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Differential Regulation of Prostaglandin $F_{2\alpha}$ Receptor Isoforms by Protein Kinase C

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ABSTRACT

Prostaglandin $F_{2\alpha}$ receptors (FP) are G protein-coupled receptors that bind prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), resulting in the activation of an inositol phosphate (IP) second messenger pathway. Alternative mRNA splicing generates two FP receptor isoforms. These isoforms, designated FP_A and FP_B, are otherwise identical except for their carboxyl termini. FP_B is essentially a truncated version of FP_A that lacks the 46 carboxyl-terminal amino acids, including four putative protein kinase C (PKC) phosphorylation sites. Until now, functional differences between these FP receptor isoforms have not been identified. We now report that pretreatment with the PKC inhibitor bisindolylmaleimide I enhanced $PGF_{2\alpha}$-stimulated IP accumulation in transfected cells stably expressing the FP_A isoform but not in cells stably expressing the FP_B isoform. Whole-cell phosphorylation experiments showed a strong agonist-dependent phosphorylation of the FP_A isoform but little or no phosphorylation of the FP_B. Pretreatment of cells with bisindolylmaleimide I decreased $PGF_{2\alpha}$-stimulated phosphorylation of the FP_A isoform consistent with a PKC-dependent phosphorylation. In vitro phosphorylation of an FP_A carboxyl-terminal fusion protein by recombinant PKCα showed that the carboxyl terminus of the FP_A is a substrate for PKC. These results suggest that PKC-dependent phosphorylation is responsible for differential regulation of second messenger signaling by FP prostanoid receptor isoforms.

Neurotransmitter and hormonal signaling through G protein-coupled receptors (GPCR) is a major mechanism of intercellular communication. In addition to activating second-messenger pathways, the agonist-induced activation of GPCRs may induce a state of diminished agonist responsiveness known as desensitization. Phosphorylation of GPCRs by regulatory kinases has often been implicated in desensitization in which the phosphorylated receptor becomes uncoupled from its cognate G protein and/or is internalized. Kinases that have been shown to be involved in GPCR desensitization include cAMP-dependent protein kinase, calcium-dependent protein kinase (PKC), and GPCR kinase (GRK) (Freedman and Lefkowitz, 1996).

Given the diversity of GPCRs, it is not surprising that there is considerable variation in the regulation of GPCRs by kinases and that for many GPCRs, the details of their kinase-dependent regulation are unknown. Among the subfamily of prostanoid receptors, for example, there is little known either about their desensitization or about the kinases that might be involved. There are five major subtypes of prostanoid receptors (EP, DP, FP, IP and TP) that correspond to the five major prostanoid metabolites [prostaglandins E_2, D_2, $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin, and thromboxane A_2, respectively]. To date, only the TP and IP receptors have been shown to undergo agonist-dependent phosphorylation (Habib et al., 1997; Smyth et al., 1998). In the case of TP receptors, a GRK may be involved, whereas PKC is important for phosphorylation of both TP and IP receptors.

FP prostanoid receptors are GPCRs that bind endogenous $PGF_{2\alpha}$, resulting in the activation of an inositol phosphate (IP) second-messenger pathway. Previously, we cloned an alternative mRNA splice variant of the FP receptor (termed FP_B) from an ovine large-cell corpus luteum library (Pierce et al., 1997). This FP_B receptor isoform is identical with the original FP receptor (termed FP_A) throughout its entire amino acid sequence except for the carboxyl terminus. Thus, the first nine amino acids of the carboxyl terminus are identical, but then the FP_A isoform continues for another 46 amino acids, whereas FP_B terminates after one. Essentially, therefore, FP_B represents a truncated isoform of FP_A. Functionally, both isoforms have similar pharmacological profiles

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ABBREVIATIONS: GPCR, G protein-coupled receptor; PKC, protein kinase C; GRK, G protein-coupled receptor kinase; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; IP, inositol phosphate; HEK, human embryonic kidney; TMX, thymeleatoxin; BIM, bisindolylmaleimide I; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione-*S*-transferase; PCR, polymerase chain reaction; HA, hemagglutinin; RIPA, radioimmunoprecipitation assay; PAGE, polyacrylamide gel electrophoresis; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate.

and stimulate IP accumulation to a similar extent (Pierce et al., 1997). To elucidate possible differences in signal transduction and/or regulation, the FP_A and FP_B receptor isoforms were stably expressed in human embryonic kidney (HEK) cells. We now report differential regulation of these isoforms by PKC. Thus, PKC inhibits $PGF_{2\alpha}$-stimulated IP formation by the FP_A isoform but not by the FP_B isoform. In addition the FP_A isoform, but not FP_B, showed robust agonist dependent phosphorylation that was mediated by PKC, and an FP_A carboxyl-terminal fusion protein could be directly phosphorylated by PKCα.

Experimental Procedures

Materials. Human recombinant protein kinase Cα, 1-oleoyl-2-acetyl-*sn*-glycerol, thymeleatoxin (TMX) and bisindolylmaleimide I (BIM) were obtained from Calbiochem (San Diego, CA). Histone type III-SS, ATP, L-α-phosphatidyl-L-serine, lithium chloride, HEPES, FLAG-M2 affinity gel, and BSA were from Sigma Chemical (St. Louis, MO), and $PGF_{2\alpha}$ was obtained from Cayman Chemical Co. (Ann Arbor, MI). Dulbecco's modified Eagle's medium (DMEM), phosphate-free DMEM, hygromycin B, geneticin and gentamicin reagent solution were from Life Technologies/BRL (Gaithersburg, MD). Dowex AG1-X8 anion exchange resin was obtained from Bio-Rad (Hercules, CA). myo-[2-^{3}H]Inositol (1 mCi/ml) and [γ-^{32}P]ATP (10 mCi/ml) were from Amersham Corp. (Arlington Heights, IL). A glutathione-*S*-transferase (GST)/FP_A carboxyl terminal fusion protein was prepared as described previously (Anthony et al., 1998).

Preparation of Cells Stably Expressing the FP_A and FP_B Isoforms. HEK cells (293-EBNA; Invitrogen, Carlsbad, CA) stably expressing either the ovine FP_A or FP_B receptor isoforms were prepared as follows. cDNA encoding either the FP_A isoform (Graves et al., 1995) or the FP_B isoform (Pierce et al., 1997) was subcloned into the *Xho*I/*Not*I sites of pCEP4 (Invitrogen) to yield the plasmids pCEP4/FP_A and pCEP4/FP_B. For each plasmid, 20 μg of cesium-purified plasmid DNA was used to transfect one 10-cm plate of HEK cells using the calcium phosphate method with glycerol shock. Two days after transfection, the cells were treated with 200 μg/ml of hygromycin B; approximately 8 days later, hygromycin B-resistant clones were individually selected and transferred to 24-well plates. After reaching confluence, the cells were transferred to 6-well plates and further expanded to 10-cm plates. Cells were maintained at 37°C with 5% CO_2/95% air and in DMEM containing 10% fetal bovine serum, 250 μg/ml geneticin, 200 μg/ml of hygromycin B, and 100 μg/ml gentamicin.

Preparation of FLAG-Tagged Ovine FP_A and FP_B Receptor Isoforms. FLAG epitopes were introduced into the amino termini of the ovine FP_A and FP_B receptor isoforms by a two-step polymerase chain reaction (PCR) followed by a triple ligation reaction. In the first PCR, using the ovine FP_A as a template, a product was generated using a sense primer [5′-ATC TTC TGC CTG GTG TTC GCC GAC TAC AAG GAC GAT GAT GAC GCT AGC ACG AAC AAT TCT GTA CAG-3′ (underlining shows the FLAG epitope sequence)] encoding the FLAG epitope, part of a hemagglutinin (HA) signal sequence, and the amino terminus of the ovine FP_A (nucleotides 106–123; Pierce et al., 1997); and an antisense primer [5′-GGT CCT TGA AGC TTG AAT TTT-3′ (underlining shows *Hin*dIII site)] containing a unique *Hin*dIII site from nucleotides 631–651 of the ovine FP_A. This first product was resolved by agarose gel electrophoresis and purified using Geneclean (Bio101, Vista, CA). It was then used as the template in a second PCR with the same antisense primer as before and a new sense primer [5′-CC CTG CAG GCC ACC **ATG** AAG ACC ATC ATC GCC CTG AGC TAC ATC TTC TGC CTG GTG TTC GCC-3′ (underlining shows *Pst*I site; bold and underlining show the initiator methionine and start of the HA signal sequence)] that partially overlapped the previous sense primer and encoded the rest of the HA signal sequence, a Kozak consensus sequence, and a unique *Pst*I site. Both reactions were done using Deep Vent DNA polymerase (Boehringer Mannheim, Mannheim, Germany) with the following thermocycler conditions: after an initial denaturation for 8 min at 99°C, the samples were subjected to 40 cycles consisting of 1 min at 99°C, 1 min at 55°C, and 1 min at 75°C, followed by a final extension for 7 min at 75°C.

The product of the second PCR reaction was then used in a three-way ligation to yield the final FLAG-tagged FP_A and FP_B constructs as follows. The second PCR product was purified as before and was digested with *Hin*dIII and *Pst*I and repurified. The ovine FP_A in pBluescript KS(+) was digested with *Hin*dIII and *Eco*RI and the smaller fragment encoding the 3′ end of the FP_A was purified (nucleotides 639-1710; Pierce et al., 1997). A modified pBluescript KS(+) in which the *Hin*dIII site was previously removed was digested with *Pst*I and *Eco*RI and the 2.9-kilobase plasmid backbone was purified. These three fragments were then ligated together using a rapid ligation kit (Boehringer Mannheim) and were then used to transform DH5-α cells (Life Technologies-BRL). Plasmids encoding the FLAG-tagged FP_A (FP_A-FLAG/KS+) were identified by mini-prep and restriction enzyme analysis. Plasmids encoding the FLAG-tagged FP_B (FP_B-FLAG/KS+) were prepared in an identical manner using the product obtained from a *Hin*dIII/*Eco*RI digestion of the ovine FP_B in pBluescript KS(+) (nucleotides 639-1430; Pierce et al., 1997). The final constructs were verified by DNA sequencing.

HEK cells stably expressing the FLAG-tagged FP_A and FP_B isoforms were prepared as described above for the non–FLAG-tagged isoforms. Briefly, the FLAG-tagged FP_A and FP_B isoforms in pBluescript KS(+) were digested with *Not*I and *Xho*I and were subcloned sequentially into the corresponding sites in pCDNA3 and then pCEP4. The constructs in pCEP4 were used to transfect HEK cells and positive clones were selected by hygromycin resistance and limiting dilution. Expression of the FLAG epitope was verified by immunofluorescence microscopy using the FLAG-M2 monoclonal antibody. Functionality of the receptors was confirmed by radioligand binding and agonist stimulated IP formation.

Inositol Phosphates (IP) Assay. Receptor stimulated total IP accumulation was determined by anion exchange chromatography in cells that were preincubated with *myo*-[2-^{3}H]inositol (Anthony et al., 1998). Cells were plated in 10-cm dishes (10^7 cells/dish) in DMEM with 10% fetal bovine serum, and incubated overnight with 3 μCi/ml *myo*-[2-^{3}H]inositol. Cells were trypsinized and centrifuged, and aliquots of 0.5 to 1.0 $\times$ 10^7 cells were resuspended in 500 μl of DMEM containing 10 mM LiCl. After drug additions, the cells were incubated for 1 h at 37°C and 2.5 ml of chloroform/methanol/water (1:1:0.5) was added. Nine hundred microliters of the aqueous phase was removed and mixed with 2 ml of water and applied to a 2.5-ml column of AG1-X8 anion exchange resin. After three washes with 5 ml of water and two washes with 5 ml of 5 mM borax/60 mM sodium formate buffer, the ^{3}H-labeled IPs were eluted with 2 ml of 0.2 M ammonium formate/0.1 M formic acid and radioactivity was determined by liquid scintillation counting.

In Vivo Phosphorylation Assay. Ten-centimeter plates ($\sim$2.0 $\times$ 10^7 cells) of HEK cells stably expressing the FLAG-tagged FP_A and FP_B receptors were washed twice in phosphate-free DMEM and incubated with 5 ml of phosphate-free DMEM (without serum) containing 10 mM HEPES, pH 7.4, and 0.1% BSA for 16 h at 37°C. After aspirating the media, cells were incubated at 37°C for 1 h in 2 ml of phosphate-free DMEM containing 200 μCi of [^{32}P]orthophosphate. The cells were then incubated for 5 min at 37°C with either vehicle or drugs and washed twice with ice-cold PBS containing Ca^{2+} and Mg^{2+}. Cells were scraped with 1 ml of radioimmunoprecipitation assay (RIPA) buffer and were transferred to microfuge tubes. The RIPA buffer consisted 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 NaF, 10 mM disodium pyrophosphate, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 10 μg/ml of leupeptin, and 10 μg/ml of aprotinin. The samples were rotated for 2 h at 4°C and were centrifuged at 16,000*g* for 15 min. The equivalent of 2 mg

of protein from the supernatant was immunoprecipitated with 20 μl of FLAG-M2 antibody-affinity gel for 16 h at 4°C. The affinity gel was washed three times each with 1 ml of the RIPA buffer (without protease inhibitors) and was then mixed with 30 μl of Laemmli buffer. The samples were incubated at 65°C for 15 min and were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Gels were dried for 2 h at 80°C and autoradiographs were obtained after a 3- to 5-day exposure at −80°C.

In Vitro Phosphorylation Assay. Proteins were mixed in a reaction solution consisting of 20 mM HEPES, pH 7.4, 100 μM $CaCl_2$, 10 mM $MgCl_2$, 100 μM ATP, 100 μg/ml phosphatidylserine, 50 μg/ml 1-oleoyl-2-acetyl-*sn*-glycerol and 0.03% Triton X-100. Five microliters of recombinant PKCα (1 ng/μl) and 0.1 μl of [γ-^{32}P]ATP (10 mCi/ml) were then added to give a final volume of 100 μl and were incubated at 30°C for 10 min. The samples were precipitated with 20 μl of 1% BSA and 1 ml of 10% trichloroacetic acid. After a 5-min incubation on ice, the samples were centrifuged in a microfuge at maximal speed for 10 min, washed three times with acetone, and subjected to electrophoresis through a 10% SDS-polyacrylamide gel. Phosphorylated products were visualized by autoradiography using Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY).

Results

Inhibition of PKC Enhances $PGF_{2\alpha}$ Stimulated IP Formation in FP_A-Expressing Cells but Inhibits in FP_B-Expressing Cells. HEK cells stably expressing the ovine FP_A and FP_B prostanoid receptors were prepared as described under *Experimental Procedures* and used for the characterization of the signal transduction properties of these isoforms. Cell lines were selected that had comparable levels of expression based on the whole-cell radioligand binding of [^{3}H]$PGF_{2\alpha}$ (FP_A, 3.55 ± 0.28 pmol/mg protein; FP_B, 4.09 ± 0.49 pmol/mg protein). Agonist-stimulated IP accumulation in these cell lines was examined under control conditions or after inhibition of PKC by BIM. Figure 1, A and B, shows that $PGF_{2\alpha}$ stimulated IP accumulation to the same maximal level and with a similar EC_{50} value in both the FP_A- and FP_B-expressing cells (12 nM and 19 nM for the FP_A and FP_B, respectively). After a 5-min pretreatment with BIM, however, the EC_{50} value for $PGF_{2\alpha}$-stimulated IP accumulation in FP_A-expressing cells (Fig. 1A) was shifted to the left (EC_{50}, 6.9 nM). In contrast, the EC_{50} value in FP_B-expressing cells (Fig. 1B) was shifted to the right (EC_{50}, 27 nM) and there was an approximately 20% decrease in maximal IP accumulation. Although this leftward shift of the EC_{50} values for $PGF_{2\alpha}$-stimulated IP accumulation in BIM-treated FP_A cells was small, it was maximal at physiological concentrations of $PGF_{2\alpha}$ (e.g., 1–10 nM) and it was very reproducible (Figs. 1–3). Likewise, the inhibition observed in BIM-treated FP_B cells was very consistent.

The differential effect of PKC inhibition on the FP_A and FP_B receptor isoforms was further characterized by stimulation of IP accumulation with a fixed concentration of $PGF_{2\alpha}$ (10 nM) after pretreatment of the cells with several concentrations of BIM. Figure 2 again shows that IP accumulation was enhanced in FP_A cells treated with 1 μM and 10 μM BIM, whereas in FP_B cells, BIM either had no effect or inhibited $PGF_{2\alpha}$-stimulated IP accumulation. This series of experiments was repeated with another inhibitor of PKC (Go 6976) with virtually identical results (data not shown).

Activation of PKC Inhibits $PGF_{2\alpha}$-Stimulated IP Formation in Both FP_A- and FP_B-Expressing Cells. Because inhibition of PKC enhanced $PGF_{2\alpha}$-stimulated IP formation in FP_A-expressing cells but not in FP_B-expressing cells, we hypothesized that the additional carboxyl terminal sequence present in the FP_A isoform contained potential inhibitory PKC phosphorylation sites. To test this, PKC was activated with several concentrations of TMX and $PGF_{2\alpha}$-mediated IP accumulation was examined in the presence and absence of BIM. Figure 3A shows that in cells expressing the FP_A isoform, TMX produced a dose-dependent inhibition of $PGF_{2\alpha}$-stimulated IP accumulation that could be reversed by simultaneous pretreatment with BIM. Unexpectedly, pretreatment with TMX also inhibited $PGF_{2\alpha}$-stimulated IP accumulation in FP_B-expressing cells (Fig. 3B), which suggests that activation of PKC may inhibit agonist-stimulated IP formation by both FP receptor isoforms. Figure 3C, however, shows that in control (untransfected) HEK cells, TMX produced a clear dose-dependent inhibition of basal IP formation that could be reversed by simultaneous pretreatment with BIM. This strongly suggests that activation of PKC by TMX inhibits phospholipase C (PLC) activity and that the effects of PKC activation on the FP_A and FP_B isoforms occurs through inhibition of PLC rather than by a direct effect on the receptors. Nearly identical results were obtained in similar experiments using phorbol 12-myristate 13-acetate (PMA) (data not shown).

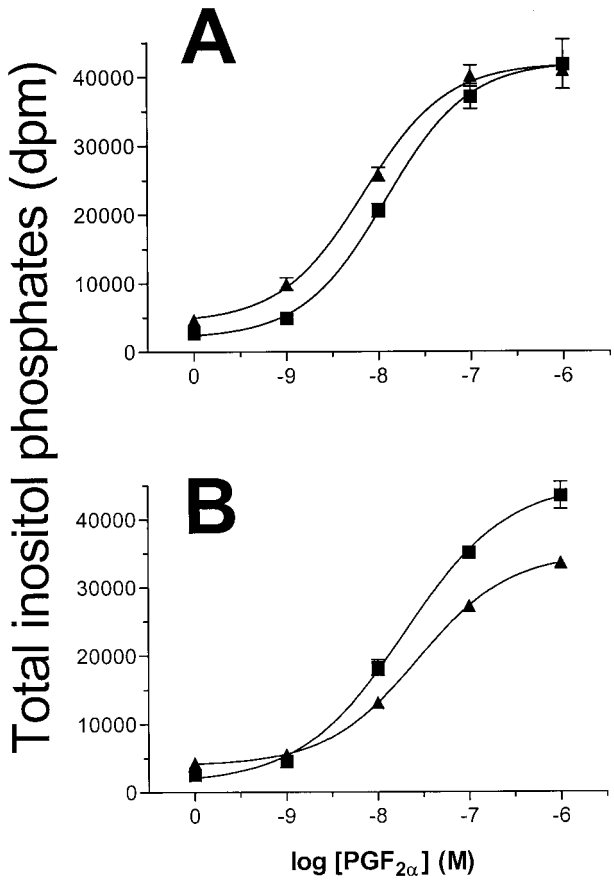

Fig. 1. Effects of BIM on $PGF_{2\alpha}$-stimulated total IP formation in cell lines stably expressing the FP_A or FP_B prostanoid receptor isoforms. HEK 293 cells stably expressing the FP_A receptor (A) or FP_B receptor (B) were pretreated with either vehicle (■) or 10 μM BIM (▲) for 5 min before treatment with the indicated concentrations of $PGF_{2\alpha}$ for 60 min. Total [^{3}H]inositol phosphates were determined as described under *Experimental Procedures*. Data are the means ± S. D. of one of three independent experiments each performed in duplicate.

In Vivo Phosphorylation of the FP_A and FP_B Isoforms. Cell lines stably expressing recombinant FP_A and FP_B receptor isoforms containing FLAG epitopes were prepared as described under *Experimental Procedures* to directly examine agonist-dependent phosphorylation of these receptors. Cell lines were selected that had comparable levels of expression, both with each other and with the wild-type FP_A- and FP_B-expressing cells, based on agonist stimulated IP formation and on the radioligand binding (e.g., FP_A, 3.56 ± 0.04 pmol/mg protein; FP_B, 2.15 ± 0.17 pmol/mg protein). Phosphorylation of the receptors was examined in whole cells that had been radiolabeled with [^{32}P]orthophosphate followed by immunoprecipitation of the receptors with an anti-FLAG antibody as described under *Experimental Procedures*. Figure 4 shows the effects of increasing concentrations of $PGF_{2\alpha}$ on the incorporation of ^{32}P into the FLAG-tagged FP_A and FP_B receptor isoforms. Although the FP_A isoform clearly showed a dose-dependent increase in phosphorylation that appeared maximal at 1 μM $PGF_{2\alpha}$, agonist-dependent phosphorylation of the FP_B was nearly absent. To examine the possibility that this phosphorylation was PKC-dependent, cells were pretreated with 10 μM BIM and then stimulated with 1 μM $PGF_{2\alpha}$. Figure 5 again shows the strong agonist-dependent phosphorylation of the FP_A isoform compared with the FP_B and it shows that pretreatment with BIM significantly decreased phosphorylation of the FP_A in response to stimulation with $PGF_{2\alpha}$. For the FP_B isoform, it seems that there may be a low level of agonist-dependent phosphorylation that is decreased to background levels by pretreatment with BIM.

In Vitro Phosphorylation of a GST/FP_A Carboxyl Terminal Fusion Protein. To examine the potential of the carboxyl terminus of the FP_A to serve as a substrate for PKC, an in vitro phosphorylation experiment was performed using recombinant PKCα and a GST fusion protein containing the 46 amino acids present in the carboxyl terminus of the FP_A that are absent in the FP_B. Figure 6 shows that after a 10-min incubation at 30°C, PKCα directly phosphorylated the GST/FP_A carboxyl terminal fusion protein (Fig. 6, lane 2) but not GST itself (Fig. 6, lane 3). PKCα also phosphorylated histone (Fig. 6, lane 4), which served as a positive control.

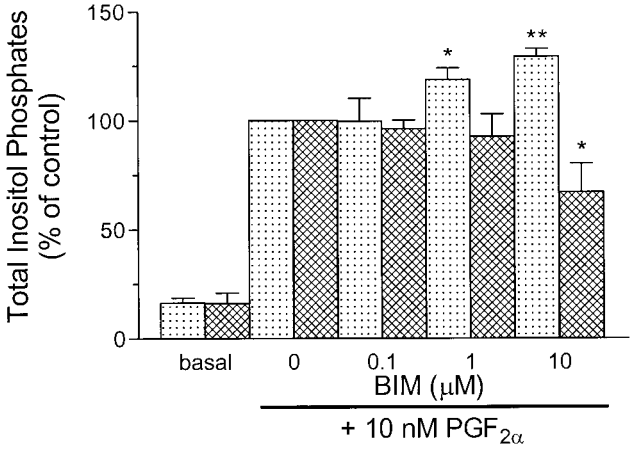

Fig. 2. Effects of increasing concentrations of BIM on total IP formation induced by 10 nM $PGF_{2\alpha}$ in cells stably expressing the FP_A (▨) or the FP_B (▩) prostanoid receptor isoforms. Cells were pretreated with the indicated concentrations of BIM for 5 min before treatment with vehicle (basal) or 10 nM $PGF_{2\alpha}$ for 60 min. Total ^{3}H-labeled IPs were determined as described under *Experimental Procedures*. Data are the means ± S.D. of three independent experiments each performed in duplicate. The asterisks indicate significant differences from 0 μM BIM (ANOVA; * $p < .05$; ** $p < .01$).

Discussion

Previously, we reported the cloning of a novel alternative mRNA splice variant of the ovine FP prostanoid receptor that we defined as FP_B (Pierce et al., 1997). This FP_B isoform differed from the previously cloned isoform (defined as FP_A) in that it lacked the carboxyl terminal 46 amino acids of FP_A;

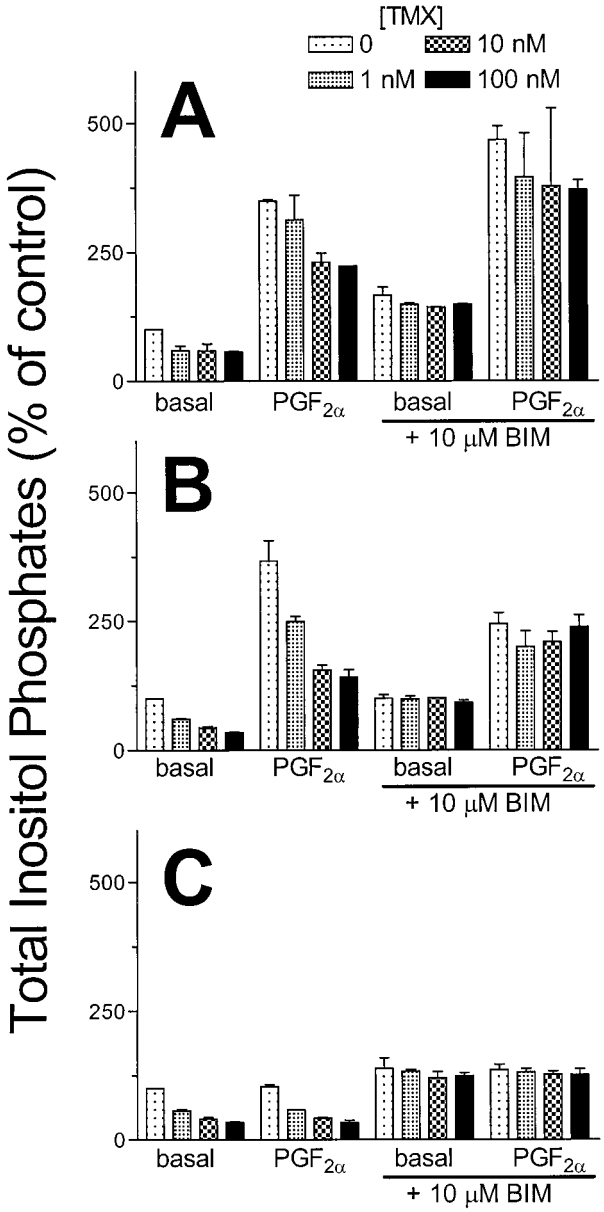

Fig. 3. Effects of increasing concentrations of TMX in the presence and absence of 10 μM BIM on $PGF_{2\alpha}$-stimulated IP formation in cells stably expressing the FP_A and FP_B prostanoid receptor isoforms (A and B, respectively) or in control (untransfected) HEK cells (C). Where indicated, cells were pretreated with 10 μM BIM for 5 min, followed by pretreatment with the indicated concentrations of TMX for 20 min, and were then stimulated with either vehicle alone (basal) or 10 nM $PGF_{2\alpha}$ for 60 min. Total ^{3}H-labeled IPs were determined as described under *Experimental Procedures*. Data are the means ± S.D. of one of three independent experiments each performed in duplicate.

therefore, it represented essentially a truncated form of FP_A. Heretofore, functional differences between these isoforms were unknown, but we now report that FP_A is preferentially phosphorylated by PKC and, in contrast to the FP_B isoform, which is unaffected, FP_A is subject to a rapid negative feedback by PKC.

Cell lines stably expressing the ovine FP_A and FP_B receptors were prepared to study possible functional differences between these isoforms. Two cell lines were selected that had comparable levels of expression as judged by radioligand binding and by $PGF_{2\alpha}$-stimulated IP accumulation. Both of the receptor isoforms shared a similar level of maximal IP formation and similar EC_{50} values for $PGF_{2\alpha}$. Interestingly, when these cells were treated with the PKC inhibitors BIM or Go 6976, there was a shift to the left in the dose-response curve for $PGF_{2\alpha}$-timulated IP formation in cells expressing the FP_A isoform but not in cells expressing the FP_B isoform. A possible explanation for this differential sensitivity is the presence of potential PKC phosphorylation sites in the 46 amino acids that are unique to the FP_A isoform. Analysis of this domain shows four consensus sites for PKC phosphorylation at residues 319 (T), 337 (S), 341 (S),and 353 (T). Our findings with whole cells showing PKC-dependent phosphorylation of the FP_A isoform, combined with the results showing in vitro phosphorylation of a carboxyl terminal FP_A fusion protein by recombinant PKCα, strongly suggest that one or more of these sites are phosphorylated in vivo.

Given that inhibition of PKC enhanced $PGF_{2\alpha}$-stimulated IP accumulation in FP_A-expressing cells, we speculated that activation of PKC would selectively inhibit $PGF_{2\alpha}$-stimulated IP formation in FP_A-expressing cells but not in FP_B-expressing cells. Contrary to expectations, however, pretreating cells with either TMX or PMA inhibited IP accumulation in both FP_A- and FP_B-expressing cells. There are a couple of possible explanations for this. One is that there are additional PKC phosphorylation sites in the common region of these receptors; a second is that stimulation of PKC down-regulates another component of the IP second-messenger pathway. As it concerns the first possibility, there are two additional PKC consensus sites in the second intracellular loop of these FP receptor isoforms. It is possible that phosphorylation of these common sites by a strong stimulus, such as TMX or PMA, could inhibit the activity of these receptors. The possibility of PKC-mediated down-regulation of another component of the IP pathway is more likely, however, as suggested by the results obtained in untransfected HEK cells. In these cells, TMX pretreatment caused a clear dose-dependent decrease in basal IP accumulation that was reversed by simultaneous pretreatment with BIM. This is essentially the same pattern observed in the FP_A- and FP_B-transfected cells, which implies that another component of the signaling pathway, such as G_q or PLC is inhibited. In fact, previous studies have shown that PLCβ1 is phosphorylated by PKC (Ryu et al., 1990) and that phosphorylation of PLCβ3 by PKC inhibits platelet-activating-factor–stimulated IP accumulation (Ali et al., 1997).

The involvement of cytoplasmic carboxyl terminal domains in the regulation of GPCR signaling and desensitization is well established. It seems that several members of the prostanoid receptor family have exploited this in the form of alternative mRNA splicing to create receptor isoforms that have common amino terminal and transmembrane domains, but divergent carboxyl termini. Thus, thirteen carboxyl terminal isoforms of the EP_3 receptor may exist, as well as two isoforms of the TP receptors and two of the FP receptors

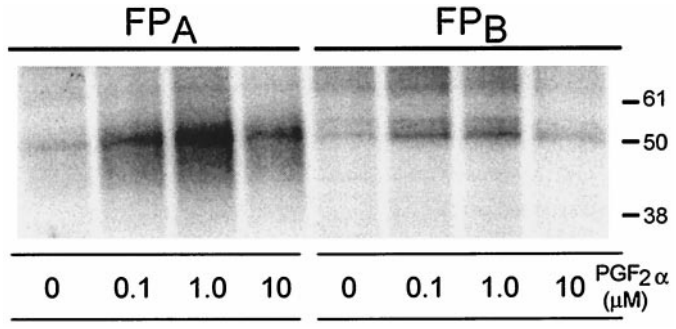

Fig. 4. $PGF_{2\alpha}$-induced phosphorylation of FLAG-tagged FP_A and FP_B prostanoid receptors. Cells stably expressing the FP_A and FP_B isoforms were prelabeled with ^{32}P and were stimulated with the indicated concentrations of $PGF_{2\alpha}$ for 5 min at 37°C. This was followed by immunoprecipitation with an anti-FLAG affinity gel, SDS-PAGE, and autoradiography as described under *Experimental Procedures*. Positions of molecular mass markers are indicated on the right in kiloDaltons.

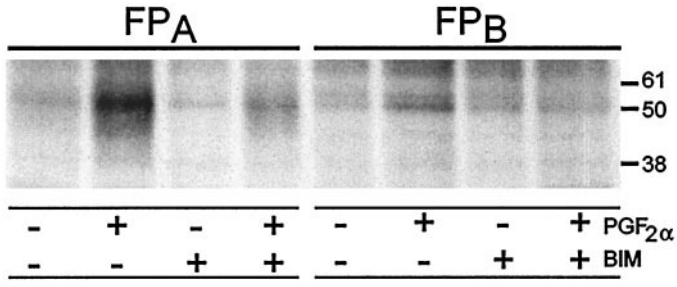

Fig. 5. The effects of BIM on $PGF_{2\alpha}$-induced phosphorylation of FLAG-tagged FP_A and FP_B prostanoid receptors. Cells stably expressing the FP_A and FP_B isoforms were prelabeled with ^{32}P and were either not stimulated (−) or were stimulated (+) with 1 μM $PGF_{2\alpha}$ either in the absence (−) or presence (+) of 10 μM BIM. This was followed by immunoprecipitation with an anti-FLAG affinity gel, SDS-PAGE, and autoradiography as described under *Experimental Procedures*. The positions of three of the molecular mass markers are indicated on the right in kiloDaltons. The results are representative of three independent experiments.

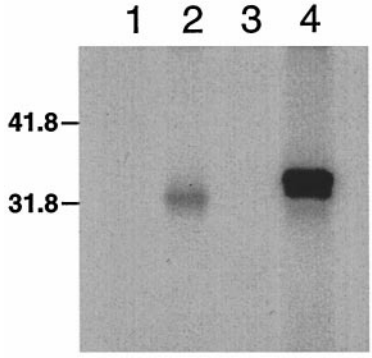

Fig. 6. In vitro phosphorylation of a GST/FP_A-carboxyl terminal fusion protein by recombinant PKCα. Phosphorylation reactions were conducted with [γ-^{32}P]ATP as described under *Experimental Procedures* using recombinant PKCα alone (lane 1), recombinant PKCα plus a GST/FP_A carboxyl terminal fusion protein (lane 2), GST (lane 3), or histone (lane 4). This is the autoradiograph obtained after SDS-PAGE. The positions of two of the molecular mass markers are indicated on the left in kiloDaltons. The predicted Mr of the GST/FP_A carboxyl terminal fusion protein and GST are 31.6 and 26.5, respectively. The results are representative of three independent experiments.

(Pierce and Regan, 1998). In all of these isoforms, the alternative splicing occurs at a conserved splice site that is approximately nine amino acids from the end of the seventh transmembrane domain. In the case of the EP_3 receptor isoforms, the divergent carboxyl termini give rise to differences in G protein coupling and desensitization. For example, studies with the mouse $EP_{3\alpha}$ and $EP_{3\beta}$ isoforms show that the $EP_{3\alpha}$ isoform undergoes rapid desensitization, whereas the $EP_{3\beta}$ isoform does not (Negishi et al., 1993). In the case of the TP receptor isoforms, the divergent carboxyl termini have been shown to affect coupling to adenylyl cyclase (Hirata et al., 1996), and recently it has been found that the TP_β isoform, which has a longer carboxyl terminus, undergoes agonist-promoted internalization, whereas the TP_α isoform does not (Parent et al., 1999).

The present findings with the FP receptor isoforms indicate that the unique carboxyl terminal domain of the FP_A provides for a rapid negative feedback by PKC that most probably involves phosphorylation of its carboxyl terminus by PKC. This essentially represents a rapid desensitization that makes both isoforms equally responsive to $PGF_{2\alpha}$ under the conditions of the present IP assay or after heterologous activation of PKC, for example by TMX or PMA. One could imagine, however, that under conditions of acute activation or in situations in which PKC becomes down-regulated, the FP_A isoform might be more responsive than the FP_B isoform to submaximally stimulating concentrations of agonist.

Recently, it has been shown that agonist-induced desensitization of both the TP receptor (Spurney, 1998) and the IP receptor (Smyth et al., 1998) seems to involve phosphorylation by PKC in the carboxyl terminal domains of these receptors. Thus IP receptors in which the PKC consensus sites were mutated showed impaired desensitization, and a carboxyl terminal deletion mutant did not desensitize at all. Likewise, mutation of carboxyl terminal PKC sites in the TP receptor impaired desensitization, and inhibitors of PKC could block phosphorylation of the receptor. This is consistent with the present findings and suggests that PKC-dependent phosphorylation of the carboxyl terminus is an important mechanism for the regulation of prostanoid receptors.

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