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Differential Regulation of Phosphorylation of the cAMP Response Element-Binding Protein after Activation of EP_2 and EP_4 Prostanoid Receptors by Prostaglandin E_2

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ABSTRACT

The EP_2 and EP_4 prostanoid receptors are G-protein-coupled receptors whose activation by their endogenous ligand, prostaglandin (PG) E_2, stimulates the formation of intracellular cAMP. We have previously reported that the stimulation of cAMP formation in EP_4-expressing cells is significantly less than in EP_2-expressing cells, despite nearly identical levels of receptor expression (*J Biol Chem* **277:**2614–2619, 2002). In addition, a component of EP_4 receptor signaling, but not of EP_2 receptor signaling, was found to involve the activation of phosphatidylinositol 3-kinase (PI3K). In this study, we report that PGE_2 stimulation of cells expressing either the EP_2 or EP_4 receptor results in the phosphorylation of the cAMP response element binding protein (CREB) at serine-133. Pretreatment of cells with *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline (H-89), an inhibitor of protein kinase A (PKA), attenuated the PGE_2-mediated phosphorylation of CREB in EP_2-expressing cells, but not in EP_4-expressing cells. Pretreatment of cells with wortmannin, an inhibitor of PI3K, had no effects on the PGE_2-mediated phosphorylation of CREB in either EP_2- or EP_4-expressing cells, although it significantly increased the PGE_2-mediated activation of PKA in EP_4-expressing cells. However, combined pretreatment with H-89 and wortmannin blocked PGE_2-mediated phosphorylation in EP_4-expressing cells as well as in EP_2-expressing cells. PGE_2-mediated intracellular cAMP formation was not affected by pretreatment with wortmannin, or combined treatment with wortmannin and H-89, in either the EP_2- or EP_4-expressing cells. These findings suggest that PGE_2 stimulation of EP_4 receptors, but not EP_2 receptors, results in the activation of a PI3K signaling pathway that inhibits the activity of PKA and that the PGE_2-mediated phosphorylation of CREB by these receptors occurs through different signaling pathways.

The EP_2 and EP_4 prostanoid receptors are members of the superfamily of G-protein-coupled receptors (GPCRs) and are two of the four subtypes of receptors for prostaglandin (PG) E_2 (Regan, 2003). After the initial cloning of these receptors, their functional significance was unclear because they both were preferentially activated by PGE_2 and they both seemed to signal exclusively through the activation of adenylyl cyclase (Honda et al., 1993; Regan et al., 1994). It was subsequently determined, however, that they differed with respect to PGE_2-mediated receptor desensitization and internalization. Thus, EP_4 receptors were found to undergo rapid agonist-induced desensitization, whereas EP_2 receptors did not (Nishigaki et al., 1996). Likewise, EP_4 receptors were found to undergo rapid PGE_2-mediated internalization, and EP_2 receptors did not (Desai et al., 2000).

The first evidence of differences in the signaling potential of the EP_2 and EP_4 receptors involved the observation that PGE_2 could stimulate the phosphorylation of glycogen synthase kinase-3 (GSK-3) and T cell factor (Tcf) transcriptional activation in cells stably expressing these receptors (Fujino et al., 2002). Although both receptors possessed these activities, the stimulation of GSK-3 phosphorylation and Tcf transcriptional activation by the EP_2 receptor was primarily through a PKA-dependent pathway, whereas, for the EP_4 receptor, these effects were mediated primarily through a phosphatidylinositol 3-kinase (PI3K)-dependent pathway. It was further shown that PGE_2 treatment of EP_4-expressing cells, but not EP_2-expressing cells, resulted in the induction of early growth response factor-1 (EGR-1) by a pathway involving the activation of PI3K and the extracellular signal-regulated kinases (ERKs) (Fujino

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; PG, prostaglandin; GSK, glycogen synthase kinase; Tcf, T cell factor; PI3K, phosphatidylinositol 3-kinase; EGR, early growth response factor; ERK, extracellular signal-regulated kinase; CRE, cAMP response element; CREB, cAMP response element-binding protein; PKA, protein kinase A; HEK, human embryonic kidney; Me_2SO, dimethyl sulfoxide; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PD98059, 2′-amino-3′-methoxyflavone.

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et al., 2003). Additional evidence of EP_4 receptor signaling through a PI3K-dependent pathway has also been reported for colorectal carcinoma cells. Thus, PGE_2 was found to increase the growth and motility of human adenocarcinoma cells (LS-174) through activation of PI3K via stimulation of EP_4 receptors (Sheng et al., 2001). Likewise, it has been reported that stimulation of EP_4 receptors by PGE_2 in mouse colon adenocarcinoma cells (CT26) activates PI3K and ERKs signaling and is associated with cell growth in the absence of any detectable increase in intracellular cAMP formation (Pozzi et al., 2004).

An important function of $G_{\alpha s}$-coupled receptors is the transcriptional regulation of genes whose promoters contain cAMP response elements (CREs). In this signaling cascade, the release of $G_{\alpha s}$ after stimulation of the receptor leads to the activation of adenylyl cyclase and increased formation of intracellular cAMP. The subsequent activation of PKA by cAMP can result in the phosphorylation of the CRE binding protein (CREB), which is a transcription factor that interacts with CREs and is central to the regulation cAMP responsive gene expression (Mayr and Montminy, 2001; Johannessen et al., 2004). Among the many genes whose expression can be regulated by cAMP is cyclooxygenase-2, whose catalytic product, PGH_2, is the immediate precursor for the biosynthesis of the prostaglandins and thromboxanes. It is interesting that bradykinin has been found to increase cAMP-dependent cyclooxygenase-2 promoter activity in human pulmonary artery smooth muscle cells through an autocrine signaling pathway involving the activation of endogenous EP_2 and EP_4 prostanoid receptors (Bradbury et al., 2003).

The phosphorylation of CREB by PKA occurs at serine-133 and results in the recruitment of the CREB-binding protein and/or its paralogue, p300, which function with phospho-CREB as coactivators of gene transcription (Mayr and Montminy, 2001; Johannessen et al., 2004). However, phosphorylation at Ser-133 does not occur exclusively by way of cAMP signaling and PKA. For example, CREB may be phosphorylated at Ser-133 by the calcium/calmodulin-dependent kinases and by members of the $pp90^{rsk}$ family kinases after the activation of calcium signaling and growth factor-mediated mitogenic signaling, respectively (Mayr and Montminy, 2001; Johannessen et al., 2004). In addition, the phosphorylation of CREB at Ser-133 has been reported to occur in a PI3K-dependent manner after the activation of the ERKs and Akt signaling pathways (Mayr and Montminy, 2001; Johannessen et al., 2004).

Given the ability of EP_2 and EP_4 receptors to activate cAMP signaling pathways and the additional ability of EP_4 receptors to activate PI3K signaling pathways, we were interested in the potential phosphorylation of CREB at Ser-133 by these receptors. We now show that stimulation of both the human EP_2 and EP_4 receptors by PGE_2 can lead to the phosphorylation of CREB at Ser-133. In EP_2-expressing cells, the mechanism is primarily cAMP- and PKA- dependent. In EP_4-expressing cells, the mechanism is more complex and involves a PI3K-dependent pathway. A novel finding is that PGE_2 stimulation of EP_4-expressing cells negatively regulates the activity of PKA through the activation of PI3K signaling.

Materials and Methods

Western Blotting. Cell lines stably expressing the EP_2 and EP_4 receptors were prepared using HEK-293 EBNA cells (Invitrogen, Carlsbad, CA) and the mammalian expression vector pCEP4 (Invitrogen) as described previously (Fujino et al., 2002, 2003). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 250 μg/ml G-418 (Geneticin), 100 μg/ml gentamicin, and 200 μg/ml hygromycin B (all from Invitrogen). Sixteen hours before the immunoblotting experiments, cells were switched from their regular Dulbecco's modified Eagle's medium to Opti-MEM (Invitrogen) containing 250 μg/ml G-418 and 100 μg/ml gentamicin. Cells were incubated at 37°C with either vehicle (0.1% Me_2SO) or 1 μM PGE_2 (Cayman) for the time indicated in the figures. For experiments involving the use of signaling inhibitors, cells were pretreated with either vehicle (0.1% Me_2SO) or with 10 μM H-89 (Calbiochem) or with 100 nM wortmannin (Sigma) or with the combination of 10 μM H-89 and 100 nM wortmannin for 15 min at 37°C. Cells were then 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 μM 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 μg of protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes as described previously (Fujino et al., 2002; Fujino et al., 2003). Membranes were incubated in 5% nonfat dry milk for 1 h and were then washed and incubated for 16 h at 4°C in 5% bovine serum albumin containing anti-phospho-CREB (Ser-133) antibody (Cell Signaling Technology, Beverly, MA) at a dilution of 1:1000. Membranes were washed three times and incubated for 1 h at room temperature in 5% nonfat milk with a 1:2000 dilution of anti-rabbit secondary antibodies conjugated with horseradish peroxidase. After three washes, immunoreactivity was detected by chemiluminescence as described previously (Fujino et al., 2002, 2003). To ensure equal loading of proteins, the membranes were stripped and reprobed with a 1:1000 dilution of anti-CREB antibody (Cell Signaling Technology) as described above for the anti-phospho-CREB antibody.

PKA Kinase Activity Assay. Cells were cultured in 12-well plates and were pretreated with either vehicle (0.1% Me_2SO) or inhibitors (10 μM H-89, 100 nM wortmannin, or the combination thereof) for 15 min at 37°C followed by treatment with either vehicle (0.1% Me_2SO) or 1 μM PGE_2 for 10 min. The cells were washed with ice-cold phosphate-buffered saline and were placed on ice. Two hundred microliters of lysis buffer (20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) was added; after a 10-min incubation on ice, the cells were scraped off and transferred to microcentrifuge tubes. The cell lysates were centrifuged for 15 min at 16,000*g*, and aliquots of the supernatants containing ~0.2 μg of protein were assayed for PKA activity according to the manufacturer's instructions using an enzyme-linked immunosorbent assay kit and a synthetic peptide substrate for PKA (Stressgen Biotechnologies, San Diego, CA).

cAMP Assay. Cells were cultured in 12-well plates and were replaced with fresh Opti-MEM containing 0.1 mg/ml 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO). Cells were pretreated with either vehicle (0.1% Me_2SO) or inhibitors (10 μM H-89, 100 nM wortmannin, or the combination thereof) for 15 min at 37°C followed by treatment with either vehicle (0.1% Me_2SO) or 1 μM PGE_2 for 10 min. The media were removed and the cells were placed on ice. Two hundred microliters of TE buffer (50 mM Tris-HCl and 4 mM EDTA, pH 7.5) was added and the cells were scraped off and

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transferred to microcentrifuge tubes. The samples were boiled for 8 min, placed on ice, and centrifuged for 1 min at 16,000*g*. Two microliters of the supernatants (representing ~10^4 cells) were transferred to microcentrifuge tubes containing 48 μl of TE, 50 μl of [^{3}H]cAMP (Invitrogen) and 100 μl of 0.06 mg/ml PKA (Sigma-Aldrich). The samples were vortexed, incubated on ice for 2 h, followed by the addition of 100 μl of TE buffer containing 2% bovine serum albumin and 26 mg/ml powdered charcoal (Sigma-Aldrich). After vortexing and centrifugation for 1 min at 16,000*g*, the radioactivity in 200-μl aliquots of the supernatants was determined by liquid scintillation spectrometry. The amount of cAMP present was calculated from a standard curve prepared using unlabeled cAMP and was expressed as picomoles per 10^4 cells.

Results

PGE_2-Stimulated Phosphorylation of CREB in EP_2- or EP_4-Transfected HEK Cells and in Untransfected HEK Cells. cAMP-regulated gene expression by GPCRs typically involves the PKA-mediated phosphorylation of the transcription factor CREB after the activation of PKA by increases in intracellular cAMP (Mayr and Montminy, 2001; Johannessen et al., 2004). We have previously characterized the stimulation of intracellular cAMP formation by PGE_2 in HEK cells stably expressing either the human EP_2 or EP_4 prostanoid receptor (Fujino et al., 2002). Although the levels of receptor expression were similar (~100 fmol/mg of whole-cell protein), the maximal extent of stimulation of cAMP formation in the EP_4-expressing cell line was only ~20% of that obtained in the EP_2-expressing cell line. However, for a separate measure of signaling activity (PGE_2 stimulation of Tcf-mediated transcriptional activation) both cell lines yielded similar maximal extents of activation (Fujino et al., 2002). We were interested, therefore, in possible similarities or differences in the ability of PGE_2 to stimulate the phosphorylation of CREB at Ser-133 in these same EP_2- or EP_4-expressing cell lines. For these experiments, cells expressing either the human EP_2 or EP_4 prostanoid receptor or untransfected HEK cells were treated with 1 μM PGE_2 for various times ranging from 5 to 60 min and were then immunoblotted with antibodies that recognized either phospho-CREB (pCREB/pATF1) or total CREB (phosphorylated and nonphosphorylated). As shown in Fig. 1, A and B, treatment with PGE_2 resulted in a rapid time-dependent phosphorylation of CREB in both the EP_2 and EP_4 receptor expressing cell lines. The time course of CREB phosphorylation was similar for both cell lines and seemed to reach a similar maximum. As shown in Fig. 1C, there was no significant effect of PGE_2 on the phosphorylation of CREB in the untransfected HEK cells. Figure 1 also shows that when these blots were stripped and re-probed with antibodies that recognized both the phosphorylated and nonphosphorylated forms of CREB, nearly identical amounts of CREB were present throughout the time course of treatment and among the three cell lines.

PGE_2-Stimulated cAMP Formation and Activation of PKA in EP_2- or EP_4-Transfected HEK Cells. As noted for the experiments depicted in Fig. 1, the maximal extent of PGE_2-stimulated CREB phosphorylation seemed to reach a similar maximum in both the EP_2- and EP_4-expressing cells. This is somewhat surprising given that in previous studies, we had found that the maximal extent of PGE_2-stimulated cAMP formation was significantly greater after 1 h in EP_2-expressing cells compared with EP_4-expressing cells. Thus, one might expect a greater activation of PKA in EP_2-expressing cells and perhaps a greater extent of CREB phosphorylation in EP_2-expressing cells. We decided, therefore, to examine the formation of cAMP and activation of PKA after 10 min of stimulation with PGE_2 in cells expressing either the EP_2 or EP_4 receptor. As confirmed in Fig. 2A, the maximal extent of PGE_2-stimulated cAMP accumulation was approximately 3-fold greater in EP_2-expressing cells compared with EP_4-expressing cells. Likewise, as shown in Fig. 2B, PGE_2-stimulated PKA activity was approximately 3.5 times greater in EP_2-expressing cells than in EP_4-expressing cells. These findings suggest, therefore, that the similar levels of PGE_2-stimulated CREB phosphorylation observed for both cell lines might reflect limited substrate availability (relative to the activity of PKA) or the activation of an alternative signaling pathway in EP_4-expressing cells, which also leads to the phosphorylation of CREB.

Effects of H-89 and Wortmannin on PGE_2-Stimulated Phosphorylation of CREB in EP_2- or EP_4-Transfected HEK Cells. As reviewed in the introduction, we have found previously that PGE_2 can stimulate the phosphorylation of GSK-3 and can increase Tcf transcriptional activation in cells stably transfected with the human EP_2 or EP_4 prostanoid receptor (Fujino et al., 2002). In EP_2-expressing cells, however, these activities are mediated primarily by a PKA-dependent pathway, whereas in EP_4-expressing cells, these activities primarily involve the activation of a PI3K-dependent pathway. Therefore, we decided to use H-89, an inhibitor of PKA, and wortmannin, an inhibitor of PI3K, to investigate the contributions of PKA signaling and PI3K signaling, respectively, to the PGE_2-stimulated phosphorylation of CREB in these cells. For these experiments, the cells were pretreated with either vehicle, 10 μM H-89, or 100 nM wortmannin for 15 min followed by treatment with 1 μM PGE_2 for 10 min and were then immunoblotted with antibodies to phospho-CREB (pCREB/pATF1) or total CREB. As depicted in Fig. 3A, after the pretreatment of EP_2-expressing cells with H-89 there was a significant decrease of PGE_2-stimulated phosphorylation of CREB, suggesting the involvement of PKA in this phosphorylation. On the other hand, pretreatment of EP_4-expressing cells with H-89 had no significant effect on PGE_2-mediated CREB phosphorylation. Figure 3B shows that, somewhat unexpectedly, pretreatment with wortmannin had no effect on PGE_2-stimulated CREB phosphorylation in either EP_2- or EP_4-expressing cells. We have shown in prior studies that pretreatment of EP_4-expressing cells with 100 nM wortmannin for 15 min can inhibit the PGE_2-mediated phosphorylation of GSK-3, as well as the PGE_2-mediated stimulation of Tcf reporter gene activity and induction of EGR-1 (Fujino et al., 2002, 2003). As an additional precaution, however, we repeated the experiments depicted in Fig. 3B with the PI3K inhibitor LY294002. As with wortmannin, pretreatment of EP_2- and EP_4-expressing cells with 30 μM LY294002 for 15 min had no effect on the PGE_2-stimulated phosphorylation of CREB (data not shown).

Effects of the Combination of H-89 and Wortmannin on PGE_2-Stimulated Phosphorylation of CREB in EP_2- or EP_4-Transfected HEK Cells. Although the individual effects of H-89 and wortmannin pretreatment on EP_4-expressing HEK cells was without effect on the PGE_2-stimulated phosphorylation of CREB, we considered the possibility that there might be some kind of interaction between the

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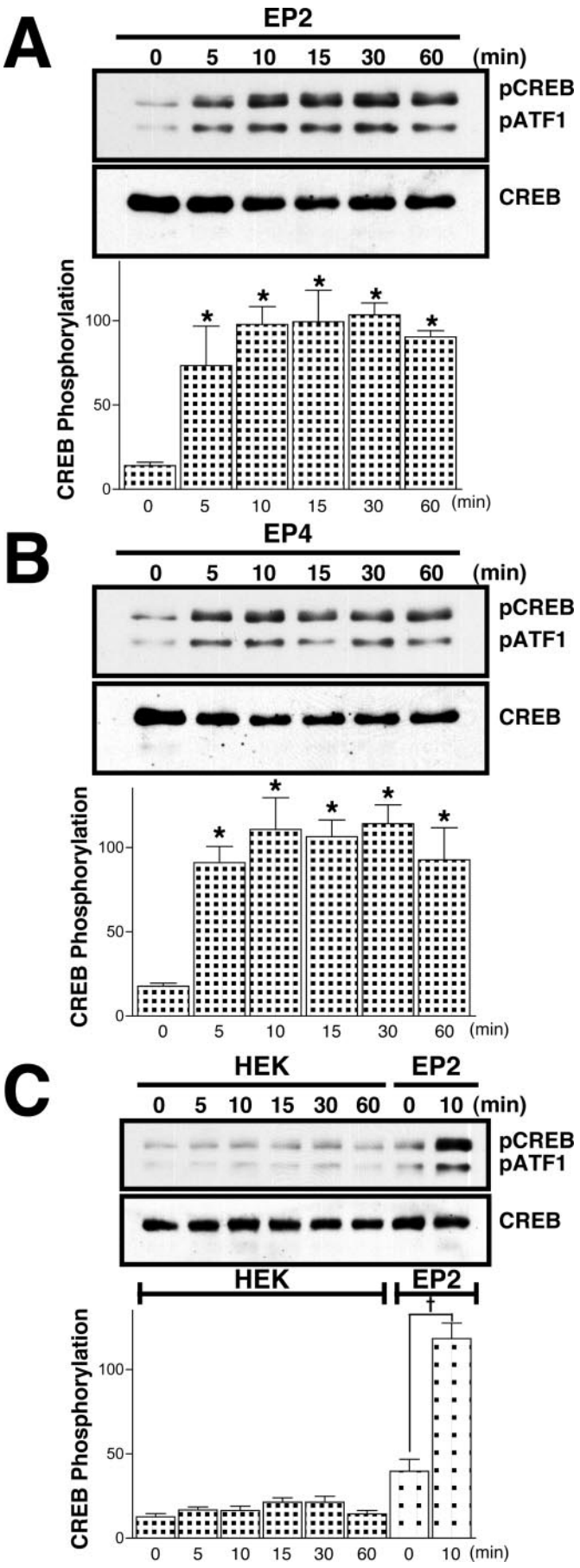

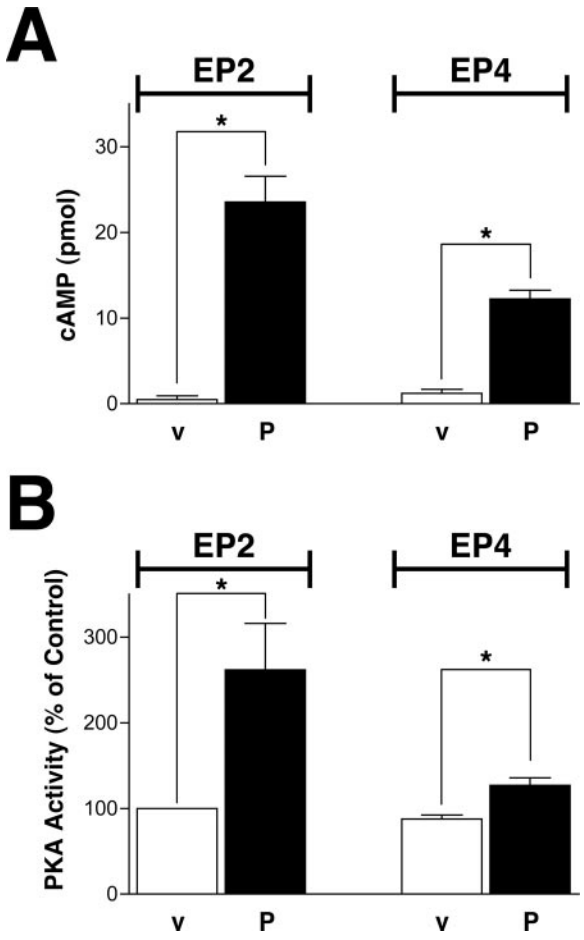

Fig. 2. PGE_2-stimulated cAMP accumulation (A) and PKA activity (B) in HEK cells transfected with either the human EP_2 or EP_4 prostanoid receptor. Cells were treated with either vehicle (v) or 1 μM PGE_2 (P) for 10 min at 37°C and were assayed for cAMP accumulation or for PKA activity as described under *Materials and Methods*. Data are the mean ± S.E.M. from three independent experiments. PKA activity data are normalized to the vehicle-treated EP_2-expressing cells as 100%. *, $p < 0.05$, *t* test. These data are the same as shown in Figs. 5 and 6 for control cells that were not treated with inhibitors.

PKA and PI3K signaling pathways. We decided, therefore, to examine the effect of these inhibitors in combination. Fig. 4 shows that, as expected, the combination of H-89 and wortmannin pretreatment of EP_2-expressing cells resulted in the inhibition of PGE_2-stimulated CREB phosphorylation to an extent similar to that observed by pretreatment with H-89 alone (compare Fig. 3A). It is interesting that the combination of H-89 and wortmannin pretreatment of EP_4-expressing cells resulted in a complete inhibition of PGE_2-stimulated

Fig. 1. Immunoblots of the time course of PGE_2-stimulated phosphorylation of CREB in HEK cells transfected with either the human EP_2 (A) or EP_4 (B) prostanoid receptor and in untransfected HEK cells (C). Cells were incubated with 1 μM PGE_2 for the indicated times and were subjected to immunoblot analysis as described under *Materials and Methods*. The upper of the two immunoblots in each panel represents results obtained with antibodies against phospho-CREB (pCREB). This antibody also detects the phosphorylated form of the CREB-related protein known as ATF1 (pATF1). The lower of the two immunoblots shown in each panel represent the results obtained with antibodies that recognize total CREB (phosphorylated and nonphosphorylated). The histograms represent the ratio of pCREB to total CREB as assessed by the pooled densitometry data (mean ± S.E.M.) from three independent experiments. *, $p < 0.05$, analysis of variance; †, $p < 0.05$, *t* test.

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CREB phosphorylation even though pretreatment with either inhibitor alone had no effect (compare Fig. 4 with Fig. 3, A and B). The experiments depicted in Fig. 4 were repeated with the combination of H-89 and the wortmannin inhibitor LY294002 and virtually identical results were obtained. Thus, pretreatment of EP_4-expressing cells with the combination of 30 μM LY294002 and 10 μM H-89 for 15 min resulted in a nearly complete blockade of the PGE_2-stimulated phosphorylation of CREB (data not shown). These findings are consistent with an interaction between the PKA and PI3K signaling pathways with respect to the PGE_2-mediated phosphorylation of CREB in EP_4-expressing cells.

Effects of H-89, Wortmannin and the Combination of H-89 and Wortmannin on PGE_2 Stimulated PKA Activity in EP_2 or EP_4 Transfected HEK Cells. To further explore the potential interaction of the PKA and PI3K signaling pathways, we examined the effects of inhibitors of these pathways, both alone and in combination, on PGE_2-stimulated PKA activity in cells stably expressing either the human EP_2 or EP_4 prostanoid receptor. As noted previously in Fig. 2, Fig. 5 shows that in the absence of any inhibitor pretreatment, there was a 2.6-fold stimulation of PKA activity in EP_2 cells treated for 10 min with 1 μM PGE_2 and a 1.5-fold stimulation of PKA activity in EP_4-expressing cells. Pretreatment of both EP_2- and EP_4-expressing cells with

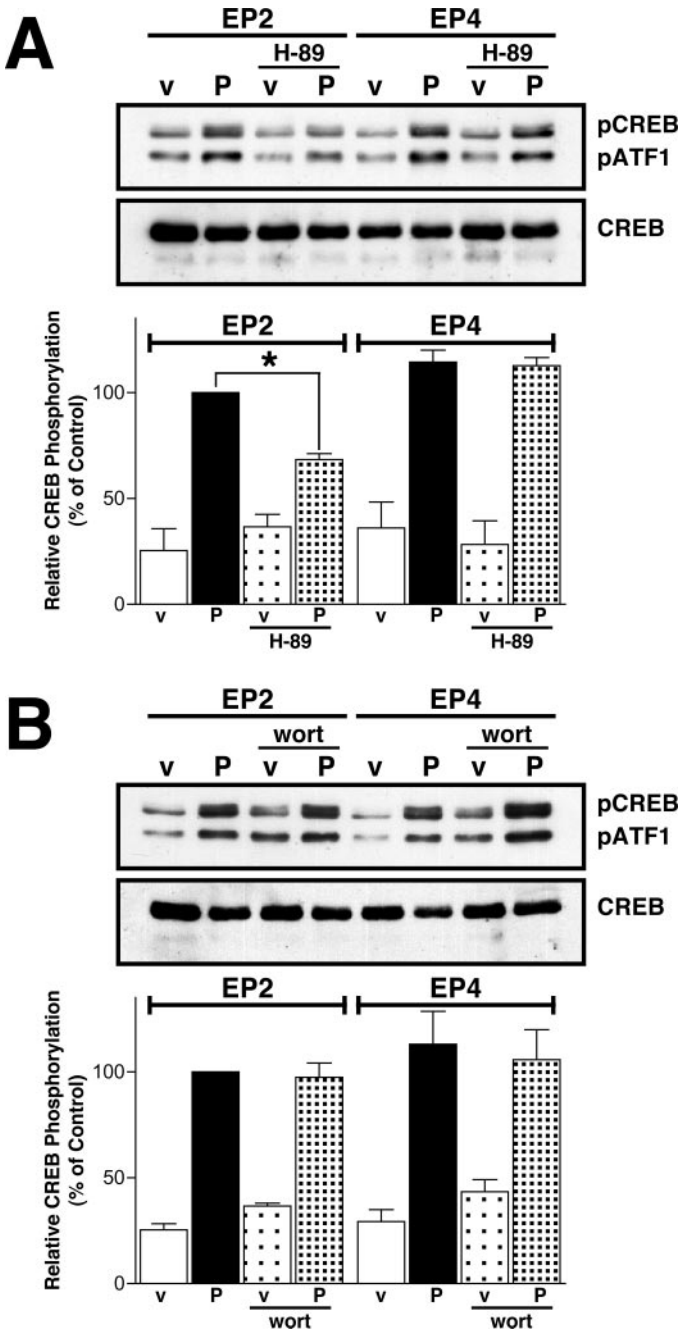

Fig. 3. The effects of H-89 (A) and wortmannin (B) on PGE_2-stimulated phosphorylation of CREB in HEK cells transfected with either the human EP_2 or EP_4 prostanoid receptor. Cells were pretreated with either vehicle or 10 μM H-89 or 100 nM wortmannin (wort) for 15 min followed by treatment with either vehicle (v) or 1 μM PGE_2 (P) for 10 min at 37°C and were then immediately subjected to immunoblot analysis as described under *Materials and Methods*. The upper of the two immunoblots shown in each panel represents results obtained with antibodies against phospho-CREB (pCREB). This antibody also detects the phosphorylated form of the CREB-related protein known as ATF1 (pATF1). The lower of the two immunoblots shown in each panel contains the results obtained with antibodies that recognize total CREB (phosphorylated and nonphosphorylated). The histograms represent the ratio of pCREB to total CREB as assessed by the pooled densitometry data (mean ± S.E.M.) from three independent experiments. Data are normalized to the PGE_2-treated EP_2-expressing cells as 100%. *, $p < 0.05$, t test.

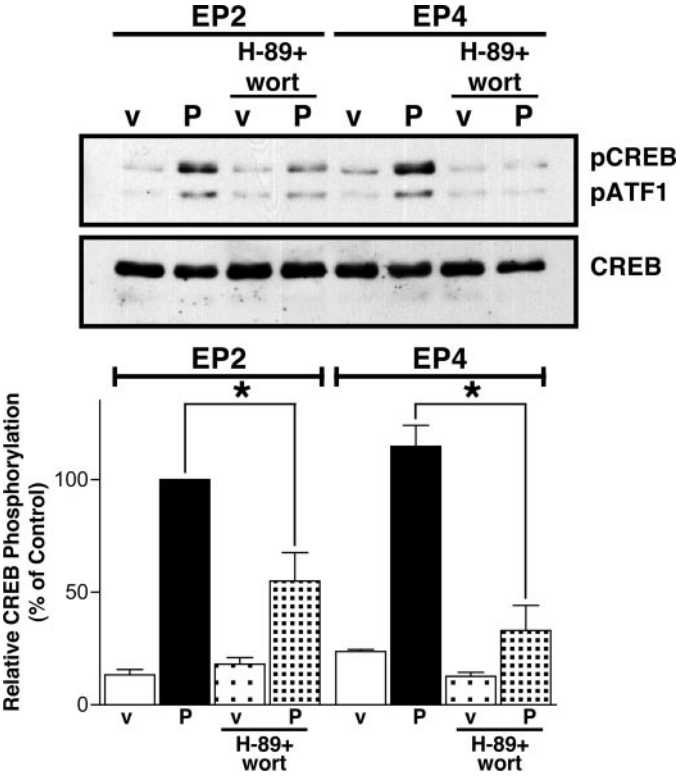

Fig. 4. The effects of the combination of H-89 and wortmannin on PGE_2-stimulated phosphorylation of CREB in HEK cells transfected with either the human EP_2 or the human EP_4 prostanoid receptor. Cells were pretreated with either vehicle or the combination of 10 μM H-89 and 100 nM wortmannin (wort) for 15 min followed by treatment with either vehicle (v) or 1 μM PGE_2 (P) for 10 min at 37°C and were then immediately subjected to immunoblot analysis as described under *Materials and Methods*. The upper immunoblot shows the representative results obtained with antibodies against phospho-CREB (pCREB). This antibody also detects the phosphorylated form of the CREB-related protein known as ATF1 (pATF1). The lower immunoblot shows the results obtained with antibodies that recognize total CREB (phosphorylated and nonphosphorylated). The histogram represents the ratio of pCREB to total CREB as assessed by the pooled densitometry data (mean ± S.E.M.) from three independent experiments. Data are normalized to the PGE_2-treated EP_2-expressing cells as 100%. *, $p < 0.05$, t test.

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H-89 alone resulted in a nearly complete inhibition of PGE_2-stimulated PKA activity in both cell lines. The inhibition of PGE_2-stimulated PKA activity in EP_2-expressing cells correlated nicely with the inhibition of PGE_2-stimulated CREB phosphorylation by H-89 in the EP_2-expressing cells (compare Fig. 3A). On the other hand, the inhibition of PGE_2-stimulated PKA activity in EP_4-expressing cells did not correlate with the effects of H-89 on PGE_2-stimulated CREB phosphorylation in EP_4-expressing cells, which was not inhibited by H-89 pretreatment (compare Fig. 3A). These findings further support the conclusion that the PGE_2-mediated phosphorylation of CREB in EP_4-expressing cells does not occur through a PKA-dependent pathway.

As shown in Fig. 5, pretreatment with wortmannin had essentially no effect on PGE_2-stimulated PKA activity in EP_2-expressing cells, but in EP_4-expressing cells, it caused a significant increase in PGE_2-stimulated PKA activity compared with untreated cells. Thus, after pretreatment of EP_4-expressing cells with wortmannin, PGE_2-stimulated PKA activity increased approximately 2.7-fold, whereas in untreated cells, the stimulation was approximately 1.5-fold. These data may explain the failure of wortmannin pretreatment to block PGE_2-stimulated CREB phosphorylation in EP_4-expressing cells (Fig. 3B) because the PGE_2-mediated activation of PI3K signaling in EP_4-expressing cells inhibits the activity of PKA. Therefore, pretreatment of EP_4-expressing cells with wortmannin alone relieves this PI3K-mediated inhibition, resulting in a PKA-dependent phosphorylation of CREB. As expected, Fig. 5 shows that pretreatment of EP_2- and EP_4-expressing cells with the combination of H-89 and wortmannin inhibited the PGE_2 stimulation of PKA activity in both cell lines. These findings are consistent with the inhibition of PGE_2-mediated CREB phosphorylation in both the EP_2 and EP_4 expressing cell lines after pretreatment with the combination of H-89 and wortmannin (compare Figure 4).

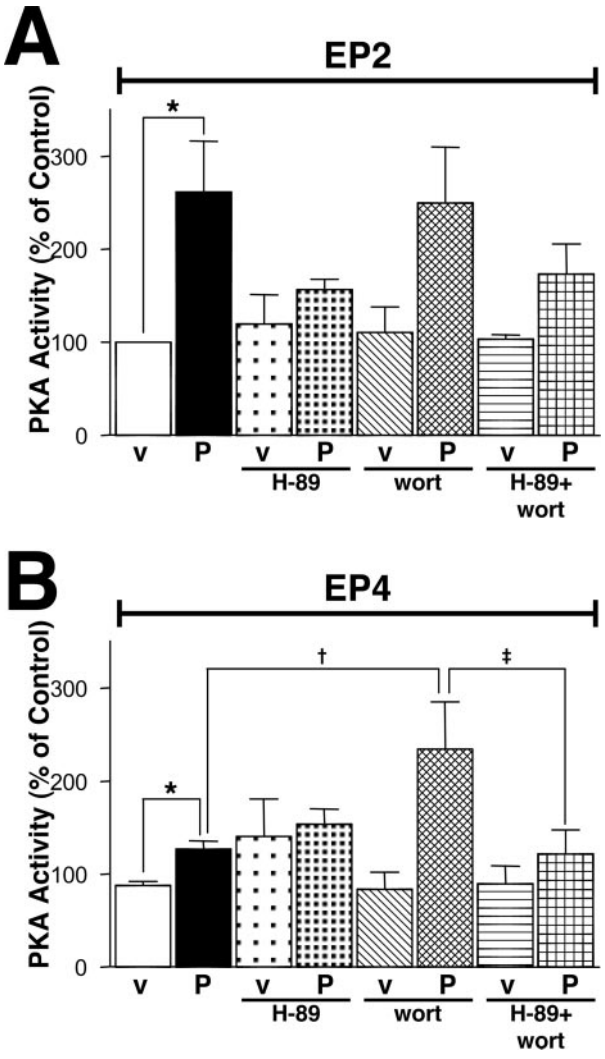

Fig. 5. The effects of H-89, wortmannin, and the combination of H-89 and wortmannin on PGE_2-stimulated PKA activity in HEK cells transfected with either the human EP_2 (A) or EP_4 (B) prostanoid receptor. Cells were pretreated with either vehicle or 10 μM H-89 or 100 nM wortmannin (wort) or the combination of 10 μM H-89 and 100 nM wortmannin for 15 min followed by treatment with either vehicle (v) or 1 μM PGE_2 (P) for 10 min at 37°C and were then assayed for PKA activity as described under *Materials and Methods*. Data are the mean ± S.E.M. from three independent experiments and are normalized to the vehicle treated EP_2-expressing cells as 100%. *, $p < 0.05$, *t* test; †, $p < 0.05$, *t* test; ‡, $p < 0.05$, *t* test.

Effects of H-89, Wortmannin, and the Combination Thereof on PGE_2-Stimulated cAMP Formation in EP_2- or EP_4-Transfected HEK Cells. Given that the activity of PKA is regulated by cAMP, we decided to examine PGE_2-stimulated cAMP formation in EP_2 and EP_4 cells under control conditions and after treatment with inhibitors of the PKA and PI3K pathways. This was of particular interest with respect to wortmannin's effect of increasing PGE_2-stimulated PKA activity in EP_4-expressing cells, because this could reflect either a PI3K-mediated inhibition of PKA activity or a PI3K-mediated increase in intracellular cAMP accumulation. As shown previously in Fig. 2, Fig. 6 shows that in the absence of pretreatment with inhibitors, there was a 23-fold stimulation of cAMP accumulation in EP_2 cells treated for 10 min with 1 μM PGE_2 and a 7.4-fold stimulation of cAMP accumulation in EP_4-expressing cells. For both EP_2- and EP_4-expressing cells, the PGE_2-mediated stimulation of cAMP accumulation correlated reasonably well with the PGE_2-mediated stimulation of PKA activity shown in Fig. 5. Figure 6 also shows that pretreatment with either H-89, wortmannin, or both had virtually no effect on PGE_2-stimulated cAMP accumulation in either EP_2- or EP_4-expressing cells. The evidence that wortmannin pretreatment of EP_4-expressing cells had little effect on PGE_2-mediated cAMP accumulation supports the conclusion that the PGE_2-mediated activation of PI3K signaling in EP_4-expressing cells inhibits the activity of PKA by a mechanism that does not involve a decreased formation of intracellular cAMP.

Discussion

The stimulation of intracellular cAMP formation through the activation of adenylyl cyclase by $G_{\alpha s}$-coupled GPCRs is, in evolutionary terms, perhaps the oldest and most widespread second-messenger pathway used by this superfamily of receptors. For example, of the eight most closely related human prostanoid receptor subtypes, four of them (EP_2, EP_4, IP, and DP_1) couple primarily to this signaling pathway (Hata and Breyer, 2004). Three of the remaining prostanoid receptor subtypes (EP_1, FP, and TP) couple primarily to $G_{\alpha q}$ and activate the inositol phosphate signaling pathway; one (EP_3) couples to $G_{\alpha i}$ and decreases the formation of intracel-

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lular cAMP through the inhibition of adenylyl cyclase. It is interesting that the phylogeny of these prostanoid receptors shows that the subtypes that couple to $G_{\alpha s}$ are all more closely related to each other and form a distinct subfamily compared with the subtypes that couple to $G_{\alpha q}$ and $G_{\alpha i}$ (Regan et al., 1994; Toh et al., 1995). The fact that EP receptor subtypes are present in both major subfamilies suggests that the primordial receptor was an EP subtype and that the initial evolution of these receptors was based on their ability to activate different signal transduction pathways (Regan et al., 1994).

Of the four prostanoid receptor subtypes that couple to $G_{\alpha s}$, two (EP_2 and EP_4) are activated by PGE_2 and two (IP and DP_1) are activated by prostacyclin and PGD_2, respectively. As the discovery of these prostanoid receptor subtypes unfolded, it seemed likely that the IP and DP_1 receptors evolved to subserve different signaling molecules, which could also be said of the EP_2 and EP_4 receptors as a group, but obviously not as individual subtypes. In fact, the EP_2 and EP_4 receptors do not seem to have evolved together as their own subfamily, because the phylogeny shows that the EP_2, IP, and DP_1 receptors are actually more related to each other and form a subgroup distinct from the EP_4 receptor (Toh et al., 1995). This suggests an evolutionary divergence of an ancestral $G_{\alpha s}$-coupled EP receptor into two descendants. One of these descendants was a $G_{\alpha s}$-coupled EP receptor that eventually gave rise to the EP_2, IP, and DP_1 receptor subtypes; the second descendant was a $G_{\alpha s}$-coupled EP receptor that evolved into the present day EP_4 receptor subtype. Thus, the evolution of the EP_2, IP, and DP_1 receptor subtypes, presumably on the basis of their ability to discriminate between their respective endogenous ligands, seems to have come after an earlier event that eventually gave rise to the EP_2 and EP_4 subtypes. The underlying basis for the initial divergence of the ancestral $G_{\alpha s}$ coupled EP receptor is speculative, but it is reasonable to suppose that it may have been a consequence of functional differences involving receptor regulation or signal transduction.

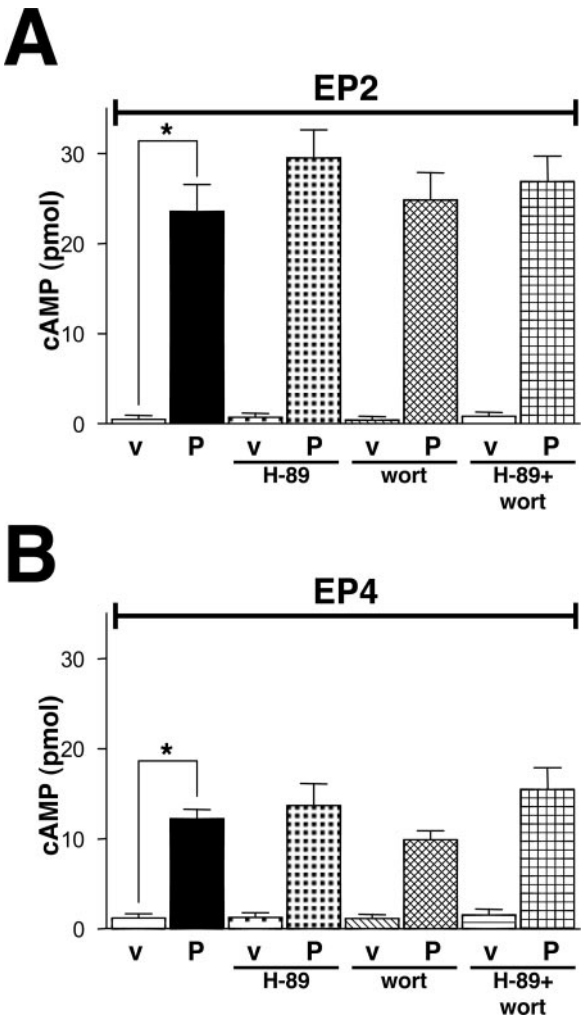

Fig. 6. The effects of H-89, wortmannin, and the combination of H-89 and wortmannin on PGE_2-stimulated cAMP accumulation in HEK cells transfected with either the human EP_2 (A) or EP_4 (B) prostanoid receptor. Cells were pretreated with either vehicle or 10 μM H-89 or 100 nM wortmannin (wort) or the combination of 10 μM H-89 and 100 nM wortmannin for 15 min followed by treatment with either vehicle (v) or 1 μM PGE_2 (P) for 10 min at 37°C and were then assayed for cAMP accumulation as described under *Materials and Methods*. Data are the mean ± S.E.M. from three independent experiments, each performed in duplicate, and are normalized to the vehicle-treated EP_2-expressing cells as 100%. *, $p < 0.05$, *t* test.

As reviewed in the Introduction, the initial characterization of the EP_2 and EP_4 receptors subtypes revealed no significant functional differences between these receptors; they were both preferentially activated by PGE_2 and they both stimulated the formation of intracellular cAMP (Honda et al., 1993; Regan et al., 1994). Thereafter, it was found that there were differences in the desensitization (Nishigaki et al., 1996) and internalization (Desai et al., 2000) of these receptors; more recently, it has become clear that there are significant differences in the signaling properties of the EP_2 and EP_4 prostanoid receptors (Fujino and Regan, 2003). As shown in Fig. 7, what seems to be emerging with respect to the signaling differences is that the EP_2 receptor subtype couples to a classic cAMP signaling pathway involving a marked stimulation of intracellular cAMP formation and activation of PKA. The EP_4 receptor, on the other hand, can activate the cAMP/PKA pathway but it is less robust, and there is a concomitant activation of the PI3K and ERK signaling pathways. The stimulation of either receptor subtype often leads to the activation of the same downstream effectors, albeit by different pathways. For example, both the EP_2 and EP_4 receptors can stimulate Tcf transcriptional activation, but the EP_2 receptor uses primarily a cAMP/PKA-dependent pathway, whereas the EP_4 receptor uses primarily a PI3K-dependent pathway (Fujino et al., 2002). However, stimulation of the EP_4 receptor can also result in the selective activation of downstream effectors that are not activated after the stimulation of EP_2 receptors. For example, PGE_2 stimulation of the EP_4 receptor induces the expression of EGR-1, which does not occur after PGE_2 stimulation of the EP_2 receptor (Fujino et al., 2003).

The present findings further highlight the unique signaling properties of the EP_2 and EP_4 receptors and are another example in which the activation of either receptor leads to a similar result on a downstream effector but is achieved through the activation of different signaling pathways. As shown in Fig. 7, PGE_2 stimulation of either receptor results in the phosphorylation of CREB on Ser-133, but the EP_2 receptor does this solely through the activation of a cAMP/PKA-dependent pathway, whereas the EP_4 receptor can use both the cAMP/PKA and PI3K pathways. In addition, it was

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found that PGE_2 stimulation of the EP_4 receptor inhibited the activity of PKA by a PI3K-dependent mechanism. Therefore, because of this PI3K-mediated inhibition of PKA activity, under normal conditions the phosphorylation of CREB after PGE_2 stimulation of the EP_4 receptor occurs primarily by way of the PI3K pathway. These findings are similar to results that have been reported for the β_2-adrenergic receptor in rat cardiomyocytes, which is also a $G_{\alpha s}$ coupled receptor that can activate cAMP/PKA signaling (Jo et al., 2002). Thus, inhibition of PI3K with wortmannin resulted in a marked increase in the PKA-mediated phosphorylation of phospholamban after agonist stimulation of the β_2-adrenergic receptor. The apparent increase in PKA activity occurred in the absence of any change in intracellular cAMP formation and suggests that agonist stimulation of the β_2-adrenergic receptor negatively regulates the activity of PKA through the activation of a PI3K signaling pathway.

The phosphorylation of CREB on Ser-133 is central to the regulation of CREB-mediated transcriptional activation and correlates well with the extent of target gene activation (Mayr and Montminy, 2001; Johannessen et al., 2004). The stimulation of intracellular cAMP formation and activation of PKA by $G_{\alpha s}$-coupled GPCRs is a key mediator of the Ser-133 phosphorylation of CREB; as reviewed in the introduction, however, it is not the only mechanism. This is clearly exemplified by our present findings, which show that the EP_2 receptor-mediated phosphorylation of CREB on Ser-133 is mainly PKA-dependent, whereas the EP_4-mediated CREB phosphorylation is not and involves a PI3K-dependent pathway. The activation of Ras signaling pathways by growth factor receptors has been shown to promote the phosphorylation of CREB on Ser-133, and we have reported previously the PI3K-dependent activation of ERKs 1 and 2 by the EP_4 receptor but not the EP_2 receptor (Fujino et al., 2003). However, inhibition of mitogen-activated protein kinase kinase signaling with PD98059, did not affect the PGE_2-mediated phosphorylation of CREB in either the EP_2- or EP_4-expressing cells (data not shown). We found previously that the inhibition of MEK by PD98059 in EP_4-expressing cells blocked the ERK-mediated induction of EGR-1 expression, which suggests that activation of a mitogen-activated protein kinase kinase/ERK signaling pathway is not involved in the PGE_2-mediated phosphorylation of CREB in EP_4-expressing cells.

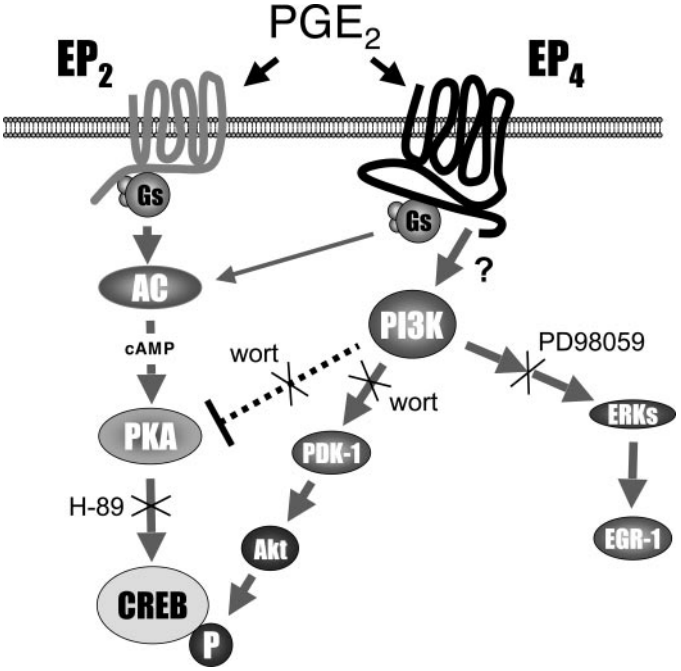

Fig. 7. Model for PGE_2-mediated phosphorylation of CREB by the human EP_2 and EP_4 prostanoid receptors. After the binding of PGE_2, both receptors can activate G_s, which in turn activates adenylyl cyclase (AC). Increased intracellular cAMP formation then activates PKA, which can phosphorylate CREB on serine-133. The EP_4 receptor also activates a PI3K signaling pathway, which leads to the phosphorylation of CREB on serine-133, possibly through the sequential activation of PDK-1 and protein kinase B (Akt). H-89 inhibits the activity of PKA and blocks EP_2 receptor-mediated CREB phosphorylation, but does not block EP_4 receptor-mediated CREB phosphorylation because of signaling through the PI3K pathway. Inhibition of PI3K by wortmannin (wort) alone does not block EP_4 receptor-mediated CREB phosphorylation because signaling through the PKA pathway is still active and because wortmannin relieves a PI3K-mediated inhibition of PKA. Although activation of mitogen-activated protein kinase (ERK) signaling has previously been implicated in CREB phosphorylation, it does not seem to be involved in the EP_4-receptor mediated phosphorylation of CREB because phosphorylation was not blocked by the mitogen-activated protein kinase kinase/ERK inhibitor PD98059. The mechanism of activation of PI3K by EP_4 remains unknown.

It is well established that the serine/threonine kinase, protein kinase B (Akt), can phosphorylate CREB on Ser-133 in response to a variety of stressful stimuli (Mayr and Montminy, 2001; Johannessen et al., 2004). Akt itself is phosphorylated and activated by the phosphoinositide-dependent kinase-1 (PDK-1) as a downstream consequence of the activation of PI3K (Toker, 2000). We have shown previously that treatment of EP_4-expressing cells with PGE_2 stimulates the phosphorylation of Akt and that this phosphorylation can be blocked by pretreatment with wortmannin (Fujino et al., 2002). As shown in Fig. 7, it is plausible that the PGE_2-stimulated phosphorylation of CREB in EP_4-expressing cells is mediated by Akt after the activation of PI3K signaling. The specific PI3K and the mechanism of its activation by the EP_4 receptor remains unknown. PI3K actually comprises a family of enzymes, most of which can be inhibited by wortmannin (Vanhaesebroeck and Waterfield, 1999). GPCRs have been shown to activate PI3K by several mechanisms including direct activation by G-protein $\beta\gamma$-subunits and transactivation through the epidermal growth factor receptor. Further studies will be needed to elucidate the mechanism of PI3K activation after stimulation the EP_4 receptor with PGE_2.

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