Cellular Conditioning and Activation of β-Catenin Signaling by the FP_B Prostanoid Receptor*

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Prostaglandin F_{2α} (PGF_{2α}) is an important autacoid that regulates a variety of physiological processes such as inflammation, cardiac hypertrophy, intraocular pressure, and regression of corpus luteum. PGF_{2α} is synthesized from arachidonic acid by the cyclooxygenases and binds to FP prostanoid receptors. The FP receptor splice variants are designated FP_A and FP_B. We have recently documented another fundamental difference between these FP receptor isoforms (4). We have found that stimulation of FP_B-expressing cells with prostaglandin F_{2α} produces a marked activation of Tcf/β-catenin-mediated transcriptional activation, which is not observed in FP_A-expressing cells (3).

We have recently documented another fundamental difference between these FP receptor isoforms (4). We have found that the FP_A isoform undergoes a classic agonist-induced and clathrin-dependent internalization, whereas the FP_B isoform undergoes an agonist-independent constitutive internalization that does not involve clathrin. We now report a molecular mechanism that we believe links the constitutive agonist-independent internalization of the FP_A isoform with the selective activation of Tcf/β-catenin signaling by the FP_B isoform. A key observation is that agonist-induced Tcf/β-catenin transcriptional activation by the FP_B isoform was blocked by inhibition of Rho-mediated cellular shape change. Furthermore, we have found an association of the FP_A isoform, but not the FP_B isoform, with phosphatidylinositol 3-kinase (PI3K), which may explain its agonist-independent constitutive internalization. We hypothesize that constitutive internalization of the FP_A isoform involving PI3K conditions FP_A-expressing cells to subsequently agonist-induced Tcf/β-catenin signaling by increasing the cellular content of cytosolic β-catenin.

**Experimental Procedures**

**Immunoprecipitation and Western Blotting—HEK-293 cells stably expressing FP_A and FP_B prostanoid receptor isoforms (1), as well as FLAG-tagged FP_A and FP_B-expressing cell lines (5), were generated and cultured as described previously. Cells were pretreated with either vehicle (0.1% Me_{2}SO or water), or 100 nM wortmannin (Sigma) for 15
min, or 40 μg/ml C3-toxin for 48 h at 37 °C. Cells were then incubated at 37 °C with either vehicle (0.002% sodium carbonate) or 1 μg PGF2α (Amersham Biosciences) for 1 h and then washed and resuspended in 1 ml of vehicle (0.1% Me2SO) or water, 100 nm wortmannin for 15 min, or 40 μg/ml C3-toxin for 48 h at 37 °C. Cell viability was determined by filtration through Whatman GF/C glass filters using a luminometer as described previously (3) using 10 μg of protein per sample. Measurements were corrected for background activity by subtraction of the TOP flash values from the corresponding TOP flash values.

**RESULTS**

Interaction of PI3K with the FPβ Prostanoid Receptor—We have previously shown that PGF2α can activate Tcf/β-catenin signaling in FPβ-expressing cells but not in FPβα-expressing cells (3). This selective activation of Tcf/β-catenin signaling by the FPβ isoform was associated with an agonist-mediated increase in cytoplasmic β-catenin and a decrease in its phosphorylation status. Decreased phosphorylation stabilizes β-catenin levels by preventing its degradation and thereby promotes nuclear translocation and Tcf transcriptional activation. However, the molecular mechanisms leading to the decrease in β-catenin phosphorylation following stimulation of the FPβ receptor were unclear.

One possible mechanism leading to decreased phosphorylation of β-catenin would involve the sequential activation of PI3K and Akt resulting in the phosphorylation of glycogen synthase kinase-3 (GSK-3). Phosphorylation of GSK-3 inhibits its kinase activity and decreases β-catenin phosphorylation. The potential involvement of PI3K with FPβ-mediated signaling was, therefore, explored in HEK cells stably expressing FLAG-tagged FPβ and FPβ receptors that were treated with 1 μg PGF2α and then examined by a combination of immunoblotting with antibodies to p85 PI3K (Fig. 1, A and B) and meas-

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Cells were split and grown in six-well plates containing 22-mm round glass cover slips for 3–4 days. Cells were pretreated with either vehicle (0.1% Me2SO) or 100 nm wortmannin for 15 min at 37 °C followed by the addition of a 1:500 dilution of anti-FLAG M2 antibodies in vehicle (0.002% sodium carbonate) or 1:500 dilution of anti-FLAG M2 antibodies in 1 μg PGF2α, for 10 min at 37 °C. The cells were fixed, permeabilized, and labeled with fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibodies as previously described (4). The cells were then examined by scanning confocal microscopy as described previously (4).
FLAG-tagged FP_A and FP_B receptors were expressed in these cells were then lysed and immunoprecipitated (IP) with anti-FLAG M2 affinity gel, and immunoblotting (IB) was performed as described under “Experimental Procedures” using anti-PI3K p85 antibody (top). To verify the immunoprecipitation, the membranes were stripped and reprobed with anti-FLAG M2 antibody (bottom). The panels represent one of three independent experiments. B, immunoprecipitations were performed as in A following pretreatment of FP_A- and FP_B-expressing cells with vehicle or 100 nM wortmannin (wort) for 15 min followed by treatment with vehicle (v) or 1 μM PGF_2α (P) for 1 h. Shown is a representative immunoblot and histogram of the expression of PI3K p85 as assessed by the pooled densitometry data (means ± S.E.) from three independent experiments. C, cellular PI3K activity of FP_A- and FP_B-expressing cells that were pretreated with either vehicle or 100 nM wortmannin (wort) for 15 min followed by treatment with either vehicle (v) or 1 μM PGF_2α (P) for 1 h. Shown is a representative autoradiograph and a histogram of the pooled PI3K activity (means ± S.E.) from three independent experiments. Data are normalized to the vehicle-treated FP_B cells as 100%. *, p < 0.05, as compared with vehicle-treated FP_A-expressing cells. Orig, origin; PIP, phosphatidylinositol phosphate.

Fig. 1. Interaction of PI3K with the FP_B prostanoid receptor. A, FLAG-tagged FP_A- and FP_B-expressing cells were treated with either vehicle (v) or 1 μM PGF_2α (P) for the indicated times. The cells were then lysed and immunoprecipitated (IP) with anti-FLAG M2 affinity gel, and immunoblotting (IB) was performed as described under “Experimental Procedures” using anti-PI3K p85 antibody (top). To verify the immunoprecipitation, the membranes were stripped and reprobed with anti-FLAG M2 antibody (bottom). The panels represent one of three independent experiments. B, immunoprecipitations were performed as in A following pretreatment of FP_A- and FP_B-expressing cells with vehicle or 100 nM wortmannin (wort) for 1 h. Shown is a representative immunoblot and histogram of the expression of PI3K p85 as assessed by the pooled densitometry data (means ± S.E.) from three independent experiments. C, cellular PI3K activity of FP_A- and FP_B-expressing cells that were pretreated with either vehicle or 100 nM wortmannin (wort) for 15 min followed by treatment with either vehicle (v) or 1 μM PGF_2α (P) for 1 h. Shown is a representative autoradiograph and a histogram of the pooled PI3K activity (means ± S.E.) from three independent experiments. Data are normalized to the vehicle-treated FP_B cells as 100%. *, p < 0.05, as compared with vehicle-treated FP_A-expressing cells. Orig, origin; PIP, phosphatidylinositol phosphate.

Inhibition of PI3K Inhibits Agonist-independent Internalization of FP_B Receptors—It has been reported that the endocytosis of E-cadherin is regulated by Rac1 and that this may involve the activation of PI3K (7, 8). Furthermore, it appears that the endocytosis of E-cadherin involves a clathrin-independent mechanism (9). Recently we have found that the FP_B receptor isoform undergoes an agonist-independent constitutive inter-
nalization that is also clathrin-independent, whereas, the FP\textsubscript{A} isoform undergoes agonist-induced internalization that is clathrin-dependent (4). Given the association of PI3K with the FP\textsubscript{B} isoform (shown in Fig. 1) we were interested in the potential role of PI3K with respect to the agonist-independent constitutive internalization of the FP\textsubscript{B} isoform. For these experiments HEK cells stably expressing the FP\textsubscript{A} and FP\textsubscript{B} receptor isoforms were pretreated with either vehicle or 100 nM wortmannin followed by treatment with either vehicle or 1 \mu M PGF\textsubscript{2\alpha}. Cell lines expressing wild type FP receptors were selected as reported previously (5) that had comparable levels of expression based on agonist-stimulated IP formation and on the radioligand binding (e.g. FP\textsubscript{A} 3.55 \pm 0.28 pmol/mg of protein, FP\textsubscript{B} 4.09 \pm 0.49 pmol/mg of protein). Receptor desensitization and internalization were then assessed, respectively, by the whole cell binding of [\textsuperscript{3}H]PGF\textsubscript{2\alpha}, (Fig. 2A) and by immunofluorescence confocal microscopy with anti-FLAG M2 antibodies (Fig. 2B).

As shown in Fig. 2A, treatment with PGF\textsubscript{2\alpha}, resulted in a 50% decrease in [\textsuperscript{3}H]PGF\textsubscript{2\alpha} binding in both FP\textsubscript{A} and FP\textsubscript{B}-expressing cells (2). Interestingly pretreatment with wortmannin, alone, increased [\textsuperscript{3}H]PGF\textsubscript{2\alpha} binding in FP\textsubscript{B} cells, but not in FP\textsubscript{A} cells. This sensitization of [\textsuperscript{3}H]PGF\textsubscript{2\alpha} binding following wortmannin pretreatment, although slight, was statistically significant and was obtained consistently and repeatedly in FP\textsubscript{B} cells, but not in FP\textsubscript{A} cells. Fig. 2B shows the results of immunofluorescence microscopy of the FLAG-tagged FP\textsubscript{A} and FP\textsubscript{B} receptor isoforms using live cell labeling of cell surface receptors (4). A comparison of panels a and b shows that the FP\textsubscript{A} isoform is localized primarily on the cell surface membrane in vehicle-treated FP\textsubscript{A} cells and that following treatment with PGF\textsubscript{2\alpha}, the receptor undergoes extensive internalization. A comparison of panels c and d shows that pretreatment of FP\textsubscript{A} cells with wortmannin did not affect this pattern of receptor localization. In contrast, examination of panels e and f shows that the FP\textsubscript{B} isoform is localized both intracellularly and on the cell surface regardless of PGF\textsubscript{2\alpha} treatment. This, as we have previously reported, reflects constitutive agonist-independent internalization of the FP\textsubscript{B} isoform. Comparison of panels e and g further shows that pretreatment with wortmannin reduced the intracellular localization of the FP\textsubscript{B} isoform in vehicle-treated FP\textsubscript{B}-expressing cells; this suggests that inhibition of PI3K blocked the constitutive agonist-independent internalization of the FP\textsubscript{B} isoform. This is consistent with the sensitization of [\textsuperscript{3}H]PGF\textsubscript{2\alpha} binding observed above following pretreatment of the FP\textsubscript{B} cells with wortmannin.

Inhibition of PI3K Increases Membrane Association of E-Cadherin, \(\beta\)-Catenin, and PI3K—The interaction of E-cadherin with \(\beta\)-catenin is well established (10), and recent studies have shown additional interaction between E-cadherin and PI3K (8). Having established an association between PI3K and the FP\textsubscript{B} receptor (Fig. 1) and an effect of wortmannin on FP\textsubscript{B} receptor localization (Fig. 2), we sought to determine the effects of wortmannin on the localization of E-cadherin and \(\beta\)-catenin in FP\textsubscript{B}-expressing HEK cells. Fig. 3 shows representative immunobLOTS (A) and the pooled densitometric analyses (B) for the expression of membrane associated (particulate) E-cadherin and \(\beta\)-catenin and for the expression of cytosolic \(\beta\)-catenin following pretreatment of FP\textsubscript{A}- and FP\textsubscript{B}-expressing cells with 100 nM wortmannin for 15 min. As we have previously reported, the expression of \(\beta\)-catenin is higher in FP\textsubscript{B}-expressing cells as compared with FP\textsubscript{A}-expressing cells in both the particulate and cytosolic fractions (3). We now show that the expression of particulate E-cadherin is also higher in FP\textsubscript{B}-expressing cells. Fig. 3B shows that wortmannin pretreatment of vehicle-treated FP\textsubscript{B} cells slightly, but significantly, increased the expression of membrane-associated (particulate) E-cadherin and \(\beta\)-catenin, while simultaneously decreasing the expression of cytosolic \(\beta\)-catenin. These increases in membrane-associated E-cadherin and \(\beta\)-catenin are very similar to the sensitization of [\textsuperscript{3}H]PGF\textsubscript{2\alpha} binding to the FP\textsubscript{B} receptors observed in Fig. 2A.

**Fig. 2.** FP receptor isoform desensitization and internalization as assessed by whole cell binding of [\textsuperscript{3}H]PGF\textsubscript{2\alpha} (A) and immunofluorescence confocal microscopy (B). A. FP\textsubscript{A}- and FP\textsubscript{B}-expressing cells were pretreated with either vehicle or 100 nM wortmannin (wort) for 15 min followed by treatment with either vehicle or 1 \mu M PGF\textsubscript{2\alpha}. (P) for 1 h and were then washed and assayed for the binding of [\textsuperscript{3}H]PGF\textsubscript{2\alpha}, as described under “Experimental Procedures.” Data are normalized to the vehicle-treated FP\textsubscript{A}- and FP\textsubscript{B}-expressing cells as 100% and are the means \pm S.E. of four independent experiments each performed in duplicate. *, \(p<0.05\), as compared with vehicle-treated FP\textsubscript{B}-expressing cells. B. FLAG-tagged FP\textsubscript{A}- and FP\textsubscript{B}-expressing cells were pretreated with either vehicle or 100 nM wortmannin for 15 min followed by treatment with either vehicle or 1 \mu M PGF\textsubscript{2\alpha} for 10 min concurrently with anti-FLAG M2 antibodies. Shown are representative photomicrographs obtained from one of three independent experiments. Scale bars represent 10 \mu m.
following pretreatment with the PI3K inhibitor, wortmannin. As noted above, PI3K has recently been found to bind E-cadherin (8). The immunoblot shown in Fig. 3C confirms this interaction by showing co-immunoprecipitation of the p85 subunit of PI3K with E-cadherin in both FPA- and FPB-expressing HEK cells. This figure also shows that PGF2α treatment of FPB-expressing cells increased the co-immunoprecipitation of the p85 subunit with E-cadherin suggesting that PGF2α treatment, which we have shown in Fig. 1C to inhibit PI3K activity, increases its association with E-cadherin. This conclusion is corroborated by the effects of the wortmannin pretreatment of vehicle-treated FPB cells, which also increased the co-immunoprecipitation of the p85 subunit with E-cadherin. Notably, such corresponding changes were not observed in the FPA-expressing cells.

Activation of PI3K Is Not Sufficient to Explain PGF2α Stimulation of Tcf/β-Catenin Signaling by the FPB Receptor—One of the most pronounced effects of the treatment of FPA-expressing cells with PGF2α is a dramatic reorganization of the β-catenin as evidenced by immunofluorescence microscopy using antibodies to β-catenin (3). Fig. 4 shows this effect in which the localization of β-catenin is green and the nuclear staining of 4′,6-diamidino-2-phenylindole (DAPI) is blue. Thus, a comparison of panels e and f shows that treatment with PGF2α causes a marked increase in β-catenin immunofluorescence in regions of cell to cell contact. A comparison of panels a and b shows that a corresponding reorganization of β-catenin does not occur in FPA-expressing cells after treatment with PGF2α. Pretreatment with wortmannin was used to determine the possible influence of PI3K activity on this PGF2α-induced reorganization of β-catenin. Comparison of panels e and g shows that wortmannin pretreatment of vehicle-treated FPB cells produced a slight increase in β-catenin immunofluorescence in regions of cell to cell adhesion. However, as shown by comparing panels g and h, wortmannin pretreatment of FPB-expressing cells did not have any major effect on the PGF2α-induced reorganization of β-catenin.

Stabilization of β-catenin expression and the promotion of Tcf transcriptional activation involves the decreased phosphorylation of β-catenin and is directly influenced by the activity of GSK-3β. The activity of GSK-3β, in turn, is a function of its state of phosphorylation, and it is known that phosphorylation of GSK-3β at serine 9 inhibits its kinase activity (11). Immunoblotting of phospho-GSK-3β and phospho-β-catenin (Fig. 5A) and direct measurement of Tcf transcriptional activation (Fig. 5B) following pretreatment of FPB-expressing cells with wort-
null localization in FPA and FPB cells. Effect on the phosphorylation of GSK-3β/H9252 partially unaffected in FPA-expressing cells as shown previously. As one might expect, there were corresponding increases in the phosphorylation of cytosolic β-catenin (panel c). On the other hand, in FPA-expressing cells treatment with PGF2α had no apparent effect on the phosphorylation of GSK-3β (panel a), and there was a marked increase in the phosphorylation of cytosolic β-catenin (panel c). It would be expected, therefore, that followed treatment with PGF2α, the degradation of β-catenin would be favored in FPA-expressing cells, whereas, the stabilization of β-catenin and potential activation of transcription would be favored in FPB-expressing cells. In fact, as shown in Fig. 5B, this is what was found when transcriptional activation was measured with the use of the Tcf-responsive luciferase reporter gene. Thus, luciferase activity was stimulated 3-fold in PGF2α-treated FPB-expressing cells, whereas it was essentially unaffected in FPA-expressing cells as shown previously (3).

Fig. 5 also shows that pretreatment with wortmannin reduced the basal levels of GSK-3β phosphorylation in both the vehicle-treated FPA- and FPB-expressing cells. As one might expect, there were corresponding increases in the phosphorylation of cytotic β-catenin in both the vehicle-treated FPA- and FPB-expressing cells that were pretreated with wortmannin. When these same cells were treated with PGF2α, there were slight increases in the phosphorylation of GSK-3β in both FPA- and FPB-expressing cells, and, correspondingly, slight decreases in the phosphorylation of β-catenin. Surprisingly, however, the Tcf-responsive reporter gene activity was only modestly inhibited (~17%) in wortmannin-pretreated FPB-expressing cells that were treated with PGF2α. This indicates that PGF2α-induced activation of Tcf/β-catenin signaling by the FPB isoform cannot be simply explained by direct activation of a PI3K/GSK-3β pathway.

Activation of Rho Is Required for PGF2α Stimulation of Tcf/β-catenin Signaling by the FPB Receptor—Because the activation of PI3K alone was not sufficient to explain the stimulation of Tcf/β-catenin signaling by the FPB receptor, we decided to examine the influence of Rho. We have previously established that activation of Rho by PGF2α in FPA- and FPB-expressing cells leads to morphological changes consisting of the loss of filopodia, cell rounding, and the formation of cell aggregates...
These morphological changes are reversible in FP A-expressing cells following the removal of PGF2\sub{α}/H9251, but, remarkably, they are not reversible in FP B-expressing cells (2). However, these morphological changes can be blocked in both FP A- and FP B-expressing cells by pretreatment with C3-toxin, a bacterial exoenzyme that ADP-ribosylates and inactivates Rho. In this series of experiments we used C3-toxin to examine its effects on PGF2\sub{α}-stimulated inositol phosphates formation (Fig. 6), cellular reorganization of β-catenin immunofluorescence (Fig. 7), phosphorylation of GSK-3β and β-catenin (Fig. 8A), and Tcf-responsive reporter gene activity (Fig. 8B) in HEK cells stably expressing the FP A and FP B receptor isoforms.

Fig. 6A shows phase-contrast microscopy of FP A- and FP B-expressing cells that were pretreated with either vehicle or 40 μg/ml C3-toxin for 48 h followed by treatment with either vehicle or 1 μM PGF2\sub{α}/H9251 for 1 h. As we have previously reported (1), pretreatment with C3-toxin completely blocked PGF2\sub{α}-induced shape change in both FP A-expressing cells (compare panels b and d) and in FP B-expressing cells (compare panels f and h). C3-toxin did not inhibit the PGF2\sub{α}-stimulated formation of inositol phosphates in either the FP A-expressing cells or the FP B-expressing cells. In fact, pretreatment with C3-toxin appears to have slightly enhanced the formation of inositol phosphates, indicating that the effects of C3-toxin on PGF2\sub{α}-induced shape changes were not a consequence of nonspecific cellular toxicity.

Fig. 7 shows the immunofluorescence localization of β-catenin (green) and the nuclear staining of DAPI (blue) in FP A- and FP B-expressing HEK cells that were pretreated as above with vehicle or 40 μg/ml C3-toxin for 48 h followed by treatment with either vehicle or 1 μM PGF2\sub{α}/H9251 for 1 h. As we have previously reported (1), pretreatment with C3-toxin completely blocked PGF2\sub{α}-induced shape change in both FP A-expressing cells (compare panels b and d) and in FP B-expressing cells (compare panels f and h). Fig. 6B further shows that pretreatment with C3-toxin did not inhibit the PGF2\sub{α}-stimulated formation of inositol phosphates in either the FP A-expressing cells or the FP B-expressing cells. In fact, pretreatment with C3-toxin appears to have slightly enhanced the formation of inositol phosphates, indicating that the effects of C3-toxin on PGF2\sub{α}-induced shape changes were not a consequence of nonspecific cellular toxicity.

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**Fig. 8.** Effects of C3-toxin on PGF<sub>2α</sub> stimulation of Tcfβ-catenin signaling by FP receptors. FP<sub>A</sub> and FP<sub>B</sub> expressing cells were pretreated with either vehicle or 40 μg/ml PGF<sub>2α</sub> (P) for 48 h followed by treatment with either vehicle (v) or 1 μM C3-toxin (P) for 1 h. A. Immunoblotting (IB) with antibodies to: phospho-GSK-3β (pGSK3); GSK-3β (GSK3); phospho-serine and phospho-threonine (pS/pT); and β-catenin (β-cat). Prior to immunoblotting for phospho-serine and phospho-threonine, samples were immunoprecipitated (IP) with antibodies to β-catenin. Shown is a representative immunoblot from one of three independent experiments. B. Tcf-responsive luciferase reporter gene activity obtained 16 h after the above treatment with C3-toxin and PGF<sub>2α</sub>. Cells were extensively washed as described under “Experimental Procedures” immediately following the 1-h treatment with vehicle (v) or PGF<sub>2α</sub> (P). Shown is a histogram of the pooled luciferase activity (means ± S.E.) from three independent experiments each performed in duplicate. Data are normalized to the vehicle-treated FP<sub>B</sub>-expressing cells as 100%. *, p < 0.05, as compared with vehicle-treated FP<sub>B</sub>-expressing cells.

**Fig. 9.** Two-step model of the mechanism of Tcfβ-catenin signaling in cells expressing FP<sub>B</sub> prostanoid receptors. Step 1 (Conditioning): In the absence of PGF<sub>2α</sub>, there is an association of PI3K with the FP<sub>B</sub> receptor that leads to activation of the kinase. This activation of PI3K leads to a constitutive (agonist-independent) internalization of the FP<sub>B</sub> receptor in a membrane complex containing E-cadherin and β-catenin. The escape of β-catenin from this complex increases the concentration of cytosolic β-catenin. Step 2 (Activation): In the presence of PGF<sub>2α</sub>, activation of the FP<sub>B</sub> receptor leads to activation of the protein kinase C and Rho signaling pathways. In addition, PI3K dissociates from the receptor leading to a decrease of kinase activity. The activation of the Rho signaling pathway leads to cell rounding and a dramatic increase of E-cadherin and β-catenin in the region of cell-cell adhesion. This morphological and molecular reorganization further increases the concentration of cytosolic β-catenin to a point that exceeds the capacity of the cell to degrade it, leading to Tcf transcriptional activation. This does not occur in FP<sub>A</sub>-expressing cells, because they lack the conditioning step and have a lower initial concentration of cytoplasmic β-catenin.

cells would be unaffected by pretreatment with C3-toxin. Fig. 8B, however, shows that C3-toxin pretreatment of FP<sub>B</sub>-expressing cells almost totally abolished PGF<sub>2α</sub>-stimulated Tcf-responsive reporter gene activity. These results clearly establish the importance of Rho activation, or possibly shape change itself, for the PGF<sub>2α</sub> stimulation of Tcfβ-catenin signaling in cells expressing the FP<sub>B</sub> receptor. It may be recalled that pretreatment with C3-toxin did not block PGF<sub>2α</sub>-stimulated inositol phosphates formation (Fig. 6B), therefore, these effects of C3-toxin on Tcfβ-catenin signaling are unlikely to represent nonspecific cellular toxicity.

**DISCUSSION**

In this report we have explored the molecular mechanisms that are involved in the selective activation of Tcfβ-catenin signaling by the FP<sub>B</sub> prostanoid receptor isoform. We have several major findings. The first is that the FP<sub>B</sub> receptor can interact with the p85 subunit of PI3K, and both the activation of PI3K and its interaction with the receptor are disrupted in the presence of PGF<sub>2α</sub>. The second finding is that inhibition of PI3K inhibits the constitutive, agonist-independent, internalization of the FP<sub>B</sub> receptor and this is associated with an accumulation of the FP<sub>B</sub> receptor on the cell surface membrane. Inhibition of PI3K activity is also accompanied by an increase in the membrane-associated expression of E-cadherin,
As illustrated in Fig. 9 these findings support the notion of a macromolecular complex involving the FP<sub>B</sub> receptor, PI3K, E-cadherin, and β-catenin. This complex undergoes constitutive internalization in the absence of PGF<sub>2α</sub> and can be blocked when PI3K activity is inhibited with wortmannin. Although we could not immunoprecipitate E-cadherin or β-catenin with FP<sub>B</sub> receptor (data not shown), this might be explained if the complex is not tightly associated and especially if PI3K functions as a scaffold between the receptor and E-cadherin.

Two other major findings are as follows. First, a PI3K pathway, alone, is not sufficient to explain the PGF<sub>2α</sub>-induced stimulation of Tcf transcriptional activation by the FP<sub>B</sub> receptor. The second is that activation of Rho is clearly required for FP<sub>B</sub> receptor-mediated Tcf transcriptional activation. There are several additional observations to note in terms of developing a molecular mechanism to explain the selective activation of Tcf/β-catenin signaling by the FP<sub>B</sub> receptor. One is that the expression of both particulate and cytosolic E-cadherin and β-catenin are significantly greater in FP<sub>B</sub>-expressing cells compared with FP<sub>A</sub>-expressing cells. We have previously observed that PGF<sub>2α</sub> stimulation of FP<sub>A</sub>-expressing cells, but not FP<sub>B</sub>-expressing cells, causes a major reorganization of β-catenin in which there is an increased accumulation of β-catenin along regions of cell to cell contact (3). In addition, PGF<sub>2α</sub> stimulation of the FP<sub>A</sub> receptor does not activate Tcf/β-catenin signaling, nor does it induce the reorganization of β-catenin, even though it activates Rho and induces cell rounding (1).

To explain these findings we propose a two-step mechanism in which FP<sub>A</sub>-expressing cells first undergo an agonist-independent conditioning step involving constitutive internalization of the FP<sub>B</sub> receptor followed by an agonist-dependent activation step leading to Tcf transcriptional activation. FP<sub>A</sub>-expressing cells fail to activate Tcf/β-catenin signaling, because they lack the initial conditioning step. This two-step mechanism is illustrated in more detail in Fig. 9. In support of the conditioning step we have previously shown agonist-independent constitutive internalization of the FP<sub>B</sub> receptor, but not the FP<sub>A</sub> receptor (4). Furthermore, this constitutive internalization of the FP<sub>B</sub> was found to be clathrin-independent. It is also well established that E-cadherin can associate with β-catenin, and recently it has been shown that the endocytosis of E-cadherin is clathrin-independent (9) and involves the activation of PI3K (7, 8). In this study we have found that the FP<sub>B</sub> receptor isoform, but not the FP<sub>A</sub>, can associate with PI3K. We hypothesize that this interaction results in the activation of PI3K and thereby promotes binding to E-cadherin, which leads to the internalization of this protein complex. In other words, the constitutive internalization of the FP<sub>B</sub> receptor is a PI3K/E-cadherin-dependent, but clathrin-independent process. Further consequences of this constitutive internalization is an increase in cytosolic β-catenin, which we have documented, as well as an increase in the basal level of agonist-independent Tcf transcriptional activation (3) (see also Figs. 5B and 8B). Therefore as compared with FP<sub>A</sub>-expressing cells, FP<sub>B</sub>-expressing cells have increased levels of cellular E-cadherin and β-catenin and are likely to have more active cycling of these proteins between the cell membrane and cytosolic compartments.

The activation step is initiated through the specific binding of PGF<sub>2α</sub> to the FP<sub>B</sub> receptor. The immediate consequence of this is stimulation of phosphatidylinositol and Rho signaling as well as dissociation of PI3K from the FP<sub>B</sub> receptor and a decrease in cellular PI3K activity. In both FP<sub>A</sub>- and FP<sub>B</sub>-expressing cells this is followed by cell rounding and aggregation. However, in FP<sub>B</sub> cells, but not FP<sub>A</sub> cells, there is a critical accumulation of E-cadherin and β-catenin at regions of cell to cell contact that occurs simultaneously with cell rounding and aggregation. Similarly in FP<sub>B</sub>-expressing cells, but not in FP<sub>A</sub>-expressing cells, there is stimulation of Tcf transcriptional activation. It is clear that there is an absolute requirement for stimulation of the FP<sub>B</sub> receptor. Thus, cell rounding and aggregation can be induced in FP<sub>B</sub>-expressing cells by treatment with lysophosphatidic acid, but these effects are reversible and do not activate Tcf/β-catenin signaling (2).

The increased levels of E-cadherin and β-catenin that occur during the conditioning step also appear to be absolute requirements for the agonist-induced activation of Tcf/β-catenin signaling in FP<sub>B</sub>-expressing cells. It would be expected that the loss of filopodia, cell rounding, and aggregation would dramatically decrease cellular surface area and essentially concentrate and further increase the functional consequences of the increased expression of E-cadherin and β-catenin. For reasons that we still do not fully appreciate, the cell rounding, aggregation, and reorganization of β-catenin become self-sustaining in FP<sub>B</sub> receptor-expressing cells. Our hypothesis is that the capacity of GSK-3β to phosphorylate β-catenin is overwhelmed by the combination of the increased expression of β-catenin (conditioning) and by the agonist-induced shape change and reorganization of β-catenin (activation). Although the activation step also results in the increased phosphorylation and inhibition of GSK-3β, the effects of C3-toxin (Fig. 8) show that agonist-induced Tcf transcriptional activation is actually independent of GSK-3β activity and can be mediated solely through a Rho-dependent pathway. This sequence of conditioning and activation increases the concentration of E-cadherin and β-catenin in regions of cell-cell adhesion and maintains the aggregated phenotype by generating free cytosolic β-catenin at a rate that exceeds the capacity of the cell to degrade it. The resulting increase in Tcf transcriptional activation could then initiate a positive feedback loop; for example, by inducing further expression of E-cadherin or some other adhesion molecule.

Our present findings and conclusions regarding the mechanism of FP<sub>B</sub> prostanoid receptor signaling have general significance with regards to GPCR signaling. Most importantly it clearly illustrates the potential of a receptor to alter the cellular signaling environment by virtue of its constitutive activity. The fact that receptors may have agonist-independent constitutive activity has been known for some time, and its importance has been recognized, as, for example, in mutations of the luteinizing hormone receptor that are responsible for precocious puberty (12). In this example, however, constitutive agonist-independent activity represents the same activity as that obtained with agonist, which is also the case for virtually all previous descriptions of constitutive activity. The agonist-independent conditioning of FP<sub>B</sub>-expressing cells that we have described differs importantly in this regard. Thus, it represents an independent signaling pathway, i.e., coupling to PI3K, that is actually disrupted by treatment with agonist. Additionally this constitutive activity profoundly alters the signaling potential of the agonist itself. Thus, PGF<sub>2α</sub> treatment activates Rho and induces cellular shape change in both FP<sub>A</sub>- and FP<sub>B</sub>-expressing cells, but it only activates Tcf/β-catenin signaling in FP<sub>B</sub>-expressing cells. We believe it is likely that other GPCRs will have similar agonist-independent constitutive activities. Furthermore, such activities may be best revealed through the application of gene microarray technology and other approaches that do not rely on conventional assumptions about GPCR signaling.

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