Molecular Pharmacology Fast Forward. Published on October 4, 2005 as doi:10.1124/mol.105.017749

MOLPHARM/2005/017749

EP_4 PROSTANOID RECEPTOR COUPLING TO A PERTUSSIS TOXIN SENSITIVE INHIBITORY G-PROTEIN

Hiromichi Fujino and John W. Regan*

Department of Pharmacology & Toxicology

College of Pharmacy

The University of Arizona

Tucson, AZ, U.S.A. 85721-0207

Running title: EP_4 Prostanoid Receptor Coupling to $G_{\alpha i}$

Corresponding author: John W. Regan, Department of Pharmacology & Toxicology, College of Pharmacy, The University of Arizona, Tucson, AZ, U.S.A. 85721-0207; Tel 520-(626)-2181; Fax 520-(626)-2466; e-mail: regan@pharmacy.arizona.edu

number of text pages: 25

number of tables: 0

number of figures: 3

number of references: 33

number of words in *Abstract*: 171

number of words in *Introduction*: 619

number of words in *Discussion*: 1993

Abbreviations: PGE_2, prostaglandin E_2; cAMP, cyclic-3,5-adenosine monophosphate; Tcf, T-cell factor; PKA, cAMP dependent protein kinase A; PI3K, phosphatidylinositol 3-kinase; GSK-3, glycogen synthase kinase-3; EGR-1, early growth response factor-1; ERKs, extracellular signal-regulated kinases.

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

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 following stimulation with PGE_2 can increase the formation of intracellular cyclic 3,5-adenosine monophosphate (cAMP). In addition, PGE_2 stimulation of the EP_4 receptor can activate phosphatydilinositol 3-kinase (PI3K) leading to phosphorylation of the extracellular signal-regulated kinases (ERKs) and induction of early growth response factor-1 (EGR-1) (Fujino H, Xu W and Regan JW (2003) *J Biol Chem* **278**:12151-12156). 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 pertussis toxin sensitive inhibitory G-protein ($G_{\alpha i}$), which can inhibit cAMP dependent signaling and activate PI3K/ERKs dependent signaling.

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

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, $PGF_{2\alpha}$, etc.) 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 guanine nucleotide binding proteins (G-proteins). There are four major subfamilies of G-proteins that are defined by their alpha subunits (G_α) 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 cyclic 3,5-adenosine monophosphate (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, while 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 phosphates formation (Regan, unpublished), suggesting coupling to members of the $G_{\alpha q}$ subfamily. The EP_3 receptors are classically 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

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

mRNA splicing and their coupling to G-proteins is complex (Kotani et al., 1995). For example, in humans there are eight isoforms and at least two of these isoforms, the $EP_{3\text{-}II}$ and $EP_{3\text{-}IV}$, appear 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 phosphates formation.

Stimulation of the human EP_2 and EP_4 receptors with PGE_2 increases intracellular cAMP formation indicating that both these isoforms can couple to $G_{\alpha s}$ to stimulate adenylyl cyclase (Regan, 2003). However, it appears that functional coupling to the cAMP signaling pathway is more efficient for the human EP_2 receptor subtype as compared with 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 20-50% of that achieved by the EP_2 subtype (Fujino et al., 2002; Fujino et al., 2005). Recently, it has also been found 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; Fujino et al., 2003; Fujino et al., 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 as compared with the EP_2 receptor subtype.

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Cell Culture-- Cell lines stably expressing the EP_2 or EP_4 receptors were prepared using HEK-293-EBNA cells and the mammalian expression vector $pCEP_4$ (Invitrogen) as previously described (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 and sixteen hours prior to the immunoblotting experiments, cells were switched from their regular culture medium to Opti-MEM (Invitrogen) containing 250 µg/ml geneticin and 100 µg/ml gentamicin. Cells were pretreated with either vehicle (water) or 100 ng/ml pertussis toxin (PTX, Calbiochem) for 16 hrs at 37 °C. Cells were then treated with 0.1 mg/ml isobutylmethyl-xanthine (Sigma) for 15 min followed by treatment with either vehicle (0.1% Me_2SO), 1 µM PGE_2 (Cayman), 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 following the initial treatments with PTX or drugs. Experiments were terminated by the removing the media and placing the cells on ice. Two hundred µl of TE buffer (50 mM Tris-HCl, 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 µl of the supernatants (representing ~5 x 10^4 cells) was added to new tubes containing 50 µl of [^{3}H]cAMP (PerkinElmer Life Sciences) and 100 µl of 0.06 mg/ml PKA (Sigma product P5511). The mixture was vortexed and incubated on ice for 2 h, followed by the addition of 100 µl of TE buffer containing 2% bovine serum albumin (BSA) and 26 mg/ml powdered charcoal. After vortexing and centrifugation for 1 min at 14,000 rpm, 100 µl aliquots of the supernatants were

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

removed and radioactivity was measured by liquid scintillation counting. The amount of cAMP present was calculated from a standard curve prepared using cold cAMP and was expressed as pmol per 5 x 10^4 cells.

Western Blotting-- Sixteen hours prior to the immunoblotting experiments, cells were switched from their regular culture medium to Opti-MEM (Invitrogen) containing 250 µg/ml geneticin and 100 µg/ml gentamicin. Cells were pretreated with either vehicle (water) or 100 ng/ml PTX for 16 hrs at 37 °C. Cells were then treated with either vehicle (0.1% Me_2SO), 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 x *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 hrs at 4 °C with primary antibodies using the following conditions. For the ERKs, incubations were done in 3% nonfat milk containing either a 1:1,000 dilution of antiphospho-ERK1/2 antibody (9106, Cell Signaling); or mixture of a 1:500 dilution of anti-ERK1 antibody and a 1:10,000 dilution of anti-ERK2 antibody (sc-93 and sc-154, Santa Cruz Biotechnology). For EGR-1, incubations were done in 3% nonfat milk containing a 1:1,000 dilution of anti-EGR-1 antibody (sc-110, Santa Cruz Biotechnology). After incubating with the primary antibody, membranes were washed three times and incubated for 1 hr 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 previously reported that the maximal levels of PGE_2 stimulated cAMP formation is significantly lower in HEK cells stably expressing the human EP_4 prostanoid receptor as 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). Previously we had hypothesized 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 $G_{\alpha i}$, much like has been shown for cardiac β_2-adrenergic receptors (Xiao et al., 1999a; Xiao et al., 1999b). To test this hypothesis we pretreated cells with pertussis toxin (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 hrs with PTX and were then treated for 10 min with various concentrations of PGE_2. As shown in Figure 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 vs 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

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

lower for EP_4 expressing cells as compared with EP_2 expressing cells (0.4 nM vs 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 Cell 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 following PTX pretreatment of EP_4 expressing cells is not a consequence of the activation of endogenous $G_{\alpha i}$ coupled EP_3 receptors. Never the less, 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 Figure 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 $G_{\alpha i}$ were responsible for this increase, similar increases in forskolin stimulated cAMP formation should have been observed following 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 ERKs Phosphorylation and Induction of EGR-1 Expression. We have previously shown that PGE_2 stimulation of the human EP_4 receptor, but not the human EP_2 receptor, can induce the functional expression of early growth response factor-1 (EGR-1) through the activation of the PI3K and

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

ERKs 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 if 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 hrs 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. The upper panel of Figure 3A shows that in the absence of PTX pretreatment, PGE_2 stimulated ERKs phosphorylation in EP_4 expressing cells, but not in EP_2 expressing cells, and that pretreatment with PTX completely abolished this effect. Similarly, the upper panel of Figure 3B 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. Additionally, the lower panels of Figures 3A and 3B show that nearly identical amounts of ERKs 1 and 2 were present under all conditions and in both cell lines. These data support the conclusion that the activation of ERKs 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.

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

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 was 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, like 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, while 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 $G_{\alpha i}$.

We have previously reported 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 utilize 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. Similarly, 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 which 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 which 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 appear 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 where 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

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

activation of PI3K and ERKs 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, while the β-adrenergic receptor subtypes were considered for a long time 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 much like the EP_4 receptor, results in the inhibition cAMP formation and activation of PI3K and ERKs signaling cascades (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 over expression of β_1-adrenergic receptors in mice leads to cardiac hypertrophy, heart failure, and early death; whereas, over expression 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 co-expressed in the same tissues and it is likely that there is a functional basis for this co-expression.

One possibility as it concerns the co-expression 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

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

for the EP_4 receptor as compared to the EP_2 receptor (Fujino et al., 2002; Kiriyama et al., 1997; Abramovitz et al., 2000). 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. Additionally, 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 only take place 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

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

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 ERKs signaling pathways by the EP_4 receptor. To date human EP_2 prostanoid receptors appear 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 $G_{\alpha i}$. As 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 following 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 (Figure 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.

It is the $G_{\alpha i}$ mediated activation of PI3K signaling that further differentiates the signaling properties of the EP_4 receptor as compared with the EP_2 receptor. Thus, we have previously shown that PGE_2 stimulation of the EP_4 receptor leads to the PI3K dependant activation of ERKs signaling pathways, which is not observed following PGE_2 stimulation of the EP_2 receptor

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

(Fujino et al., 2003). In spite of these differences some of the downstream signaling consequences following PGE_2 stimulation of the EP_2 or EP_4 receptors appear 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 (CREB) phosphorylation by the EP_4 receptor is mainly through a PI3K dependant 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 EP_4 receptor mediated signaling as opposed to EP_2 receptor mediated signaling.

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 is probably through the release of $G_{\beta\gamma}$ subunits after coupling of the receptor to $G_{\alpha i}$. We have discussed studies which show that PGE_2 stimulation of endogenous EP_4 receptors in native cell systems can activate PI3K and ERKs signaling by mechanisms that appear to be independent of coupling to $G_{\alpha s}$. These findings suggest the coupling of endogenous 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/ERKs signaling. For example, in an elegant study of prostanoid receptor mediated signaling in human airway smooth muscle cells it was found that the effects of EP_4 receptor stimulation could be explained solely by activation of

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

a cAMP/PKA dependent pathway (Clarke et al., 2005). We speculate that the specific signaling pathways utilized by more "promiscuous" GPCRs, such as the EP_4 and β_2-adrenergic, will be very cell type dependent as compared with more dedicated "monogamous" receptors, such as the EP_2 and β_1-adrenergic.

REFERENCES

Abramovitz M, Adam M, Boie Y, Carriere M-C, Denis D, Godbout C, Lamontagne S, Rochette C, Sawyer N, Tremblay NM, Belley M, Gallant M, Dufresne C, Gareau Y, Ruel R, Juteau H, Labelle M, Ouimet N and Metters KM (2000) The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. *Biochim Biophys Acta* **1483**:285-293.

Akaogi J, Yamada H, Kuroda Y, Nacionales DC, Reeves WH and Satoh M (2004) Prostaglandin E_2 receptors EP_2 and EP_4 are up-regulated in peritoneal macrophages and joints of pristane-treated mice and modulate TNF-α and IL-6 production. *J Leukocyte Biol* **76**:227-236.

Butcher RW and Baird CE (1968) Effects of prostaglandins on adenosine 3',5'-monophosphate levels in fat and other tissues. *J Biol Chem* **243**:1713-1717.

Caristi S, Piraino G, Cucinotta M, Valenti A, Loddo S and Teti D (2005) Prostaglandin E_2 induces interleukin-8 gene transcription by activating C/EBP homologous protein in human T lymphocytes. *J Biol Chem* **280**:14433-14442.

Chesley A, Lundberg MS, Asai T, Xiao R-P, Ohtani S, Lakatta EG and Crow MT (2000) The β_2-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G_i-dependent coupling to phosphatidylinositol 3'-kinase. *Circ Res* **87**:1172-1179

Clarke DL, Belvisi MG, Smith SJ, Hardaker E, Yacoub MH, Meja KK, Newton R, Slater DM and Giembycz MA (2005) Prostanoid receptor expression by human airway smooth muscle cells and regulation of the secretion of granulocyte colony-stimulating factor. Am J Physiol Lung Cell Mol Physiol **288**:L238-L250

Coleman RA, Smith WL and Narumiya S (1994) VIII. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* **46**:205-229.

Daaka Y, Luttrell LM and Lefkowitz RJ (1997) Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390**:88-91.

Foerster K, Groner F, Matthes J, Koch WJ, Birnbaumer L and Herzig S (2003) Cardioprotection specific for the G protein G_{i2} in chronic adrenergic signaling through β_2-adrenoceptors. *Proc Natl Acad Sci* **100**:14475-14480.

Fujino H, Salvi S and Regan JW (2005) Differential regulation of phosphorylation of the cAMP response element binding protein following activation of EP_2 and EP_4 prostanoid receptors by prostaglandin E_2. *Mol Pharmacol* **68**:251-259.

Fujino H, West KA and Regan JW (2002) Phosphorylation of glycogen synthase kinase-3 and stimulation of T-cell factor signaling following activation of EP_2 and EP_4 prostanoid receptors by prostaglandin E_2. *J Biol Chem* **277**:2614-2619.

Fujino H, Xu W and Regan JW (2003) Prostaglandin E_2 induced functional expression of early growth response factor-1 by EP_4, but not EP_2, prostanoid receptors via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinases. *J Biol Chem* **278**:12151-12156.

Funk CD, Furci L, FitzGerald GA, Grygorczyk R, Rochette C, Bayne MA, Abramovitz M, Adam M and Metters KM (1993) Cloning and expression of a cDNA for the human prostaglandin E receptor EP_1 subtype. *J Biol Chem* **268**:26767-26772.

Harizi H, Grosset C and Gualde N (2003) Prostaglandin E_2 modulates dendritic cell function via EP_2 and EP_4 receptor subtypes. *J Leukocyte Biol* **73**:756-763.

Hata AN and Breyer RM (2004) Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacol Ther* **103**:147-166.

Hepler JR and Gilman AG (1992) G proteins. *Trends Biochem Sci* **17**:383-387.

Jo S-H, Leblais V, Wang PH, Crow MT and Xiao R-P (2002) Phosphatidylinositol 3-kinase functionally compartmentalizes the concurrent G_s signaling during β_2-adrenergic stimulation. *Circ Res* **91**:46-53.

Kim S, Jin J and Kunapuli SP (2004) Akt activation in platelets depends on G_i signaling pathways. *J Biol Chem* **279**:4186-4195.

Kiriyama M, Ushikubi F, Kobayashi T, Hirata M, Sugimoto Y and Narumiya S (1997) Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. *Br J Pharmacol* **122**:217-224.

Kotani M, Tanaka I, Ogawa Y, Usui T, Mori K, Ichikawa A, Narumiya S, Yoshimi T, and Nakao K (1995) Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP_3 subtype generated by alternative messenger RNA splicing: multiple second messenger systems and tissue-specific distributions. *Mol Pharmacol* **48**:869-879.

Kuehl FA and Humes JL (1972) Direct evidence for a prostaglandin receptor and its application to prostaglandin measurements. *Proc Natl Acad Sci* **69**:480-484.

Moore WV and Wolff J (1973) Binding of prostaglandin E_1 to beef thyroid membranes. *J Biol Chem* **248**:5705-5711.

Naraba H, Yokoyama C, Tago N, Murakami M, Kudo I, Fueki M, Oh-ishi S and Tanabe T (2002) Transcriptional regulation of the membrane-associated prostaglandin E_2 synthase gene. Essential role of the transcription factor Egr-1. *J Biol Chem* **277**:28601-28608.

Pozzi A, Yan X, Macias-Perez I, Wei S, Hata AN, Breyer RM, Morrow JD and Capdevila JH (2004) Colon carcinoma cell growth is associated with prostaglandin E2/EP4 receptor-evoked ERK activation. *J Biol Chem* **279**:29797-29804.

Regan JW (2003) EP_2 and EP_4 prostanoid receptor signaling. *Life Sci* **74:**143-153.

Reno F and Cannas M (2005) Effect of prostaglandin E2 on PMA-induced macrophage differentiation. *Prostaglandins Other Lipid Mediat* **75**:13-24.

Rodbell M (1980) The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* **284**:17-22.

Sheng H, Shao J, Washington MK and DuBois RN (2001) Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. *J Biol Chem* **276**:18075-18081.

Ui M (1984) Islet-activating protein, pertussis toxin: a probe for functions of the inhibitory guanine nucleotide regulatory component of adenylate cyclase. *Trends Pharmacol Sci* **5**:277-279.

Wilson RJ, Rhodes SA, Wood RL, Shield VJ, Noel LS, Gray DW and Giles H (2004) Functional pharmacology of human prostanoid EP_2 and EP_4 receptors. *Eur J Pharmacol* **501**:49-58.

Xiao R-P, Avdonin P, Zhou Y-Y, Cheng H, Akhter SA, Eschenhagen T, Lefkowitz RJ, Koch WJ and Lakatta EG (1999a) Coupling of β_2-adrenoceptor to G_i proteins and its physiological relevance in murine cardiac myocytes. *Circ Res* **84**:43-52.

Xiao R-P, Cheng H, Zhou Y-Y, Kuschel M and Lakatta EG (1999b) Recent advances in cardiac β_2-adrenergic signal transduction. *Circ Res* **85**:1092-1100.

Yart A, Chap H and Raynal P (2002) Phosphoinositide 3-kinases in lysophosphatidic acid signaling: regulation and cross-talk with the Ras/mitogen-activated protein kinase pathway. *Biochem Biophys Acta* **1582**:107-111.

FOOTNOTES

Support for this work was provided by the National Institutes of Health (EY11291) and Allergan Inc.

FIGURE LEGENDS

Figure 1. The effects of pertussis toxin (PTX) on PGE_2-stimulated cAMP formation 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 hrs, 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. *$p < 0.01$, ANOVA, followed by Bonferroni post testing.

Figure 2. The effects of pertussis toxin (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 hrs, 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.

Figure 3. The effects of pertussis toxin (PTX) on PGE_2-stimulated phosphorylation of the extracellular signal-regulated kinases (pERK 1/2) (*Panel A*) and on the expression of early growth response factor-1 (EGR-1) (*Panel 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 hrs, followed by treatment with either vehicle (v) or 1 μM PGE_2 for 10 min (*Panel A*) or for 60

min (*Panel B*). Cells were then subjected to immunoblot analysis as described under *Materials and Methods*. *UpperPanel A*, immunoblotting with antibodies against phospho-ERKs 1 and 2 (*pERK1/2*). *Lower Panel A*, the blots shown in the *upper panel* were stripped and re-probed with antibodies against ERKs 1 and 2 (*ERK1/2*). *Upper Panel B*, immunoblotting with antibodies against EGR-1 (*EGR1*). *Lower Panel B*, the blots shown in the *upper panel* 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.

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

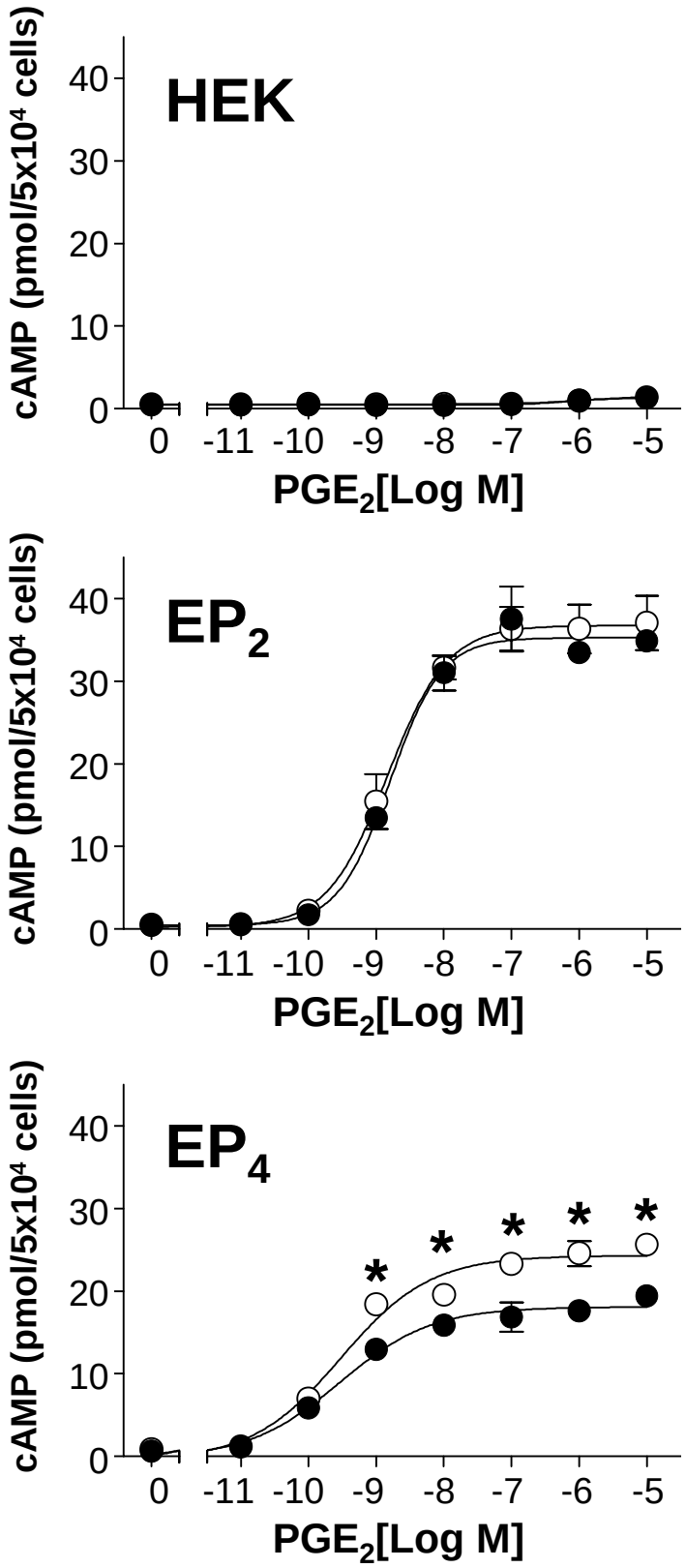

Figure 1

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

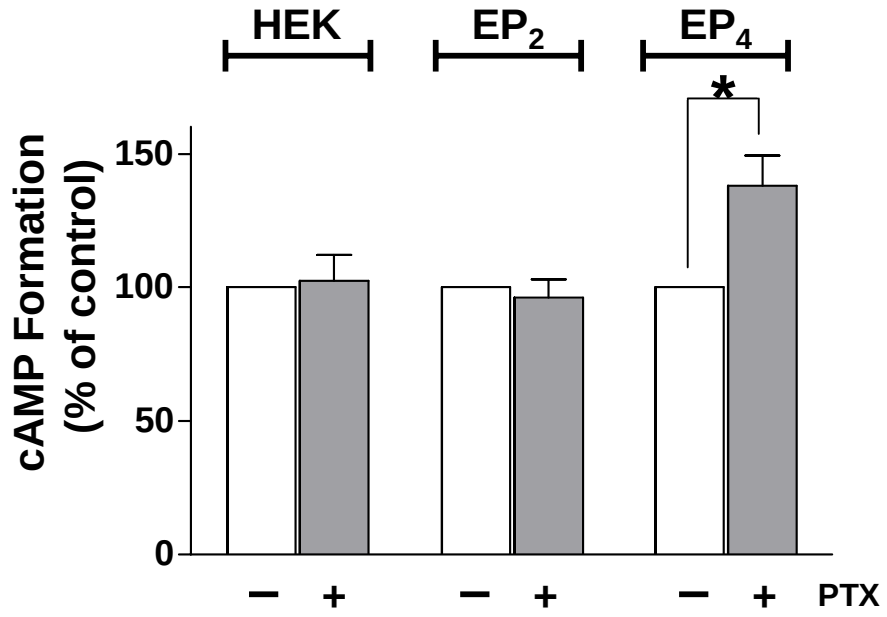

Figure 2

Molecular Pharmacology Fast Forward. Published on October 4, 2005 as DOI: 10.1124/mol.105.017749
This article has not been copyedited and formatted. The final version may differ from this version.

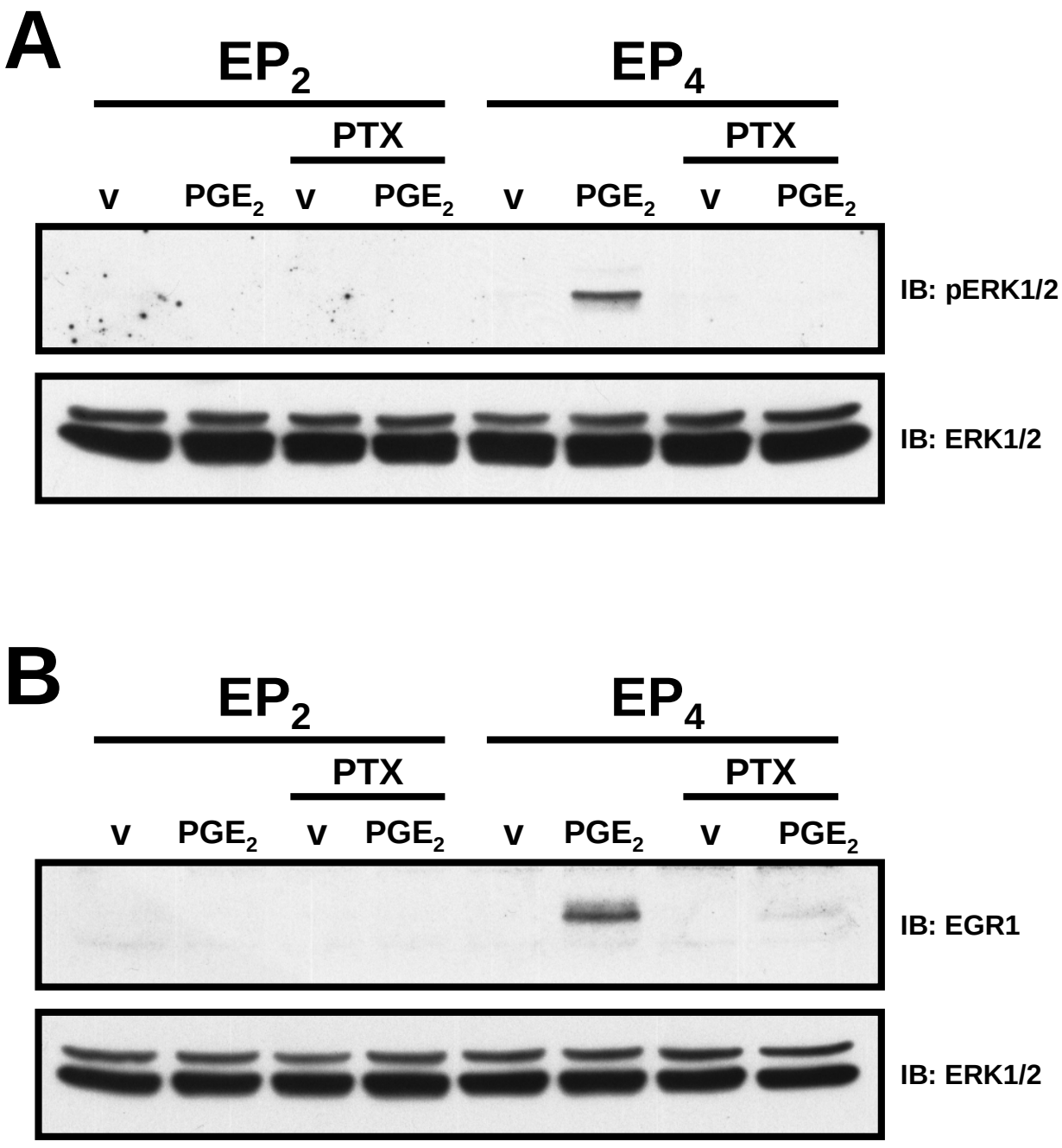

Figure 3