: 4559-80.

[13] Houle S, Larrivée JF, Bachvarova M, Bouthillier J, Bachvarov DR, Marceau F. Antagonist-induced intracellular sequestration of rabbit bradykinin B_2 receptor. Hypertension 2000; 35: 1319-25.

[14] Bawolak MT, Gera L, Morissette G, Stewart JM, Marceau F. B-9972 (D-Arg-[Hyp^3,Igl^5,Oic^7,Igl^8]-bradykinin) is an inactivation-resistant agonist of the bradykinin B_2 receptor derived from the peptide antagonist B-9430 (D-Arg-[Hyp^3,Igl^5,D-Igl^7,Oic^8]-bradykinin): pharmacologic profile and effective induction of receptor degradation. J Pharmacol Exp Ther 2007; 323: 534-46.

[15] Bernier V, Lagacé M, Lonergan M, Arthus MF, Bichet DG, Bouvier M. Functional rescue of the constitutively internalized V2 vasopressin receptor mutant R137H by the pharmacological chaperone action of SR49059. Mol Endocrinol 2007; 18: 2074-84.

[16] Bawolak MT, Lodge R, Morissette G, Marceau F. Bradykinin B_2 receptor-mediated transport into intact cells: anti-receptor antibody-based cargoes. Eur J Pharmacol 2011; 668: 107-14.

[17] Hamdan FF, Rochdi MD, Breton B, Fessart D, Michaud DE, Charest PG, Laporte SA, Bouvier M. Unraveling G protein-coupled receptor endocytosis pathways using real-time monitoring of agonist-promoted interaction between β-arrestins and AP-2. J Biol Chem 2007; 282: 29089-100.

[18] Bachvarov DR, Houle S, Bachvarova M, Bouthillier J, Adam A, Marceau F. Bradykinin B_2 receptor endocytosis, recycling, and down-regulation assessed using green fluorescent protein conjugates. J Pharmacol Exp Ther 2001; 297: 19-26.

[19] Stenmark H, Parton RG, Steele-Mortimer O, Luetcke A, Gruenberg J, Zerial M. Inhibition of Rab5 GTPase activity stimulates membrane fusion in endocytosis. EMBO J 1994; 13: 1287–96

[20] Innamorati G, Le Gouill C, Balamotis M, Birnbaumer M. The long and the short cycle: alternative intracellular routes for trafficking of G-protein-coupled receptors. J Biol Chem 2001; 276: 13096–103.

[21] Kreuzer OJ, Krisch B, Dery O, Bunnett NW, Meyerhof W. Agonist-mediated endocytosis of rat somatostatin receptor subtype 3 involves β-arrestin and clathrin-coated vesicles. J Neuroendocrinol 2001; 13: 279–287.

[22] Fan GH, Lapierre LA, Goldenring JR, Richmond A. Differential regulation of CXCR2 trafficking by Rab GTPases. Blood 2003; 101: 2115–24.

[23] Stanasila L, Abuin L, Diviani D, Cotecchia S. Ezrin directly interacts with the α1b-adrenergic receptor and plays a role in receptor recycling. J Biol Chem 2006; 281: 4354-63.

Legends for figures

Fig. 1. Analysis of the fluorescence distribution in intact HEK 293 cells. Examples of predominantly peripheral distributions are shown. These particular cells expressed B_2R-GFP. Epifluorescence, original magnification 1000×.

Fig. 2. Effect of drugs on BK-induced endocytosis of B_2R-GFP in HEK 293 cells stably expressing this fusion protein. Green epifluorescence (original magnification 1000×), with duplicate typical fields shown for agonist-treated cells. The cells were counted in two categories: those with predominantly peripheral fluorescence (whether or not condensed in structures near the cell surface) as opposed to those with a predominantly intracellular labeling, with variable discontinuity in the plasma membrane labeling. Histograms at the right represent the proportion of cells with a peripheral labeling, the effect of BK (100 nM, 30 min, grey histograms) being of decreasing this proportion. Numbers at the right of histograms represent the numbers of cells evaluated during 5 days of experiment. χ^2 statistics: * $P<0.01$, ** $P<10^{-4}$ *vs.* the control cells treated with drug vehicle (top histogram); † $P<10^{-4}$ *vs.* vehicle and BK-treated cells (2^{nd} histogram from the top).

Fig. 3. Effect of IMZ-602 on the BK-induced condensation of β-arrestin$_2$-GFP in HEK 293a cells transiently co-expressing this fusion protein and myc-B_2R. Green epifluorescence (original magnification 1000×), with duplicate typical fields shown for agonist-treated cells. The proportion of cells with peripheral localization of the endosomal structures is represented to the right for BK-treated cells (grey bars); these structures were very rare in unstimulated cells (numerical data not shown). Numbers at the right of histograms represent the numbers of cells evaluated during 2 days

of experiment. χ^2 statistics for the effect of IMZ-602 on BK-induced localization of condensed fluorescent structures: * $P<10^{-4}$.

Fig. 4. Effect of IMZ-602 on the subcellular distribution of a fluorescent B_2R agonist, FTC-B-9972, in HEK 293a cells that expressed myc-B_2R. Presentation as in Fig. 3. * $P<0.001$.

Fig. 5. Lack of effect of tubulin ligands IMZ-602 and paclitaxel on the BK-induced clearance of myc-B_2R from the HEK 293a cell surface, as assessed by fluorescent anti-myc antibodies applied after cell stimulation (15 min incubation at room temperature). Non-transfected cells were used as control for cell autofluorescence and/or non-specific binding of the antibodies. Microscopic images are representative results of 2 days of experiments and are corroborated with cytofluorometry of 10,000 cells (fluorescence distribution shown side by side). Histograms at the right represent the median cell fluorescence in duplicated cytofluorometry experiments.

Fig. 6. Effect of drugs (applied at -2.5 h) on recycling of B_2R-GFP to the cell surface in HEK 293 cells stably expressing this fusion protein and previously stimulated with BK (100 nM, - 3 h). Presentation as in Fig. 2. Numbers at the right of histograms represent the numbers of cells evaluated during 2 days of experiment. The effect of BK was evaluated using χ^2 statistics relative to the appropriate controls with the same drug pretreatment (* $P<0.05$; ** $P<10^{-4}$).

Fig. 7. Binding of [^{3}H]BK (3 nM) to HEK 293 cells stably expressing B_2R-GFP as affected by pretreatment (37°C, in culture medium) with unlabeled BK (3 h before the assay) with or without co-treatment with cytochalasin D (applied 2.5 h before the binding assay). Values are the means ±

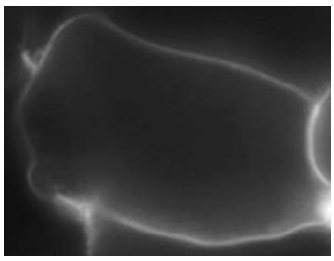
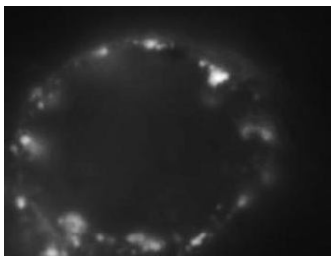
S.E.M. of 5 experiments composed of duplicate determinations. ANOVA indicated that the four groups were heterogeneous ($P < 0.001$). Further comparison with the Tukey-Kramer multiple comparison test were applied to selected pairs of value as indicated by brackets.

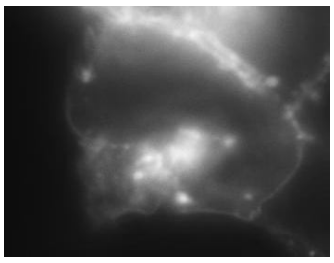
Fig. 8. Transiently expressed GDP-locked Rab proteins tagged with the Cherry fluorescent protein recapitulate the effects of drugs active on the cytoskeleton on the cycling of B_2R-GFP (stably expressed in HEK 293 cells). Control cells were transfected with Cherry. Only cells exhibiting the two transgenes, based on fluorescence colors, were evaluated. Presentation as in Fig. 2, with the addition of color-coded histograms for BK treatments. The effect of each Rab construction was evaluated using χ^2 statistics relative to the appropriate condition of BK treatment in cells transfected with Cherry (* $P<10^{-4}$). The 3-h time point was not studied in cells that expressed Rab5-Cherry. Numbers at the right of histograms represent the numbers of cells evaluated during up to 4 days of experiment.

Fig. 9. Schematic representation of the role of cytoskeletal elements and Rab propteins in BK B_2R cycling. The marker 1 designates a state of endocytosis of the receptor-ligand-β-arrestin complex proximal to the plasma membrane but inaccessible to an extracellular antibody; marker 2 is next to an endosome that has migrated inward.

Figure 1

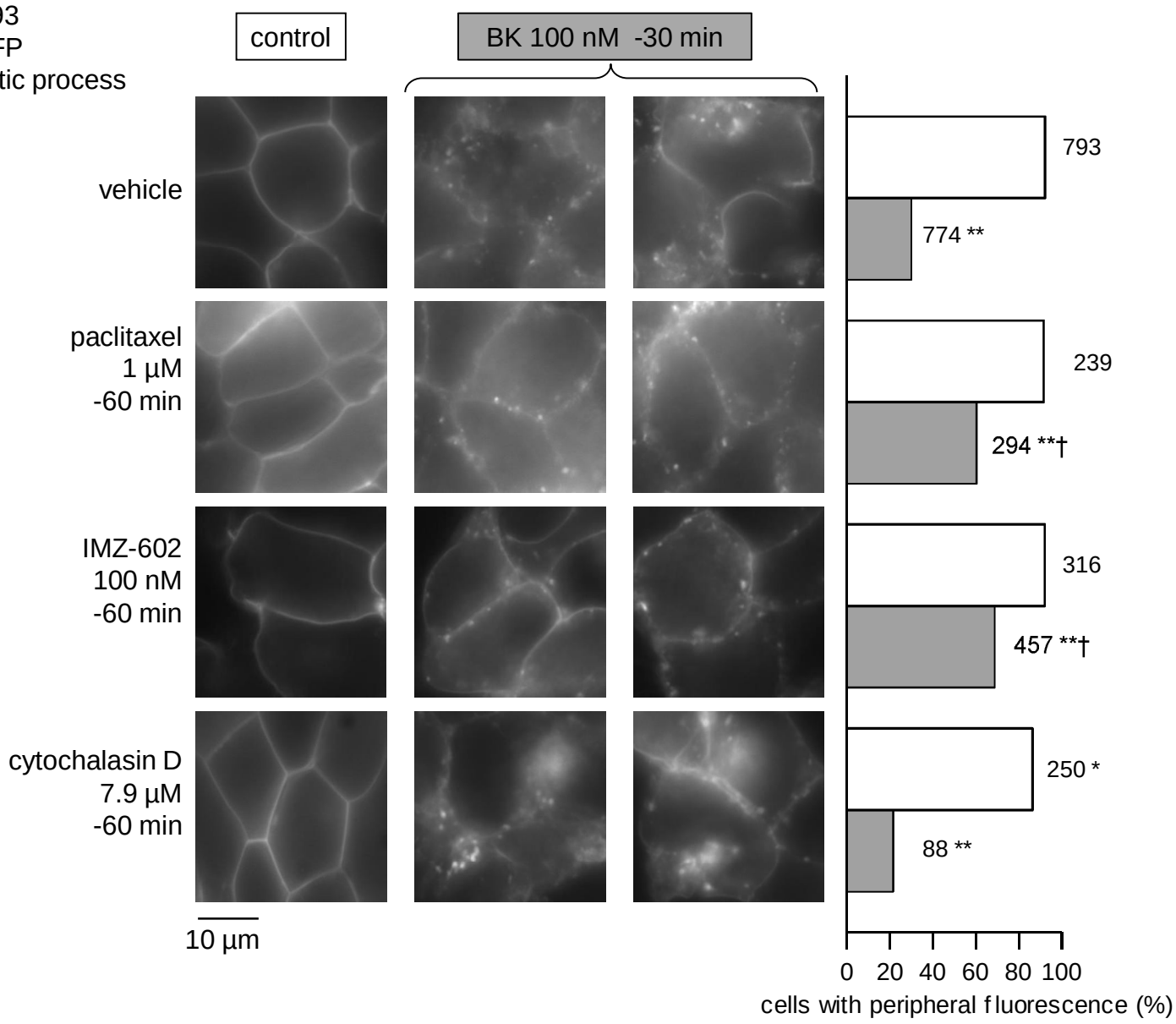
Fig. 1

peripheral fluorescence:  or 

non-peripheral fluorescence: 

10 µm

Fig. 2


HEK 293
B_2R-GFP
endocytic process
control
BK 100 nM -30 min
vehicle
paclitaxel
1 µM
-60 min
IMZ-602
100 nM
-60 min
cytochalasin D
7.9 µM
-60 min
10 µm
793
774 **
239
294 **†
316
457 **†
250 *
88 **
0 20 40 60 80 100
cells with peripheral fluorescence (%)

HEK 293a
β-arrestin$_2$-GFP
endocytic process

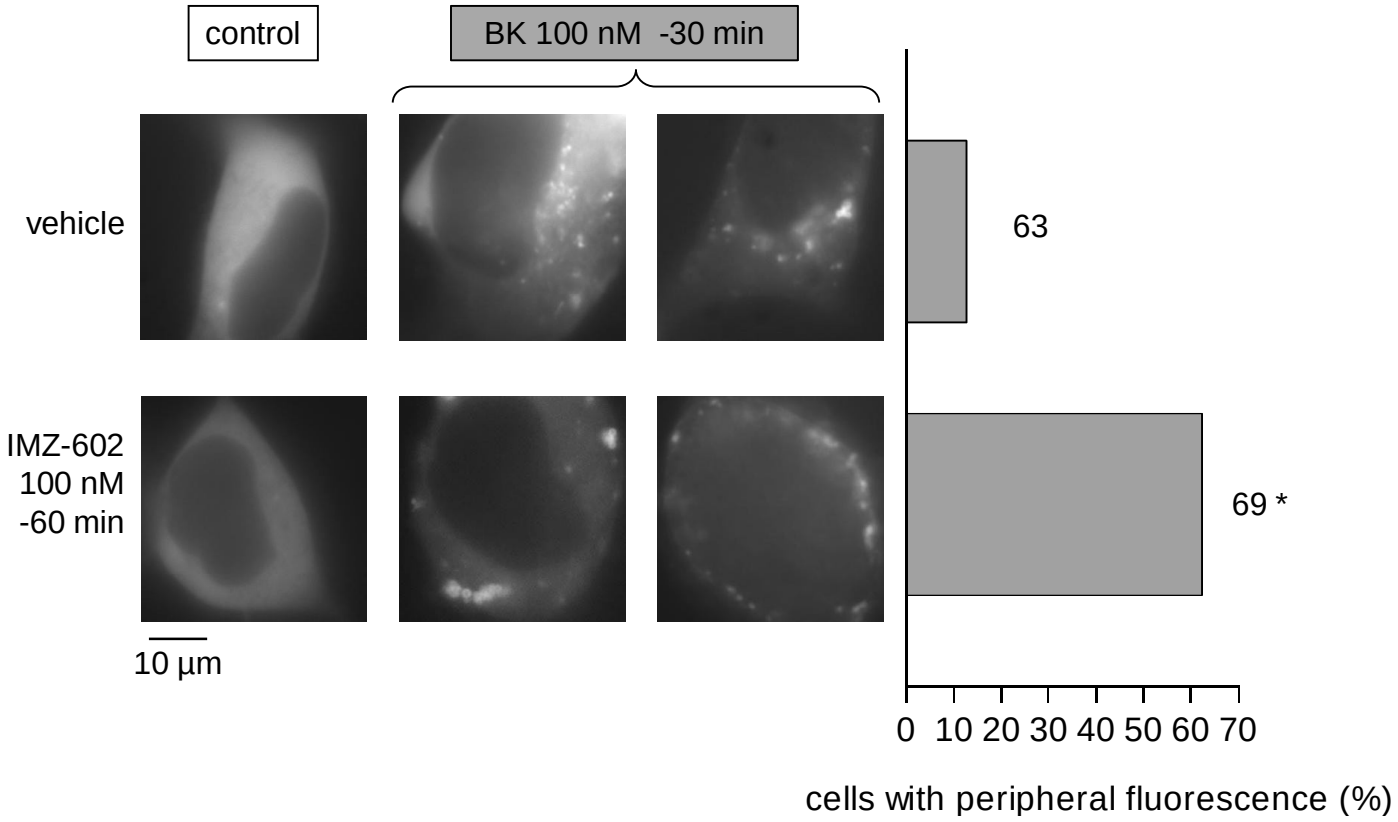


Figure 4

Fig. 4

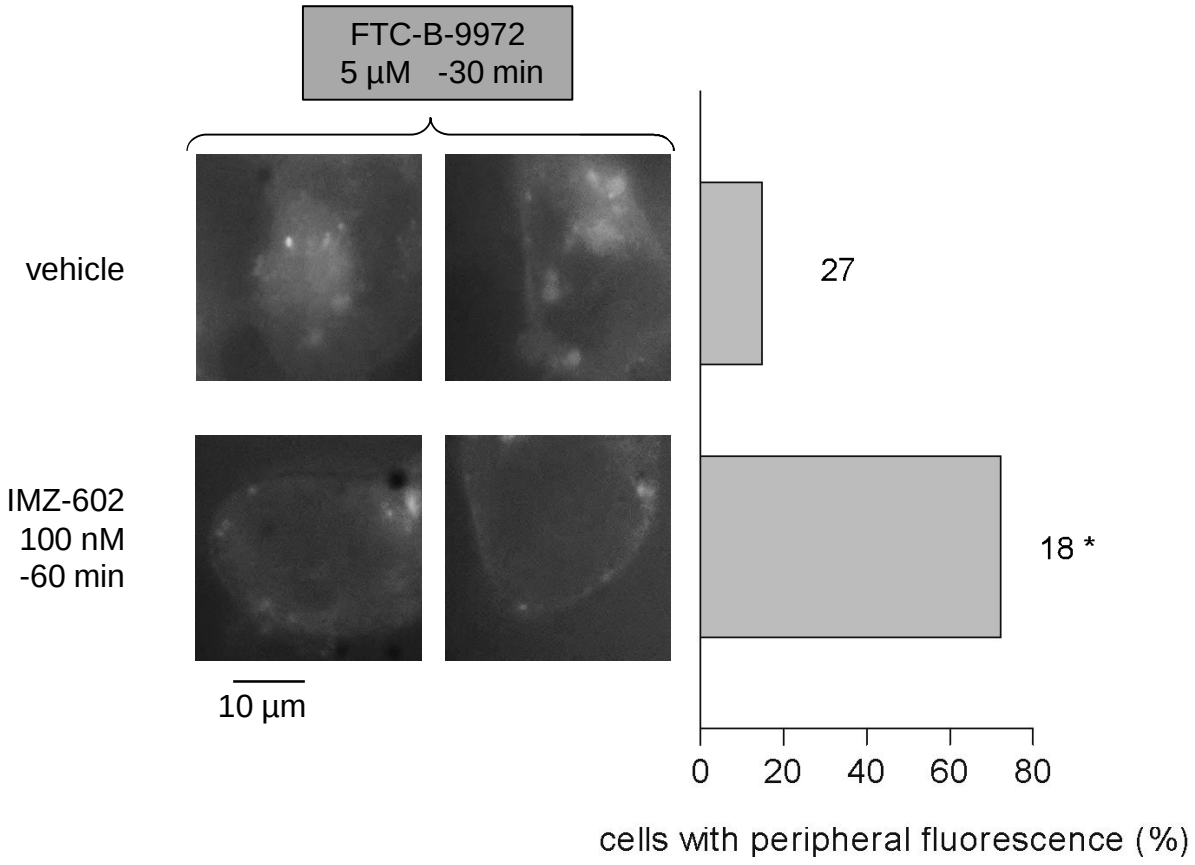


Figure 5

Fig. 5

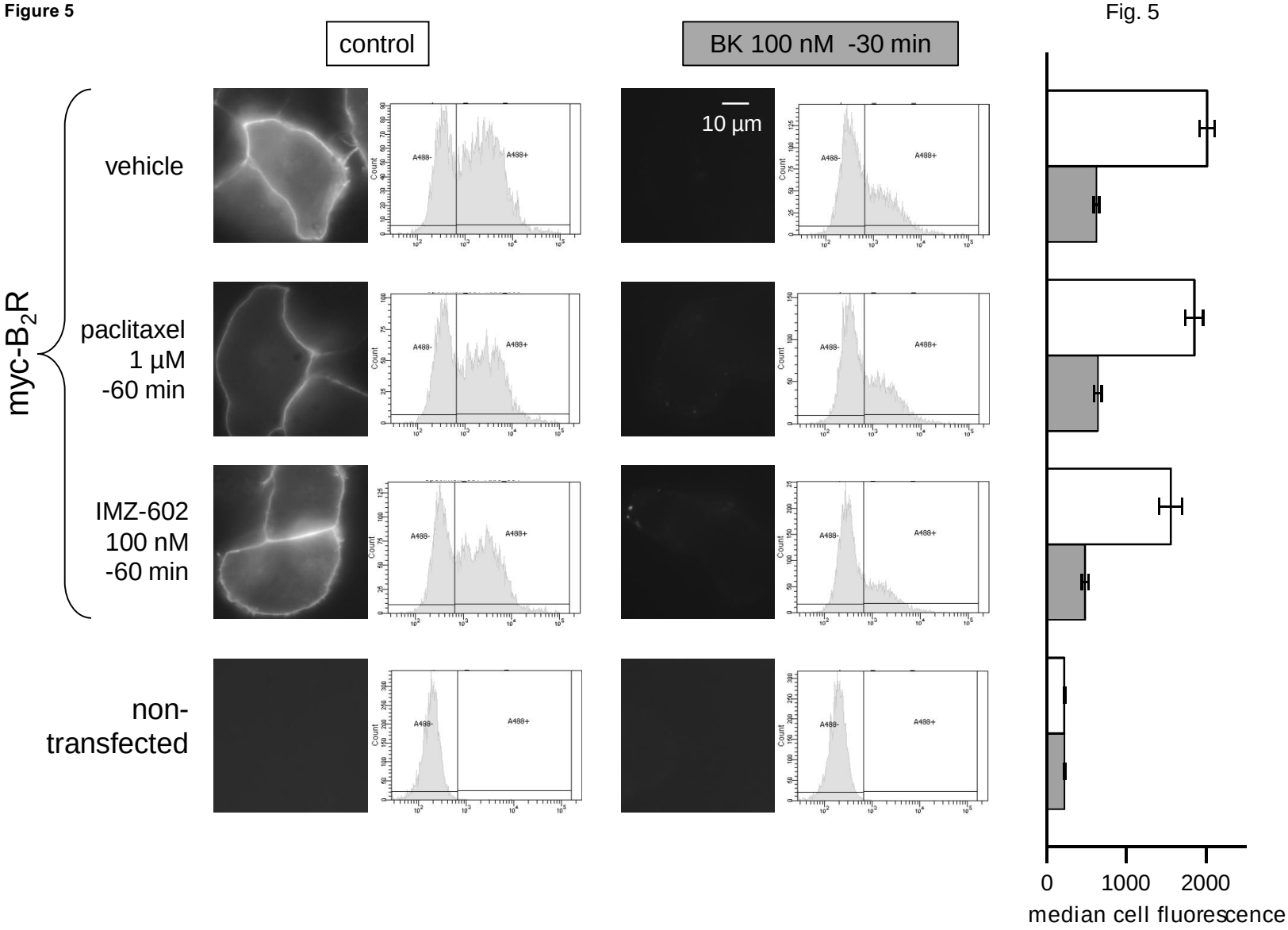


Fig. 6

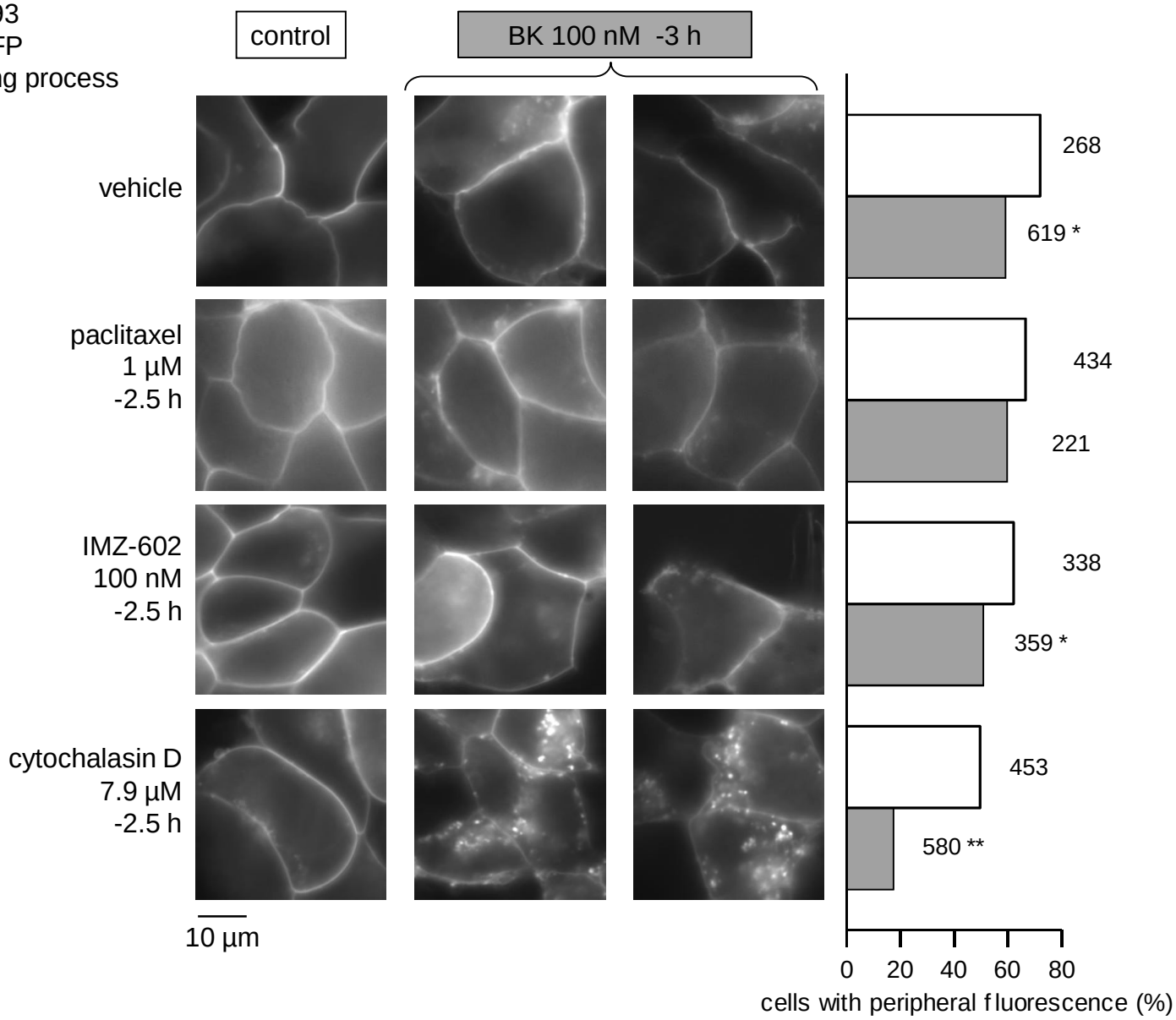

HEK 293
B_2R-GFP
recycling process
control
BK 100 nM -3 h
vehicle
paclitaxel
1 µM
-2.5 h
IMZ-602
100 nM
-2.5 h
cytochalasin D
7.9 µM
-2.5 h
10 µm
268
619 *
434
221
338
359 *
453
580 **
0
20
40
60
80
cells with peripheral fluorescence (%)

Figure 7

Fig. 7

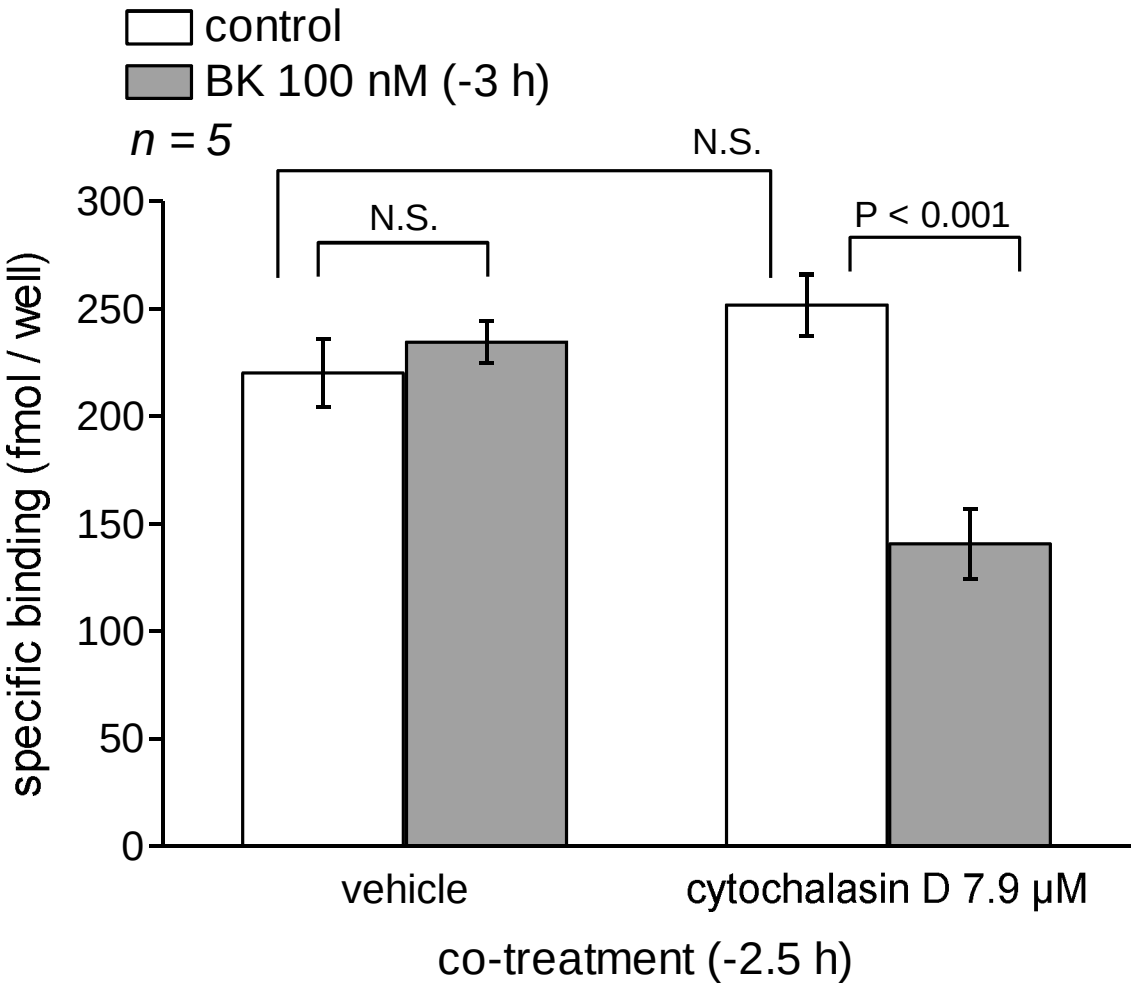


Figure 8

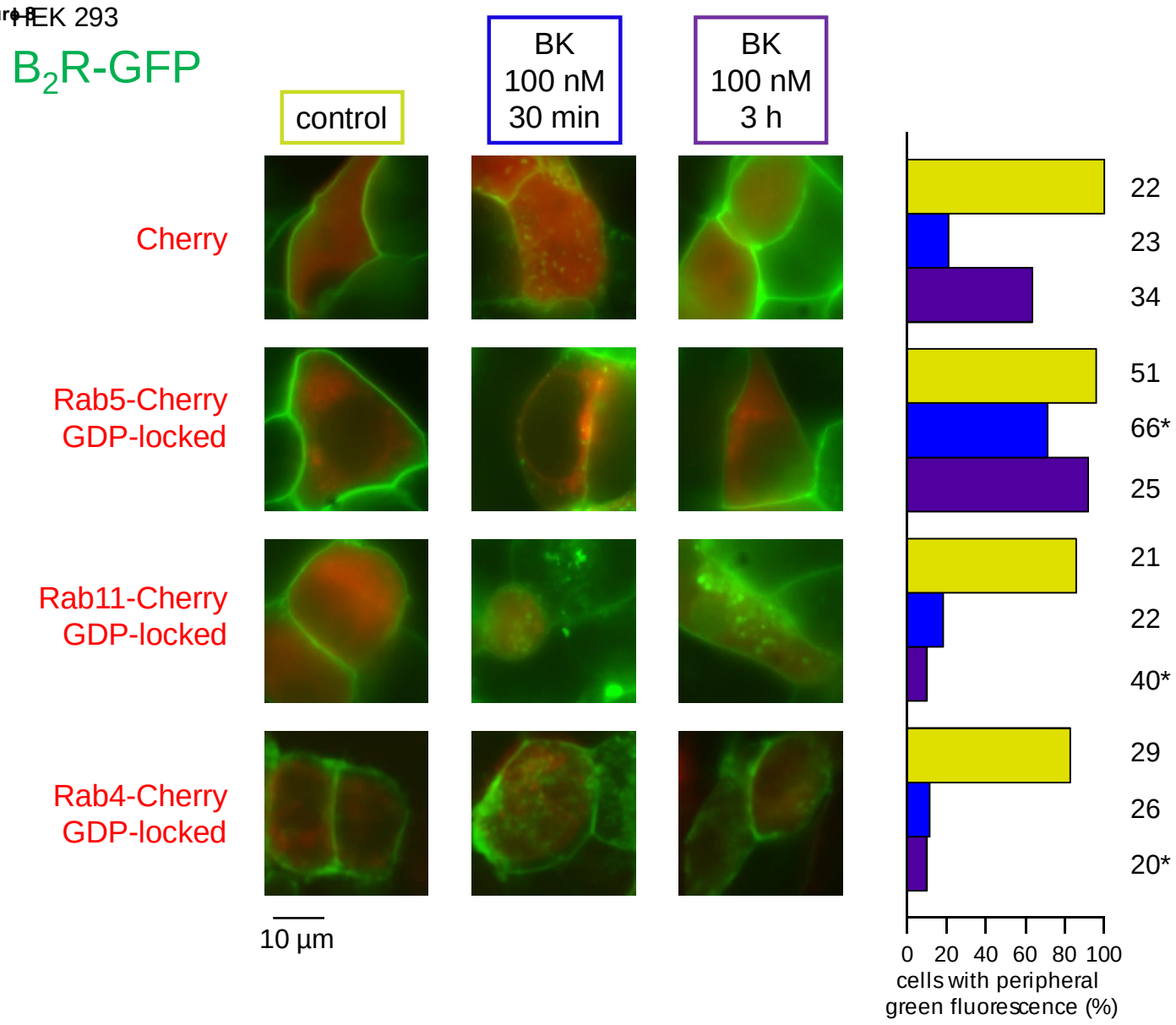
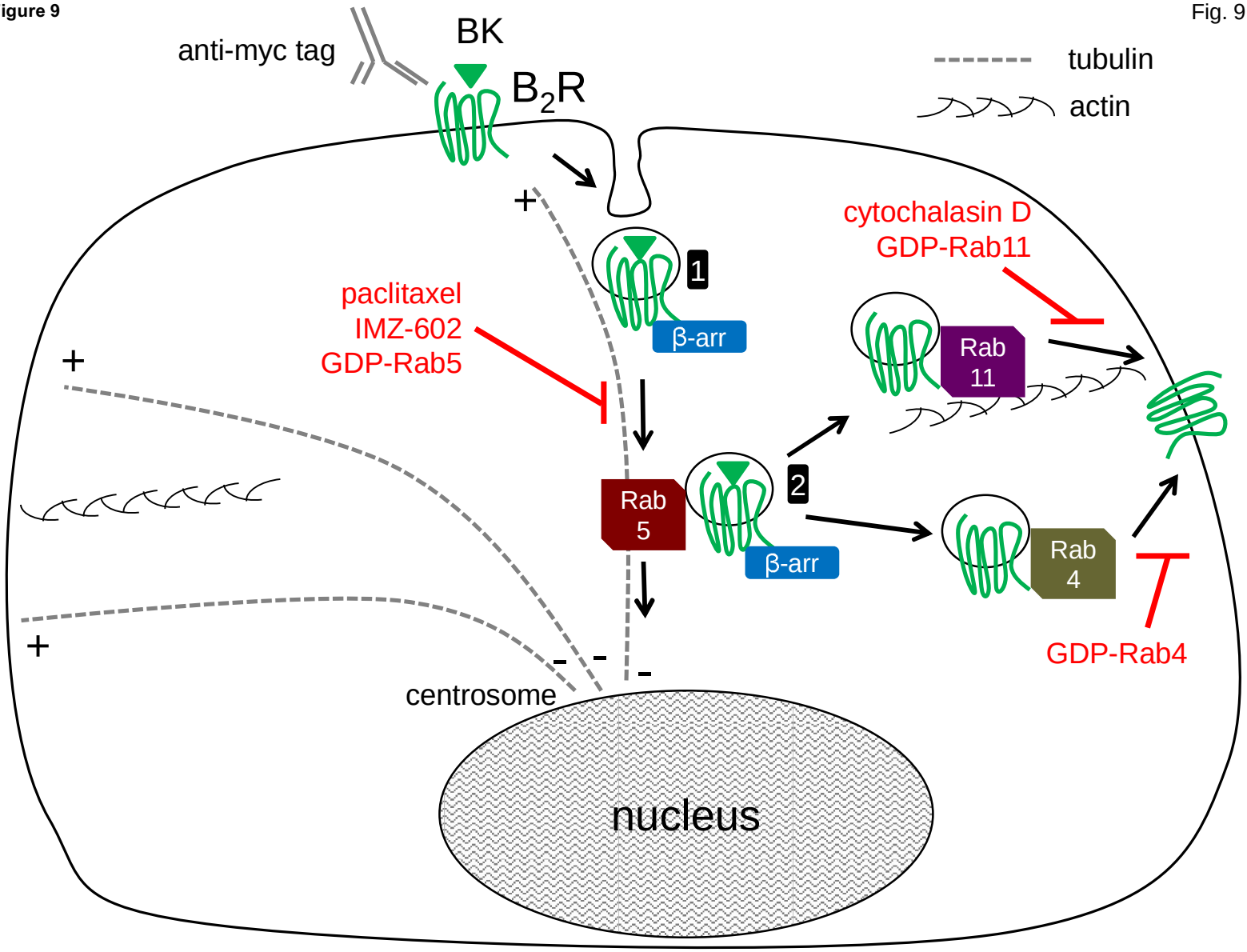

HEK 293
B2R-GFP
control
BK
100 nM
30 min
BK
100 nM
3 h
Cherry
Rab5-Cherry
GDP-locked
Rab11-Cherry
GDP-locked
Rab4-Cherry
GDP-locked
10 µm
22
23
34
51
66*
25
21
22
40*
29
26
20*
0 20 40 60 80 100
cells with peripheral
green fluorescence (%)

Figure 9

Fig. 9



***Graphical Abstract (for review)**