Wortmannin alters the intracellular trafficking of the bradykinin B₂ receptor: role of phosphoinositide 3-kinase and Rab5

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Wortmannin reportedly induces the formation of enlarged cytoplasmic endosomes. Such vesicles were observed in a definite time window after wortmannin treatment (250 nM) in HEK-293 cells stably expressing a B₂R (B₂ receptor)–green fluorescent protein conjugate and other cell types. The alternative PI3K (phosphoinositide 3-kinase) inhibitor LY 294002 (100 µM) and a dominant-negative form of the enzyme (p85α ΔiSH₂) induce a more modest vesicle enlargement. PI3K inhibition by drugs did not affect agonist-induced [³H]arachidonate release. The wortmannin-induced formation of giant endosomes also involves Rab5 activity, since a dominant-negative form of this GTPase (Rab5 S34N) partially inhibits the wortmannin effect and a constitutively active form of Rab5 (Rab5 Q79L) induces the formation of enlarged endosomes. Moreover, agonist stimulation targeted B₂R–green fluorescent protein towards the periphery of the giant vesicles and led to partial receptor degradation only

in wortmannin-treated cells. Receptor degradation was decreased by protease inhibitors and by bafilomycin A1, a drug that inhibits lysosome function. Accumulation of fluorescent material inside the enlarged endosomes was observed in cells treated with bafilomycin A1, wortmannin and an agonist. [³H]Bradykinin binding was decreased in HEK-293 cells treated with both wortmannin and the agonist, but not with either separately. Furthermore, a wortmannin-induced functional down-regulation of B₂R was observed in rabbit jugular veins after repeated agonist stimulation (contractility assay). This is the first report of a G-protein-coupled receptor down-regulation induced by an alteration of its usual routing in the cell. These results suggest that both PI3K and Rab5 influence B₂R intracellular trafficking.

Key words: endosome, green fluorescent protein, receptor degradation.

INTRODUCTION

Intracellular trafficking of cell-surface receptors is one of the most crucial components of GPCR (G-protein-coupled receptor) regulation, because it ultimately controls their fate [1]. The complexity of the intracellular trafficking machinery is just being appreciated as several types of vesicular organelles and particular trafficking pathways are discovered and characterized [2]. Classically, cell-surface receptors are targeted towards two major trafficking pathways after endocytosis: back to the plasma membrane through recycling endosomes or to lysosomes for degradation through late endosomes [1].

Wortmannin induces the formation of enlarged cytoplasmic vesicles in several cell types [3–8]. The observed enlarged vesicles are known to exhibit a number of properties associated with the early endosomes, the recycling endocytic pathway and perhaps the late endosomes [4,6,7]. These seemingly divergent endosomal characteristics probably result from inhibition of a PI3K (phosphoinositide 3-kinase)-dependent mechanism involved in protein sorting to different trafficking pathways from endosomes [4,6,7]. However, there is no consensus on the mechanism of giant endosome formation induced by wortmannin (a direct activation of Rab5 independent of PI3K [3,5]; an indirect and PI3K-dependent inhibition of the early recycling pathway for which the effector is Rab4 [6]). Wortmannin influences agonist-induced endocytosis of receptors: for epidermal growth factor receptors or angiotensin AT₁ receptors, agonist–receptor complexes were targeted towards the giant endosomes, but the final fate of these

receptor types was not changed as they were still degraded in the lysosomes in the first case (perhaps to an increased extent) and largely recycled to the cell surface in the second [3,5,6]. The endocytic pathway of the transferrin receptor was also affected by wortmannin as the receptor internalization rate increased, the formation of transferrin-receptor-rich enlarged endosomes was observed and recycling was either inhibited or decreased [7,8].

The bradykinin (BK) B₂ receptor (B₂R) is a GPCR constitutively and widely expressed in mammalian tissues, particularly in the cardiovascular and renal systems where it mediates prominent and diversified physiological effects (vasodilation, natriuresis, etc.). It could play a role in some types of inflammatory reactions [9]. As for many of the GPCRs, the B₂R undergoes rapid desensitization and subcellular redistribution/endocytosis after agonist binding. Specific molecular reactions, such as phosphorylation of C-terminal serine and threonine residues by GPCR kinases, are involved in these phenomena [10–14]. B₂R endocytosis and trafficking differ from those of the AT₁ receptor, as it is apparently initiated in caveolae and largely dynamin- and arrestin-independent [15–17]. The B₂R recycling to the plasma membrane is essentially complete [13]. Prolonged agonist stimulation does not down-regulate B₂Rs, at least in transfected HEK-293 cells; hypothetical mechanisms by which a physiological or pathological B₂R down-regulation would occur are unclear, although extracellular proteases can initiate B₂R destruction [13,18–20].

In the present study, the main objective was to assess the effect of PI3K inhibition, particularly by wortmannin, on HEK-293 cells stably expressing a previously reported, fully functional

Abbreviations used: B₂R, B₂ receptor; BK, bradykinin; CHO, Chinese-hamster ovary; CHX, cycloheximide; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; PI3K, phosphoinositide 3-kinase; PLA₂, phospholipase A₂; SMC, smooth-muscle cell lines.

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rabbit B₂R–GFP (green fluorescent protein) conjugate [13,21]. Specifically, endocytosis, intracellular trafficking, signalling and fate of the stimulated B₂R–GFP were investigated. The role of Rab5 in B₂R–GFP endocytosis and its involvement in the wortmannin effect were also examined. The physiological consequences of wortmannin treatment on a population of B₂Rs expressed at a physiological density have been verified by using the contractility of the rabbit jugular vein.

EXPERIMENTAL

Chemicals and antibodies

BK was purchased from Bachem (Plymouth Meeting, PA, U.S.A.) and the transfection reagent Ex-Gen 500 from MBI Fermentas (Flamborough, ON, Canada). Wortmannin, LY 294002 and all other reagents were obtained from Sigma (St. Louis, MO, U.S.A.). The monoclonal anti-GFP antibody and the secondary antibodies (pre-adsorbed goat anti-mouse or anti-rabbit IgG–horseradish peroxidase) were purchased from Clontech (Palo Alto, CA, U.S.A.) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) respectively. The polyclonal anti-phospho-AKT (Ser⁴⁷³) antibody was obtained from New England Biolabs (Mississauga, ON, Canada). [Phe⁸Ψ(CH₂-NH)Arg⁹]BK [22], a B₂R agonist with a modified peptide backbone resistant to peptidases such as the angiotensin I-converting enzyme, was a gift from Professor D. Regoli (Institute of Pharmacology, University of Sherbrooke, Sherbrooke, Canada). A dominant-negative form of the bovine PI3K p85α subunit (pSG5-bov p85α ΔiSH₂ [23]) was a gift from Dr M. D. Waterfield (Ludwig Institute for Cancer Research, Royal Free and University College Medical School, London, U.K.). A dominant-negative and a constitutively active form of Rab5 (Rab5 S34N and Rab5 Q79L respectively [24]) were kindly provided by Dr M. Zerial (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany).

Cell culture and cell transfection

The derivation and properties of an HEK-293 cell line stably expressing the rabbit B₂R–GFP conjugate is described elsewhere [13,21]. Non-transfected HEK-293 cells were used in control experiments. These cells were grown in α-minimal essential medium, supplemented with foetal bovine serum (5 %, v/v), horse serum (5 %, v/v), penicillin–streptomycin (1 %, v/v) and Genteticin (500 µg/ml; except for non-transfected HEK-293 cells). Chinese-hamster ovary (CHO) cells were used in comparative experiments and were grown in Dulbecco's modified Eagle's medium, supplemented with foetal bovine serum (10 %) and antibiotics. Primary smooth-muscle cell lines (SMC) were established from the aorta of healthy rabbits and cultured as described elsewhere [25]. In some experiments, HEK-293 or CHO cells were transiently transfected with either GFP or B₂R–GFP by using the transfection reagent Ex-Gen 500 as directed by the manufacturer. In some cases, HEK-293 cells were co-transfected with GFP or B₂R–GFP and the dominant-negative form of the bovine PI3K p85α subunit, Rab5 S34N or Rab5 Q79L.

Phase-contrast and confocal microscopy

HEK-293 or CHO cells (transiently transfected or not), HEK-293 cells stably expressing B₂R–GFP and rabbit SMCs were observed without fixation or drug washout using a Bio-Rad 1024 confocal microscope (× 40 objective with oil immersion for phase-contrast microscopy; × 60 objective with oil immersion,

emission wavelength 488 nm and detection above 510 nm for fluorescence microscopy). All treatments were made in the complete culture medium; CHX (cycloheximide; 71 µM) was always added to the medium to avoid synthesis of new receptors that would interfere with the observations. Experiments consisted of treatment of cells with wortmannin (250 nM), LY 294002 (100 µM) or the ethanol vehicle (0.1 %) for the defined time periods (from 30 min to 24 h), followed or not by a B₂R agonist stimulation (10 or 100 nM BK; from 15 min to 3 h). Other experiments involved the addition of bafilomycin A1 (300 nM) during the cell treatment period and the use of a metabolically resistant B₂R agonist [Phe⁸Ψ(CH₂-NH)Arg⁹]BK (100 nM).

PLA₂ (phospholipase A₂) assay

An arachidonic acid release assay was performed as described in [21] to evaluate whether PI3K inhibition affects agonist-induced PLA₂ activation in HEK-293 cells stably expressing B₂R–GFP (24-well plates).

Immunoblots

Immunoblots for B₂R–GFP were performed as described previously [13]. Briefly, HEK-293 cells or HEK-293 cells stably expressing B₂R–GFP grown in 25 cm² flasks (approx. 80 % confluence) were treated with wortmannin (250 nM), LY 294002 (10–100 µM) or the ethanol vehicle (0.1 %), and optionally stimulated with BK (100 nM) or a metabolically resistant B₂R agonist [Phe⁸Ψ(CH₂-NH)Arg⁹]BK (100 nM). CHX (71 µM) was always added to inhibit interference from newly synthesized receptors. In some experiments, HEK-293 cells stably expressing B₂R–GFP were treated with wortmannin and bafilomycin A1 or various protease inhibitors before the agonist stimulation, to inhibit wortmannin-facilitated B₂R–GFP degradation. Total cell extracts were recovered and analysed after SDS/PAGE (9 % gel) and protein transfer using a GFP-specific monoclonal antibody or a polyclonal anti-phospho-AKT antibody.

Binding assay

The binding of 2 nM [³H]BK (90 Ci/mmol; PerkinElmer Life Sciences, Boston, MA, U.S.A.) to adherent intact HEK-293 cells stably expressing B₂R–GFP (24-well plates) was evaluated as described previously [21]. The assay was applied to evidence B₂R down-regulation after stimulation with the agonist BK in serum-containing culture medium under specific experimental conditions.

Contractility assay

A local ethics committee approved the experiments involving rabbits. Jugular veins were collected and prepared as described in [26]. Isometric contraction of jugular-vein strips submitted to BK (10 nM) stimulation was recorded to evaluate the effect of wortmannin (250 nM) or the ethanol vehicle (0.1 %) on the wild-type B₂R expressed at a physiological density. Tissues were bathed in Krebs solution whose composition was NaCl 117.5 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.18 mM, CaCl₂ 2.5 mM, NaHCO₃ 25.0 mM and D-glucose 5.5 mM. The tissues were continuously treated with CHX (71 µM) to inhibit synthesis of new receptors. Contractility results are expressed as a percentage of the first response to BK. Results are expressed as means ± S.E.M. Statistical analysis was performed with the

InStat 2.0 computer program (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Wortmannin-induced formation of enlarged cytoplasmic vesicles

Using phase-contrast microscopy, we have verified whether wortmannin actually induced the formation of enlarged cytoplasmic vesicles in the cell types used in our experiments (HEK-293 cells, HEK-293 cells stably expressing B₂R-GFP, rabbit vascular SMCs or CHO cells) as reported for other cell lines [3–5,7]. These enlarged vesicles were observed in all four cell types treated with wortmannin (250 nM, 90 min; Figure 1). The giant vesicles appeared in the cells within minutes (< 30 min) of treatment, with a maximal abundance at approx. 60–90 min and progressive disappearance beyond 3 h (Figure 1; results not shown). No visible giant vesicle could be observed in HEK-293 cells stably expressing B₂R-GFP (Figure 1) 24 h after the initial treatment with wortmannin. The ethanol vehicle (0.1 %, 90 min) or the alternative PI3K inhibitor LY 294002 (100 µM, 90 min) did not produce the same effect (Figure 1; results not shown).

We assessed the effectiveness of wortmannin and LY 294002 as PI3K inhibitors over time (from 60 min to 24 h) by measuring phosphorylated AKT, a PI3K product, in total cell extracts. HEK-293 cells stably expressing B₂R-GFP cultured in serum-containing medium showed the presence of a baseline concentration of phospho-AKT (Figure 2). Treatment with wortmannin (250 nM, 1 h) or LY 294002 (100 µM, 1 h), but not with the ethanol vehicle (0.1 %, 1 h), decreased phospho-AKT in total cell extracts (Figure 2), confirming their pharmacological identity as PI3K inhibitors. Wortmannin (250 nM) was a noticeably weaker inhibitor if applied 3 or 24 h before cell extraction, and LY 294002 was only partially active at 10 µM (Figure 2).

Effect of PI3K inhibitors on endocytosis and intracellular trafficking of B₂R-GFP

Fluorescent receptors found at the cell surface of HEK-293 cells stably expressing B₂R-GFP were generally excluded from the large hollow structures in resting cells treated with wortmannin (Figures 1 and 3). B₂R-GFP-expressing cells treated with wortmannin (250 nM, 30 min or 24 h), the alternative PI3K inhibitor LY 294002 (100 µM, 30 min) or the ethanol vehicle (0.1 %, 30 min) exhibited receptor endocytosis and intracellular trafficking up to 60 min after BK (10 nM) stimulation, but no more at the 3 h time point (Figure 3). This reversibility has been attributed previously to BK destruction by peptidases in serum-containing culture medium, followed by completion of the receptor recycling process [13]. Thus inhibition of PI3K by wortmannin or LY 294002 did not affect the fact that B₂R-GFP-agonist complexes undergo endocytosis and intracellular trafficking in a definite time frame. However, presence of B₂R-GFP was still important 60 min after the agonist stimulation in wortmannin-treated (30 min) cells compared with control or LY 294002-treated cells (Figure 3). Moreover, the morphology difference was striking as fluorescent receptors were rapidly found at the periphery of enlarged cytoplasmic vesicles in cells pretreated with wortmannin for 30 min, whereas they were observed in small and ill-defined structures in cells pretreated for 30 min with the ethanol vehicle (Figure 3). LY 294002 at 100 µM (but not at 10 µM; results not shown) caused an intermediate level of endosome enlargement, with occasional well-defined

Figure 1 Wortmannin-induced formation of large cytoplasmic vesicles

HEK-293 cells stably expressing B₂R-GFP were treated with wortmannin (250 nM) for the defined time periods (A). Non-transfected HEK-293 cells (B), rabbit SMCs (C) or CHO cells (D) were treated with wortmannin (250 nM) or the ethanol vehicle (0.1 %) for 90 min. Cells were then visualized by phase-contrast microscopy (square fields with sides of 54 µm) or by confocal fluorescence microscopy (square fields with sides of 40 µm). The selected confocal planes are halfway through the thickness of most cells. Arrowheads point out representative features described in the text.

Figure 2 Immunoblot of total cell extracts based on specific polyclonal anti-phospho-AKT antibodies: inhibition by wortmannin and LY 294002 of AKT phosphorylation

HEK-293 cells stably expressing B₂R-GFP and cultured in a medium containing 10 % foetal bovine serum (lane 1 from left, untreated cells) were further treated with the ethanol (EtOH) vehicle (0.1 %, 1 h; lane 2), LY 294002 (10 µM, 1 h, lane 3; 100 µM, 1 h, lane 4; 100 µM, 3 h, lane 5) or wortmannin (250 nM, 1 h, lane 6; 250 nM, 3 h, lane 7; 250 nM, 24 h, lane 8).

hollow vesicles. The distribution of the fluorescent receptors and that of most of the enlarged cytoplasmic vesicles observed in phase-contrast microscopy coincide in cells treated with both wortmannin and BK (overlay of phase-contrast frames with fluorescent frames; results not shown). This supports the notion that these giant vesicles are part of the endocytic pathway. Moreover, this particular wortmannin effect on B₂R-GFP subcellular distribution is limited in time, since a 24 h treatment did not induce the

Figure 3 Effect of PI3K inhibitors on endocytosis and intracellular trafficking of B₂R-GFP

HEK-293 cells stably expressing B₂R-GFP were treated with the ethanol vehicle (0.1 %, 30 min), wortmannin (250 nM, 30 min or 24 h) or LY 294002 (100 μ M, 30 min) before BK (10 nM) stimulation for the defined time periods (from 0 to 180 min). Cells were then observed in confocal fluorescence microscopy. Presentation as in Figure 1.

formation of enlarged fluorescent vesicles after BK stimulation (Figure 3).

Molecular mechanism of the wortmannin-induced formation of enlarged cytoplasmic vesicles

As observed by confocal fluorescence microscopy, the wortmannin-induced enlarged cytoplasmic vesicles excluded GFP, thus being seen in negative, in HEK-293 or CHO cells transiently transfected with a GFP coding vector (Figures 4 and 5 respectively). Moreover, B₂R-GFP, transiently expressed in HEK-293 or CHO cells that were also pretreated with wortmannin, appeared at the periphery of the enlarged vesicles after BK stimulation, but not in resting cells (Figures 4 and 5 respectively). LY 294002 produced similar effects but with much less intensity (vesicles approx. three times smaller; Figure 4). Given the discrepancy between effects of both PI3K inhibitors at concentrations proven to be effective, we have investigated the involvement of Rab5 in the wortmannin effect as proposed by Chen and Wang [5].

Using the same general approach as outlined above, HEK-293 cells were transiently co-transfected with expression vectors coding for either GFP or B₂R-GFP and the dominant-negative form of the bovine PI3K p85 α subunit, Rab5 S34N or Rab5 Q79L. Transient expression of the dominant-negative form of the PI3K p85 α subunit in HEK-293 cells led to the formation of cytoplasmic vesicles similar in size to those produced by LY 294002 (Figure 4), suggesting an additional interaction between wortmannin and another factor for the formation of enlarged cytoplasmic vesicles. The enlargement of endosomes after Rab5 Q79L expression has been reported previously in the literature

Figure 4 Formation of large cytoplasmic vesicles in HEK-293 cells transiently expressing B₂R-GFP or GFP alone

Cells were treated with wortmannin (250 nM), LY 294002 (100 μ M) or the ethanol vehicle (0.1 %) for a period of 90 min. BK was present in some cases for the last 60 min of incubation before visualization of the treated cells in confocal fluorescence microscopy. In some experiments, HEK-293 cells were co-transfected with either GFP or B₂R-GFP and the dominant-negative form of the bovine PI3K p85 α subunit, Rab5 S34N or Rab5 Q79L. Presentation as in Figure 1. Arrowheads point out representative features (in some cases enlarged in insets) described in the text.

Figure 5 Wortmannin-induced formation of large cytoplasmic vesicles in CHO cells transiently expressing B₂R-GFP or GFP

Cell treatments and presentation are as in Figure 4.

Figure 6 [³H]Arachidonate release by HEK-293 cells stably expressing B₂R-GFP as a function of PI3K inhibition (drugs applied 30 min before the agonist) and of BK stimulation (20 min)

Results are expressed as means $\pm$ S.E.M. ($n = 5-6$).

and is the result of extensive fusion between endosomes forced by the Rab5 activity [5,24,27]. Rab5 Q79L expression led to the formation of numerous vesicles of variable sizes in GFP-expressing cells. Surprisingly, in B₂R-GFP-expressing cells, the stimulated fluorescent receptors were only found at the periphery of the smallest vesicles (similar in size to those produced by LY 294002 or the dominant-negative form of the PI3K p85 α subunit). Since B₂R-GFP does not appear at the periphery of the largest vesicles in agonist-stimulated Rab5 Q79L-expressing cells, the action of Rab5 may be limited to the initial steps of the B₂R-GFP intracellular trafficking. The presence of the larger vesicles in cells expressing both GFP and Rab5 Q79L probably suggests an active role for Rab5 in the fluid-phase uptake into the cells. Moreover, the expression of Rab5 S34N inhibited in part the wortmannin-induced formation of enlarged cytoplasmic vesicles as the number and the size of the vesicles decreased, suggesting the involvement of Rab5 in the general effect of wortmannin. Rab5 S34N alone did not lead to the appearance of large vesicles and did not exert any visible effect on B₂R-GFP endocytosis. Overall, these results support a mechanism involving Rab5 activation and PI3K inhibition for the wortmannin-induced formation of enlarged cytoplasmic vesicles, since Rab5 activation or PI3K inhibition alone led to the formation of smaller-sized endosomes.

Effect of PI3K inhibition on B₂R-GFP signalling

PI3K inhibition by treatments with wortmannin (250 nM) or LY 294002 (100 μ M) did not affect PLA₂ activation for a range of BK concentrations in HEK-293 cells stably expressing B₂R-GFP (Figure 6).

Effect of bafilomycin A1 on wortmannin-induced alteration of B₂R-GFP trafficking

We have characterized previously the B₂R-GFP fusion protein as a 101–105 kDa protein using two different anti-GFP antibodies [13,21], and have confirmed this result using a third antibody, a monoclonal antibody with low background (Figure 7). GFP presence in the construction allows the detection of smaller molecular mass C-terminal fragments resulting from receptor cleavage [20]. We have shown previously that the B₂R-GFP population could undergo endocytosis without degradation for at least 3 h after BK stimulation when cells were pretreated with the

Figure 7 Immunoblot of total HEK-293 cell extracts based on monoclonal anti-GFP antibodies

(A) Wortmannin-induced degradation of B₂R-GFP after agonist stimulation in HEK-293 cells stably expressing B₂R-GFP. Extracts of B₂R-GFP-expressing cells were obtained after a 3.5 h treatment with the ethanol (EtOH) vehicle (0.1 %, lane 2 from left), LY 294002 (100 μ M, lane 3) or wortmannin (250 nM, lane 4) to evaluate the effect of these drugs on the stability of the fluorescent receptors. The same type of cells were also stimulated with a metabolically resistant agonist [Phe⁸ Ψ (CH₂-NH)Arg⁹]BK (100 nM) for 3 h after treatment for 30 min with the ethanol vehicle (lane 5), LY 294002 (lane 6) or wortmannin (lane 7). Shorter agonist stimulations were evaluated on wortmannin-treated cells (60 min, lane 8; 30 min, lane 9). An extract from non-transfected HEK-293 cells treated with wortmannin (3.5 h) was also immunoblotted as a control (lane 1). Results are representative of two independent experiments. (B) Lysosomal proteases mediate wortmannin-induced degradation of B₂R-GFP after agonist stimulation. Extracts of HEK-293 cells stably expressing B₂R-GFP were obtained after treatment for 30 min with the ethanol vehicle (0.1 %, lanes 2 and 4 from left) or wortmannin (250 nM, lanes 3 and 5), followed by incubation for 3 h with (lanes 4 and 5 respectively) or without (lanes 2 and 3 respectively) a metabolically resistant B₂R agonist [Phe⁸ Ψ (CH₂-NH)Arg⁹]BK (100 nM). Bafilomycin A1 (300 nM, lane 6), E-64 (100 μ M, lane 7), leupeptin (100 μ g/ml, lane 8) or pepstatin A (10 μ M, lane 9) were added to wortmannin 30 min before the 3 h agonist stimulation. An extract from non-transfected HEK-293 cells treated with wortmannin (3.5 h) was also immunoblotted as a control (lane 1). Results are representative of two independent experiments.

peptidase inhibitor captopril to prevent agonist breakdown [13]. The use of a metabolically resistant B₂R agonist [Phe⁸ Ψ (CH₂-NH)Arg⁹]BK reproduced the prolonged effect, as this agonist (100 nM) stimulated B₂R-GFP internalization in cytoplasmic vesicles for at least 3 h [19]. In experiments reported in the present study, cells treated with the ethanol vehicle (0.1 %), LY 294002 (100 μ M) or wortmannin (250 nM) exhibited minimal B₂R-GFP degradation in the absence of the agonist (Figure 7A). However, partial degradation of the fluorescent receptors could be detected in wortmannin-treated cells (mostly 70–80 kDa

fragments as well as GFP-sized fragments, approx. 27–30 kDa in mass) only if cells were also stimulated with the metabolically resistant B₂R agonist [Phe⁸Ψ(CH₂-NH)Arg⁹]BK (100 nM) for a period of 3 h, but not for shorter periods of time (30 or 60 min; Figure 7A). Moreover, no agonist-induced receptor degradation could be detected under these conditions when cells were initially treated for 24 h with wortmannin (results not shown), suggesting that fluorescent receptors must reach the enlarged vesicles to be targeted for degradation. Also, no accelerated form of degradation could be observed in cells treated with the ethanol vehicle or LY 294002 plus the agonist (Figure 7A). Non-transfected HEK-293 cells were treated with wortmannin as a control and exhibited minimal background reactivity (Figures 7A and 7B). This experiment was also performed with BK as the agonist and produced similar results but with less intense receptor degradation (results not shown).

In another experiment, we have attempted to inhibit agonist-induced B₂R-GFP degradation by pretreating cells with various inhibitors along with wortmannin before the agonist stimulation. The effect of these inhibitors is to be compared with the receptor degradation observed in cells treated with both wortmannin (250 nM) and the metabolically resistant B₂R agonist [Phe⁸Ψ(CH₂-NH)Arg⁹]BK (100 nM; Figure 7B). Bafilomycin A1 (300 nM), which inhibits the functioning of lysosomes, E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; 100 μM], a cysteine protease inhibitor, and leupeptin (100 μg/ml), a cysteine and serine protease inhibitor, all prevented B₂R-GFP degradation, whereas pepstatin A (10 μM), an aspartate protease inhibitor, did not prevent B₂R-GFP degradation (Figure 7B). These results suggest that at least a fraction of the fluorescent receptors is actually targeted towards the lysosomes for degradation when cells are pretreated with wortmannin.

We have observed, by confocal fluorescence microscopy, the effect of bafilomycin A1 on HEK-293 cells stably expressing B₂R-GFP, treated with wortmannin (250 nM) and further stimulated for 3 h with BK (100 nM) or the B₂R agonist [Phe⁸Ψ(CH₂-NH)Arg⁹]BK (100 nM). BK-induced B₂R-GFP presence in cells is not observed 3 h after stimulation owing to BK degradation by peptidases and completion of receptor recycling and degradation in the presence of wortmannin (Figure 3). However, concomitant bafilomycin A1 treatment reveals the accumulation of receptor-associated fluorescence in coarse vesicles in these cells (Figure 8); presumably the enlarged endosomes filled with B₂R-GFP stopped in their trafficking before their degradation in the lysosomes. Similar observations could be made in cells stimulated with the metabolically resistant agonist, but the accumulation of fluorescent material in coarse vesicles is more intense (Figure 8).

Wortmannin-facilitated agonist-induced down-regulation of B₂R-GFP in HEK-293 cells

Intact HEK-293 cells stably expressing B₂R-GFP bound [³H]BK (2 nM) in a specific manner (Figure 9A); the binding was slightly attenuated by treatment for 3.5 h with CHX (71 μM; Figure 9A, control), but addition of the agonist BK (100 nM, last 3 h) did not change the density of the binding sites at the cell surface (BK has a half-life of 8 min in serum-containing medium at 37 °C and is not believed to be present at the end of the pretreatment period to compete with the radioligand [13]). Pretreatment with wortmannin alone (250 nM, 3.5 h combined with CHX) had no effect, but adding the agonist BK (last 3 h) significantly decreased the number of surface receptors as assessed by radioligand binding (Figure 9A). A combined LY 294002 (100 μM) and BK treatment

Figure 8 Effect of bafilomycin A1 on the wortmannin-induced degradation of B₂R-GFP as assessed by cellular fluorescence distribution

HEK-293 cells stably expressing B₂R-GFP were treated with wortmannin (250 nM) and bafilomycin A1 (300 nM) or its DMSO vehicle (0.04 %) for 30 min. Cells were then stimulated with BK (100 nM) or a metabolically resistant B₂R agonist [Phe⁸Ψ(CH₂-NH)Arg⁹]BK (100 nM) for 3 h before visualization in confocal fluorescence microscopy. Presentation as in Figure 1. Results are representative of two experiments.

Figure 9 Facilitation of agonist-induced B₂R down-regulation by wortmannin

(A) Specific binding of [³H]BK (2 nM) to HEK-293 cells stably expressing B₂R-GFP. Cells were either not treated (control) or treated with CHX (71 μM, 3.5 h). Concomitant treatments consisted of wortmannin (250 nM, 3.5 h), LY 294002 (100 μM, 3.5 h) and/or BK (100 nM, last 3 h). Results are the means ± S.E.M. for 5–9 determinations. The effect of BK was tested by the Mann–Whitney test relative to the CHX-pretreated cells (**P* < 0.05). Non-specific binding, determined in the presence of 1 μM unlabelled BK, amounted to less than 5 % of the specific binding. 'LY', LY 294002. (B) Wortmannin-induced functional B₂R down-regulation in rabbit jugular veins. Rabbit jugular-vein strips were continuously treated with CHX (71 μM) to inhibit synthesis of new receptors. The ethanol vehicle (0.1 %, open bars) or wortmannin (250 nM, black bars) were also constantly present in the Krebs solution. Three contractile responses to BK (10 nM) were recorded at 30 min intervals with ample tissue washing between each response. Values are the means ± S.E.M. for 4–6 determinations and are expressed as a percentage of the initial response. The paired non-parametric test showed that the second- and third-response amplitudes decreased significantly when compared with the first response in tissues treated with wortmannin, but not in those treated with the ethanol vehicle (**P* < 0.05). See the text for further analysis.

did not significantly decrease, but somewhat attenuated, [³H]BK binding to the cell surface.

Wortmannin-facilitated B₂R down-regulation in rabbit jugular veins

Rabbit jugular-vein strips were prepared to evaluate the effect of wortmannin on a function (contraction) mediated by the wild-type B₂R expressed at a physiological density. Tissues were pretreated with the ethanol vehicle (0.1 %) or wortmannin (250 nM) 30 min before three successive BK (10 nM) stimulations given at 30 min intervals. CHX (71 µM) was constantly present in the Krebs solution to inhibit synthesis of new receptors that would interfere with the possible down-regulation process. The veins responded by a contraction to 10 nM BK (0.38 ± 0.13 g for the control-ethanol vehicle), not significantly modified by the presence of wortmannin (0.43 ± 0.13 g). Relative to the first response, the second and third responses to BK decreased significantly in tissues treated with wortmannin (up to 40 %; paired non-parametric test, *P* < 0.05; Figure 9B), whereas the repeated responses to BK were not significantly different from the first ones in tissues treated with the ethanol vehicle.

DISCUSSION

It has been demonstrated previously that wortmannin, a potent but not fully specific PI3K inhibitor [28–30], induced the formation of enlarged cytoplasmic vesicles in several cell types [3–8]. These vesicles are common to early endosomes, the recycling pathway and the degradative pathway [4,6,7]. However, there is no consensus on the mechanism of their formation. Chen and Wang [3,5] suggested that the wortmannin-induced formation of enlarged cytoplasmic vesicles results from Rab5 activation independent of PI3K inhibition. Others have suggested that such vesicle formation is the result of an indirect and PI3K-dependent inhibition of the early recycling pathway, for which the effector is Rab4 [6]. Interestingly, in the first-case scenario, LY 294002 does not induce the formation of cytoplasmic vesicles as large as those produced by wortmannin (approx. three times smaller), whereas in the second case, it elicits similar effects [5,6].

We have characterized previously the internalization/recycling process of B₂R–GFP [13]. After agonist stimulation, this receptor is internalized within minutes (< 15 min) in the form of small and ill-defined fluorescent particles. These particles can be observed up to 60 min after the initial agonist stimulation; eventually, complete recycling of the internalized receptors takes place within 3 h of stimulation. As reported previously for the epidermal growth factor receptor or the angiotensin AT₁ receptor [3,6], PI3K blockade using wortmannin or LY 294002 did not significantly change the acute signalling (Figure 6) or trafficking kinetics of the B₂R–GFP (Figure 3). The latter drug is perhaps more useful in this demonstration because its inhibitory effect is still strong 3 h post-application (Figure 2). Still, B₂R–GFP presence might have been more persistent 60 min after the agonist stimulation in cells treated with wortmannin for 30 min when compared with control or LY 294002-treated cells (Figure 3). This observation suggests that wortmannin may be slowing down the intracellular trafficking of the B₂R–GFP, perhaps by inhibiting the rapid recycling pathway [6].

However, the morphology of B₂R–GFP-containing vesicles in agonist-treated cells is quite different from that of controls when cells were pretreated with wortmannin. The fluorescent receptors are rapidly found at the periphery of enlarged vesicles after agonist stimulation. Artificial enlargement of endocytic vesicles using wortmannin allows the unequivocal visualization of agonist-induced receptor endocytosis in cellular systems

exhibiting a high fluorescence background, such as transiently transfected cells (Figures 4 and 5). Consistent with their formation through endocytic membrane influx [4], wortmannin induced the formation of enlarged cytoplasmic vesicles that excluded cytosolic GFP expressed in HEK-293 cells (Figure 4). This peculiar action of wortmannin is not exclusively dependent on PI3K inhibition in this experimental system, based on the similar but less intense effect obtained with LY 294002 or a dominant-negative form of the PI3K p85α subunit (Figure 4). The effect of wortmannin on PI3K has been described as irreversible in the literature [31]. It was not appreciated until now that the wortmannin-induced formation of enlarged cytoplasmic vesicles involved a particular time window. In fact, longer incubations (> 3 h) with wortmannin are associated with a loss of giant vesicles and an apparent restoration of the normal B₂R–GFP cycling process (vesicular morphology, lack of receptor degradation based on immunoblots). The reversible effect of wortmannin is probably due to the known instability of the drug in aqueous solutions [31].

It has been reported in the literature that a constitutively active form of Rab5 (Rab5 Q79L) also induces the formation of giant endosomes [5,24,27]. Our results confirm these observations, although we have observed a decreased number and variable size of the endosomes in cells expressing Rab5 Q79L compared with wortmannin-treated cells. Stimulated B₂R–GFP are only found at the periphery of the smallest endosomes (similar in size to those induced by LY 294002 or the dominant-negative form of the PI3K p85α subunit), suggesting a modest participation of Rab5 in the formation of large endosomes related to receptor endocytosis compared with those resulting from cell fluid-phase uptake. Rab5 S34N partially inhibited the wortmannin-induced formation of giant vesicles, approximately to the level of LY 294002 effects, supporting the involvement of Rab5 in the mechanism of formation of enlarged vesicles. Interestingly, Rab5 S34N did not appear to affect B₂R–GFP endocytosis, in contrast with what was reported for other GPCRs [32–34].

Wortmannin not only affected the B₂R–GFP intracellular trafficking by inducing the formation of larger vesicles, but also changed the routing of at least a fraction of the internalized fluorescent receptors. Instead of being recycled back to the plasma membrane, some receptors were targeted towards the lysosomes for degradation (immunoblotting, binding assay). The lower efficacy of LY 294002 compared with wortmannin for formation of large vesicles had been noted previously [5] and may be further correlated with the lack of significant agonist-induced B₂R–GFP down-regulation (Figures 7A and 9A). The fact that wortmannin apparently slows down the intracellular trafficking of internalized B₂R–GFP could facilitate this new routing. The fast-paced entry of internalized receptors into early endosomes resulting in Rab5 activation combined with the inhibition of PI3K could favour the presence of receptors in the giant endosomes, leading to partial receptor breakdown. This degradation was prevented by lysosomal protease inhibitors (immunoblotting) as well as bafilomycin A1 (immunoblotting, accumulation of fluorescent material in vesicles observed in microscopy). The digestion of the densely expressed B₂R–GFP in agonist-stimulated wortmannin-treated HEK-293 cells is far from complete and is not particularly rapid (3 h), but the down-regulation of wild-type B₂Rs was functionally evident in rabbit jugular-vein strips that underwent repeated BK stimulations during treatment with wortmannin. The relevant cell type, rabbit SMCs, exhibited enlarged vesicular structures when treated with wortmannin in a cell culture system (Figure 1), suggesting that wortmannin acts similarly in the rabbit jugular vein as in our B₂R–GFP-expressing cellular system.

In conclusion, we have shown that wortmannin modifies the intracellular routing of the B₂R–GFP by targeting the activated fluorescent receptors towards enlarged cytoplasmic vesicles that facilitated their degradation in the lysosomes. This particular wortmannin effect is dependent on complete PI3K inhibition and on Rab5 activation. Observations in a more physiological system (jugular-vein contractility assay) support that the facilitation of GPCR down-regulation by wortmannin treatment is quantitatively significant.

Note added in proof (received 8 August 2003)

We have recently observed that HEK-293 cells co-transfected with the constitutively active Rab-5 Q79L and B₂R–GFP and further treated with LY 294002 (100 μ M) and the agonist BK (10 nM) exhibit giant vesicles positive at their periphery for the fluorescent receptors (confocal microscopy), thus fully reconstituting the effect of wortmannin combined to an agonist.

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