

Differential Regulation of Phosphorylation of the cAMP Response Element Binding Protein Following Activation of EP₂ and EP₄ Prostanoid Receptors by Prostaglandin E₂

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List of abbreviations: PGE₂, prostaglandin E₂; CREB, cAMP response element binding protein; 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; .

Abstract

The EP₂ and EP₄ prostanoid receptors are G-protein coupled receptors whose activation by their endogenous ligand, prostaglandin E₂ (PGE₂), stimulates the formation of intracellular cyclic 3,5-adenosine monophosphate (cAMP). We have previously reported that the stimulation of cAMP formation in EP₄ expressing cells is significantly less than in EP₂ expressing cells, despite nearly identical levels of receptor expression (Fujino, H., West, K. A., and Regan, J. W. (2002) *J. Biol. Chem.* 277, 2614-2619). In addition, a component of EP₄ receptor signaling, but not of EP₂ receptor signaling, was found to involve the activation of phosphatidylinositol 3-kinase (PI3K). In this study, we report that PGE₂ stimulation of cells expressing either the EP₂ or EP₄ receptors results in the phosphorylation of the cAMP response element binding protein (CREB) at serine-133. Pretreatment of cells with H-89, an inhibitor of protein kinase A (PKA), attenuated the PGE₂ mediated phosphorylation of CREB in EP₂ expressing cells, but not in EP₄ expressing cells. Pretreatment of cells with wortmannin, an inhibitor of PI3K, had no effects on the PGE₂ mediated phosphorylation of CREB in either EP₂ or EP₄ expressing cells; although it significantly increased the PGE₂ mediated activation of PKA in EP₄ expressing cells. However, combined pretreatment with H-89 and wortmannin blocked PGE₂ mediated phosphorylation in EP₄ expressing cells, as well as in EP₂ expressing cells. PGE₂ mediated intracellular cAMP formation was not affected by pretreatment with wortmannin, or combined treatment with wortmannin and H-89, in either the EP₂ or EP₄ expressing cells. These findings suggest that PGE₂ stimulation of EP₄ receptors, but not EP₂ receptors, results in the activation of a PI3K signaling pathway that inhibits the activity of PKA and that the PGE₂ mediated phosphorylation of CREB by these receptors occurs through different signaling pathways.

Introduction

The EP₂ and EP₄ prostanoid receptors are members of the superfamily of G-protein coupled receptors (GPCRs) and are two of the four subtypes of receptors for prostaglandin E₂ (PGE₂) (Regan, 2003). Following the initial cloning of these receptors, their functional significance was unclear since they both were preferentially activated by PGE₂ and they both appeared 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 PGE₂ mediated receptor desensitization and internalization. Thus, EP₄ receptors were found to undergo rapid agonist induced desensitization, whereas EP₂ receptors did not (Nishigaki et al., 1996). Similarly, EP₄ receptors were found to undergo rapid PGE₂ mediated internalization and EP₂ receptors did not (Desai et al., 2000).

The first evidence of differences in the signaling potential of the EP₂ and EP₄ receptors involved the observation that PGE₂ 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 EP₂ receptor was primarily through a PKA-dependent pathway, whereas, for the EP₄ receptor these effects were mediated primarily through a phosphatidylinositol 3-kinase (PI3K)-dependent pathway. It was further shown that PGE₂ treatment of EP₄ expressing cells, but not EP₂ 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 et al., 2003). Additional evidence of EP₄ receptor signaling through a PI3K dependent pathway has also been reported for colorectal carcinoma cells. Thus, PGE₂ was found to increase the growth and motility of human adenocarcinoma cells (LS-174) through activation of PI3K via stimulation of EP₄ receptors (Sheng et al., 2001). Similarly, it has been reported that stimulation of EP₄ receptors by PGE₂ 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_{αs} coupled receptors is the transcriptional regulation of genes whose promoters contain cAMP response elements (CREs). In this signaling cascade the release of G_{αs} following 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 (COX-2), whose catalytic product, PGH₂, is the immediate precursor for the biosynthesis of the prostaglandins and thromboxanes. Interestingly, it has been found that bradykinin can increase cAMP dependent COX-2 promoter activity in human pulmonary artery smooth muscle cells through an autocrine signaling pathway involving the activation of endogenous EP₂ and EP₄ prostanoid receptors (Bradbury et al., 2003).

The phosphorylation of CREB by PKA occurs at serine-133 (ser-133) and results in the recruitment of the CREB-binding protein (CBP) and/or its paralogue, p300, that function with phospho-CREB as co-activators of gene transcription (Mayr and Montminy, 2001; Johannessen et al., 2004). However, phosphorylation at ser-133 is not 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 following 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 following the activation of the ERKs and Akt signaling pathways (Mayr and Montminy, 2001; Johannessen et al., 2004).

Given the ability of EP₂ and EP₄ receptors to activate cAMP signaling pathways and the additional ability of EP₄ 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 EP₂ and EP₄ receptors by PGE₂ can lead to the phosphorylation of CREB at ser-133. In EP₂ expressing cells the mechanism is primarily cAMP and PKA dependent. In EP₄ expressing cells the mechanism is more complex and involves a PI3K dependent pathway. A novel finding is that PGE₂

stimulation of EP₄ expressing cells negatively regulates the activity of PKA through the activation of PI3K signaling.

Materials and Methods

Western Blotting. Cell lines stably expressing the EP₂ and EP₄ receptors were prepared using HEK-293 EBNA cells (Invitrogen) and the mammalian expression vector pCEP4 (Invitrogen) as described previously (Fujino et al., 2002; Fujino et al., 2003). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum, 250 µg/ml geneticin (Invitrogen), 100 µg/ml gentamicin (Invitrogen) and 200 µg/ml hygromycin B (Invitrogen). Sixteen hours prior to the immunoblotting experiments, cells were switched from their regular DMEM medium to Opti-MEM (Invitrogen) containing 250 µg/ml geneticin and 100 µg/ml gentamicin. Cells were incubated at 37°C with either vehicle (0.1% Me₂SO) or 1 µM PGE₂ (Cayman) for the time indicated in the figures. For experiments involving the use of signaling inhibitors, cells were pretreated with either vehicle (0.1% Me₂SO) 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 x 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% non-fat dry milk for 1 hour and were then washed and incubated for 16 hours at 4°C in 5% bovine serum albumin (BSA) containing anti-phospho-CREB (ser-133) antibody (Cell Signaling, catalog number 9191) at a dilution of 1:1,000. Membranes were washed three times and incubated for 1 hour a room temperature in 5% non-fat milk with a 1:2,000 dilution of anti-rabbit secondary antibodies conjugated with horseradish peroxidase. After washing three times, immunoreactivity was detected by chemiluminescence as described previously (Fujino et al., 2002; Fujino et al., 2003). To ensure equal loading of proteins, the membranes were stripped and reprobed with a

1:1000 dilution of anti-CREB antibody (Cell Signaling, catalog number 9192) 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% Me₂SO) 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% Me₂SO) or 1 μM PGE₂ for 10 min. The cells were washed with ice-cold phosphate buffered saline (PBS) and were placed on ice. Two hundred μl 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 and following 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 x 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 ELISA-based assay kit and a synthetic peptide substrate for PKA (Stressgen Bioreagents, catalog number EKS-390A).

cAMP Assay. Cells were cultured in 12-well plates and were replaced with fresh Opti-MEM containing 0.1 mg/ml isobutylmethylxanthine (Sigma). Cells were pretreated with either vehicle (0.1% Me₂SO) 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% Me₂SO) or 1 μM PGE₂ for 10 min. The media were removed and the cells were placed 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 16,000 x g. Two μl of the supernatants (representing ~10⁴ cells) were transferred to microcentrifuge tubes containing 48 μl of TE, 50 μl of [³H]cAMP (Life Sciences) and 100 μl of 0.06 mg/ml PKA (Sigma, catalog number P-5511). The samples were vortexed, incubated on ice for 2 hours, followed by the addition of 100 μl of TE buffer containing 2% BSA and 26 mg/ml powdered charcoal (Sigma). After vortexing and centrifugation for 1 min at 16,000 x 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 cold cAMP and was expressed as pmol per 10⁴ cells.

Results

PGE₂ Stimulated Phosphorylation of CREB in EP₂ or EP₄ 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, following 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 PGE₂ in HEK cells stably expressing either the human EP₂ or EP₄ prostanoid receptors (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 EP₄ expressing cell line was only ~20% of that obtained in the EP₂ expressing cell line. However, for a separate measure of signaling activity (PGE₂ stimulation of Tcf mediated transcriptional activation) both cell lines yielded similar maximal extents of activation (Fujino et al., 2002). We were, therefore, interested in possible similarities or differences in the ability of PGE₂ to stimulate the phosphorylation of CREB at ser-133 in these same EP₂ or EP₄ expressing cell lines. For these experiments cells expressing either the human EP₂ or EP₄ prostanoid receptors or untransfected HEK cells were treated with 1 μM PGE₂ 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 non-phosphorylated). As shown in panels A and B of Figure 1, treatment with PGE₂ resulted in a rapid time-dependent phosphorylation of CREB in both the EP₂ and EP₄ receptor expressing cell lines. The time course of CREB phosphorylation was similar for both cell lines and appeared to reach a similar maximum. As shown in panel C, there was no significant effect of PGE₂ on the phosphorylation of CREB in the untransfected HEK cells. Panels A, B and C of Figure 1 also show that when these blots were stripped and re-probed with antibodies that recognized both the phosphorylated and non-phosphorylated forms of CREB, nearly identical amounts of CREB were present throughout the time course of treatment and among the three cell lines.

PGE₂ Stimulated cAMP Formation and Activation of PKA in EP₂ or EP₄ Transfected HEK Cells. As noted for the experiments depicted in Figure 1, the maximal extent of PGE₂ stimulated CREB phosphorylation appeared to reach a similar maximum in both the EP₂ and EP₄ expressing cells. This is somewhat surprising given that in previous studies we had found that the maximal extent of PGE₂ stimulated cAMP formation was significantly greater, after 1 hour, in EP₂ expressing cells as compared with EP₄ expressing cells. Thus, one might expect a greater activation of PKA in EP₂ expressing cells and perhaps a greater extent of CREB phosphorylation in EP₂ expressing cells. We decided, therefore, to examine the formation of cAMP and activation of PKA after 10 min of stimulation with PGE₂ in cells expressing either the EP₂ or EP₄ receptors. As confirmed in panel A of Figure 2, the maximal extent of PGE₂ stimulated cAMP accumulation was approximately 3 fold greater in EP₂ expressing cells as compared with EP₄ expressing cells. Similarly, as shown in panel B of Figure 2, PGE₂ stimulated PKA activity was approximately 3.5 times greater in EP₂ expressing cells as compared with EP₄ expressing cells. These findings suggest, therefore, that the similar levels of PGE₂ 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 EP₄ expressing cells which also leads to the phosphorylation of CREB.

Effects of H-89 and Wortmannin on PGE₂ Stimulated Phosphorylation of CREB in EP₂ or EP₄ Transfected HEK Cells. As reviewed in the introduction, we have previously found that PGE₂ can stimulate the phosphorylation of GSK-3 and can increase Tcf transcriptional activation in cells stably transfected with either the human EP₂ or EP₄ prostanoid receptors (Fujino et al., 2002). In EP₂ expressing cells, however, these activities are mediated primarily by a PKA dependent pathway, whereas in EP₄ 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 respectively investigate the contributions of PKA signaling and PI3K signaling to the PGE₂ 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 μ M PGE₂ for 10 min and were then immunoblotted with antibodies to phospho-CREB (pCREB/pATF1) or total CREB. As depicted in Figure 3A, following the pretreatment of EP₂ expressing cells with H-89 there was a significant decrease of PGE₂ stimulated phosphorylation of CREB, suggesting the involvement of PKA in this phosphorylation. On the other hand, pretreatment of EP₄ expressing cells with H-89 had no significant effect on PGE₂ mediated CREB phosphorylation. Figure 3B shows that somewhat unexpectedly pretreatment with wortmannin had no effect on PGE₂ stimulated CREB phosphorylation in either EP₂ or EP₄ expressing cells. We have shown in prior studies that pretreatment of EP₄ expressing cells with 100 nM wortmannin for 15 min can inhibit the PGE₂ mediated phosphorylation of GSK-3, as well as the PGE₂ mediated stimulation of Tcf reporter gene activity and induction of EGR-1 (Fujino et al., 2002; Fujino et al., 2003). As an additional precaution, however, we repeated the experiments depicted in Figure 3B with the PI3K inhibitor LY294002. As with wortmannin, pretreatment of EP₂ and EP₄ expressing cells with 30 μ M LY294002 for 15 min had no effect on the PGE₂ stimulated phosphorylation of CREB (data not shown).

Effects of the Combination of H-89 and Wortmannin on PGE₂ Stimulated

Phosphorylation of CREB in EP₂ or EP₄ Transfected HEK Cells. Although the individual effects of H-89 and wortmannin pretreatment on EP₄ expressing HEK cells was without effect on the PGE₂ stimulated phosphorylation of CREB, we considered the possibility that there might be some kind of interaction between the PKA and PI3K signaling pathways. We, therefore, decided to examine the effect of these inhibitors in combination. As expected, Figure 4 shows that the combination of H-89 and wortmannin pretreatment of EP₂ expressing cells resulted in the inhibition of PGE₂ stimulated CREB phosphorylation to a similar extent as that observed by pretreatment with H-89 alone (*cf*, Figure 3A). Of interest, Figure 4 shows that the combination of H-89 and wortmannin pretreatment of EP₄ expressing cells resulted in a complete inhibition of PGE₂ stimulated CREB phosphorylation even though pretreatment with either inhibitor alone had no effect (*cf*, Figures 3A and 3B). The experiments depicted in Figure 4 were repeated with the combination of H-89 and the wortmannin inhibitor LY294002 and virtually identical

results were obtained. Thus, pretreatment of EP₄ expressing cells with the combination of 30 μ M LY294002 and 10 μ M H-89 for 15 min resulted in a nearly complete blockade of the PGE₂ 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 PGE₂ mediated phosphorylation of CREB in EP₄ expressing cells.

Effects of H-89, Wortmannin and the Combination of H-89 and Wortmannin on PGE₂ Stimulated PKA Activity in EP₂ or EP₄ 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 PGE₂ stimulated PKA activity in cells stably expressing either the human EP₂ or EP₄ prostanoid receptors. As noted previously in Figure 2, Figure 5 shows that in the absence of any inhibitor pretreatment there was a 2.6 fold stimulation of PKA activity in EP₂ cells treated for 10 min with 1 μ M PGE₂ and a 1.5 fold stimulation of PKA activity in EP₄ expressing cells. Pretreatment of both EP₂ and EP₄ expressing cells with H-89 alone resulted in a nearly complete inhibition of PGE₂ stimulated PKA activity in both cell lines. The inhibition of PGE₂ stimulated PKA activity in EP₂ expressing cells correlated nicely with the inhibition of PGE₂ stimulated CREB phosphorylation by H-89 in the EP₂ expressing cells (*cf.*, Figure 3A). On the other hand, the inhibition of PGE₂ stimulated PKA activity in EP₄ expressing cells did not correlate with the effects of H-89 on PGE₂ stimulated CREB phosphorylation in EP₄ expressing cells, which was not inhibited by H-89 pretreatment (*cf.*, Figure 3A). These findings further support the conclusion that the PGE₂ mediated phosphorylation of CREB in EP₄ expressing cells is not through a PKA dependent pathway.

As shown in Figure 5, pretreatment with wortmannin had essentially no effect on PGE₂ stimulated PKA activity in EP₂ expressing cells, but in EP₄ expressing cells it caused a significant increase in PGE₂ stimulated PKA activity as compared with untreated cells. Thus, following pretreatment of EP₄ expressing cells with wortmannin, PGE₂ 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 PGE₂ stimulated CREB phosphorylation in EP₄

expressing cells (*cf.*, Figure 3B) because the PGE₂ mediated activation of PI3K signaling in EP₄ expressing cells inhibits the activity of PKA. Therefore, pretreatment of EP₄ expressing cells with wortmannin alone relieves this PI3K mediated inhibition, resulting in a PKA dependent phosphorylation of CREB. As expected, Figure 5 shows that pretreatment of EP₂ and EP₄ expressing cells with the combination of H-89 and wortmannin inhibited the PGE₂ stimulation of PKA activity in both cell lines. These findings are consistent with the inhibition of PGE₂ mediated CREB phosphorylation in both the EP₂ and EP₄ expressing cell lines following pretreatment with the combination of H-89 and wortmannin (*cf.* Figure 4).

Effects of H-89, Wortmannin and the Combination of H-89 and Wortmannin on PGE₂ Stimulated cAMP Formation in EP₂ or EP₄ Transfected HEK Cells. Given that the activity of PKA is regulated by cAMP, we decided to examine PGE₂ stimulated cAMP formation in EP₂ and EP₄ cells under control conditions and following treatment with inhibitors of the PKA and PI3K pathways. This was of particular interest with respect to wortmannin's effect of increasing PGE₂ stimulated PKA activity in EP₄ expressing cells since this could reflect either a PI3K mediated inhibition of PKA activity or a PI3K mediated increase in intracellular cAMP accumulation. As shown previously in Figure 2, Figure 6 shows that in the absence of pretreatment with inhibitors there was a 23 fold stimulation of cAMP accumulation in EP₂ cells treated for 10 min with 1 μM PGE₂ and a 7.4 fold stimulation of cAMP accumulation in EP₄ expressing cells. For both EP₂ and EP₄ expressing cells, the PGE₂ mediated stimulation of cAMP accumulation correlated reasonably well with the PGE₂ mediated stimulation of PKA activity shown in Figure 5. Figure 6 also shows that pretreatment with either H-89, wortmannin or the combination of H-89 and wortmannin had virtually no effect on PGE₂ stimulated cAMP accumulation in either EP₂ or EP₄ expressing cells. The evidence that wortmannin pretreatment of EP₄ expressing cells had little effect on PGE₂ mediated cAMP accumulation supports the conclusion that the PGE₂ mediated activation of PI3K signaling in EP₄ 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_{αs} coupled GPCRs is, in evolutionarily terms, perhaps the oldest and most widespread second messenger pathway used by this superfamily of receptors. By way of example, of the eight most closely related human prostanoid receptor subtypes, four of them (EP₂, EP₄, IP and DP₁) couple primarily to this signaling pathway (Hata & Breyer, 2004). Three of the remaining prostanoid receptor subtypes (EP₁, FP and TP) are coupled primarily to G_{αq} and activate the inositol phosphate signaling pathway and one (EP₃) is coupled to G_{αi} and decreases the formation of intracellular cAMP through the inhibition of adenylyl cyclase. It is interesting that the phylogeny of these prostanoid receptors shows that the subtypes which couple to G_{αs} are all more closely related to each other and form a distinct subfamily as compared with the subtypes that couple to G_{αq} and G_{αi} (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_{αs}, two (EP₂ and EP₄) are activated by PGE₂ and two (IP and DP₁) are activated by prostacyclin and PGD₂, respectively. As the discovery of these prostanoid receptor subtypes unfolded it seemed likely that the IP and DP₁ receptors evolved to subservise different signaling molecules, which could also be said of the EP₂ and EP₄ receptors as a group, but obviously not as individual subtypes. In fact, the EP₂ and EP₄ receptors do *not* appear to have evolved together as their own subfamily because the phylogeny shows that the EP₂, IP and DP₁ receptors are actually more related to each other and form a subgroup that is distinct from the EP₄ receptor (Toh et al., 1995). This suggests an evolutionary divergence of an ancestral G_{αs} coupled EP receptor into two descendants. One of these descendants was a G_{αs} coupled EP receptor that eventually gave rise to the EP₂, IP and DP₁ receptor subtypes and the second descendant was a G_{αs} coupled EP receptor that evolved into the present day EP₄ receptor subtype. Thus, the evolution of the EP₂, IP and DP₁ receptor subtypes, presumably on the basis of their ability to discriminate between their respective endogenous ligands, appears to have come *after* an earlier event that eventually gave rise

to the EP₂ and EP₄ subtypes. The underlying basis for the initial divergence of the ancestral G_{αs} 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.

As reviewed in the introduction, the initial characterization of the EP₂ and EP₄ receptors subtypes did not reveal any significant functional differences between these receptors: they were both preferentially activated by PGE₂ and they both stimulated the formation of intracellular cAMP (Honda et al., 1993; Regan et al., 1994). Subsequently it was found that there were differences in the desensitization (Nishigaki et al., 1996) and internalization (Desai et al., 2000) of these receptors and more recently it has become clear that there are significant differences in the signaling properties of the EP₂ and EP₄ prostanoid receptors (Fujino and Regan, 2003). As shown in Figure 7, what appears to be emerging with respect to the signaling differences is that the EP₂ receptor subtype couples to a classic cAMP signaling pathway involving a marked stimulation of intracellular cAMP formation and activation of PKA. The EP₄ 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 ERKs signaling pathways. Often the stimulation of either receptor subtype leads to the activation of the same downstream effectors, albeit by different pathways. For example, both the EP₂ and EP₄ receptors can stimulate Tcf transcriptional activation, but the EP₂ receptor utilizes primarily a cAMP/PKA dependent pathway while the EP₄ receptor uses primarily a PI3K dependent pathway (Fujino et al., 2002). However, stimulation of the EP₄ receptor can also result in the selective activation of downstream effectors that are not activated following the stimulation of EP₂ receptors. For example, PGE₂ stimulation of the EP₄ receptor induces the expression of EGR-1 which does not occur following PGE₂ stimulation of the EP₂ receptor (Fujino et al., 2003).

The present findings further highlight the unique signaling properties of the EP₂ and EP₄ 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 Figure 7, PGE₂ stimulation of either receptor results in the phosphorylation of CREB on ser-133, but the EP₂ receptor does this solely

through the activation of a cAMP/PKA dependent pathway, whereas the EP₄ receptor can utilize both the cAMP/PKA and PI3K pathways. Additionally, it was found that PGE₂ stimulation of the EP₄ 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 following PGE₂ stimulation of the EP₄ receptor occurs primarily by way 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 _{α_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 following 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 _{α_s} coupled GPCRs is a key mediator of the ser-133 phosphorylation of CREB, but as reviewed in the introduction, it is not the only mechanism. This is clearly exemplified by our present findings which show that the EP₂ receptor mediated phosphorylation of CREB on ser-133 is mainly PKA dependent, while the EP₄ 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 previously reported the PI3K dependent activation of ERKs 1 and 2 by the EP₄ receptor, but not the EP₂ receptor (Fujino et al., 2003). However, inhibition of MAP kinase kinase (MEK) signaling with PD98059, did not affect the PGE₂ mediated phosphorylation of CREB in either the EP₂ or EP₄ expressing cells (data not shown). Previously we had found that the inhibition of MEK by PD98059 in EP₄ expressing cells blocked the ERKs mediated induction of EGR-1 expression, which suggests that activation of a MEK/ERKs

signaling pathway is not involved in the PGE₂ mediated phosphorylation of CREB in EP₄ expressing cells.

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 previously shown that treatment of EP₄ expressing cells with PGE₂ stimulates the phosphorylation of Akt and that this phosphorylation can be blocked by pretreatment with wortmannin (Fujino et al, 2002). As shown in Figure 7, it is plausible that the PGE₂ stimulated phosphorylation of CREB in EP₄ expressing cells is mediated by Akt following the activation of PI3K signaling. The specific PI3K and the mechanism of its activation by the EP₄ receptor is presently unknown. PI3K actually comprises a family of enzymes, most of which can be inhibited by wortmannin (Vanhaesebroeck & Waterfield, 1999). GPCRs have been shown to activate PI3K by several mechanisms which include direct activation by G-protein βγ-subunits and transactivation through the epidermal growth factor receptor. Further studies will be needed to elucidate the mechanism of PI3K activation following stimulation the EP₄ receptor with PGE₂.

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Figure Legend

Figure 1. Immunoblots of the time course of PGE₂-stimulated phosphorylation of CREB in HEK cells transfected with either the human EP₂ (Panel A) or EP₄ (Panel B) prostanoid receptors and in untransfected HEK cells (Panel C). Cells were incubated with 1 μM PGE₂ for the indicated times and were subjected to immunoblot analysis as described under “*Materials and Methods*”. The upper of the two immunoblots shown in each panel are 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 of the two immunoblots shown in each panel are the results obtained with antibodies that recognize total CREB (phosphorylated and non-phosphorylated). 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, ANOVA. †p < 0.05, t-test.

Figure 2. PGE₂-stimulated cAMP accumulation (Panel A) and PKA activity (Panel B) in HEK cells transfected with either the human EP₂ or EP₄ prostanoid receptors. Cells were treated with either vehicle (v) or 1 μM PGE₂ (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 EP₂ expressing cells as 100%. *p < 0.05, t-test. These data are the same as shown in Figures 5 and 6 for control cells that were not treated with inhibitors.

Figure 3. The effects of H-89 (Panel A) and wortmannin (Panel B) on PGE₂-stimulated phosphorylation of CREB in HEK cells transfected with either the human EP₂ or EP₄ prostanoid receptors. 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 PGE₂ (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 are 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 of the two immunoblots shown in each panel are the results obtained with antibodies that recognize total CREB (phosphorylated and non-phosphorylated). The histograms represent the ratio of pCREB to total CREB as assessed by the pooled densitometry data (mean \pm S.E.M.) from three independent experiments. Data are normalized to the PGE₂ treated EP₂ expressing cells as 100%. **p* < 0.05, t-test.

Figure 4. The effects of the combination of H-89 and wortmannin on PGE₂-stimulated phosphorylation of CREB in HEK cells transfected with either the human EP₂ or EP₄ prostanoid receptors. Cells were pretreated with either vehicle or the combination of 10 μ M H-89 and 100 nM wortmannin (*wort*) for 15 min followed by treatment with either vehicle (*v*) or 1 μ M PGE₂ (*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 non-phosphorylated). The histogram represents the ratio of pCREB to total CREB as assessed by the pooled densitometry data (mean \pm S.E.M.) from three independent experiments. Data are normalized to the PGE₂ treated EP₂ expressing cells as 100%. **p* < 0.05, t-test.

Figure 5. The effects of H-89, wortmannin, and the combination of H-89 and wortmannin, on PGE₂-stimulated PKA activity in HEK cells transfected with either the human EP₂ (Panel A) or EP₄ (Panel B) prostanoid receptors. Cells were pretreated with either vehicle or 10 μ M H-89 or 100 nM wortmannin (*wort*) or the combination of 10 μ M H-89 and 100 nM wortmannin for 15 min followed by treatment with either vehicle (*v*) or 1 μ M PGE₂ (*P*) for 10 min at 37°C and were then assayed for PKA activity as described under “*Materials and Methods*”. Data are the mean \pm S.E.M. from three independent experiments and are normalized to the vehicle treated EP₂ expressing cells as 100%. **p* < 0.05, t-test. †*p* < 0.05, t-test. ‡*p* < 0.05, t-test.

Figure 6. The effects of H-89, wortmannin, and the combination of H-89 and wortmannin, on PGE₂-stimulated cAMP accumulation in HEK cells transfected with either the human EP₂ (Panel A) or EP₄ (Panel B) prostanoid receptors. 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 PGE₂ (*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 EP₂ expressing cells as 100%. *p < 0.05, t-test.

Figure 7. Model for prostaglandin-E₂ (PGE₂) mediated phosphorylation of the cAMP response element binding protein (CREB) by the human EP₂ and EP₄ prostanoid receptors. Following the binding of PGE₂, both receptors can activate the stimulatory guanine nucleotide binding protein (G_s), which in turn activates adenylyl cyclase (AC). Increased intracellular cAMP formation then activates protein kinase A (PKA) which can phosphorylate CREB on serine-133. The EP₄ receptor also activates a phosphatidylinositol 3-kinase (PI3K) signaling pathway, which leads to the phosphorylation of CREB on serine-133; possibly through the sequential activation of phosphoinositide-dependent kinase-1 (PDK-1) and protein kinase B (Akt). H-89 inhibits the activity of PKA and blocks EP₂ receptor mediated CREB phosphorylation, but does not block EP₄ receptor mediated CREB phosphorylation because of signaling through the PI3K pathway. Inhibition of PI3K by wortmannin (*wort*) alone does not block EP₄ 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 MAP kinase (ERKs) signaling has previously been implicated in CREB phosphorylation, it does not appear to be involved in the EP₄ receptor mediated phosphorylation of CREB because phosphorylation was not blocked by the MEK/ERKs inhibitor, PD98059. The mechanism of activation of PI3K by the EP₄ is presently unknown (?).

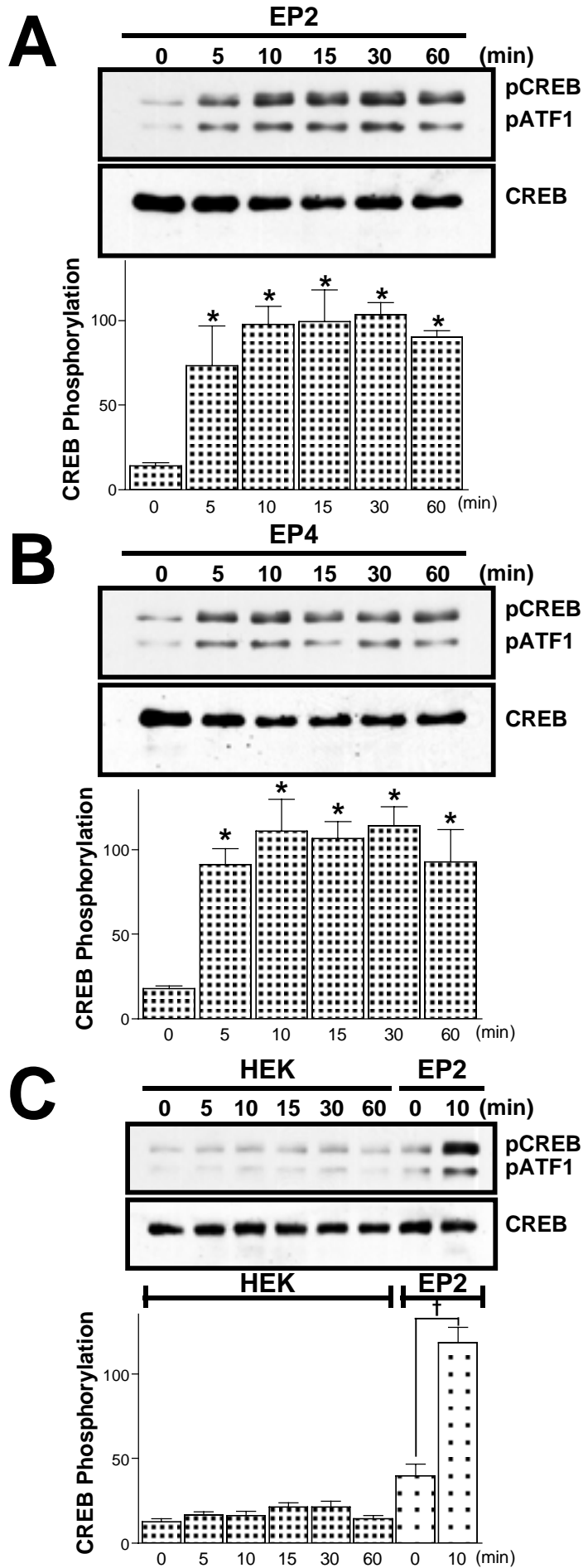


Figure 1

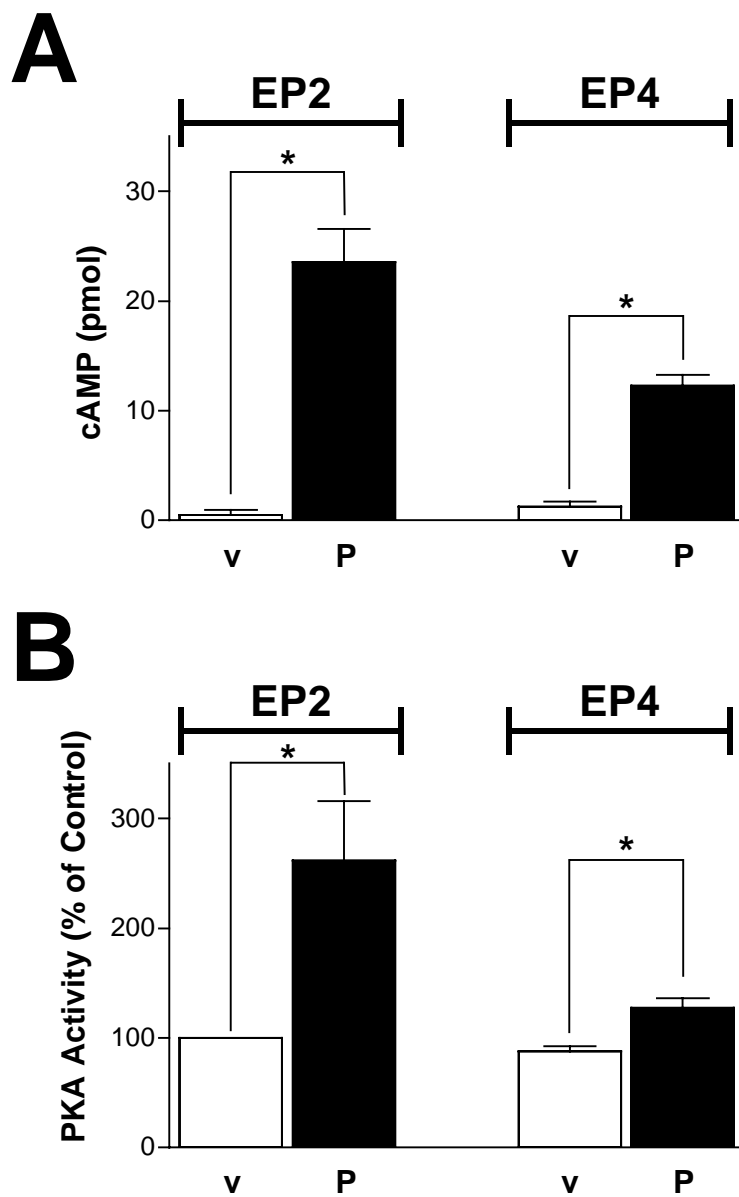
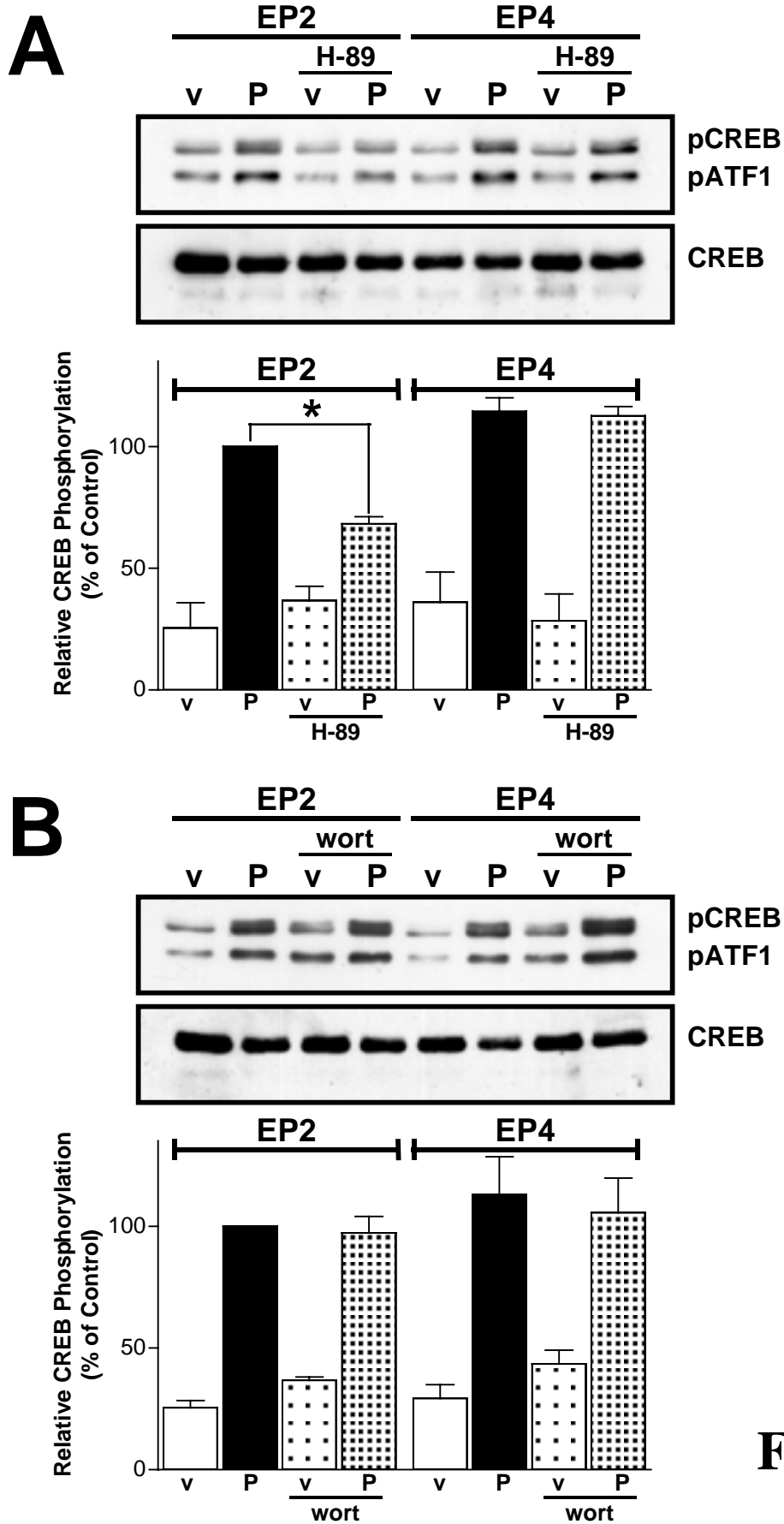


Figure 2



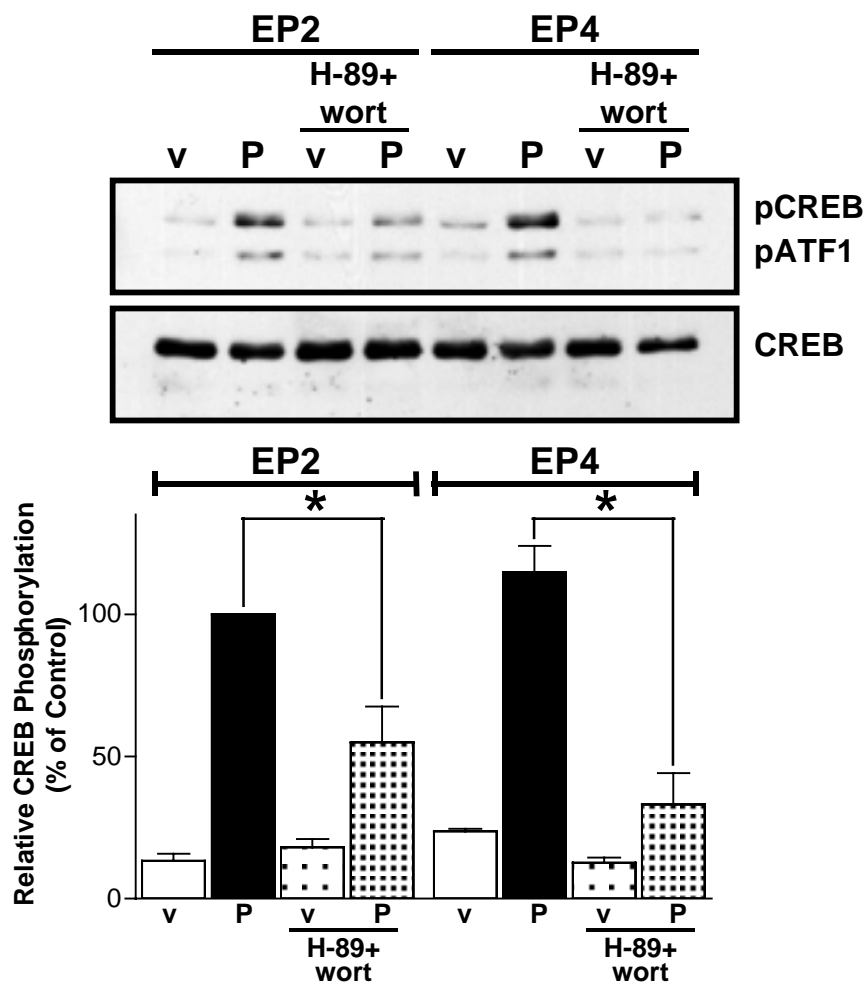


Figure 4

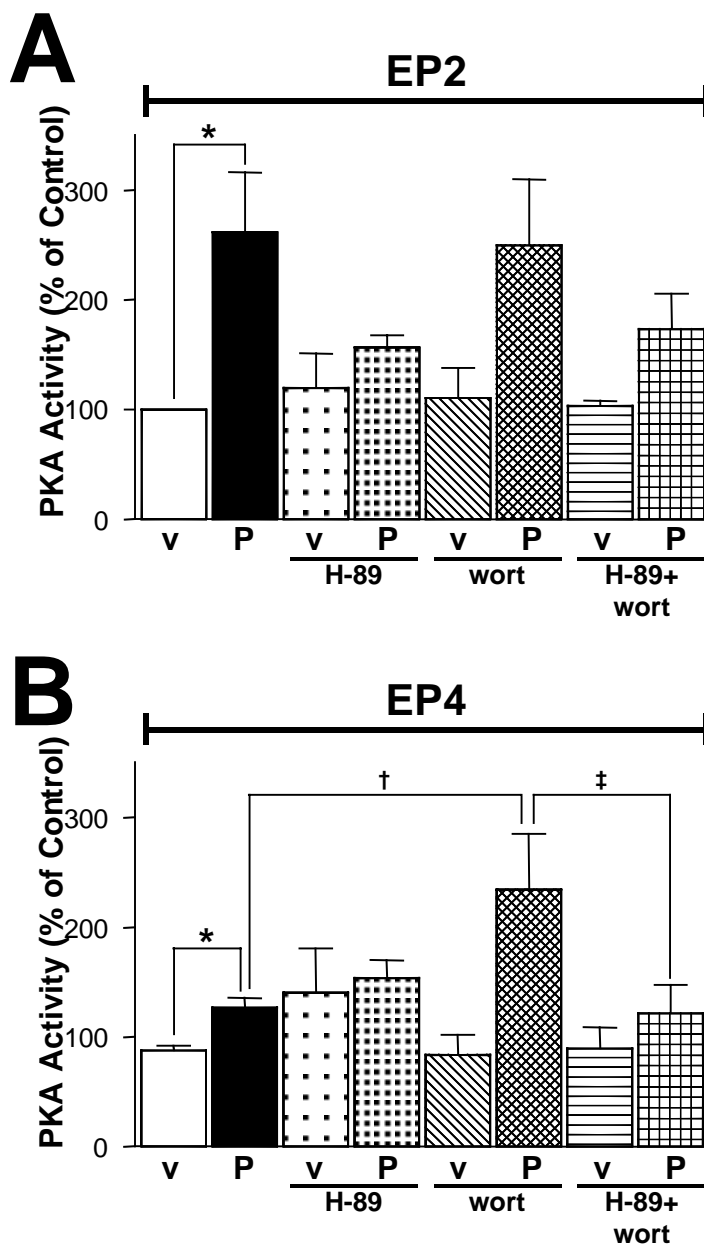


Figure 5

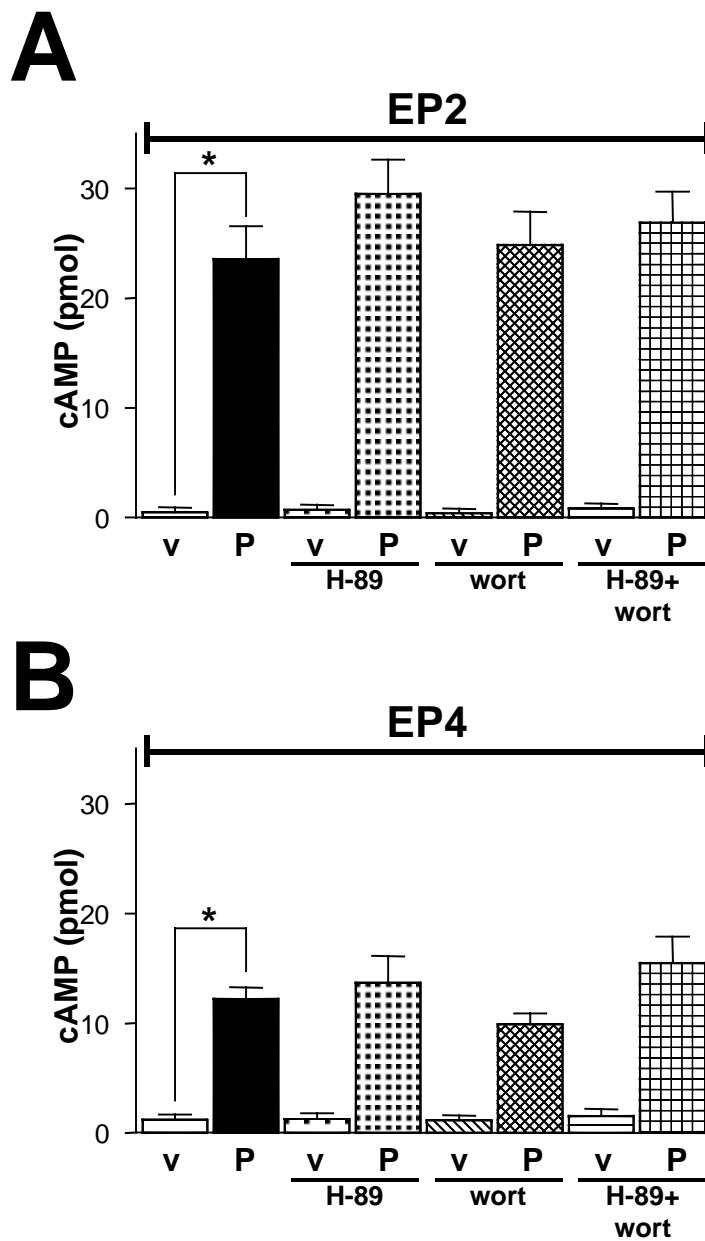


Figure 6

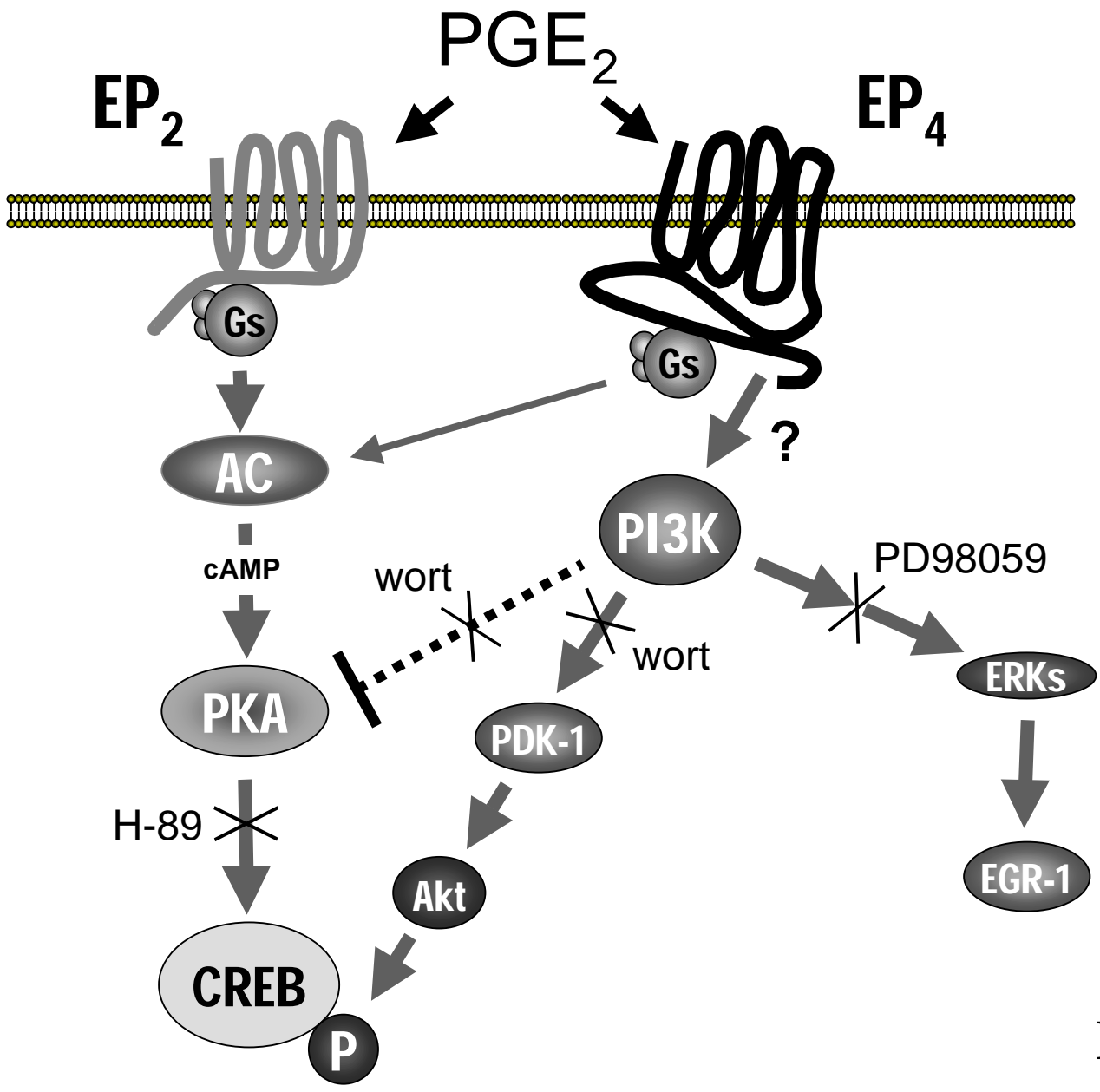


Figure 7