Activation of mitogen-activated protein kinase by the bradykinin B_2 receptor is independent of receptor phosphorylation and phosphorylation-triggered internalization

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Abstract Recent evidence suggests that serine/threonine phosphorylation and internalization of β_2-adrenergic receptors play critical roles in signalling to the mitogen-activated protein kinase cascade. To investigate whether this represents a general mechanism employed by G protein-coupled receptors, we studied the requirement of these processes in the activation of mitogen-activated protein kinase by $G\alpha_q$-coupled bradykinin B_2 receptors. Mutant B_2 receptors impaired in receptor phosphorylation and internalization are fully capable to activate mitogen-activated protein kinase. Bradykinin-induced long-term effects on mitogenic signalling monitored by measuring the transcriptional activity of Elk1 were identical in cells expressing the wild-type or mutant B_2 receptors. Therefore, G protein-coupled bradykinin receptors activate the mitogen-activated protein kinase pathway independently of receptor phosphorylation and internalization.

Key words: Bradykinin B_2 receptor; Mitogenic signalling; Mitogen-activated protein kinase; Internalization; Phosphorylation

1. Introduction

G protein-coupled receptors (GPCRs) are integral membrane proteins involved in the transmission of signals from a variety of extracellular ligands to the cytoplasm. Through heterotrimeric G proteins, they activate effector enzymes such as adenylyl cyclases, phospholipases or ion channels leading to changes in the level of second messengers and diverse cellular responses [1]. Activation of several GPCRs has been shown to elicit growth-promoting effects including stimulation of mitogenic signalling pathways, regulation of transcription, induction of DNA synthesis and cell proliferation. In addition, naturally occurring mutants of GPCRs and G proteins can induce cellular transformation when overexpressed in cultured fibroblasts [2,3].

The molecular mechanisms underlying GPCR-induced mitogenic signal transmission have been studied to some extent. Several reports indicate that many GPCRs can activate the Ras/mitogen-activated protein (MAP) kinase pathway, which is sufficient and necessary for the control of proliferation in different cell systems [2]. Translocation of the Grb2/Sos complex in the vicinity of membrane-associated Ras represents a common mechanism by which different GPCRs lead to Ras and MAP kinase activation [4]. This translocation is accomplished by activation of protein tyrosine kinases, such as Src family kinases, Bruton's tyrosine kinase, Pyk2, epidermal growth factor (EGF) or platelet-derived growth factor receptor kinases, which in turn lead to phosphorylation of the adaptor protein Shc and its association with the Grb2/Sos complex or by direct binding of Grb2 to tyrosine-phosphorylated EGF receptor or Pyk2 [4–7]. Mechanisms by which GPCRs activate protein tyrosine kinases appear to be different. $G\alpha_i$-coupled receptors preferentially utilize a $G\beta\gamma$-dependent route via PI3 kinase γ [8], whereas $G\alpha_q$-coupled receptors employ protein kinase C and/or calcium signals to activate protein tyrosine kinases and the MAP kinase pathway [4,7,9].

Recent reports have indicated that also serine/threonine phosphorylation and internalization of GPCRs might be involved in the modulation of these signalling pathways thus affecting the activation of MAP kinase. For example, an involvement of β-arrestin/dynamin-dependent receptor sequestration was reported to be important for the association with Src and the activation of MAP kinase by the $G\alpha_s$-coupled β_2-adrenergic receptor [10–12]. In addition, protein kinase A-mediated β_2-adrenergic receptor phosphorylation, formerly thought to be mainly responsible for heterologous receptor desensitization [13], has been shown to lead to a switch in G protein-coupling from G_s to G_i which permits signalling to the MAP kinase module in a $G\beta\gamma$-dependent manner [14]. However, a role of receptor phosphorylation and internalization in linking other prototypic GPCRs with MAP kinase activation has not yet been studied.

Therefore, we have investigated whether phosphorylation and/or internalization of the $G\alpha_q$-coupled bradykinin B_2 receptor (B_2R) is important for agonist-induced MAP kinase activation. B_2Rs have been implicated in the regulation of blood pressure and cell proliferation in response to kinin peptides [15–18]. They have been shown to activate the MAP kinase cascade via protein kinase C and/or calcium-dependent pathways, involving protein tyrosine kinases Pyk2, Src and EGF receptor [6,7,19,20]. Recently, we have demonstrated that B_2R internalization requires phosphorylation on three

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Abbreviations: B_2R, bradykinin B_2 receptor; EGF, epidermal growth factor; ERK2, extracellular-regulated kinase 2; GPCR, G protein-coupled receptor; HA, hemagglutinin; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; PMA, phorbol-12-myristate-13-acetate

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serines and two threonine residues in the carboxy-terminal tail [21]. In this report, we analyzed the ability of the wild-type B_2R or phosphorylation- and internalization-deficient receptor mutants to activate MAP kinase and regulate transcription in HEK 293T cells. Our data indicate that bradykinin-induced activation of MAP kinase and Elk-mediated transcription are not dependent on serine/threonine phosphorylation and phosphorylation-mediated internalization of B_2R.

2. Materials and methods

2.1. Materials

Rainbow protein marker, [γ-^{32}P]ATP (>4000 Ci/mmol) and [^{32}P]H_3PO_4 (285 Ci/mg P) were from Amersham, bradykinin was from Bachem, aprotinin (Trasylol) was from Bayer, Bradford protein assay kit was from Bio-Rad, leupeptin was from Boehringer Mannheim, Pefabloc was from FLUKA, Lipofectamine and protein ladder markers (10–200 kDa) were from Life Technologies, ATP, bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and myelin basic protein (MBP) were from SIGMA and protein A-agarose was from Zymed. All other chemicals of analytical grade were from Merck or SIGMA.

2.2. Plasmids

Human B_2R was cloned as previously described and subcloned into the mammalian expression vector pcDNA3 [22]. Mutants were generated using the Transformer site-directed mutagenesis kit from Clontech. In $B_2\Delta ST$ mutant, serines at positions 339, 346 and 348 as well as threonine at 342 and 345 were substituted by alanine and in B_2tI334, a stop codon was introduced after isoleucine 334 [21]. Hemaglutinin (HA)-tagged extracellular-regulated kinase 2 (ERK2) was kindly provided by C. Marshall, Institute of Cancer Research (London, UK).

2.3. Cell Culture and transfection

Human embryonic kidney cells HEK 293T were grown on six well plates to about 50% confluence in DMEM containing 10% FBS. Transfections were done in serum-free medium with 1–2 μg/well of the indicated cDNAs by the Lipofectamine method according to the supplier's manual. Cells were kept in a humidified 5% CO_2, 95% air atmosphere at 37°C. About 24 h after transfection, cells were serum-starved in DMEM containing 0.3% BSA for another 24 h and used for experiments.

2.4. Immunoprecipitation of HA-ERK2 and in vitro kinase reaction

Transfected and serum-starved cells were stimulated for 0.5–60 min with different concentrations of bradykinin at 37°C. After two washes with PBS, cells were scraped into 0.5 ml of ice-cold lysis buffer containing 50 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 2 mM sodium orthovanadate, 1% (w/v) Triton X-100, 10% (w/v) glycerol and protease inhibitors (1 mM Pefabloc, 10 μg/ml leupeptin and 1% Trasylol). Following 45 min at 4°C with gentle rocking, a soluble fraction was prepared by centrifugation at 4°C for 15 min at 13 000 rpm. Equal amounts of lysates (about 200 μg protein, measured by the Bradford assay) was subjected to immunoprecipitation with 5 μl of a polyclonal antiserum against the HA-tag (provided by S. Ekman, Ludwig Institute for Cancer Research, Uppsala, Sweden). Incubations were carried out with 35 μl protein A-agarose slurry for 3 h at 4°C. Resultant precipitates were washed three times with lysis buffer (see above) followed by two washes with kinase buffer containing 10 mM Tris, pH 7.5, 10 mM $MgCl_2$. Kinase reactions were started by addition of 30 μl kinase buffer including 200 μM ATP, 1 μCi [γ-^{32}P]ATP and 10 μg MBP. Reactions were performed for 20 min at room temperature and stopped by addition of 30 μl SDS sample buffer and heating for 2 min at 98°C. Proteins were separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and gels were cut above the 30 kDa marker band. Upper parts were transferred onto nitrocellulose membranes (Schleicher and Schuell) using a semi-dry unit (Bio-Rad) and probed with 0.5 μg/ml anti-ERK2 antibodies (Santa Cruz) in 5% BSA/TBS to check equal immunoprecipitation of HA-ERK2 in all samples. Lower parts were stained with 0.2% Coomassie brilliant blue R250 in 10% acetic acid, 12.5% isopropanol to monitor the amounts of MBP. Substrate phosphorylation was analyzed using a Phosphoimager (Fuji, BAS2000).

2.5. Receptor phosphorylation

Phosphorylation analysis of B_2R and its mutants in 293T cells was done as previously described [23]. For immunoprecipitation of radiolabelled receptors, a polyclonal anti-peptide antibody against the C-terminal domain was used [23]. For the B_2tI334 mutant lacking this epitope, a mixture of polyclonal antibodies against GST fusion proteins of the N-terminal and of the third extracellular domain was applied [24]. Precipitation of receptor protein was followed by parallel [^{35}S] labelling experiments.

2.6. Inositol phosphate generation and receptor internalization

The measurement of inositol phosphate generation triggered by kinin receptors and the analysis of receptor internalization were done as previously detailed [22].

2.7. Elk luciferase assay

The luciferase assays were done using pFA-Elk1 and pFR-Luc reporter plasmids of the PathDetect Reporting System (Stratagene) and the Luciferase Assay System kit (Promega). HEK 293T cells grown on 12 well plates to 50% confluence were transfected using the lipofectamine method, with the following amounts of cDNA per well: 200 ng receptor, 100 ng β-galactosidase, 50 ng luciferase (pFR-Luc) and 50 ng Elk1 (pFA-Elk1). After 6 h incubation, 10% FBS was added and cells were allowed to regenerate for 12 h. Thereafter, cells were serum-starved for 24 h, stimulated with 1 μM bradykinin for 12 h and lysed thereafter in 150 μl/well of 1% Triton X-100, 10% glycerol, 25 mM Tris phosphate pH 7.8, 2 mM dithiothreitol, 2 mM *trans*-1,2-diaminocyclohexane-*N,N,N',N'* tetra acetic acid. Luciferase activity in cell lysates was measured using the Luciferase Assay System from Promega and a Wallac 1420 multilabel counter. To determine the transfection efficiency, part of the lysates were analyzed for β-galactosidase activity.

3. Results

3.1. Functional characterization of B_2 receptor mutants in 293T cells

To investigate the role of phosphorylation and/or internalization of $G\alpha_q$-coupled receptors for MAP kinase activation, we used the following bradykinin B_2 receptor mutants: B_2tI334 with a C-terminal truncation after isoleucine at position 334 and a $B_2\Delta ST$ in which five serine and threonine residues within the carboxy-terminal domain were mutated to alanine. These mutant receptors are significantly impaired in their internalization capacity in COS-7 and CHO cells [21]. In this study, we employed HEK 239T cells since they do not express any endogenous kinin receptor (unlike COS-7 cells), they can be transiently transfected with high yields and they are a well-established cell model to study mitogenic signalling pathways [5,25].

Initially, we tested the functionality of the wild-type and mutant B_2R in transiently transfected 293T cells by measuring their coupling to phospholipase C and to the receptor phosphorylation and internalization machinery. Phospholipase C activation was studied by following bradykinin-induced generation of inositol phosphates [22]. Both B_2R mutants used in this study were able to stimulate phospholipase C activity to a similar extent as wild-type receptor, reflected by a 3–4-fold increase in the cellular level of inositol phosphates after 5 min of stimulation with 1 μM bradykinin (Fig. 1A).

We next analyzed the agonist-induced phosphorylation of B_2R variants in 293T cells. Following [^{32}P] labelling of transiently transfected 293T cells and immunoprecipitation of B_2R, receptor phosphorylation was visualized by autoradiography [23,24]. B_2R in 293T cells showed a weak basal phos-

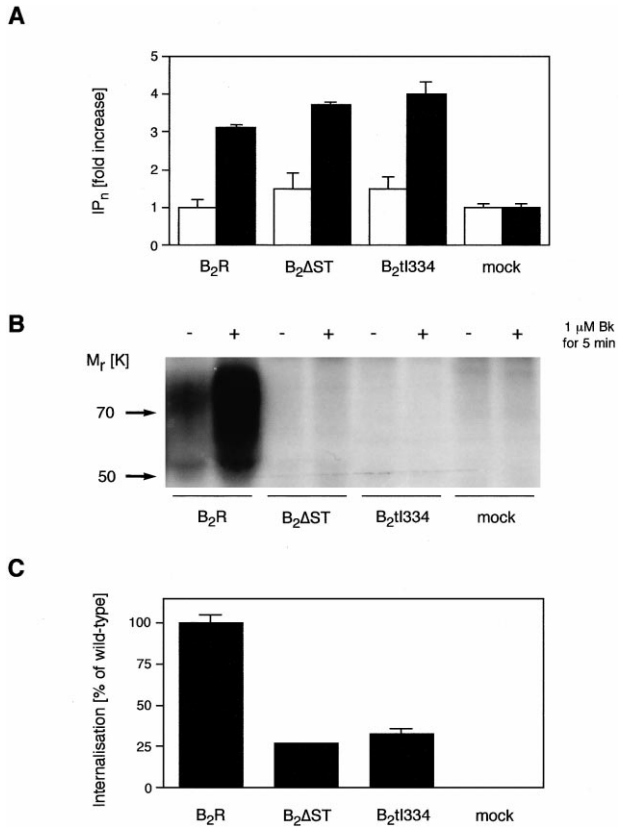

Fig. 1. Phospholipase C activation, bradykinin-induced phosphorylation and internalization of B_2R mutants. HEK 293T cells were transiently transfected with wild-type B_2R, $B_2\Delta ST$ or B_2tI334 or empty plasmid (mock). A: Increase of total inositol phosphates (IP_n) was measured without (white bars) and with 5 min of 1 μM bradykinin stimulation (gray bars). Means ± S.D. from five independent experiments are presented. B: Receptor phosphorylation was determined after 6 h of [^{32}P] labelling with 0.5 mCi/ml orthophosphate. Cells were lysed and B_2R was immunoprecipitated using polyclonal antibodies against the C-terminal domain (for wild-type and $B_2\Delta ST$) or to the N-terminal and third extracellular domain (B_2tI334). Precipitated proteins were separated by 10% SDS-PAGE and [^{32}P] incorporation was visualized by autoradiography. C: Receptor internalization was monitored by incubation of cells with 2 nM [^{3}H]bradykinin for 15 min. Following removal of extracellular radioactivity by a low pH wash [22], internalized ligand-receptor complexes were measured after cell lysis using a β-counter. Data are means ± S.D. of three experiments.

phorylation which increased 2–4-fold after 5 min of stimulation with 1 μM bradykinin (Fig. 1B). In contrast, the $B_2\Delta ST$ and B_2tI334 mutants failed to undergo any detectable agonist-induced phosphorylation (Fig. 1B). Comparable levels of expression and immunoprecipitated receptor proteins were confirmed by parallel binding and [^{35}S] labelling experiments (data not shown).

We then measured B_2R internalization in 293T cells closely following the kinetics of bradykinin-induced MAP kinase activation (see below). Transiently transfected 293T cells were incubated with 2 nM [^{3}H]bradykinin at 37°C for 15 min and surface-bound versus intracellular ligand was determined. Under these experimental conditions, 54 ± 3% of the radioactive bradykinin specifically bound to the wild-type B_2R was found intracellular in 293T cells. The $B_2\Delta ST$ as well as the B_2tI334 mutant were markedly impaired in their ability to undergo ligand-induced internalization reflected by a 3–4-fold reduction of intracellular localized B_2R (Fig. 1C). Cells transfected with empty expression vector (mock) had no specific [^{3}H]bradykinin binding and failed to respond to stimulation with 1 μM bradykinin in all three assays presented in Fig. 1.

3.2. Time- and dose-dependent activation of ERK2 in 293T cells by B_2 receptors

To characterize bradykinin-inducible MAP kinase activation, 293T cells were co-transfected with B_2R and HA-tagged ERK2 and stimulated with the indicated bradykinin concentrations for varying time periods. HA-ERK2 was immunoprecipitated from lysates and ERK2 kinase activity was determined by an in vitro MBP phosphorylation assay. The precipitation efficiency was controlled by immunoblotting with an ERK2 antibody. B_2R-mediated ERK2 activation displayed typical transient monophasic kinetics, with a peak in response after 5 min followed by a slow decrease over 60 min (Fig. 2A) and return to baseline at 120 min (not shown). Quantitative analysis of incorporated [^{32}P] revealed a 30–40-fold increase in ERK2 activity after 5 min of bradykinin stimulation. There was no increase in ERK2 kinase activity in cells stimulated with bradykinin when B_2R cDNA was omitted from the transfection mixtures consistent with the absence of endogenous receptors in 293T cells. MAP kinase activation was dependent on the dose of bradykinin applied, with an apparent EC_{50} of about 5 nM (Fig. 2B). Immunoblotting with ERK2 antibodies confirmed an equal precipitation efficiency for HA-ERK2 throughout the experiments (Fig. 2, lower panels). Both the kinetics and dose-dependency of bradykinin-induced ERK2 activation is similar in PC12 and HF-15 cells expressing endogenous B_2R (data not shown). Thus,

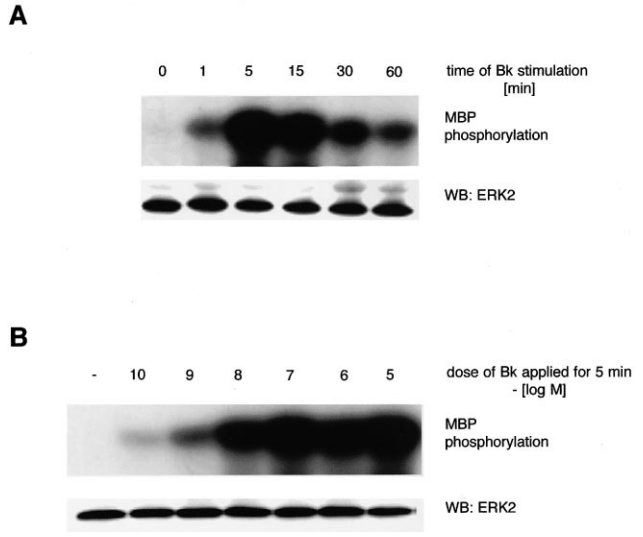

Fig. 2. Time- and dose-dependent ERK2 activation by B_2R in 293T cells. HEK 293T cells grown on six well plates were co-transfected with B_2R and HA-tagged ERK2. Following 24 h of serum starvation, cells were stimulated with 1 μM bradykinin for 1–60 min (A) or with 0.1 nM to 10 μM bradykinin for 5 min (B). ERK2 activity was determined after immunoprecipitation of the kinase with a polyclonal anti-HA antibody and using MBP as a substrate. Reactions were carried out for 20 min at room temperature. Western blots with a polyclonal ERK2 antibody were performed to confirm that equal amounts of ERK2 were present in the precipitates (lower panels) and phosphorylated MBP was detected by autoradiography.

transfected bradykinin receptors are functionally coupled to the ERK/MAP kinase pathway in 293T cells.

3.3. *ERK2 activation and Elk1-driven transcription induced by B_2 receptor are independent of receptor phosphorylation and internalization in 293T cells*

To test whether receptor phosphorylation and internalization are necessary for B_2R-induced MAP kinase stimulation and Elk1 transcriptional activation, we analyzed ERK2 kinase and Elk1 luciferase activity in 293T cells transfected with the wild-type or mutant B_2R. Stimulation of the $B_2\Delta$ST, which is phosphorylation-incompetent and shows a reduced internalization capacity, resulted in an ERK2 activation comparable to the parental B_2R (Fig. 3A). Similarly, challenge of truncated B_2tI334 receptors defective in ligand-induced phosphorylation and internalization led to a full ERK2 activation (Fig. 3A). For control, the levels of HA-ERK2 in the immunoprecipitates were analyzed by Western blotting using an antibody against ERK2 (Fig. 3A, center panel).

It has previously been shown that MAP kinase activation leads to phosphorylation of transcription factors of the Elk family and to an increase in their transcriptional activity [26]. We therefore studied long-term effects of bradykinin-induced MAP kinase activation by monitoring Elk1 transcriptional activation using a luciferase reporter assay. Robust Elk1 activation was observed when control cells were treated with 10% FBS/1 μM phorbol-12-myristate-13-acetate (PMA), a mixture known to strongly activate the MAP kinase cascade (Fig. 3B). Bradykinin stimulation of B_2R for 12 h led to a 12-fold increase in the Elk1-driven luciferase activity. Agonist challenge of truncated B_2tI334 receptors or $B_2\Delta$ST mutants increased the Elk1 transcriptional activity to a comparable level as the parental B_2R (Fig. 3B). Taken together, these results suggest that both short- and long-term effects induced by MAP kinase activation are independent on $G\alpha_q$ protein-coupled B_2R phosphorylation and internalization.

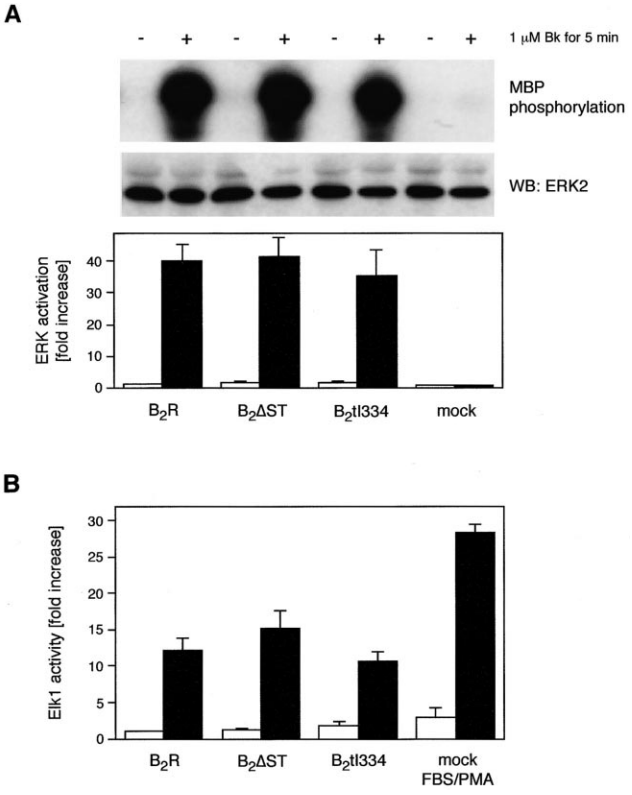

Fig. 3. Bradykinin-induced ERK2 and Elk1 activation by B_2R and mutants. A: HEK 293T cells were co-transfected with empty plasmid (mock), wild-type B_2R, $B_2\Delta$ST or B_2tI334 and HA-tagged ERK2. After 5 min of 1 μM bradykinin stimulation, ERK2 activities (upper panel) and kinase levels (center panel) were analyzed. Substrate phosphorylations with (gray bars) or without (white bars) agonist stimulation were quantified using a phosphoimager (lower panel). Means ± S.D. of three independent experiments are shown. B: For Elk1 activation, a reporter gene assay was used. Luciferase activities in cells stimulated with 1 μM bradykinin for 12 h (gray bars) were measured and compared with background levels (white bars). For control, mock-transfected cells were stimulated with 10% FBS and 1 μM PMA. Data ± S.D. were calculated from six independent experiments.

4. Discussion

Several intracellular pathways which link GPCRs with the activation of the Ras/MAP kinase cascade have been described, including activation of protein kinase C, increase in intracellular calcium, activation of PI3 kinase γ and both receptor and non-receptor tyrosine kinases as well as translocation of the Grb2/Sos complex [4,6,7,9]. More recently, it was suggested that receptor phosphorylation on serine/threonine residues and internalization play also an important role in modulating the MAP kinase activity by the β_2-adrenergic receptor [10–12,14]. β_2-adrenergic receptor internalization appears to be necessary for the assembly of signalling proteins involved in the MAP kinase activation [10,14], pointing to a novel mechanism by which GPCRs may be linked to mitogenic signalling machinery.

In this study, we examined whether the $G\alpha_q$-coupled bradykinin B_2 receptor utilizes a similar phosphorylation- and internalization-dependent mechanism to activate the Ras/MAP kinase pathway. Bradykinin-induced MAP kinase activation appears to be a fast and monophasic process (Fig. 2). Similarly, B_2R phosphorylation occurs within minutes of exposure to ligand [23] and therefore, it could be involved in regulation of the MAP kinase pathway. However, the B_2R mutants $B_2\Delta$ST and B_2tI334 which are completely impaired in phosphorylation are still fully functional in coupling to the MAP kinase pathway and phospholipase C signalling (Figs. 1 and 2). Given the fact that the acute MAP kinase activation induced by bradykinin in in vitro kinase assays (maximum at 5 min) significantly precedes B_2R internalization (maximum at about 60 min), we have also tested the long-term effects of MAP kinase activation by monitoring Elk1 transcriptional activity in the nucleus. The wild-type and mutant B_2Rs activated Elk1 to the similar extent after 12 h stimulation indicating that the capacity to induce Elk transcriptional activity was independent of phosphorylation-mediated internalization of B_2R.

We have previously shown that B_2R phosphorylation occurs in the carboxy-terminal tail and that this phosphorylation is required for full agonist-induced internalization [21,23]. Agonist-induced phosphorylation was shown to be causally linked to internalization for several GPCRs, including the β_2-adrenergic [27] and the m_2-muscarinic acetylcholine receptor [28]. Phosphorylated GPCRs can bind arrestin which in turn targets receptors to clathrin coated pits and subsequent endocytosis [29]. A recent study revealed a new function of β-arrestin, it can act as an adapter protein, recruiting acti-

vated Src to the agonist-occupied β_2-adrenergic receptor and thereby initiating the MAP kinase cascade [10]. In PC12 cells, bradykinin-induced complex formation between Pyk2 and Src leads to Src activation [7], suggesting that B_2R might use other mechanisms than arrestin-mediated internalization to activate Src and subsequently the MAP kinase cascade in 293T cells. This is consistent with our observations that phosphorylation- and internalization-impaired mutants of B_2R are fully capable of activating MAP kinase. It appears therefore that different GPCRs such as β_2-adrenergic or bradykinin B_2 receptors might employ internalization-dependent or -independent pathways to activate MAP kinase, respectively.

Taken together, our data show that receptor phosphorylation and phosphorylation-mediated internalization are not required for bradykinin-induced MAP kinase activation and Elk1 transcriptional control in 293T cells. Additional studies with other types of GPCRs are needed to further explore whether phosphorylation and internalization play a more common role in regulation of the MAP kinase pathway by different GPCRs.

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