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Delayed Reversal of Shape Change in Cells Expressing FP_B Prostanoid Receptors

POSSIBLE ROLE OF RECEPTOR RESENSITIZATION*

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Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptors are G-protein-coupled receptors consisting of two alternative mRNA splice variants, named FP_A and FP_B. As compared with the FP_A isoform, the FP_B isoform lacks the last 46 amino acids of the carboxyl terminus and, therefore, represents a truncated version of the FP_A. We recently found (Pierce, K. L., Fujino, H., Srinivasan, D., and Regan, J. W. (1999) *J. Biol. Chem.* 274, 35944–35949) that stimulation of both isoforms with $PGF_{2\alpha}$ leads to activation of a Rho signaling pathway, resulting in tyrosine phosphorylation of p125 focal adhesion kinase, formation of actin stress fibers, and cell rounding. Although the activation of Rho and subsequent cell rounding occur at a similar rate for both isoforms, we now report that following the removal of $PGF_{2\alpha}$ the reversal of cell rounding is much slower for cells expressing the FP_B isoform as compared with the FP_A isoform. Thus, in HEK-293 cells that stably express the FP_A isoform, the reversal of cell rounding appears to be complete after 1 h, whereas for FP_B-expressing cells there is essentially no reversal even after 2 h. Similarly, the disappearance of stress fibers and dephosphorylation of p125 focal adhesion kinase following removal of agonist are much slower in FP_B-expressing cells than in FP_A-expressing cells. The mechanism of this differential reversal appears to involve a difference in receptor resensitization following the removal of agonist. Based upon whole cell radioligand binding, agonist-induced stimulation of inositol phosphate formation, and mobilization of intracellular Ca^{2+}, the FP_B isoform resensitizes more slowly than the FP_A isoform. These findings suggest that the carboxyl terminus of the FP_A is critical for resensitization and that the slower resensitization of the FP_B isoform leads to prolonged signaling. This differential signaling distinguishes the FP_A and FP_B receptor isoforms and could be important toward understanding the physiological actions of $PGF_{2\alpha}$.

FP prostanoid receptors are G-protein-coupled receptors that bind endogenous prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$),[1] resulting in the stimulation of phosphoinositide turnover, mobilization of intracellular calcium, and activation of protein kinase C (PKC) via G_q. There are two known alternative mRNA splice variants of the sheep FP prostanoid receptors that have been defined as the FP_A and FP_B (1). These FP prostanoid receptor isoforms have common amino termini and seven-transmembrane-spanning domains but differ in their carboxyl termini in the same manner as the EP_3 and thromboxane A_2 prostanoid receptor alternative mRNA splice variants (2–5). Thus, both the FP_A and FP_B isoforms share the same nine initial amino acids of their carboxyl termini after which the FP_A continues for another 46 amino acids and the FP_B just one. Except for this one amino acid, therefore, the FP_B isoform is a truncated version of the FP_A isoform.

Initial studies of these isoforms indicated that both receptors had similar ligand binding profiles and similar stimulation of agonist-induced phosphoinositide turnover (1). Recently, however, we have found that the FP_A isoform, but not the FP_B, is subject to a rapid negative feedback regulation by PKC (6). This negative feedback by PKC probably involves phosphorylation at one or more of three PKC phosphorylation consensus sites that are present in the 46 amino acids that are unique to the carboxyl terminus of the FP_A. Indeed, whole cell phosphorylation showed that the FP_A was phosphorylated in an agonist-dependent fashion, whereas the FP_B was not.

Besides coupling to G_q and the phosphoinositides signaling pathway, we have also found that the FP_A and FP_B prostanoid receptor isoforms activate Rho, a member of the Ras family of small G-proteins, to cause cell rounding, actin stress fiber formation, and tyrosine phosphorylation of p125 focal adhesion kinase (FAK) (7). This activation of Rho and subsequent shape change is independent of G_q and probably occurs via activation of G_{12} and/or G_{13}. This is consistent with the findings for other G_q/G_{11}-coupled receptors, such as those for thrombin, thromboxane A_2, and lysophosphatidic acid (LPA) in which the agonist-induced formation of actin stress fibers has been shown to involve either G_{12} or G_{13} (8). While no significant differences between the FP_A and FP_B receptor isoforms were found with respect to their activation of Rho and cell rounding, we have now found marked differences between these isoforms with respect to the reversal of cell rounding following removal of agonist. Thus, reversal of cell rounding, loss of stress fibers,

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[1] The abbreviations used are: $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; FAK, focal adhesion kinase; PKC, Ca^{2+}-dependent protein kinase; LPA, lysophosphatidic acid; TRITC, Texas Red isothiocyanate; MES, 2-(*N*-morpholino)ethanesulfonic acid.

and dephosphorylation of p125 FAK is much slower for cells expressing the FP_B prostanoid receptor isoform than for the FP_A. Based on the results of radioligand binding, agonist-stimulated inositol phosphate accumulation, and mobilization of intracellular calcium, it appears that this slower reversal is related to slower resensitization of the FP_B isoform following agonist-induced desensitization. Therefore, while both isoforms initially desensitize to a similar extent following treatment with $PGF_{2\alpha}$, the FP_B resensitizes more slowly than the FP_A. We hypothesize that the slower resensitization of the FP_B isoform is associated with prolonged signaling following the removal of agonist.

EXPERIMENTAL PROCEDURES

Cell Culture and Imaging—HEK-293 cells stably expressing the FP_A and FP_B prostanoid receptor isoforms were generated and cultured as described previously (6, 7). These two cell lines have comparable levels of receptor expression judged both by radioligand binding (FP_A, 3.55 ± 0.28 pmol/mg protein; FP_B, 4.09 ± 0.49 pmol/mg protein) and by $PGF_{2\alpha}$-stimulated inositol phosphate formation. Cells were plated in six-well tissue culture dishes (Falcon) under half-confluent conditions (~35,000 cells/well) and were grown without any changes of media for 3–4 days in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum, 250 μg/ml Geneticin, 200 μg/ml hygromycin B, and 100 μg/ml gentamicin. To examine agonist-induced cell rounding and its reversal, either vehicle (sodium carbonate, 0.002% final concentration) or agonist ($PGF_{2\alpha}$, 1 μM final concentration) was added to the media, and the cells were incubated at 37 °C for 60 min. The cells were then rapidly washed three times each with 1 ml of Opti-MEM (Life Technologies) at 37 °C and were placed either in 1 ml of Opti-MEM (to examine reversal) or in 1 ml of Opti-MEM containing $PGF_{2\alpha}$ (to examine continuous agonist treatment). One hour later, the cells were visualized by phase-contrast microscopy using an Olympus IX70 microscope. Images were obtained using a Cohu 4915 CCD video camera and processed using IPLab Spectrum (Signal Analytics).

Phalloidin Staining of Actin Stress Fibers—Cells were split and grown as above for 3–4 days in six-well dishes containing 22-mm round glass coverslips. Cells were treated with either vehicle or 1 μM $PGF_{2\alpha}$ for 60 min and were then rapidly washed three times each with 1 ml of Opti-MEM at 37 °C. Following the last wash, 1 ml of Opti-MEM or 1 ml of Opti-MEM containing 1 μM $PGF_{2\alpha}$ was added to the cells. One hour later, the cells were fixed and were stained with TRITC-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR) as described previously (7). Cells were visualized by epifluorescence microscopy using a × 60 oil objective and optical band pass filters for Texas Red. Images were acquired as above.

Immunoprecipitation and Western Blotting—Cells were split and cultured as above in six-well plates. Following vehicle or drug treatments, the cells were washed and lysed, and 300 μg of protein was used for immunoprecipitation (1 μg/μl) as described previously (7) using 3 μl (0.6 μg) of rabbit polyclonal anti-FAK antibody (C-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Lysates were rotated for 15 h at 4 °C, and 10 μl of a 1:1 slurry of protein A-agarose (Sigma) was added followed by rotation for an additional 1 h at 4 °C. The beads were washed three times, Laemmli sample buffer was added, and the samples were boiled and electrophoresed on 7.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose, and the blots were incubated with a 1:1000 dilution of a monoclonal anti-phosphotyrosine antibody (PY-20, Transduction Laboratories) for 1 h at room temperature with rotation. The blots were washed three times and incubated for 1 h at room temperature with a 1:5000 dilution of a goat anti-mouse secondary antibody (Sigma) conjugated with horseradish peroxidase. After washing three times, immunoreactivity was detected using SuperSignal enhanced chemiluminescence (Pierce). To ensure equal loading of proteins, the blots were stripped and reprobed as before with a 1:1000 dilution of anti-FAK antibody (C-20) and a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma).

Whole Cell Radioligand Binding Assay—Cells were cultured in 10-cm plates and were trypsinized, centrifuged at 500 × *g* for 2 min, and resuspended in Opti-MEM at a concentration of 10^7 cells/ml. One-milliliter aliquots of cells were incubated for 60 min at 37 °C with either vehicle or 1 μM $PGF_{2\alpha}$, and the initial levels of receptor binding activity were assessed, as described below, by the radioligand binding of [^{3}H]$PGF_{2\alpha}$ (Amersham Pharmacia Biotech). To determine the recovery of binding activity, cells were subjected to the same initial 1-h incubation with either vehicle or $PGF_{2\alpha}$ and were washed three times by centrifugation at 500 × *g* for 2 min followed by resuspension with 1 ml of Opti-MEM at 37 °C. The cells were incubated again for 60 min at 37 °C with either vehicle or 1 μM $PGF_{2\alpha}$ (continuous treatment) and were then assayed for binding activity. For the measurement of [^{3}H]$PGF_{2\alpha}$ binding activity, cells were first washed three times as before using ice-cold MES buffer consisting of 10 mM MES (pH 6.0), 0.4 mM EDTA, and 10 mM $MnCl_2$. Following the final wash, the cells were resuspended in ice-cold MES buffer at a concentration of 10^7 cells/ml, and 100 μl was added to a final assay volume of 200 μl containing 2.5 nM [^{3}H]$PGF_{2\alpha}$ alone (total binding) or 2.5 nM [^{3}H]$PGF_{2\alpha}$ plus 10 μM unlabeled $PGF_{2\alpha}$ (nonspecific binding). Incubations were for 1 h at room temperature and were terminated by filtration through Whatman GF/C glass filters using a cell harvester (M-24R, Brandel). Filters were washed five times with the ice-cold MES buffer, and radioactivity was measured by liquid scintillation counting in 7 ml of Safety-Solve (Research Products International).

[^{3}H]Inositol Phosphate Assay—Receptor-stimulated total [^{3}H]inositol phosphate accumulation was determined by anion exchange chromatography in cells that were preincubated with *myo*-[2-^{3}H]inositol (Amersham Pharmacia Biotech). Cells were cultured in 10-cm plates in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and incubated with 3 μCi/ml *myo*-[2-^{3}H]inositol overnight. Cells were trypsinized, centrifuged at 500 × *g* for 2 min, and resuspended in Opti-MEM containing 10 mM LiCl at a concentration of 10^7 cells/ml. One-milliliter aliquots of cells were incubated for 60 min at 37 °C with either vehicle or 1 μM $PGF_{2\alpha}$ for the measurement of initial [^{3}H]inositol phosphate accumulation. For the measurement of [^{3}H]inositol phosphate accumulation after wash-out, cells were subjected to the same initial treatment as above and were washed three times by centrifugation at 500 × *g* for 2 min followed by resuspension with 1 ml Opti-MEM containing 10 mM LiCl at 37 °C. The cells were then incubated for 60 min at 37 °C with either vehicle or 1 μM $PGF_{2\alpha}$ (continuous treatment), and the accumulation of total [^{3}H]inositol phosphates was determined. Assays were terminated by the addition of 2.5 ml of chloroform/methanol/water (1:1:0.5), after which 900 μl of the aqueous phase was removed and mixed with 2 ml of water and applied to a 2.5-ml column of Dowex AG1-X8 anion exchange resin (Bio-Rad). Following 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]inositol phosphates 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 7 ml of Safety-Solve (Research Products International).

Fura-2 Assay of Intracellular Ca^{2+}—Cells were grown on 10-cm plates and were rinsed off the plates with Hanks' balanced salt solution, centrifuged for 5 min at 250 × *g*, and washed once with the assay buffer consisting of 120 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 20 mM HEPES, 1 mg/ml sodium pyruvate, and 1 mg/ml glucose (pH 7.4). The cell pellet was resuspended in the assay buffer at 3 × 10^6 cells/ml and then split between two centrifuge tubes. Fura-2/AM (Sigma) was added to both tubes (1 μM final concentration). Additionally, $PGF_{2\alpha}$ (10 nM final concentration) was added to one of the tubes but not the other. After a 1-h incubation at 37 °C in a shaking water bath, the cells were washed twice with assay buffer and resuspended at 2 × 10^6 cells/ml. Aliquots (0.5 ml) of the cell suspensions were added to Eppendorf tubes, centrifuged for 5 min at 250 × *g*, and placed on ice. The cell pellets were resuspended in 3 ml of assay buffer at 37 °C approximately 5 min prior to use and were then transferred to a stirred cuvette. Calcium signals were measured using a Perkin-Elmer LS-50 fluorescence spectrometer and excitation/emission wavelengths of 340/495 nm, respectively. For calibration of the Fura-2 signal, f_{max} was obtained by lysing the cells with digitonin (10 μl of 100 mg/ml in Me_2SO) and f_{min} by quenching the signal with EGTA and sufficient 10 M NaOH to adjust the pH to 8.5.

RESULTS

Reversal of $PGF_{2\alpha}$-induced Cell Rounding Is Slower for Cells Expressing the FP_B Prostanoid Receptor Isoform than for Cells Expressing the FP_A Isoform—Recently, we have shown that treatment of the ovine FP_A and FP_B prostanoid receptor isoforms with $PGF_{2\alpha}$ leads to changes in cellular morphology consisting of the retraction of filopodia, cell rounding, and a cobblestone appearance of cell aggregates (1). These changes, which also included the agonist-induced formation of actin stress fibers and phosphorylation of p125 FAK, were found to involve activation of Rho. To see if these changes were revers-

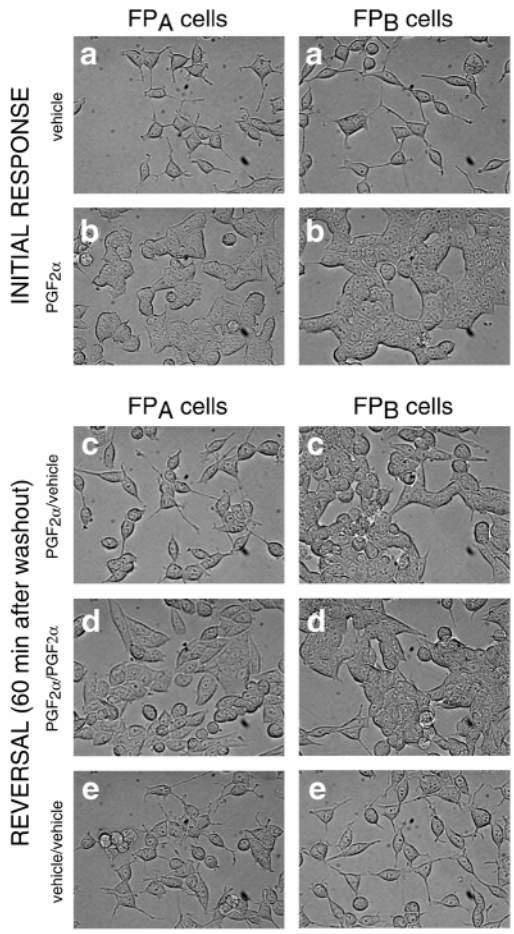

FIG. 1. **PGF$_{2\alpha}$-induced cell rounding (*top*) and its reversal following agonist wash-out (*bottom*) in HEK-293 cells stably expressing the ovine FP$_A$ and FP$_B$ prostanoid receptor isoforms.** Two plates each of FP$_A$- and FP$_B$-expressing cells were treated with either vehicle (*panels a*) or 1 μM PGF$_{2\alpha}$ (*panels b*) for 60 min at 37 °C and were then examined for their initial response to these treatments (*top*). Three plates each of FP$_A$- and FP$_B$-expressing cells were subjected to the same initial treatment with either vehicle (one plate) or 1 μM PGF$_{2\alpha}$ (two plates) and were washed out by rinsing three times with Opti-MEM followed by the addition of media containing either vehicle alone (*panels c* and *panels e*) or 1 μM PGF$_{2\alpha}$ (*panels d*). After a 60-min incubation at 37 °C, the cells were examined for reversal of cell rounding. The notation to the *left* of the *bottom panels* refers to the initial treatment/treatment after wash-out (*e.g.* in *panels c*, the initial treatment was with 1 μM PGF$_{2\alpha}$, and the treatment after wash-out was with vehicle). Images were obtained as described under "Experimental Procedures" at a magnification of × 75. These results are all from one experiment that was repeated over 10 times with virtually identical results.

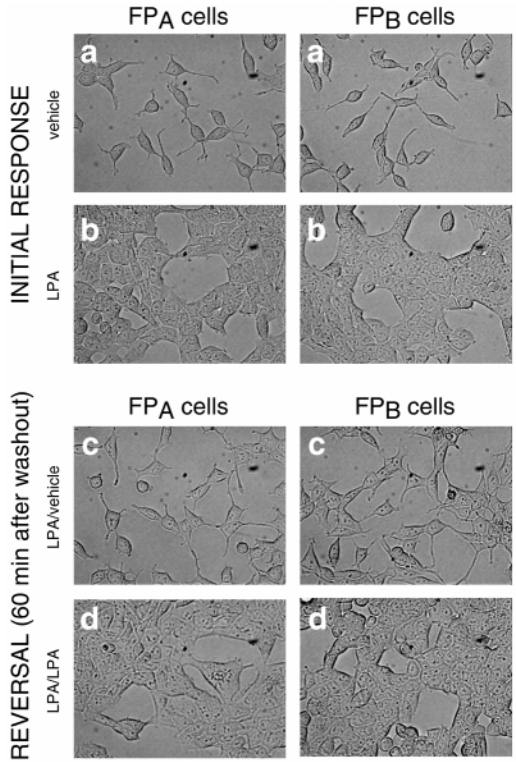

FIG. 2. **LPA-induced cell rounding (*top*) and its reversal following agonist wash-out (*bottom*) in HEK-293 cells stably expressing the ovine FP$_A$ and FP$_B$ prostanoid receptor isoforms.** Two plates each of FP$_A$- and FP$_B$-expressing cells were treated with either vehicle (*panels a*) or 10 μM LPA (*panels b*) for 60 min at 37 °C and were then examined for their initial response to these treatments (*top*). Two additional plates each of FP$_A$- and FP$_B$-expressing cells underwent an initial treatment with 1 μM PGF$_{2\alpha}$ and were then washed out by rinsing three times with Opti-MEM following by the addition of media containing either vehicle alone (*panels c*) or 10 μM LPA (*panels d*). After a 60-min incubation at 37 °C, the cells were examined for reversal of cell rounding. The notation to the *left* of the *bottom panels* refers to the initial treatment/treatment after wash-out (*e.g.* in *panels c*, the initial treatment was with 10 μM LPA, and the treatment after wash-out was with vehicle). Images were obtained as described under "Experimental Procedures" at a magnification of × 75. These results are all from one experiment that was repeated three times with virtually identical results.

ible, cells were treated with 1 μM PGF$_{2\alpha}$ for 1 h, after which they were washed and placed in fresh media. The *top* of Fig. 1 shows the appearance of FP$_A$- and FP$_B$-expressing cells by phase-contrast microscopy after the initial 1-h treatment with 1 μM PGF$_{2\alpha}$. As can be seen by comparing *panels a* and *panels b*, both the FP$_A$- and FP$_B$-expressing cells have lost their filopodia and are more rounded and aggregated in the presence of PGF$_{2\alpha}$. The *bottom* of Fig. 1 shows the appearance of the cells 1 h after wash-out, and a comparison of *panels c* reveals marked morphological differences between the FP$_A$- and FP$_B$-expressing cells. Thus, in FP$_A$-expressing cells, the filopodia have been reestablished, while in FP$_B$-expressing cells, the filopodia are absent and the cells are still aggregated. In the continuous presence of PGF$_{2\alpha}$ (*panels d*), both FP$_A$- and FP$_B$-expressing cells remain rounded and aggregated. In cells that were never exposed to PGF$_{2\alpha}$ but were subjected to the same wash-out protocol (*panels e*), the cells maintained their original stellate morphology (*cf. panels a*).

When examined 2 h after wash-out, the appearance of the cells was virtually indistinguishable from the data shown here for 1 h after wash-out (*i.e.* the FP$_A$s had fully reversed, and the FP$_B$s remained rounded and aggregated) (data not shown). Even after 24 and 48 h, there was relatively little reversal of cell rounding in the FP$_B$-expressing cells; however, reversal was also more difficult to assess because of the effects of cell crowding. Therefore, the present studies focused on the events occurring within 2 h of agonist wash-out.

HEK-293 cells are known to express endogenous receptors for LPA, whose activation causes changes in cellular morphology (9) similar to those observed here for PGF$_{2\alpha}$. To rule out the possibility of an intrinsic difference in the ability of the FP$_B$-expressing cells to undergo reversal of their morphology, we examined the reversal of LPA-induced cell rounding. The *top* of Fig. 2 shows the appearance of FP$_A$- and FP$_B$-expressing cells after an initial 1-h treatment with 10 μM LPA. As can be seen by comparing *panels a* and *panels b*, both FP$_A$- and FP$_B$-expressing cells show a loss of filopodia and cell rounding in the presence of LPA. The *bottom* of Fig. 2 shows the appearance of cells 1 h after wash-out, and as seen in *panels c*, for both

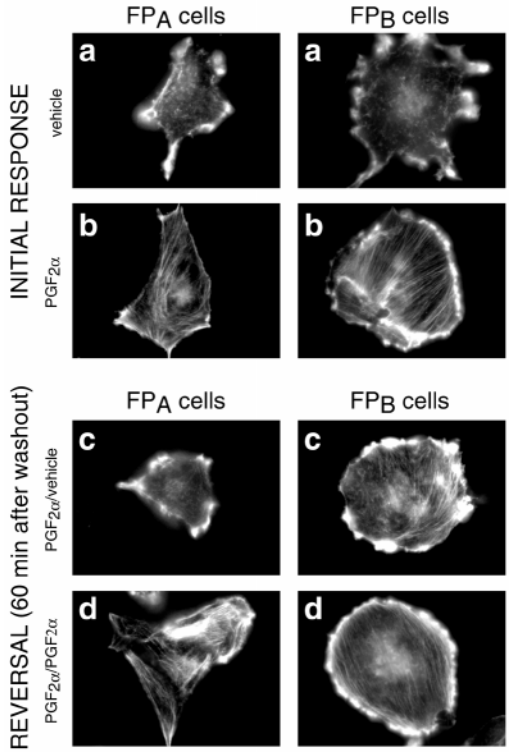

FIG. 3. **$PGF_{2\alpha}$-induced formation of actin stress fibers (*top*) and its reversal following agonist wash-out (*bottom*) in HEK-293 stably expressing the ovine FP_A and FP_B prostanoid receptor isoforms.** Two plates each of FP_A- and FP_B-expressing cells were treated with either vehicle (*panels a*) or 1 μM $PGF_{2\alpha}$ (*panels b*) for 60 min at 37 °C and were then stained with TRITC-phalloidin to examine stress fiber formation in response to these initial treatments (*upper panel*). Two additional plates each of FP_A- and FP_B-expressing cells underwent an initial treatment with 1 μM $PGF_{2\alpha}$ and were then washed out by rinsing three times with Opti-MEM followed by the addition of media containing either vehicle alone (*panels c*) or 1 μM $PGF_{2\alpha}$ (*panels d*). After a 60-min incubation at 37 °C, the cells were stained with TRITC-phalloidin to examine the reversal of stress fiber formation. The notation to the *left* of the *bottom panels* refers to the initial treatment/treatment after wash-out. Images were obtained as described under "Experimental Procedures" at a magnification of × 225. These results are all from one experiment that has been repeated over three times with virtually identical results.

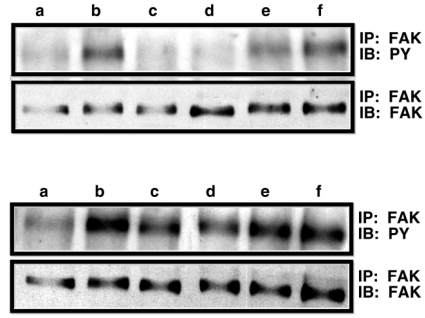

FIG. 4. **$PGF_{2\alpha}$-induced tyrosine phosphorylation of p125 FAK and its dephosphorylation following agonist wash-out in HEK-293 cells stably expressing ovine FP_A (*upper panel*) and FP_B (*lower panel*) prostanoid receptor isoforms.** Two plates each of FP_A- and FP_B-expressing cells were treated with either vehicle (*lanes a*) or 1 μM $PGF_{2\alpha}$ (*lanes b*) for 60 min at 37 °C. Four additional plates each of FP_A- and FP_B-expressing cells underwent an initial treatment with 1 μM $PGF_{2\alpha}$ and were then washed out by rinsing three times with Opti-MEM followed by the addition of media containing either vehicle alone (*lanes c* and *lanes d*) or 1 μM $PGF_{2\alpha}$ (*lanes e* and *lanes f*). After a 60 (*lanes c* and *lanes e*) or 120-min (*lanes d* and *lanes f*) incubation at 37 °C, the cells were examined for phosphorylation of p125 FAK by immunoprecipitation (*IP*) with anti-FAK antibodies and immunoblotting (*IB*) with anti-phosphotyrosine antibodies (*PY*). To ensure equal loading, the blots were stripped and reprobed with the anti-FAK antibody (*lower windows* of both *panels*). This experiment is representative of one of three independent experiments.

the FP_A- and FP_B-expressing cells, the filopodia have been reestablished. When examined 2 h after wash-out, the effects of LPA were fully reversed for both receptor isoforms (data not shown). In the continuous presence of LPA (*panels d*), both the FP_A- and FP_B-expressing cells remained rounded with a complete absence of filopodia. Thus, the slower reversal of the FP_B isoform following treatment with $PGF_{2\alpha}$ is consistent with a receptor-specific effect and is not a property of this clonal cell line.

Reversal of $PGF_{2\alpha}$-induced Stress Fiber Formation and Phosphorylation of FAK Are Also Slower for Cells Expressing the FP_B Prostanoid Receptor Isoform than for Cells Expressing the FP_A Isoform—As previously mentioned, treatment of FP_A- and FP_B-expressing cells with $PGF_{2\alpha}$ has also been found to induce actin stress fiber formation and phosphorylation of p125 FAK. To examine the reversal of these responses following agonist wash-out, FP_A- and FP_B-expressing cells were initially treated with 1 μM $PGF_{2\alpha}$ and were then washed and placed in fresh media containing either vehicle or 1 μM $PGF_{2\alpha}$. After the second incubation period, the cells were stained with TRITC-phalloidin to examine actin stress fiber formation (Fig. 3), or lysates were prepared and immunoprecipitated with anti-FAK antibodies followed by Western blotting to determine tyrosine phosphorylation of p125 FAK (Fig. 4). Fig. 3 shows that after the initial treatment with $PGF_{2\alpha}$ (*panels b*) both the FP_A- and FP_B-expressing cells showed prominent stress fiber formation (*cf. panels a*). *Panels c* of Fig. 3 show that in FP_A-expressing cells the actin stress fibers were gone 1 h after agonist wash-out. In contrast, the actin stress fibers were still present in FP_B-expressing cells, indicating that the loss of stress fibers following the wash-out of $PGF_{2\alpha}$ was slower for cells expressing the FP_B isoform as compared with the FP_A isoform.

Fig. 4 shows that there was a similar delay in the dephosphorylation of p125 FAK for FP_B-expressing cells. Thus, a comparison of *lanes a* and *b* shows that after the initial 1-h incubation with $PGF_{2\alpha}$, both FP_A-expressing cells (*upper panel*) and FP_B-expressing cells (*lower panel*) showed prominent phosphorylation of p125 FAK. However, at both 1 h (*lanes c*) and 2 h (*lanes d*) after agonist wash-out, the phosphorylation of FAK had returned to its original level for the FP_A-expressing cells (*cf. lane a*), whereas in the FP_B-expressing cells FAK remained highly phosphorylated. In the continuous presence of 1 μM $PGF_{2\alpha}$ at both 1 h (*lanes e*) and 2 h (*lanes f*) FAK remained phosphorylated in both the FP_A- and FP_B-expressing cells.

Slower Reversal of $PGF_{2\alpha}$-induced Cell Rounding for Cells Expressing the FP_B Receptor Isoform Is Not Due to Differences in the Wash-out of $PGF_{2\alpha}$ or in the Kinetics of $PGF_{2\alpha}$ Binding—Recovery of [^{3}H]$PGF_{2\alpha}$ following agonist wash-out and competitive radioligand binding were used to determine if pretreatment of FP_A- and FP_B-expressing cells with $PGF_{2\alpha}$ caused any subsequent changes in the affinity of $PGF_{2\alpha}$ for these receptors. To measure the effectiveness of the washing protocol, cells expressing either the FP_A or FP_B receptor isoforms were incubated for 1 h with 1 μM $PGF_{2\alpha}$, to which a tracer amount of [^{3}H]$PGF_{2\alpha}$ was added. The cells were then washed three times, and the recovery of radioactivity in the wash was determined. For the FP_A-expressing cells, 105 ± 3% of the initial radioactivity was recovered in the wash, while 104 ± 0.2% of the radioactivity was recovered in the wash from FP_B-expressing cells. The amount of radioactivity remaining with the cells was negligible. Therefore, the slower reversal of $PGF_{2\alpha}$-induced shape change for cells expressing the FP_B isoform is not due to

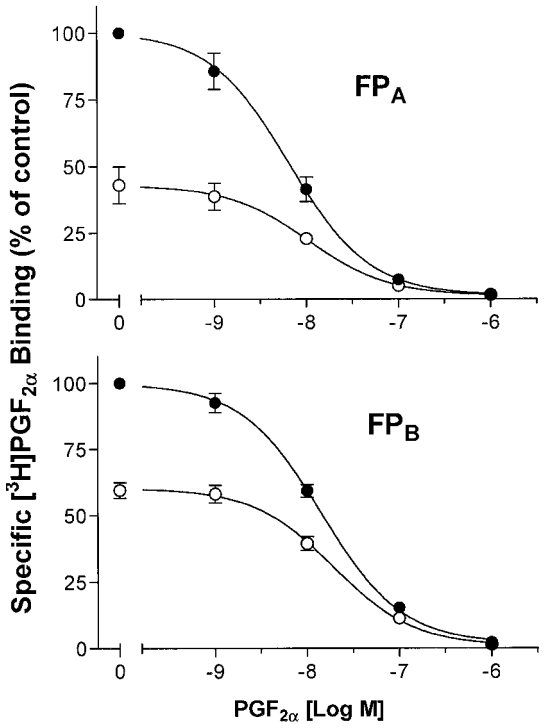

FIG. 5. **$PGF_{2\alpha}$ competition for specific whole cell binding of [^{3}H]$PGF_{2\alpha}$ following pretreatment with $PGF_{2\alpha}$ in HEK-293 cells stably expressing the ovine FP_A (*upper panel*) and FP_B (*lower panel*) prostanoid receptor isoforms.** Two plates each of FP_A- and FP_B-expressing cells were trypsinized and treated with either vehicle (●) or 1 μM $PGF_{2\alpha}$ (○) for 60 min at 37 °C and were then washed and assayed for specific binding of [^{3}H]$PGF_{2\alpha}$ as described under "Experimental Procedures." Data were normalized to vehicle in the absence of $PGF_{2\alpha}$ and were analyzed by nonlinear regression analysis. Data are the means ± S.E. of three independent experiments each performed in duplicate. EC_{50} values for the FP_A-expressing cells are 6.7 ± 1.5 nM for vehicle treatment and 11.8 ± 4.5 nM for $PGF_{2\alpha}$ treatment. For the FP_B-expressing cells, EC_{50} values are 14.3 ± 1.8 nM for vehicle treatment and 19.6 ± 4.0 nM for $PGF_{2\alpha}$ treatment.

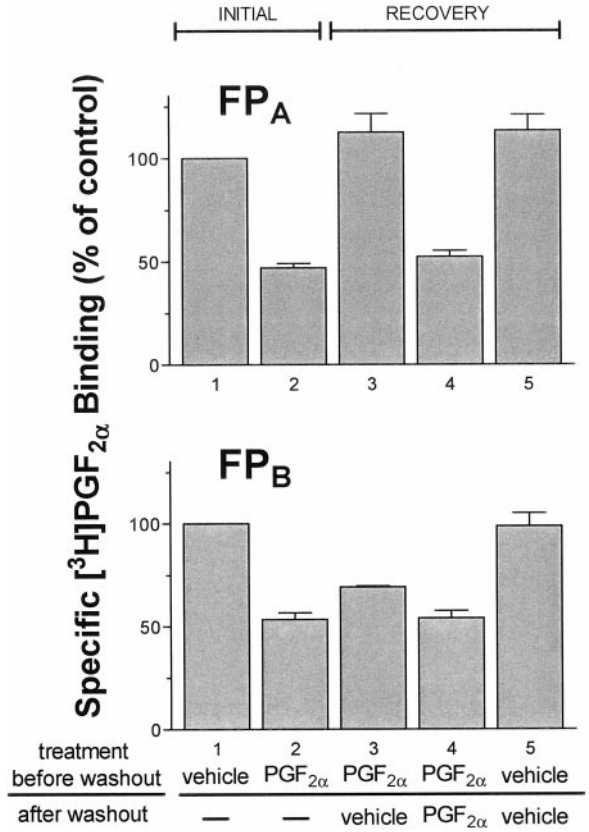

FIG. 6. **Specific whole cell binding of [^{3}H]$PGF_{2\alpha}$ following initial pretreatment with $PGF_{2\alpha}$ and after wash-out in HEK-293 cells stably expressing the ovine FP_A (*upper panel*) and FP_B (*lower panel*) prostanoid receptor isoforms.** Two plates each of FP_A- and FP_B-expressing cells were trypsinized and treated with either vehicle (*columns 1*) or 1 μM $PGF_{2\alpha}$ (*columns 2*) for 60 min at 37 °C. The cells were then washed and assayed for specific binding of [^{3}H]$PGF_{2\alpha}$ as described under "Experimental Procedures." Three plates each of FP_A- and FP_B-expressing cells were trypsinized and subjected to the same initial treatment with either vehicle (one plate) or 1 μM $PGF_{2\alpha}$ (two plates) and were then washed out by rinsing three times with Opti-MEM followed by the addition of media containing either vehicle alone (*columns 3* and *5*) or 1 μM $PGF_{2\alpha}$ (*columns 4*). After a 60-min incubation at 37 °C, the cells were washed and assayed for specific binding of [^{3}H]$PGF_{2\alpha}$. Data were normalized to the initial treatment with vehicle (*columns 1*). Each column shows the means ± S.E. of three independent experiments, each performed in duplicate.

a decreased ability to wash out $PGF_{2\alpha}$. The high efficiency of the washing procedure probably reflects the low permeability coefficient for the cellular influx of $PGF_{2\alpha}$ (10) and suggests that whole cell radioligand binding will predominantly reflect binding to cell surface receptors.

Whole cell competitive radioligand binding was done with FP_A- and FP_B-expressing cells to determine if the slower reversal of shape change in cells expressing the FP_B isoform could be correlated with an increased affinity of $PGF_{2\alpha}$ for the receptor following pretreatment with agonist. Fig. 5 shows that pretreatment with 1 μM $PGF_{2\alpha}$ for 1 h followed by wash-out resulted in approximately a 50% decrease in the maximal specific binding of [^{3}H]$PGF_{2\alpha}$ for both receptor isoforms with a slight decrease in affinity (~1.5-fold) for both isoforms. These data suggest that both the FP_A and FP_B receptor isoforms undergo a similar degree of agonist-induced loss of binding activity and that the slower reversal of shape change in cells expressing the FP_B isoform is unlikely to be the result of an increased affinity for $PGF_{2\alpha}$.

Initial Agonist-induced Loss of Binding Activity Is Similar for Both FP Prostanoid Receptor Isoforms, but Functional Desensitization Persists for the FP_B Isoform and Is Consistent with Slower Resensitization—$PGF_{2\alpha}$-induced receptor desensitization and subsequent resensitization following agonist wash-out was done to investigate possible mechanisms that might explain the slower reversal of cell rounding, actin stress fiber formation, and FAK phosphorylation by the FP_B receptor isoform. This was examined by whole cell radioligand binding with [^{3}H]$PGF_{2\alpha}$ (Fig. 6), by measurement of agonist-stimulated inositol phosphate formation (Fig. 7), and by measurement of agonist-stimulated intracellular Ca^{2+} mobilization (Fig. 8). In Fig. 6, cells were initially treated with either vehicle or 1 μM $PGF_{2\alpha}$ for 1 h and were then washed and assayed for the specific whole cell binding of [^{3}H]$PGF_{2\alpha}$. A comparison of *columns 1* and *2* shows that this initial treatment resulted in a 50% decrease in specific binding for both the FP_A-expressing cells (*upper panel*) and the FP_B-expressing cells (*lower panel*), which is likely to reflect receptor internalization. To examine the recovery of binding activity, a second group of cells underwent the same initial treatment but were then washed and reincubated for another 1 h with either vehicle (*columns 3* and *5*) or 1 μM $PGF_{2\alpha}$ (*columns 4*) and were again washed and assayed for [^{3}H]$PGF_{2\alpha}$ binding. A comparison of columns *3* and *5* shows that specific [^{3}H]$PGF_{2\alpha}$ binding recovered completely for cells expressing the FP_A isoform (*upper panel*), but was still decreased by 40% for cells expressing the FP_B isoform (*lower panel*). These data are consistent with a slower resensitization, or recycling, of the FP_B prostanoid receptor isoform as com-

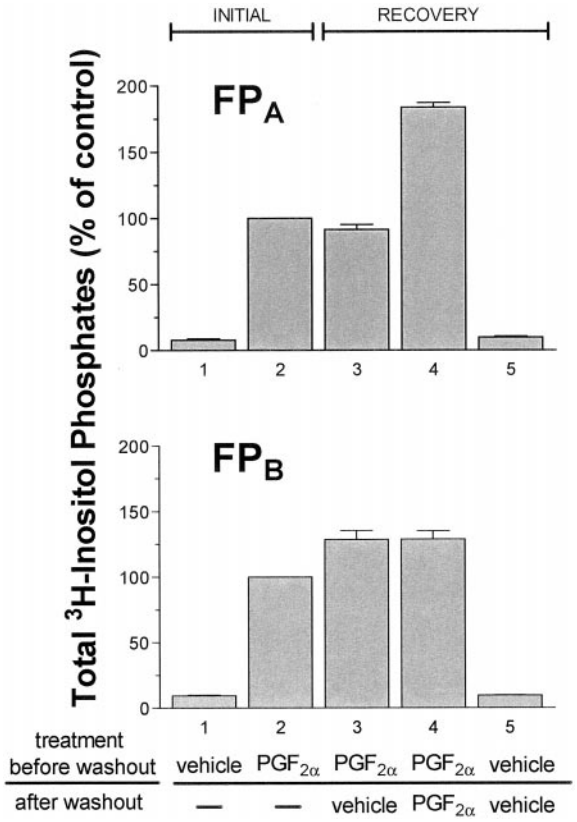

FIG. 7. **PGF$_{2\alpha}$-stimulated total [^{3}H]inositol phosphates accumulation following initial pretreatment with PGF$_{2\alpha}$ and after wash-out in HEK-293 cells stably expressing the ovine FP$_A$ (*upper panel*) and FP$_B$ (*lower panel*) prostanoid receptor isoforms.** Two plates each of FP$_A$- and FP$_B$-expressing cells were trypsinized and treated with either vehicle (*columns 1*) or 1 μM PGF$_{2\alpha}$ (*columns 2*) for 60 min at 37 °C, and total [^{3}H]inositol phosphates were determined as described under "Experimental Procedures." Three plates each of FP$_A$- and FP$_B$-expressing cells were trypsinized and subjected to the same initial treatment with either vehicle (one plate) or 1 μM PGF$_{2\alpha}$ (two plates) and were then washed out by rinsing three times with Opti-MEM followed by the addition of media containing either vehicle alone (*columns 3* and *5*) or 1 μM PGF$_{2\alpha}$ (*columns 4*). After a 60-min incubation at 37 °C, the cells were washed, and total [^{3}H]inositol phosphates were determined. Data were normalized to the initial treatment with PGF$_{2\alpha}$ (*columns 2*). Each *column* is the mean ± S.E. of three independent experiments each performed in duplicate.

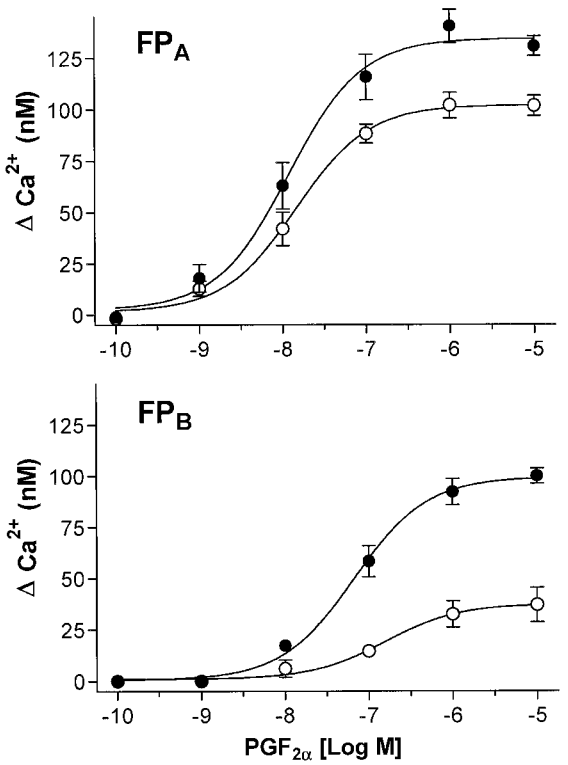

FIG. 8. **PGF$_{2\alpha}$ stimulation of intracellular calcium mobilization in HEK-293 cells stably expressing the ovine FP$_A$ (*upper panel*) and FP$_B$ (*lower panel*) prostanoid receptor isoforms.** Cells were treated with either vehicle (●) or 10 nM PGF$_{2\alpha}$ (○) for 60 min at 30 °C. The cells were then washed, and intracellular Ca^{2+} was measured by Fura-2 assay as described under "Experimental Procedures" at the concentrations of PGF$_{2\alpha}$ indicated above. Data are the means ± S.E. of three independent experiments.

pared with the FP$_A$ isoform. *Columns 4* show that continuous treatment with PGF$_{2\alpha}$ did not further decrease [^{3}H]PGF$_{2\alpha}$ binding activity (*cf. columns 2*), and *columns 5* show that the incubation and washing conditions themselves did not decrease binding activity (*cf.* columns *1*).

Agonist-stimulated total inositol phosphate accumulation was measured following initial treatment with PGF$_{2\alpha}$ and following agonist wash-out to see if the slower reversal of cell rounding in cells expressing the FP$_B$ isoform might be associated with prolonged phosphoinositide signaling. A comparison of *columns 1* and *2* in Fig. 7 shows that in both FP$_A$- and FP$_B$-expressing cells there is a robust stimulation of total inositol phosphate accumulation after the initial 1-h treatment with 1 μM PGF$_{2\alpha}$. Following agonist wash-out in the FP$_A$-expressing cells, there is no additional accumulation of total inositol phosphates when the cells are subsequently incubated with vehicle (*cf. columns 2* and *3*), but there is additional accumulation when the cells are reexposed to 1 μM PGF$_{2\alpha}$ (*cf. columns 2* and *4*). On the other hand, following agonist wash-out in FP$_B$-expressing cells, there is a slight increase in total inositol phosphates when the cells are incubated with vehicle (*cf. columns 2* and *3*), but there is *no* additional accumulation when the cells are reexposed to PGF$_{2\alpha}$ (*cf. columns 2* and *4*). It appears, therefore, that the FP$_B$-expressing cells are functionally desensitized, whereas the FP$_A$-expressing cells are not, which is consistent with either slower resensitization of the FP$_B$ isoform or more effective coupling of FP$_A$ receptors to the phosphoinositide/Ca^{2+} signaling pathway. Furthermore, it does not appear that the slower reversal of PGF$_{2\alpha}$-induced shape change in the FP$_B$-expressing cells can be explained by prolonged phosphoinositide signaling.

To examine the effects of agonist pretreatment on intracellular Ca^{2+} signaling, FP$_A$- and FP$_B$-expressing cells were incubated for 1 h with either vehicle or 10 nM PGF$_{2\alpha}$ and were washed and assayed for PGF$_{2\alpha}$-stimulated Ca^{2+} mobilization by Fura-2 assay. Fig. 8 shows that for FP$_A$-expressing cells (*upper panel*) there was a 25% decrease in the maximally stimulated mobilization of intracellular Ca^{2+} following agonist pretreatment. In contrast, there was a 60% decrease for the FP$_B$-expressing cells (*lower panel*). As with the phosphoinositide data shown in Fig. 7, it appears that the FP$_B$-expressing cells show a greater degree of functional desensitization of agonist-stimulated intracellular Ca^{2+} signaling as compared with the FP$_A$-expressing cells. Again, this is consistent with slower resensitization of the FP$_B$ receptor isoform as compared with the FP$_A$ isoform. Also, it does not appear that enhanced intracellular Ca^{2+} signaling can explain the slower reversal of PGF$_{2\alpha}$-induced shape change in cells expressing the FP$_B$ isoform.

DISCUSSION

Previously, we have demonstrated that agonist activation of the cloned ovine FP$_A$ and FP$_B$ prostanoid receptor isoforms

expressed in HEK-293 cells leads to Rho-dependent cell rounding, including the formation of the actin stress fibers and tyrosine phosphorylation of p125 FAK (7). While no differences between the isoforms were found with respect to the induction of these responses, we now report that their reversal following agonist wash-out differs markedly between the isoforms. Thus, 1 h after the removal of $PGF_{2\alpha}$, FP_A receptor-expressing cells have essentially returned to their original morphology, whereas FP_B receptor-expressing cells are still rounded and show the presence of actin stress fibers and p125 FAK phosphorylation. These results indicate that in FP_B-expressing cells there is a persistent activation of Rho-dependent signaling even after the removal of agonist. This difference is not a consequence of differing levels of receptor expression (both cell lines express similar levels) or their ability to activate phosphoinositide turnover (both receptors stimulate inositol phosphate turnover to a similar extent). Furthermore, this difference is not due to an intrinsic difference in the ability of these two clonal cells to undergo reversal. Thus, reversal of LPA-induced cell rounding following the removal of LPA was identical for both FP_A- and FP_B-expressing cells. Instead, it appears that the slower reversal of cell rounding following agonist wash-out in FP_B-expressing cells is related to slower resensitization of the FP_B receptor. At this time, we cannot determine if the persistent activation of Rho signaling by the FP_B isoform is due to a failure to turn off the original signal or the failure to activate a termination signal. In either case, the structural basis for this difference resides in the 46 amino acids in the carboxyl terminus of the FP_A isoform that are absent in the FP_B isoform.

As it concerns these 46 amino acids, we have recently shown that they are the target for PKC-mediated phosphorylation following treatment with $PGF_{2\alpha}$ (6). The FP_B receptor isoform, on the other hand, undergoes little or no agonist-dependent phosphorylation. The carboxyl terminus has also been shown to be a critical domain for regulatory phosphorylation in other prostanoid receptors. For example, both the thromboxane A_2 receptor and the prostacyclin receptor undergo agonist-dependent phosphorylation in their carboxyl termini that is important for receptor desensitization. In the case of the thromboxane A_2 receptor, both PKC and a G-protein-coupled receptor kinase may be involved (11). For the prostacyclin receptor, however, only PKC has been implicated (12). In both cases, phosphorylation was correlated with agonist-induced desensitization of second messenger signaling. The carboxyl terminus of the EP_4 receptor has also been implicated in agonist-induced desensitization of second messenger signaling, although direct phosphorylation was not examined (13). Unfortunately, none of these studies have correlated the desensitization of signaling with radioligand binding, although the carboxyl terminus of the thromboxane A_2 receptor was shown by immunofluorescence microscopy to promote agonist-induced internalization (14). Presumably receptor internalization could account for desensitization of signaling, but this is not always the case. In fact, it is becoming evident that the process of receptor internalization itself is another aspect of signaling. Thus, agonist-induced activation of mitogen-activated protein kinase cascades by G-protein-coupled receptors appears to involve receptor internalization (15).

As compared with studies of receptor desensitization, there has been relatively little work done on receptor resensitization. One recent study, however, compared the resensitization of β_2-adrenergic receptors with vasopressin V2 receptors and found that the binding of arrestin-2 to the carboxyl termini of these receptors was a critical determinant in receptor resensitization (16). In the case of the β_2-adrenergic receptors, which resensitize quickly, arrestin-2 dissociates from the receptors prior to internalization, whereas for the V2 receptors, which resensitize slowly, arrestin-2 is internalized with the receptors. The internalization of the V2 receptors was found to depend on a cluster of three serines in its carboxyl terminus. The relationship of this work to our present studies cannot be ascertained at this time. For example, the carboxyl terminus of the FP_B receptor isoform does not contain any serines or threonines and therefore is not a substrate for a serine/threonine kinase. Yet the FP_B isoform clearly undergoes agonist-dependent desensitization with respect to both phosphoinositide turnover and mobilization of intracellular Ca^{2+}, and it appears to undergo internalization based upon whole cell radioligand binding. The FP_B isoform does contain potential phosphorylation sites in its first, second, and third intracellular loops, but all of these are common to the FP_A isoform and therefore cannot explain how these two receptor isoforms differ with respect to regulatory phosphorylation events. Clearly, phosphorylation of the carboxyl terminus could be relevant to the observed desensitization of the FP_A isoform, which leads to the conclusion that the mechanisms underlying the desensitization of the FP_A and FP_B receptor isoforms are likely to differ. Likewise, their mechanisms of resensitization are likely to differ, which is reflected by our finding that the recovery of radioligand binding activity was slower for the FP_B isoform as compared with the FP_A following pretreatment with $PGF_{2\alpha}$.

Can slower resensitization of the FP_B isoform explain the slower reversal of $PGF_{2\alpha}$-induced cell rounding? This is presently unknown, but we believe it could be a link. Thus, the differential desensitization/resensitization mechanisms of the two isoforms could be associated with differences in collateral signaling that are critical to either turning on or turning off a reversal signal. For example, if the FP_A receptor isoform is internalized via clathrin-coated pits, and the FP_B is not, this could give rise to differential signaling of a receptor tyrosine kinase pathway that is permissive for the reversal process. Likewise, if the FP_B isoform is internalized, without being phosphorylated, it may continue to signal and yet be desensitized with respect to whole cell radioligand binding. Other mechanisms that do not involve receptor desensitization or resensitization, such as differential activation of an unrecognized signal transduction pathway, may also be operative.

To date, most studies of G-protein-coupled receptor isoforms have focused on potential differences in the activation of signal transduction pathways and/or initial regulatory events such as receptor desensitization. Not surprisingly, less attention has been given to the reversal of these processes, although they are essential in a physiological system. To the best of our knowledge, this is the first report of a major difference between closely related receptor isoforms with respect to the reversal of receptor-initiated changes in cell morphology. We have found that although the time course and extent of $PGF_{2\alpha}$-induced cell rounding, actin stress fiber formation, and FAK phosphorylation are similar for the two isoforms, the reversal of these effects is much slower for the FP_B isoform than the FP_A. This difference is paralleled by slower resensitization of the FP_B isoform with respect to radioligand binding and intracellular phosphoinositide/Ca^{2+} signaling, although the causal relationship of these events to the reversal of cell rounding remains to be established.

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Delayed Reversal of Shape Change in Cells Expressing FP_B Prostanoid Receptors: POSSIBLE ROLE OF RECEPTOR RESENSITIZATION

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