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Agonist-induced internalization and mitogen-activated protein kinase activation of the human prostaglandin EP4 receptor

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Abstract We examined the pathway of prostaglandin E_2 (PGE_2)-induced internalization of the prostaglandin EP4 receptor in HEK 293 cells. Co-expression of dominant negative β-arrestin (319–418) or dynamin I (K44A) with the EP4 receptor reduced internalization. The activated receptor co-localized with GFP-arrestin 2 and GFP-arrestin 3, confirming the requirement for β-arrestins in internalization. Inhibition of clathrin-coated vesicle-mediated internalization resulted in inhibition of sequestration, whereas inhibition of caveola-mediated internalization had no effect. PGE_2 stimulation of the EP4 receptor resulted in rapid mitogen-activated protein (MAP) kinase activation. Examination of an internalization-resistant mutant and co-expression of mutant accessory proteins with EP4 revealed that MAP kinase activation proceeds independently of internalization. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Prostaglandin E_2; EP4 receptor; Internalization; Mitogen-activated protein kinase

1. Introduction

Prostaglandin E_2 (PGE_2) is involved in many physiological and pathological events including regulation of blood flow, cytoprotection and inflammation [1]. PGE_2 acts through four G protein-coupled receptors (GPCRs), designated EP1, EP2, EP3 and EP4, which are encoded by different genes [2]. The EP1 receptor couples to phospholipase C, the EP2 and EP4 receptors couple to stimulation of adenylyl cyclase, while the EP3 receptor couples to inhibition of adenylyl cyclase.

The prostaglandin EP4 receptor displays broad tissue distribution, with high levels in the small intestine, thymus, lungs, spleen, heart and uterus [3]. Gene knockout of the receptor shows that it is involved in closure of the ductus arteriosus at birth [4,5].

The EP4 receptor is a 488 amino acid protein with a long cytoplasmic tail, which has previously been implicated in agonist-induced desensitization [6]. We localized the region involved to a 14 amino acid stretch containing six serine residues [7]. Recently, we showed that the carboxy tail of the human EP4 receptor also participates in agonist-induced internalization. Successive truncation of the carboxy-terminus resulted in gradual attenuation of internalization although mutation of 10 serine and one threonine residues had no effect on internalization [8].

Internalization of GPCRs involves rapid receptor–G protein uncoupling followed by translocation of receptors from the plasma membrane to intracellular vesicles [9]. The processes underlying internalization have been widely studied, with the β_2-adrenergic receptor (β_2AR) serving as the paradigm for other GPCRs [10,11]. However, it has become increasingly apparent that the molecular events responsible for β_2AR regulation do not necessarily apply to all GPCRs. There appear to be at least two distinct pathways [12]. The pathway for which the β_2AR is the prototype involves agonist-induced phosphorylation of the receptor by G protein-coupled receptor kinases, followed by β-arrestin binding [13–15]. The receptor–β-arrestin complex binds to clathrin and is subsequently internalized in a process dependent on the GTPase, dynamin [16,17]. Another mechanism, implicated in internalization of the angiotensin II type 1A receptor, does not involve either β-arrestin or dynamin [17]. Agonist-induced internalization of some receptors can also be mediated by caveolae [18].

In the present study, we have investigated the pathway of EP4 receptor internalization in HEK 293-EBNA cells. We examined whether EP4 receptor endocytosis requires β-arrestin- and dynamin-dependent mechanisms or a caveola-mediated mechanism. In addition, recent studies have demonstrated the requirement of β-arrestin- and dynamin-dependent receptor endocytosis for activation of the mitogen-activated protein (MAP) kinase pathway [19,20]. In light of this, we examined activation of MAP kinase by the EP4 receptor and its relationship to internalization of the receptor.

2. Materials and methods

2.1. Materials

HA monoclonal antibody (HA 11) was from Babco (Richmond, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated and Alexa 594-conjugated goat anti-mouse antibodies were from Molecular Probes (Eugene, OR, USA). HEK 293-EBNA cells were from Invitrogen (Carlsbad, CA, USA). The cDNAs for green fluorescent protein (GFP)-arrestin 2, GFP-arrestin 3, dominant negative dynamin (K44A) and β-arrestin (319–418) were kindly provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA, USA).

2.2. Cell culture and transfection

HEK 293-EBNA cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 250 μg/ml geneticin to maintain expression of the EBNA-1 plasmid, in 5% CO_2 at 37°C. Transfection was carried out using Lipofectamine 2000 reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions. Stable clones were selected using 200 μg/ml hygromycin B. HA-epitope-tagged EP4 receptor and the truncated

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mutant HA-t350 were prepared as described previously [8]. To examine the effects of β-arrestin or dynamin on sequestration, cells were transfected with 1 μg/well of the dominant negative β-arrestin (319–418) or dynamin in pcDNA3.

2.3. ELISA assay

ELISA was performed as described previously [8]. Briefly, transfected cells treated with 1 μM PGE_2 or vehicle for 60 min were fixed with 2% PFA. Cells were washed, blocked and treated with 1:1000 anti-HA monoclonal antibody (HA 11) for 30 min. Thereafter, cells were washed, blocked and incubated with 1:1000 dilution of a goat anti-mouse horseradish peroxidase-conjugated secondary antibody for 30 min. Lastly, cells were incubated with *o*-phenylenediamine with 30% hydrogen peroxide solution for 15 min, aliquots placed into 96 well plates containing concentrated sulfuric acid, and absorbance read at 450 nm using a Perkin-Elmer microtiter plate reader. Results were expressed relative to values obtained with untreated cells as percent surface HA immunoreactivity.

2.4. Confocal microscopy

HEK 293-EBNA cells were grown on coverslips in six well plates until 85% confluent and then transfected. To examine the effect of GFP-arrestin 2 (β-arrestin) and GFP-arrestin 3 (β-arrestin 2) on EP4 receptor internalization, cells were co-transfected with 4 μg of receptor cDNA and 0.5 μg of either GFP-arrestin 2 or 3. On the day of assay, the cells were washed once with Hanks' buffered saline solution and incubated with DMEM buffered with 20 mM HEPES, pH 7.4. Cells were treated with either vehicle or 1 μM PGE_2 for 60 min and fixed with 2% PFA. Cells were permeabilized with 0.05% Triton X-100 in phosphate-buffered saline, labeled with a 1:500 dilution of the primary anti-HA antibody for 30 min, washed and blocked with 5% BLOTTO for 30 min at 37°C and then incubated with a goat anti-mouse Alexa 594 secondary antibody for 30 min at 37°C. Fluorescence was examined using a Leica TCS-NT confocal microscope. Single optical sections are presented, representing the average of eight scans.

2.5. MAP kinase phosphorylation

HEK 293-EBNA cells stably expressing the HA-hEP4 receptor or the truncated mutant receptor HA-t350 receptor were grown in six well plates until 50–60% confluent. On the day of the experiment, cells were serum-starved for 1 h at 37°C in DMEM and treated with 1 μM PGE_2. Reactions were stopped by rapidly removing agonist-containing medium and lysed by adding 200 μl SDS sample buffer. The samples were boiled, centrifuged, resolved by SDS–PAGE and transferred onto PVDF membranes for detection of phosphorylated MAP kinase. Membranes were probed using a phospho-p44/42 MAP kinase polyclonal antibody (1:2000) for 1 h (Cell Signaling Technology, Beverly, MA, USA) and phosphorylated protein detected by ECL.

3. Results

3.1. Effect of dominant negative dynamin I (K44A) on EP4 receptor internalization

To delineate the role of dynamin in agonist-induced internalization of EP4, we studied the effect of the dynamin I mutant K44A on HA-hEP4 receptor internalization. ELISA analysis revealed that transfected dynamin K44A reduced agonist-induced internalization of EP4 receptor from 40% to 15%, indicating that dynamin is involved in the process (Fig. 1).

3.2. Effect of dominant negative β-arrestin 1 (319–418) on EP4 internalization

To examine the involvement of arrestin in EP4 receptor internalization, we co-expressed dominant negative β-arrestin (319–418) or empty pcDNA3 vector with the HA-hEP4 receptor. The β-arrestin (319–418) mutant inhibits receptor internalization by binding constitutively to clathrin [21]. Co-expression with EP4 resulted in reduced internalization from

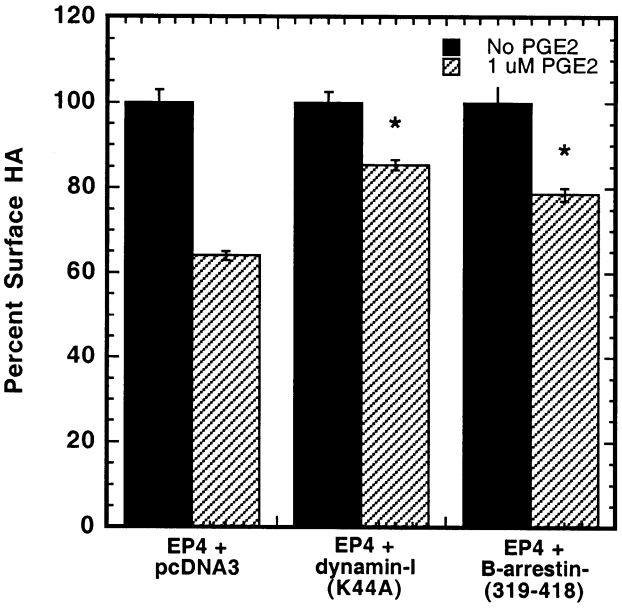

Fig. 1. Effect of dominant accessory proteins on HA-hEP4 receptor internalization. HEK 293-EBNA-HA-hEP4 cells were transfected with either pcDNA3, dominant negative β-arrestin 1 (319–418) or dynamin I (K44A). Cells were treated with vehicle or 1 μM PGE_2 for 60 min. Surface HA immunoreactivity was measured by ELISA and expressed as a percentage of samples not treated with PGE_2. Values represent the mean ± S.E.M. of four experiments, each done in triplicate. Asterisks indicate $P < 0.001$ compared with wild-type HA-hEP4 (+pcDNA3) according to a one way ANOVA.

40% to approximately 25% (Fig. 1). While the effect of β-arrestin (319–418) was not as pronounced as the effect of dynamin I (K44A), the results were statistically significant as determined by a one way ANOVA.

3.3. Colocalization of GFP-arrestin 2 and GFP-arrestin 3 with agonist-activated EP4 receptor

To examine the role of arrestins 2 and 3 in the agonist-induced internalization of the EP4 receptor, we transiently co-transfected EP4 cDNA with either GFP-arrestin 2 or GFP-arrestin 3 to visualize the localization of each protein under agonist-treated and untreated conditions. In the absence of PGE_2, the EP4 receptor is localized primarily to the plasma membrane while the arrestins are distributed throughout the cytosol (Fig. 2a–c). Upon treatment with 1 μM PGE_2 for 60 min, the receptor was internalized into punctate compartments with a corresponding loss of surface fluorescence (Fig. 2e,h). Agonist treatment also resulted in a punctate distribution of GFP-arrestin 2 (Fig. 2d) and GFP-arrestin 3 (Fig. 2g), overlapping significantly with the internalized receptor (Fig. 2f,i). These results suggest that both arrestin 2 and arrestin 3 play a role in sequestration of the EP4 receptor.

3.4. Effect of acetic acid and PMA on EP4 receptor internalization

To further examine EP4 receptor internalization via clathrin-coated vesicles, cells co-expressing both receptor and GFP-arrestin 2 were pretreated with either 5 mM acetic acid, to prevent clathrin-mediated internalization, or 1 μM phorbol 12-myristate 13-acetate (PMA) to prevent caveola-mediated internalization. Immunofluorescence confocal microscopy of cells treated with acetic acid revealed that despite

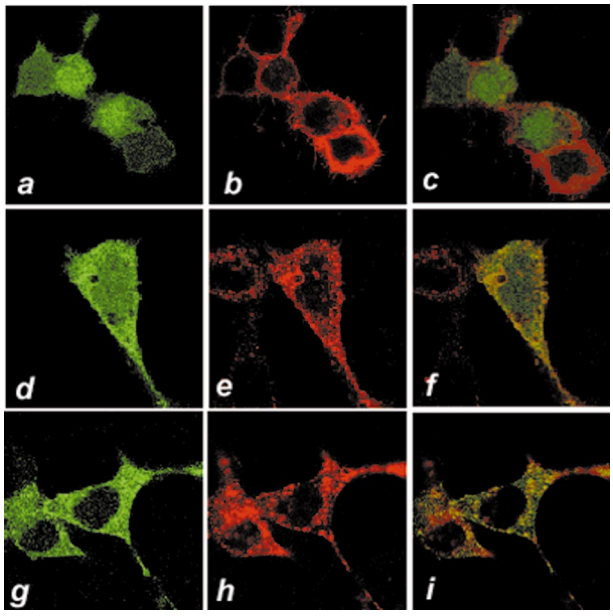

Fig. 2. Co-localization of HA-hEP4 with GFP-arrestin 2 and GFP-arrestin 3. HEK 293-EBNA cells were transiently co-transfected with HA-hEP4 and either GFP-arrestin 2 or GFP-arrestin 3 cDNAs. Forty-eight hours after transfection, the cells were treated with vehicle (a–c) or 1 μM PGE_2 (d–i) for 60 min and then processed for immunofluorescence microscopy. a,d,g: Localization of GFP-arrestin. b,e,h: Receptor localization. c,f,i: Overlay of the two individual images. Regions of overlap are in yellow. Images are representative of three to four individual experiments.

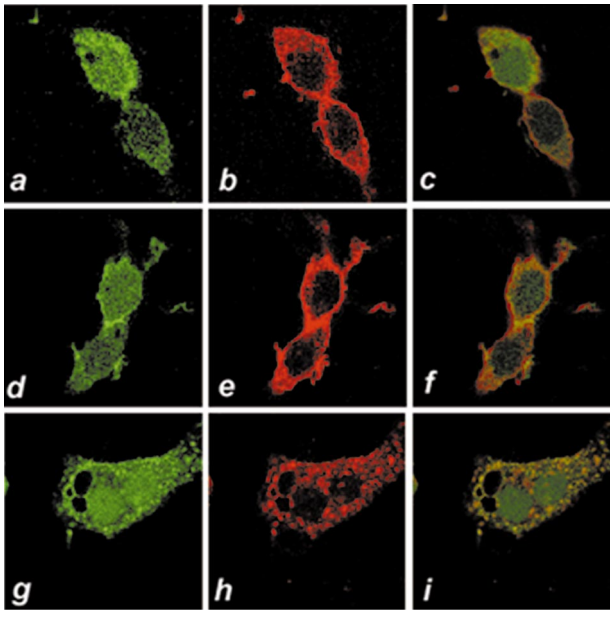

Fig. 3. Effect of acetic acid and PMA on HA-hEP4 receptor internalization. HEK 293-EBNA cells co-expressing HA-hEP4 and GFP-arrestin 2 were preincubated with either 5mM acetic acid (d–f) for 5 min or 1 μM PMA (g–i) for 30 min at 37°C and then treated with either vehicle (a–c) or 1 μM PGE_2 (d–i) for 30 min at 37°C. The cells were processed for detection of total receptor (surface and internalized) and visualized using confocal microscopy. The middle panel shows the effect of acetic acid and the bottom panel shows the effect of PMA on PGE_2-stimulated internalization. Images are representative of 2 separate experiments.

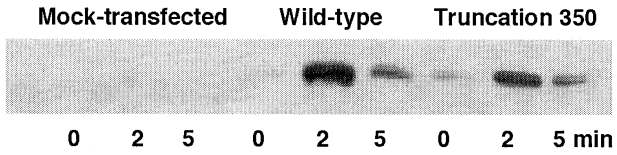

Fig. 4. Effect of HA-hEP4 receptor internalization on MAP kinase activation. HEK 293-EBNA cells stably expressing HA-hEP4, HA-t350 or pCEP4 vector were serum-starved and then stimulated with 1 μM PGE_2 for 0, 2, or 5 min at 37°C. The samples were processed for detection of phosphorylated p42 and p44 MAP kinase. Experiments were repeated three times, with similar results.

PGE_2 stimulation, the HA-hEP4 receptor was still localized to the plasma membrane, and there was also no visible translocation of GFP-arrestin 2 (Fig. 3d,e). The overlay shown in Fig. 3f shows the lack of any significant co-localization. In addition, pretreatment with PMA to prevent caveola-mediated internalization had no effect on EP4 receptor internalization as illustrated in Fig. 3g–i, and the receptor and GFP-arrestin 2 continued to co-localize, indicating that in HEK 293 cells, hEP4 receptor sequesters via clathrin-coated vesicles in an arrestin- and dynamin-dependent manner.

3.5. Role of EP4 receptor internalization in MAP kinase activation

PGE_2 activation of HEK 293-EBNA cells stably expressing the EP4 receptor resulted in rapid activation of MAP kinase within 2 min, as detected by Western blotting. The amounts of phosphorylated p42 and p44 were significantly higher than basal levels (Fig. 4). However, agonist stimulation of cells expressing the construct HA-t350, which is the severely truncated EP4 receptor mutant, resistant in internalization capability [8], also resulted in rapid phosphorylation of MAP kinase (Fig. 4), although the amount of phosphorylated MAP kinase was lower than the observed level for wild-type EP4 receptor.

To further analyze the relationship between receptor internalization and MAP kinase phosphorylation, the effect of the dominant negative mutants dynamin-I (K44A) and β-arrestin 1 (319–418) was also studied. Because both dominant negative proteins significantly reduced agonist-induced internalization of hEP4, analysis of their effect on PGE_2-stimulated MAP kinase activation via EP4 receptor would help to further examine the relationship between receptor internalization and MAP kinase activation. Agonist stimulation of cells expressing either of these mutant accessory proteins revealed that inhibition of internalization does not prevent activation of the MAP kinase cascade (Fig. 5). HEK 293-EBNA cells trans-

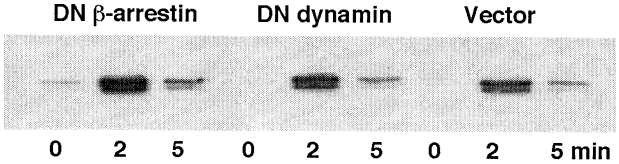

Fig. 5. Effects of dominant negative accessory proteins on PGE_2-induced MAP kinase phosphorylation. HEK 293-EBNA-HA-hEP4 cells were transiently transfected with either dominant negative β-arrestin 1 (319–418), dynamin-I (K44A) or pcDNA3 vector. Forty-eight hours after transfection, samples were processed for detection of phosphorylated MAPK. Experiments were repeated twice with similar results.

fected with pCEP4 vector alone were used as a control to validate the activation of MAP kinase in EP4 receptor transfected cells.

4. Discussion

In the present report, we have shown that agonist-induced internalization of the human EP4 receptor is mediated in a dynamin- and β-arrestin-dependent pathway in HEK 293 cells. Co-expression of dominant negative dynamin I (K44A) or β-arrestin (319–418) with the EP4 receptor reduced internalization from 40% to 15% and 25%, respectively, indicating involvement of endogenous arrestin and dynamin. Dynamin I is a GTPase that regulates the formation of clathrin-coated vesicles and is involved in the fission of endocytic vesicles from the plasma membrane [22]. Mutant dynamin I (K44A) is defective in GTP binding and blocks endocytosis at a stage after the initiation of coated vesicle formation but before sequestration into coated pits [23]. The effect of dynamin I (K44A) on EP4 receptor sequestration was more pronounced than β-arrestin (319–418). The partial effect of this mutant β-arrestin has been observed in studies with other GPCRs [24]. It is possible that the altered protein is not a very competitive inhibitor of endogenous β-arrestin 1 or that it is not expressed at a high enough concentration to produce a stronger effect. Nevertheless, the reversal of internalization by both mutants was determined to be statistically significant.

An alternative approach would be to over-express wild-type arrestin to enhance internalization. However, HEK 293 cells have high endogenous levels of arrestins [21,25] so that over-expression has a minimal effect on receptor internalization [25]. In other cell types, protein over-expression may cause non-specific effects, forcing regulatory events to occur that are not physiologically relevant.

The non-visual arrestins, arrestin 2 and arrestin 3, have been implicated in receptor-mediated endocytosis of a number of GPCRs. Particularly, they play an integral role as adapter proteins in the agonist-induced internalization of the β_2AR by binding directly to clathrin, the major component of clathrin-coated pits [14,16]. As further evidence for the involvement of arrestins in EP4 receptor internalization, we showed that GFP-β-arrestin 1 or 2 co-localize with the EP4 receptor following PGE_2 stimulation.

We used acetic acid as a probe to examine the role of clathrin. Acetic acid causes acidification of vesicles, which in turn prevents the pinching off of clathrin-coated pits to form coated vesicles [26]. Previously, acidification has been used successfully in studies with other receptors that internalize using a clathrin- and arrestin-dependent pathway. For example, internalization of m1 mAChR was determined to sequester via clathrin-coated vesicles using acetic acid to disrupt the process [27]. Treatment of EP4-expressing cells with acetic acid confirmed that the receptor internalization is mediated by β-arrestin via clathrin-coated pits.

Activators of protein kinase C such as PMA have been shown to prevent invagination of the caveolar membrane, blocking caveola-mediated internalization [28]. Pretreatment of EP4-expressing cells with 1 μM PMA had no effect on internalization and the stimulated receptor continued to co-localize with GFP-arrestin 2, indicating that caveolae are not involved.

A role for endocytosis in MAP kinase activation has been proposed for the β_2AR and the m1 mAChR [19,20]. We showed that stimulation of the EP4 receptor also leads to activation MAP kinase activation, however, internalization does not appear necessary for activation. We previously showed that the severely truncated mutant HA-t350 exhibits sharply attenuated PGE_2-induced internalization [8]. In the present work, we show that HA-t350 rapidly phosphorylates MAP kinase, independently of internalization capability. Although agonist treatment of this truncated mutant continued to activate MAP kinase, phosphorylation was somewhat reduced compared with wild-type receptor. The reduced phosphorylation may be explained by the fact that truncation of the EP4 receptor eliminates sites in the carboxy tail that may be required for MAP kinase activation. For example, a recent report showed evidence that proline-rich regions in the third intracellular loop and carboxy tail of the β_3AR [29] interact directly with the SH3 domains of Src to activate MAP kinase. Such a mechanism of activation of MAP kinase is possible because there is one PXXP motif in the carboxy tail of the EP4 receptor that is removed by truncation at amino acid 350.

We also showed that expression of dominant negative β-arrestin 1 (319–418) or dynamin I (K44A), both of which significantly reduced internalization of the EP4 receptor, had no effect on EP4-mediated MAP kinase activation. In addition to our report, studies of other receptors have also indicated that MAP kinase activation occurs independently of receptor internalization. Examples of such studies are the mu opioid receptor [24] and the α_2-adrenergic receptor [30].

In summary, we have presented evidence that the prostaglandin EP4 receptor subtype internalizes rapidly by a dynamin- and arrestin-dependent mechanism. EP4 receptor stimulation leads to activation of the MAP kinase cascade but this activation does not depend on internalization of the receptor.

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