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ACCELERATED COMMUNICATION

EP_4 Prostanoid Receptor Coupling to a Pertussis Toxin-Sensitive Inhibitory G Protein

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ABSTRACT

The EP_2 and EP_4 prostanoid receptor subtypes are G-protein-coupled receptors for prostaglandin E_2 (PGE_2). Both receptor subtypes are known to couple to the stimulatory guanine nucleotide binding protein ($G\alpha_s$) and, after stimulation with PGE_2, can increase the formation of intracellular cAMP. In addition, PGE_2 stimulation of the EP_4 receptor can activate phosphatidylinositol 3-kinase (PI3K) leading to phosphorylation of the extracellular signal-regulated kinases (ERKs) and induction of early growth response factor-1 (EGR-1) (*J Biol Chem* **278**: 12151–12156, 2003). We now report that the PGE_2-mediated phosphorylation of the ERKs and induction of EGR-1 can be blocked by pretreatment of EP_4-expressing cells with pertussis toxin (PTX). Furthermore, pretreatment with PTX increased the amount of PGE_2-stimulated intracellular cAMP formation in EP_4-expressing cells but not in EP_2-expressing cells. These data indicate that the EP_4 prostanoid receptor subtype, but not the EP_2, couples to a PTX-sensitive inhibitory G-protein ($G\alpha_i$) that can inhibit cAMP-dependent signaling and activate PI3K/ERK-dependent signaling.

Prostaglandin E_2 (PGE_2) is an endogenous signaling molecule that is produced from arachidonic acid by the sequential actions of cyclooxygenase (COX) and PGE_2 synthase. PGE_2 is also referred to as a prostanoid, which is a term that encompasses the other prostaglandins (e.g., PGD_2 and $PGF_2\alpha$) and thromboxanes. PGE_2 can bind to and stimulate four major prostanoid receptor subtypes that have been named EP_1, EP_2, EP_3, and EP_4 (Coleman et al., 1994). These receptors are all seven transmembrane-spanning receptors that activate intracellular second messenger signaling pathways by interacting with heterotrimeric G-proteins. There are four major subfamilies of G-proteins that are defined by their α subunits ($G\alpha$) and by the nature of the signaling pathways they activate (Hepler and Gilman, 1992). Perhaps the most well known are members of the $G\alpha_s$ and $G\alpha_i$ subfamilies, whose activation affects the formation of intracellular cAMP by either stimulating or inhibiting the activity of adenylyl cyclase, respectively. Members of the $G\alpha_i$ subfamily are also known as pertussis toxin (PTX) sensitive G-proteins because they can be inhibited by the actions of this toxin, which is the causative agent of whooping cough. Members of the $G\alpha_q$ subfamily activate phospholipase C to stimulate inositol phosphate and Ca^{2+} signaling, whereas members of the $G\alpha_{12}$ subfamily affect signaling pathways that involve the activation of Rho, a member of the family of small monomeric G-proteins.

The EP receptor subtypes interact with several of the subfamilies of G-proteins to activate their respective signaling pathways. PGE_2 stimulation of the human EP_1 receptor increases the concentration of free intracellular Ca^{2+} (Funk et al., 1993) and stimulates inositol phosphate formation (J. W. Regan, unpublished observations), suggesting coupling to members of the $G\alpha_q$ subfamily. The EP_3 receptors are traditionally thought to couple to $G\alpha_i$ to inhibit adenylyl cyclase. However, the EP_3 receptors actually consist of multiple isoforms that are generated by alternative mRNA splicing, and their coupling to G-proteins is complex (Kotani et al., 1995). For example, in humans, there are eight isoforms, and at

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ABBREVIATIONS: PG, prostaglandin; COX, cyclooxygenase; PTX, pertussis toxin; Tcf, T-cell factor; HEK, human embryonic kidney; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; EGR-1, early growth response factor-1; PGE_1-OH, PGE_1-alcohol; ERK, extracellular signal-regulated kinase.

least two of these isoforms, the $EP_{3\text{-}II}$ and $EP_{3\text{-}IV}$, seem to couple to $G\alpha_s$ to stimulate adenylyl cyclase. The human $EP_{3\text{-}I}$ and $EP_{3\text{-}II}$ can also couple to $G\alpha_q$ to stimulate inositol phosphate formation.

Stimulation of the human EP_2 and EP_4 receptors with PGE_2 increases intracellular cAMP formation, indicating that both of these isoforms can couple to $G\alpha_s$ to stimulate adenylyl cyclase (Regan, 2003). However, functional coupling to the cAMP signaling pathway seems to be more efficient for the human EP_2 receptor subtype than for the EP_4 subtype. Thus, when stably expressed in HEK cells at similar levels of receptor expression, the maximal stimulation of intracellular cAMP formation by the EP_4 subtype is only 20 to 50% of that achieved by the EP_2 subtype (Fujino et al., 2002, 2005). It has also been found recently that the human EP_4 receptor subtype, but not the human EP_2 subtype, can activate a phosphatidylinositol 3-kinase (PI3K) signaling pathway by a mechanism that is independent of the activation of the cAMP/protein kinase A (PKA) pathway (Fujino et al., 2002, 2003, 2005). PGE_2-mediated activation of this PI3K signaling pathway by the human EP_4 receptor leads to the induction of functional expression of early growth response factor-1 (EGR-1) (Fujino et al., 2003) and to the inhibition of the activity of PKA (Fujino et al., 2005). We now report that activation of the PI3K signaling pathway by the human EP_4 receptor involves the coupling of this receptor to a PTX-sensitive, cAMP-inhibitory G-protein ($G\alpha_i$). Coupling of the EP_4 receptor to $G\alpha_i$ explains, in part, the less efficient coupling of the EP_4 receptor to the cAMP/PKA signaling pathway compared with the EP_2 receptor subtype.

Materials and Methods

Cell Culture. Cell lines stably expressing the EP_2 or EP_4 receptors were prepared using HEK-293–Epstein-Barr virus nuclear antigen cells and the mammalian expression vector $pCEP_4$ (Invitrogen, Carlsbad, CA) as described previously (Fujino et al., 2002). Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 250 μg/ml geneticin, 100 μg/ml gentamicin, and 200 μg/ml hygromycin B.

cAMP Assay. Cells were cultured in 12-well plates; 16 h before the immunoblotting experiments, cells were switched from their regular culture medium to Opti-MEM (Invitrogen) containing 250 μg/ml G-418 (Geneticin) and 100 μg/ml gentamicin. Cells were pretreated with either vehicle (water) or 100 ng/ml PTX (Calbiochem, San Diego, CA) for 16 h at 37°C. Cells were then treated with 0.1 mg/ml 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO) for 15 min followed by treatment with either vehicle (0.1% dimethyl sulfoxide), 1 μM PGE_2 (Cayman Chemical, Ann Arbor, MI), or 1 μM PGE_1-alcohol (PGE_1-OH; Cayman) for 10 min at 37°C. In experiments using forskolin, 3 μM forskolin (Calbiochem) was added for an additional 15 min after the initial treatments with PTX or drugs. Experiments were terminated by the removing the media and placing the cells on ice. Two hundred microliters of Tris/EDTA buffer (50 mM Tris-HCl and 4 mM EDTA, pH 7.5) was added, and the cells were scraped off and transferred to microcentrifuge tubes. The samples were boiled for 8 min, placed on ice, and centrifuged for 1 min at 14,000 rpm in a microcentrifuge. Five microliters of the supernatants (representing $\sim 5 \times 10^4$ cells) was added to new tubes containing 50 μl of [^{3}H]cAMP (PerkinElmer Life and Analytical Sciences, Boston, MA) and 100 μl of 0.06 mg/ml PKA (Sigma). The mixture was vortexed and incubated on ice for 2 h, followed by the addition of 100 μl of Tris/EDTA buffer containing 2% bovine serum albumin and 26 mg/ml powdered charcoal. After vortexing and centrifugation for 1 min at 14,000 rpm, 100-μl aliquots of the supernatants were removed, and radioactivity was measured by liquid scintillation counting. The amount of cAMP present was calculated from a standard curve prepared using nonradioactive cAMP and was expressed as picomoles per 5×10^4 cells.

Western Blotting. Sixteen hours before the immunoblotting experiments, cells were switched from their regular culture medium to Opti-MEM (Invitrogen) containing 250 μg/ml G-418 and 100 μg/ml gentamicin. Cells were pretreated with either vehicle (water) or 100 ng/ml PTX for 16 h at 37°C. Cells were then treated with either vehicle (0.1% dimethyl sulfoxide), 1 μM PGE_2 for 10 min (phospho-ERKs), or 60 min (EGR-1) at 37°C. Cells were scraped into a lysis buffer (consisting of 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM sodium fluoride, 10 mM disodium pyrophosphate, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and transferred to microcentrifuge tubes. The samples were rotated for 30 min at 4°C and were centrifuged at 16,000*g* for 15 min. Aliquots of the supernatants containing 20~100 μg of protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes as described previously (Fujino et al., 2003). Membranes were incubated in 5% nonfat milk for 1 h and were then washed and incubated for 16 h at 4°C with primary antibodies using the following conditions. For the ERKs, incubations were done in 3% nonfat milk containing either a 1:1000 dilution of antiphospho-ERK1/2 antibody (Cell Signaling Technology Inc., Beverly, MA) or mixture of a 1:500 dilution of anti-ERK1 antibody and a 1:10,000 dilution of anti-ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For EGR-1, incubations were done in 3% nonfat milk containing a 1:1000 dilution of anti-EGR-1 antibody (Santa Cruz Biotechnology). After incubating with the primary antibody, membranes were washed three times and incubated for 1 h at room temperature with a 1:10,000 dilution of the appropriate secondary antibodies conjugated with horseradish peroxidase using the same conditions as described above for each of the primary antibodies. After washing three times, immunoreactivity was detected by chemiluminescence as described previously (Fujino et al., 2003). To ensure equal loading of proteins, the membranes were stripped and re-probed with appropriate antibodies under the same conditions as described above.

Results

Pertussis Toxin Potentiates PGE_2-Stimulated cAMP Formation in HEK Cells Stably Expressing the Human EP_4 Prostanoid Receptor. We have reported previously that the maximal level of PGE_2-stimulated cAMP formation is significantly lower in HEK cells stably expressing the human EP_4 prostanoid receptor compared with HEK cells stably expressing the human EP_2 receptor, even though the levels of receptor expression were very similar (Fujino et al., 2002). We have also found that the EP_4 receptor can activate a PI3K/ERKs signaling pathway to induce the expression of EGR-1, whereas the EP_2 receptor subtype does not (Fujino et al., 2003). We had hypothesized previously that the EP_4 receptor was less efficiently coupled to $G\alpha_s$, but recently we considered the possibility that the EP_4 receptor might be additionally coupled to $G\alpha_i$, as has been shown for cardiac β_2-adrenergic receptors (Xiao et al., 1999a,b). To test this hypothesis we pretreated cells with PTX, which catalyzes the transfer of ADP-ribose from NAD to $G\alpha_i$ and thereby blocks the ability of $G\alpha_i$ to inhibit the activity of adenylyl cyclase (Ui, 1984). Thus, untransfected HEK cells and HEK cells stably expressing either the human EP_2 or EP_4 receptors were pretreated for 16 h with PTX and were then treated for 10 min with various concentrations of PGE_2. As shown in Fig.

1, there was no appreciable accumulation of cAMP in untransfected HEK cells with or without PTX pretreatment. In the absence of PTX pretreatment, the maximal stimulation of cAMP formation in HEK cells expressing the EP_2 receptor was approximately twice that obtained in HEK cells expressing the EP_4 receptor (36 pmol versus 19 pmol, respectively). Pretreatment with PTX resulted in a significant 33% increase in maximal PGE_2-stimulated cAMP formation in HEK cells expressing the EP_4 receptor, whereas, in EP_2-expressing cells, pretreatment with PTX essentially had no effect. The EC_{50} for PGE_2 stimulation of cAMP formation was approximately 4-fold lower for EP_4-expressing cells compared with EP_2-expressing cells (0.4 nM versus 1.7 nM, respectively), and it was not affected by pretreatment with PTX. These data clearly support the hypothesis that the human EP_4 prostanoid receptor, but not the EP_2 receptor, can functionally couple to $G\alpha_i$ in addition to coupling to $G\alpha_s$.

Pertussis Toxin Potentiation of PGE_2-Stimulated cAMP Formation in EP_4 Cells Is Not Due to Activation of Endogenous EP_3 Receptors. The evidence that PTX treatment of EP_2-expressing cells did not potentiate PGE_2-stimulated cAMP formation suggests that the potentiation of PGE_2-stimulated cAMP formation after PTX pretreatment of EP_4-expressing cells is not a consequence of the activation of endogenous $G\alpha_i$-coupled EP_3 receptors. Nevertheless, this possibility was further examined using the EP_3/EP_4 selective agonist PGE_1-OH in cells that were treated with forskolin, which stimulates intracellular cAMP formation by the direct activation of adenylyl cyclase. As shown in Fig. 2, PTX pretreatment of untransfected HEK cells and HEK cells stably expressing EP_2 receptors had no effect on forskolin-stimulated cAMP formation in the presence of PGE_1-OH. On the other hand, in HEK cells stably expressing EP_4 receptors, pretreatment with PTX resulted in a 37% increase in forskolin-stimulated cAMP formation in the presence of PGE_1-OH. If the activation of endogenous EP_3 receptors coupled to $G\alpha_i$ was responsible for this increase, similar increases in forskolin-stimulated cAMP formation should have been observed after PTX pretreatment of the untransfected HEK cells and HEK cells expressing EP_2 receptors.

Coupling of the Human EP_4 Prostanoid Receptor to $G\alpha_i$ Mediates PGE_2-Stimulated ERK Phosphorylation and Induction of EGR-1 Expression. We have shown previously that PGE_2 stimulation of the human EP_4 receptor, but not the human EP_2 receptor, can induce the functional expression of EGR-1 through the activation of the PI3K and ERK signaling pathways (Fujino et al., 2003). It has also been reported that the β_2-adrenergic receptor can activate a PI3K signaling pathway by coupling through $G\alpha_i$ (Jo et al., 2002). We therefore decided to examine whether the PGE_2-mediated activation of PI3K/ERKs signaling and induction of EGR-1 expression occurs through a mechanism involving coupling of the EP_4 receptor to $G\alpha_i$. For these experiments, cells were either untreated or pretreated with PTX for 16 h and were then incubated with either vehicle or 1 μM PGE_2. The expression of the phospho-ERKs, total ERKs, and EGR-1 were then examined by immunoblot analysis. Figure 3A, top, shows that in the absence of PTX pretreatment, PGE_2-stimulated ERK phosphorylation in EP_4-expressing cells, but not in EP_2-expressing cells, and that pretreatment with PTX completely abolished this effect. Likewise, Fig. 3B, top, shows that in the absence of PTX pretreatment, PGE_2 stimulated the expression of EGR-1 in EP_4-expressing cells, but not in EP_2-expressing cells, and that pretreatment with PTX also blocked this action. In addition, Fig. 3, A and B, bottom, show that nearly identical amounts of ERKs 1 and 2 were present

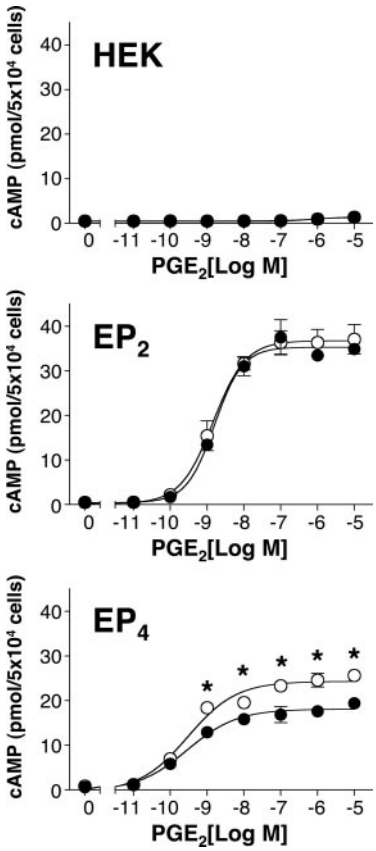

Fig. 1. The effects of PTX on PGE_2-stimulated cAMP formation in untransfected HEK cells and in HEK cells transfected with human EP_2 or EP_4 prostanoid receptors. Cells were pretreated with vehicle (●) or 100 ng/ml PTX (○) for 16 h, followed by treatment with the indicated concentrations of PGE_2 for 10 min. cAMP formation was determined as described under *Materials and Methods*. Data are the means ± S.E.M. of three independent experiments, each performed in duplicate. *, $p < 0.01$, analysis of variance, followed by Bonferroni post testing.

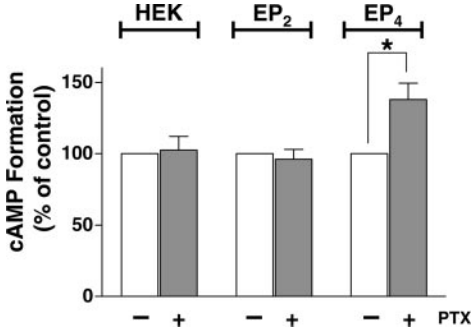

Fig. 2. The effects of PTX on forskolin-stimulated cAMP formation in the presence of the EP_3/EP_4-selective agonist PGE_1-OH in untransfected HEK cells and in HEK cells transfected with either the human EP_2 or EP_4 prostanoid receptors. Cells were pretreated with either vehicle or 100 ng/ml PTX for 16 h, followed by treatment with 1 μM PGE_1-OH for 10 min, followed by an additional incubation with 3 μM forskolin for 15 min. cAMP formation was determined as described under *Materials and Methods*. Data were normalized to the vehicle-treated controls for each group. Data are the means ± S.E.M. of three independent experiments each performed in duplicate. *, $p < 0.05$, t test.

under all conditions and in both cell lines. These data support the conclusion that the activation of ERK signaling and induction of EGR-1 by PGE_2 is mediated by coupling of the human EP_4 prostanoid receptor to a PTX-sensitive G-protein.

Discussion

The regulation of intracellular cAMP by E-type prostaglandins has been known for nearly forty years (Butcher and Baird, 1968). Thus, PGE_1 was found to lower intracellular cAMP in isolated fat pads but to increase it in several other cell types. Direct evidence for the existence of specific receptors for the E-type prostaglandins was initially obtained in radioligand binding studies with [^{3}H]PGE_1 (Kuehl and Humes, 1972), which were also used to show that the binding of [^{3}H]PGE_1 could be modulated by guanine nucleotides (Moore and Wolff, 1973). This was among the first evidence that E-type prostaglandin receptors, together with the glucagon and catecholamine receptors, interacted with G-proteins and that this interaction might constitute a general mechanism for signaling between cell surface receptors and adenylyl cyclase (Rodbell, 1980). Extensive physiological, pharmacological and molecular biological studies later defined the receptors for the E-type prostaglandins as EP receptors and classified them into the EP_1, EP_2, EP_3, and EP_4 subtypes (Coleman et al., 1994; Regan, 2003; Hata and Breyer, 2004). As reviewed in the Introduction, the EP_1 and EP_3 receptors have been generally regarded as coupling to $G\alpha_q$ and $G\alpha_i$, respectively, whereas the EP_2 and EP_4 receptors have been considered to be exclusively coupled to $G\alpha_s$. The present findings now show for the first time that in addition to coupling to $G\alpha_s$, EP_4 receptors can also couple to a PTX-sensitive G-protein to inhibit intracellular cAMP formation and activate PI3K and ERKs signaling cascades. Furthermore, the inhibition of cAMP formation by the EP_4 receptor suggests specific coupling to $G\alpha_i$.

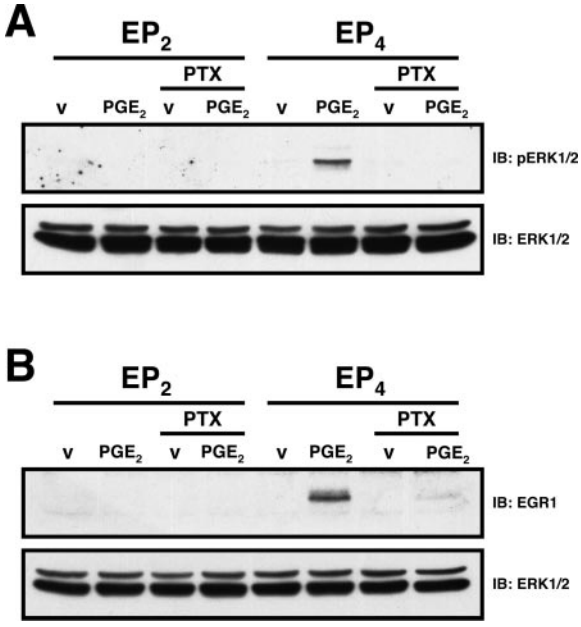

Fig. 3. The effects of PTX on PGE_2-stimulated phosphorylation of the extracellular signal-regulated kinases (pERK 1/2) (A) and on the expression of early growth response factor-1 (EGR-1) (B) in HEK cells transfected with either the human EP_2 or EP_4 prostanoid receptors. Cells were pretreated with either vehicle or 100 ng/ml PTX for 16 h, followed by treatment with either vehicle (v) or 1 μM PGE_2 for 10 min (A) or for 60 min (B). Cells were then subjected to immunoblot analysis as described under *Materials and Methods*. A, top, immunoblotting with antibodies against phospho-ERKs 1 and 2 (pERK1/2). A, bottom, the blots shown in A, top, were stripped and re-probed with antibodies against ERKs 1 and 2 (ERK1/2). B, top, immunoblotting with antibodies against EGR-1 (EGR1). B, bottom, the blots shown in B, top, were stripped and re-probed with antibodies against ERKs 1 and 2 (ERK1/2). Results are representative of three independent experiments with each antibody and condition.

We have reported previously that PGE_2 stimulation of human EP_2 and EP_4 receptors can activate Tcf/Lef signaling but that EP_2 receptors do this primarily through a cAMP/PKA pathway, whereas EP_4 receptors mainly use a PI3K pathway (Fujino et al., 2002). We have also reported that PGE_2 stimulation of human EP_4 receptors, but not EP_2 receptors, results in the functional expression of EGR-1 through the activation of PI3K and MAP kinase signaling (Fujino et al., 2003). As for the present study, these previous studies were conducted exclusively with a recombinant cell system consisting of HEK cells stably transfected with either the human EP_2 or EP_4 receptors. There is increasing evidence, however, that such observations will eventually be extended to endogenous EP_2 and EP_4 receptors in native cell systems. For example, Sheng et al. (2001) reported that PGE_2 stimulation of endogenous EP_4 receptors in human colorectal cancer cells increased cell growth and motility through the activation of PI3K and Akt. Likewise, Pozzi et al. (2004) found that PGE_2 stimulation of endogenous EP_4 receptors in mouse colon adenocarcinoma cells increased cellular proliferation by a mechanism that was independent of any measurable effect on cAMP and that involved the activation of the Akt and MAP kinases. Reno and Cannas (2005) have reported that PGE_2 stimulation of endogenous EP_2 or EP_4 receptors in human myeloid leukemia cells increased PMA-induced macrophage differentiation by a mechanism that was independent of the activation of a cAMP/PKA pathway and that involved the activation of PI3K and MAP kinase signaling. Similar findings were also obtained by Caristi et al. (2005), who found that endogenous EP_4 receptors in human T lymphocytes mediate interleukin-8 gene transcription by a mechanism that is PKA-independent and involves the activation of PI3K signaling. Thus, there are endogenous EP_4 receptors in native cell systems that can activate PI3K signaling by mechanisms that seem to be independent of coupling to $G\alpha_s$.

It is well established that GPCRs can activate PI3K and Akt signaling through the interaction of $G\beta\gamma$ subunits with either the p110β or p110γ subunits of PI3K (Yart et al., 2002). In most cases in which it has been examined, the activation of PI3K and Akt signaling involves $G\alpha_I$-coupled receptors (Kim et al., 2004). Given the present findings, it is likely that the PTX-sensitive activation of PI3K and ERK signaling by the EP_4 receptor reflects specific coupling to $G\alpha_i$ as opposed to $G\alpha_o$.

In many ways, the classification of the EP receptor subtypes and their pattern of G-protein coupling bears similarities to the adrenergic receptor subtypes. For example, the α_1- and α_2-adrenergic receptors are generally regarded as coupling to $G\alpha_q$ and $G\alpha_i$, respectively, whereas the β-adrenergic receptor subtypes were long considered to be exclusively coupled to $G\alpha_s$. It has become apparent, however, that the β_2-adrenergic receptor has additional coupling to $G\alpha_i$, which results, as in the EP_4 receptor, in the inhibition cAMP formation and activation of PI3K and ERK signaling cas-

cades (Daaka et al., 1997; Chesley et al., 2000). This is of particular functional significance for the cardiac β-receptors because it profoundly alters the consequences of persistent activation of these receptors. Thus, transgenic overexpression of β_1-adrenergic receptors in mice leads to cardiac hypertrophy, heart failure, and early death, whereas, overexpression of the β_2-adrenergic receptor actually improves cardiac function and does not adversely affect life span (Xiao et al., 1999b). Although cardiac β_2-adrenergic receptors can couple to $G\alpha_s$, it has been found that the protective effects of β_2-adrenergic receptor over expression depend upon coupling to $G\alpha_{i2}$ and $G\alpha_{i3}$ (Foerster et al., 2003). At present, the physiological and pathophysiological consequences of the unique signaling properties of the EP_4 receptor are unknown. However, like the β_1- and β_2-adrenergic receptor subtypes, the EP_2 and EP_4 prostanoid receptor subtypes are frequently coexpressed in the same tissues, and it is likely that there is a functional basis for this coexpression.

One possibility as it concerns the coexpression of the EP_2 and EP_4 receptor subtypes might be related to a cell or tissue's ability to respond to different concentrations of endogenous PGE_2. It has been clearly established that the binding affinity of PGE_2 is ~10- to 20-fold higher for the EP_4 receptor compared with the EP_2 receptor (Kiriyama et al., 1997; Abramovitz et al., 2000; Fujino et al., 2002). Furthermore, this difference in affinity is reflected in functional measures of the activation of these receptors. For example, in one detailed study of the functional pharmacology of the human EP_2 and EP_4 receptor subtypes, the EC_{50} for the stimulation of cAMP formation in cells expressing the EP_4 receptor was ~0.05 nM, whereas for the EP_2 receptor, it was ~30 nM (Wilson et al., 2004). Thus, cells expressing the EP_4 receptor are able to respond to lower concentrations of endogenous PGE_2. In addition, the pattern of intracellular signaling in cells expressing the EP_4 receptor will include the activation of both the $G\alpha_s$ and $G\alpha_i$ pathways.

The activation of a $G\alpha_i$ signaling pathway by the EP_4 receptor provides an interesting potential mechanism for further amplification of the initial PGE_2 signal. As demonstrated in the present study, the EP_4 receptor-mediated activation of $G\alpha_i$ signaling leads to the activation of the ERKs and induction of EGR-1 expression. It has been shown that EGR-1 can induce the expression of PGE_2 synthase (Naraba et al., 2002), which could be expected to increase the biosynthesis of PGE_2, perhaps to a level that would initiate the activation of EP_2 (and EP_1) receptors. This amplification of PGE_2 signaling would take place only in tissues or cells that express the EP_4 receptor subtype and would represent a mechanism for generating a differential response to low levels of endogenous PGE_2. PGE_2 is produced at low levels by a large number of cell types, and under various physiological and pathophysiological conditions, its biosynthesis is dramatically increased. This increase in PGE_2 biosynthesis is frequently correlated with the induction of COX-2, but the conditions and factors that regulate these events are unclear. Invasion of tissues by macrophages and up-regulation of their EP_4 receptors, which has been shown to occur in a mouse model of autoimmune inflammation (Akaogi et al., 2004), or up-regulation of EP_4 receptors by resident dendritic cells (Harizi et al., 2003), could provide a potential mechanism for inducing COX-2 and PGE_2 synthase expression and increasing the biosynthesis of PGE_2.

The present study further emphasizes the differences in the signaling potential of the EP_2 and EP_4 receptors and clarifies the mechanism of the activation of the PI3K and ERK signaling pathways by the EP_4 receptor. Thus far, human EP_2 prostanoid receptors seem to be exclusively coupled to $G\alpha_s$, and stimulation of these receptors by PGE_2 leads to a strong activation of the cAMP/PKA signaling pathway. On the other hand, PGE_2 stimulation of human EP_4 prostanoid receptors results in the activation of both $G\alpha_s$ and $G\alpha_i$. Compared with the EP_2 receptor, the activation of the cAMP/PKA signaling pathway by the EP_4 receptor is significantly less, which is a consequence of two mechanisms. The first is that activation of $G\alpha_i$ probably results in a direct inhibition of adenylyl cyclase, which offsets the stimulation of adenylyl cyclase through $G\alpha_s$. The second is that PGE_2-mediated activation of PI3K signaling by the EP_4 receptor inhibits the activity of PKA (Fujino et al., 2005). A similar inhibition of PKA activity has been reported after the activation of PI3K signaling by the β_2-adrenergic receptor (Jo et al., 2002). It is significant to note that even in the presence of PTX, the maximal cAMP response elicited by PGE_2 stimulation of the EP_4 receptor was less than that obtained with the EP_2 receptor (Fig. 1). This indicates that the efficiency of EP_4 receptor coupling to $G\alpha_s$-mediated signaling is less than that of the EP_2 receptor even in the absence of the activation of $G\alpha_i$ mediated signaling.

The $G\alpha_i$-mediated activation of PI3K signaling further differentiates the signaling properties of the EP_4 receptor compared with the EP_2 receptor. Thus, we have shown previously that PGE_2 stimulation of the EP_4 receptor leads to the PI3K-dependent activation of ERK signaling pathways, which is not observed after PGE_2 stimulation of the EP_2 receptor (Fujino et al., 2003). Despite these differences, some of the downstream signaling consequences after PGE_2 stimulation of the EP_2 or EP_4 receptors seem to be quite similar. For example, PGE_2 stimulation of either receptor leads to an increase Tcf transcriptional activation (Fujino et al., 2002) and in the phosphorylation of the cAMP response element binding protein (Fujino et al., 2005). However, the increase in Tcf transcription activation and cAMP response element binding protein phosphorylation by the EP_4 receptor is mainly through a PI3K-dependent mechanism, whereas for the EP_2 receptor, it is mainly through a cAMP/PKA-dependent pathway. This means that the regulation of EP_2 and EP_4 receptor signaling by cross-talk through the activation of other of types of receptors has the potential to be quite different. For example, receptors whose activation can modulate PI3K signaling will have greater potential to influence signaling mediated by the EP_4 receptor as opposed to that mediated by the EP_2 receptor.

In summary, we have shown that human EP_4 receptors, but not EP_2 receptors, can couple to PTX-sensitive G-proteins when expressed heterologously in HEK cells. Coupling of EP_4 receptors to PTX-sensitive G-proteins decreases PGE_2-mediated cAMP accumulation, suggesting specific coupling to $G\alpha_i$ rather than $G\alpha_o$. The activation of PI3K signaling by the EP_4 receptor probably occurs through the release of $G\beta\gamma$ subunits after coupling of the receptor to $G\alpha_i$. We have discussed studies showing that PGE_2 stimulation of endogenous EP_4 receptors in native cell systems can activate PI3K and ERK signaling by mechanisms that seem to be independent of coupling to $G\alpha_s$. These findings suggest the coupling of en-

dogenous EP_4 receptors to $G\alpha_i$, but clearly this will need to be further investigated. In fact, we do not believe that EP_4 receptors will be shown to have universal coupling to $G\alpha_i$ and PI3K/ERK signaling. For example, in an elegant study of prostanoid receptor-mediated signaling in human airway smooth muscle cells, Clarke et al. (2005) found that the effects of EP_4 receptor stimulation could be explained solely by activation of a cAMP/PKA-dependent pathway. We speculate that the specific signaling pathways used by more "promiscuous" GPCRs, such as EP_4 and β_2-adrenergic receptors, will be very cell-type–dependent compared with more dedicated "monogamous" receptors, such as EP_2 and β_1-adrenergic receptors.

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