A Novel Biased Allosteric Compound Inhibitor of Parturition Selectively Impedes the Prostaglandin F2α-mediated Rho/ROCK Signaling Pathway

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The prostaglandin F2α (PGF2α) receptor (FP) is a key regulator of parturition and a target for pharmacological management of preterm labor. However, an incomplete understanding of signaling pathways regulating myometrial contraction hinders the development of improved therapeutics. Here we used a peptidomimetic inhibitor of parturition in mice, PDC113.824, whose structure was based on the NH2-terminal region of the second extracellular loop of FP receptor, to gain mechanistic insight underlying FP receptor-mediated cell responses in the context of parturition. We show that PDC113.824 not only delayed normal parturition in mice but also that it inhibited both PGF2α- and lipopolysaccharide-induced preterm labor. PDC113.824 inhibited PGF2α-mediated, Gαq-dependent activation of the Rho/ROCK signaling pathways, actin remodeling, and contraction of human myometrial cells likely by acting as a non-competitive, allosteric modulator of PGF2α binding. In contrast to its negative allosteric modulating effects on Rho/ROCK signaling, PDC113.824 acted as a positive allosteric modulator on PGF2α-mediated protein kinase C and ERK1/2 signaling. This bias in receptor-dependent signaling was explained by an increase in FP receptor coupling to Gαq at the expense of coupling to Gα12. Our findings regarding the allosteric and biased nature of PDC113.824 offer new mechanistic insights into FP receptor signaling relevant to parturition and suggest novel therapeutic opportunities for the development of new tocolytic drugs.

Premature birth due to preterm delivery is the most important cause of neonatal mortality and morbidity in industrialized countries (1, 2). A common cause of preterm labor is a spontaneous increase in uterine contraction. However, little is known regarding the factors that either maintain uterine quiescence or initiate spontaneous uterine contraction. Pharmacological interventions that aim at preserving or inducing uterine quiescence remain the most attractive strategy for managing preterm labor to date.

Prostaglandins, whose synthesis is under the control of cyclooxygenases and specific prostaglandin synthases, play important roles during pregnancy and parturition (3, 4). They are initiators of physiological labor and exert their effects through different G protein-coupled receptors (GPCRs). For instance, prostaglandin F2α (PGF2α) promotes myometrial contraction through activation of PGF2α (FP) receptors (5, 6). Moreover, FP receptor null mice fail to deliver at term and are unresponsive to induced labor mediated by either PGF2α or the uterogenic hormone oxytocin (7, 8). At the molecular level, activation of FP receptor leads to inositol phosphate accumulation, protein kinase C (PKC) activation, and intracellular calcium release, consistent with coupling of FP receptor to the Gαq family of G proteins (9–12). Activation of FP receptor has also been shown to promote Rho-dependent reorganization of the cytoskeleton (13). Both signaling pathways are believed to contribute to phasic and tonic myometrial smooth muscle contraction. However, their relative contributions to uterine tissue contraction during parturition remain poorly understood. Thus, a better comprehension of PGF2α-mediated signaling mechanisms through FP receptor is not only essential to understanding parturition, but also for the potential development of drugs suppressing labor (tocolytics).

At present, the use of tocolytics to delay preterm labor is often contraindicated due to significant maternal and fetal side effects.
effects (14, 15). A new FP receptor ligand, THG113, corresponding to a peptide derived from the sequence of the second extracellular loop of FP receptor (Fig. 1A), was recently reported to inhibit preterm labor in a mouse model (16). However, the exact mechanism underlying its action on myometrial contraction remained unclear. In refining THG113 as a potential tocolytic compound specific for FP receptor with enhanced efficacy toward myometrial contraction, a peptidomimetic compound, PDC113.824, was synthesized (Fig. 1B and supplemental Fig. S1A). We used this compound to develop a better understanding of FP receptor signaling in the context of myometrial cell contraction and the regulation of parturition. We report here, that PDC113.824 is a potent tocolytic agent, inhibiting myometrial cell contraction and the regulation of parturition.

**EXPERIMENTAL PROCEDURES**

**Reagents**—[3H]PGF2α, [35S]GTPγS, and ECL were from PerkinElmer. [125I]Angiotensin was labeled as described in a previous study (17). PGF2α is from Cayman. Rabbit polyclonal anti-Gαq (C-19) and anti-Gα12 (S-20) antibodies were from Santa Cruz Biotechnology. PGF2α-bound receptors toward increased Gαq-PKC-MAPK signaling, while blocking cell contraction and cytoskeleton reorganization through inhibition of the Gα12/13-Rho-ROCK signaling pathway.

**FP Receptor Antibody Generation**—The peptide corresponding to the first extracellular loop of FP receptor was synthesized by using Fmoc ([9-fluorenylmethoxycarbonyl]) synthesis with >95% purity (Biosynthesis, Lewisville, TX) and included an additional residue (cysteine) at the NH2 terminus (NH2-CSMNNSKQLVS-COOH). The cysteine-containing peptide was conjugated to the sulfhydryl-reactive carrier protein keyhole limpet hemocyanin (Pierce). The keyhole limpet hemocyanin-conjugated peptide in complete Freund’s adjuvant (Pierce) was injected intraperitoneally and subcutaneously (total 25 μg, in 100 μl) in female BALB/c mice. Subsequent immunizations were performed every 10–13 days with keyhole limpet hemocyanin-conjugated peptide in phosphate-buffered saline. Serial dilutions of mouse serum were screened for reactivity using a solid-phase enzyme-linked immunosorbent assay, testing the original peptide (unconjugated). Several other peptides or bovine serum albumin were used as negative controls (not shown). Four days after the last immunization, splenocytes were fused with SP2/0 mouse myeloma cells following established protocols. Hybridoma supernatants were screened by enzyme-linked immunosorbent assay against unconjugated peptide or controls as above. Supernatants were further screened for binding to cell surface FP receptor by FACSscan assays, using live HEK293 cells stably transfected with FP receptor or parental HEK293 cells as a background control. Wells containing hybridomas secreting monoclonal antibodies reactive to FP receptor were subcloned twice by limiting dilution and expanded for monoclonal antibody purification. A hybridoma line producing antibody 3E12/2B2 was used in this study.

**Cell Culture and Transfection**—A stable HEK293 cell line expressing the human FP receptor was generated by transfection with pIRESP-HA-hFP. Stable lines were selected in 0.7 μg/ml puromycin. All HEK293-derived cell lines were grown at 37 °C in 5% CO2 in MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and gentamycin (100 μg/ml). hTERT-C3 myometrial cells were grown in DMEM/F-12 medium as described previously (19). For transient transfection, cells seeded at a density of 1 × 10^6 cells per 100-mm dish, or 1 × 10^5 per well in a 6-well plate, were transfected using a conventional calcium phosphate co-precipitation method. All experiments were performed 48 h post-transfection.

**Ligand Binding Experiments**—For binding experiments, 100 μg of HEK293 cells stably expressing HA–FP receptor was incubated with 10^6 cpm of [3H]PGF2α (150–240 Ci/mmol) in the presence of either vehicle (EtOH, 0.01% v/v), 1 μM cold PGF2α, 10 μM AL-8810, 1 or 10 μM PDC113.824 for 1 h at room temperature in 0.5 ml of binding buffer (described previously (16)). Potential allosteric interactions were detected using radiolabeled TGGTGG-3’) was digested with the same enzymes and inserted into pires-Puro3. The new construct was renamed pires-HA-pires-HA was linearized with BamHI and PCR-amplified FP receptor (from pcDNA3.1+/FP receptor. FWD: 5’T-TATGCTGCCGATCTCCATGAAGATCAGCCACAGATAATCGCAGTGAAGGTAAGCCA-
gland dissociation assays. Dissociation of $[^3H]PGF2\alpha$ was measured as follows: FP receptor cells (100 μg) were preincubated with 10$^6$ cpm of $[^3H]PGF2\alpha$ with or without 1 μM PDC113.824 in a total volume of 0.4 ml of binding buffer for 60 min. Ligand dissociation was then initiated by the addition of 1 μM cold PGF2\alpha for different times. Nonspecific binding was determined by addition of 1 μM PGF2\alpha for 90 min. Binding was stopped by addition of 2 ml of cold Tris-HCl 50 mM, pH 7.4, and cells were filtered on GF/B-filters. Incorporated radioactivity was measured by liquid scintillation spectrometry.

$[^3S]GTP\gamma S$ Loading Studies—FP receptor cells were co-transfected with EE-tagged-εα or -εα12. On the day of the experiment, cells were serum-starved prior to treatment with or without 0.5 μM PDC113.824 at 37 °C for 30 min. Cells were collected in ice-cold buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA) and homogenized on ice by 20 strokes with a Teflon potter. The homogenate was centrifuged at 23,000 rpm at 4 °C and resuspended in TME buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 4.8 mM MgCl$_2$, 100 mM NaCl). GDP (final concentration of 1 μM) was added to 50 μg of membranes, and the mixture was incubated on ice for 10 min. The reaction was moved to 30 °C and incubated for 5 min before the addition of $[^3S]GTP\gamma S$ (1250 Ci/mmol) to a final concentration of 5 nM. PGF2\alpha was added 30 s later, and the reaction was allowed to proceed for 5 min. Reactions were stopped with cold immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 20 mM MgCl$_2$, 150 mM NaCl, 0.5% Nonidet P-40, protease inhibitors, 100 μM GDP and GTP), and membranes were solubilized for 30 min at 4 °C. To immunoprecipitate specific complexes, 1.2 μg of anti-εα (clone C-19, Santa Cruz Biotechnology) or anti-εα (clone S-20, Santa Cruz Biotechnology) antibodies were added for 2 h at 4 °C. Protein G-agarose beads were added, and the mixture was incubated for an additional 60 min at 4 °C. Beads were then washed three times with immunoprecipitation buffer, and incorporated $[^3S]GTP\gamma S$ was measured by liquid scintillation spectrometry.

PKCB1-GFP Translocation—FP receptor cells co-transfected with PKCB1-GFP were serum-starved for 30 min and pre-treated with vehicle (water) or PDC113.824 (2 μM) for 30 min followed by treatment with increasing concentrations of PGF2\alpha (from 10$^{-14}$ to 10$^{-7}$ M) for 10 min each. Images were collected every 30 s using live-cell microscopy at 37 °C on a Zeiss LSM-510 Meta laser scanning microscope equipped with XL-3 temperature chamber with a 63× glycerol/water immersion lens in single track mode using excitation at 488 nm for GFP and emission measured with the LP505 filter set. Translocation was determined by calculating the fluorescence level at the membrane (area under the curve) divided by the fluorescence level in the cytosol, using Metamorph (Universal Imaging Corp.).

Cell Ruffling and Immunofluorescence Studies—FP receptor cells were plated on coverslips. For dominant negative εα12, εα12(Q231L/D299N) was transfected 48 h prior to the experiment. Cells were serum-starved for 30 min and pre-treated or not with either 0.1 or 1 μM PDC113.824 or the following pharmacological inhibitors: MEK1/2 (PD98059, 1 μM), phosphatidylinositol 3-kinase (LY294002, 1 μM), PKC (Go6983, 1 μM), or Rho kinase (Y27632, 1 μM) for 30 min at 37 °C or with Rho inhibitor (C3 exoenzyme, 1 μg/ml) for 4 h at 37 °C. Cells were then stimulated with 1 μM PGF2\alpha for 20 min, fixed with 4% paraformaldehyde, and stained with Fluor488-Phalloidin. Nine fields (50–75 cells/field) per coverslip were quantified to assess cellular ruffling. For FP receptor labeling, paraformaldehyde-fixed myometrial or FP receptor cells were incubated with mouse-anti-FP receptor (clone 3E12/2B2) for 90 min prior to applying goat-anti-mouse Alexa488-coupled secondary antibody for an extra 60 min.

Raichu-RBD Experiments—The Raichu-RBD probe was used as described previously (20). Briefly, Raichu-RBD was composed of a YFP moiety in the NH$_2$-terminal domain (m2Venus), a central Rho-binding domain (RBD) of Rhotekin and a CFP moiety in the COOH-terminal domain. The FP receptor or AR T cells were plated on 35-mm microscopy dishes at a density of 50,000 cells. 24 h later, cells were transfected with Raichu-RBD alone or co-transfected with εα12DN. 48 h later, cells were serum-starved for 30 min, pre-treated or not with PDC113.824 (1 μM) for 30 min or C3 exoenzyme (1 μg/ml) for 3–4 h, followed by stimulation with 1 μM PGF2\alpha for 25 min. Images were collected every 30 s for the first 6 min, then every minute until 12 min, and at 15, 20, and 25 min using live-cell microscopy at 37 °C on a Zeiss LSM-510 Meta laser scanning microscope as described above with excitation at 405 nm for CFP and the RET channel, 514 nm for YFP and with emission measured with BP420–480 for CFP, and BP530–600 for YFP and RET.

Energy transfer efficiency between the CFP (donor) and YFP (acceptor) was determined by calculating the ratio of the YFP over CFP fluorescence from three different regions of each cell, and corrected for background signal using Metamorph. RET images were translated into colored gradient images using Rainbow2 visualization in Zen Light software (Zeiss). This function translates each pixel of the image into intensity values and reports them using a color code. 24–36 cells/condition were quantified in five to eight independent experiments.

Collagen Contraction Assay—Collagen contraction assays were performed as described previously (19). Briefly, 14,000 to 17,500 hTERT-C3 myometrial cells were plated in DMEM/F-12 medium in 0.5% (v/v) fetal bovine serum on the collagen lattice and left for 2 h at 37 °C. Cells were then pre-treated with either vehicle, C3 exoenzyme (1 μg/ml), or PDC113.824 (2 μM) for 2 h. To allow contraction, each collagen lattice was detached from the bottom of the well with a small spatula and left overnight at 37 °C in the absence or presence of 1 μM PGF2\alpha or oxytocin. Contraction was stopped by fixing lattices in phosphate-buffered saline with 4% paraformaldehyde. The plate containing collagen lattices was then photographed using the Alpha Imager System. Percentage of contraction of collagen lattices was then calculated using Metamorph, using the following equation: % contraction = 100 – (area of lattice*100/area of the well).

Murine Preterm Labor Models and Ex Vivo Myometrial Contraction Assay—Timed-pregnant CD-1 mice at 16 days gestational (normal term is 19.2 days) were anesthetized with isoflurane (2%). Primed osmotic pumps (Alzet pump, Alzet, Cupertino, CA) containing either saline or PDC113.824 (10 mg/day/animal) were subcutaneously implanted on the backs of the animals; infusion of PDC113.824 was immediately pre-
ceded by bolus injection of PDC113.824 (0.1 mg/animal intraperitoneally). Within 15 min after placement of the pumps, animals were injected with PGF2α or lipopolysaccharide (LPS) Escherichia coli endotoxin (50 μg/animal intraperitoneally) to mimic the inflammatory/infectious component of human preterm labor. In a separate group of animals, PGF2α or LPS was injected 4–7 h prior to administration of PDC113.824. Animals were inspected every hour for the first 18 h and every 2 h thereafter to document the timing of birth. All experiments were approved by the Animal Care Committee of Centre Hospitalier Universitaire Sainte-Justine (Montreal, Quebec, Canada).

Ex vivo myometrial contraction assay was performed as previously described (16). Briefly, uteri from mice were obtained from animals immediately following term delivery. Myometrial strips (2–3 mm wide and 1–2 cm long) from both were suspended in organ baths containing Krebs buffer equilibrated with 21% oxygen at 37 °C with an initial tension at 2 g. After 1 h of equilibration, changes in mean basal tension, as well as peak, duration, and frequency of spontaneous contraction in the absence or presence of PGF2α and PDC113.824 were recorded with a Kent digital polygraph system.

Statistical Analysis—Statistical tests were performed with GraphPad Prism 4.3 software. Assumptions of normality and equal variance were met for all data analyzed. One-way analysis of variance with Dunnett’s correction was used in Fig. 3, B and D (comparing all results to vehicle treatment), Fig. 5, B and E (comparing all results to PGF2α treatment), and supplemental Fig. S2. Two-way analysis of variance with repeated measures was used in Fig. 8, E and F, with Bonferroni correction. Independent t-tests were used in Figs. 2 (C and E), 4C, 6 (B–D), 8A, and 9 (A–F), to compare between vehicle and PDC113.824. A one-sample t test (Figs. 4 (A and D), 7A, and 9 (C–F)) was used when the data were normalized, and basal levels were considered with respect to the hypothetical value (1 or 100). The Fisher’s exact test was used in Fig. 2 (A, B, and D). A two-tailed p value of <0.05 was considered significant. All results are expressed as means ± S.E. Sample size (n) and p values are given in the figure legends.

RESULTS

Design and Optimization of the Peptidomimetic PDC113.824—Conversion of the THG113 sequence (Ile-Leu-Gly-His-Arg-Asp-Tyr-Lys) into the peptide mimic (Fig. 1 and supplemental Fig. S1A) involved, in brief, a systematic analysis of the sequence using alanine and enantiomeric amino acid scans, which highlighted the importance of the Arg and Asp side chains; replacement of the hydrophobic termini with hydrocarbon pharmacophores and the Gly-His residue by different
indolizidinone turn mimics (21–24); and refinement near the Arg-Asp residue using different amino acid substitutions to arrive at the pyridylalanine-β-homophenylalanine surrogate. The 3-phenylacetamido indolizidin-2-one 9-carboxyl and PDC113.824 (Fig. 1B) are thus believed to mimic the active β-turn geometry about the Gly residue and the signaling pharmacophore of the Arg-Asp-Tyr triad in the parent peptide, respectively.

**Tocolytic Effects of PDC113.824 in Normal and Preterm Labor Models**—We first verified that the peptide mimetic, PDC113.824, acted as a tocolytic agent in vivo in normal parturition. Mice near term (gestational day 17.5) were treated or not with PDC113.824, and delivery was assessed in the animals (Fig. 2A). Results showed that PDC113.824 significantly delayed delivery compared with untreated animals who all delivered at term (day 19). Indeed, at day 19 only 50% of PDC113.824-treated animals had delivered. Delivery of all PDC113.824-treated animals was delayed to day 20.

We also tested if PDC113.824 would also block provoked preterm labor using LPS, known to promote a general inflammatory state, which results in prostaglandin synthesis, and induce premature delivery (16). Results showed that for all the animals tested, delivery occurred within 12 h following LPS injection into mice at gestational day 16 (Fig. 2, B and C). Pretreatment with PDC113.824 prior to LPS injection significantly delayed delivery, such that by gestational day 17, only 20% of treated animals delivered. As was the case for normal term delivery (e.g. saline treatment), PDC113.824-treated mice did not deliver until day 19 even when treated with LPS. Hence, PDC113.824 significantly extended the average time of delivery following LPS treatment by ~20 h as compared with untreated animals (Fig. 2C). We also verified whether PDC113.824 interfered specifically with PGF2α-induced labor. Animals were treated or not with PDC113.824 prior to injection with PGF2α at gestational day 15.5 (Fig. 2D). All PGF2α-treated animals delivered rapidly by 2 h post-injection. Again, PDC113.824 treatment markedly delayed delivery in PGF2α-injected animals, as around 40% of the animals had delivered by days 16~17 post-injection (Fig. 2D). Accordingly, the mean time of delivery was significantly increased in the presence of PDC113.824 (Fig. 2F).

To ensure that the observed labor-delaying effect of our synthetic tocolytic was mediated through its actions on the uterus, and to dissociate its potential effects on luteolysis, which would decrease levels of the natural tocolytic progesterone produced in the ovary (7), we isolated myometrium from spontaneous post-partum mice and assessed the direct effects of PDC113.824 on spontaneous and PGF2α-induced contraction (supplemental Fig. S2). Results showed that PDC113.824 significantly reduced both the strength and duration of both PGF2α-induced and spontaneous myometrial contraction in a dose-dependent manner, consistent with the increased expression of FP in the uterus during labor, which occurs even in rodents (6). Taken together, our results suggest that PDC113.824 delays both term and preterm labor, at least in part through the inhibition of uterine contraction.

**PDC113.824 Negatively Modulates PGF2α-mediated Myometrial Cell Contraction and Rho/ROCK Signaling**—The putative inhibitory effects PDC113.824 on PGF2α-mediated contraction were next examined in myometrial cells. Human myometrial smooth muscle cells (hTERT-C3) were used, because they have been previously shown to respond to uterotonic factors and to retain their contractile properties (19). As a prelude to these experiments, the expression of endogenous FP receptor in these cells was confirmed using a monoclonal antibody raised against the receptor (clone 3E12/2B2, Fig. 3A). Antibody labeling was shown to be specific for FP receptor,

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**FIGURE 3.** PDC113.824 inhibits PGF2α-induced cellular contraction in myometrial cells. A, confocal images of FP receptor staining on hTERT-C3 myometrial cells using a mouse monoclonal anti-FP antibody (clone 3E12/2B2). Top left: FP receptor staining in stably expressing FP receptor cells; top right: FP receptor in hTERT-C3 cells; and bottom left: labeling in naïve HEK293 cells; top right: FP receptor staining in stably expressing FP receptor cells; bottom left: labeling in naïve HEK293 cells. Scale bar represents 10 μm. B, specific binding of [3H]PGF2α to myometrial cells incubated with: vehicle (binding buffer), 10 μM AL8810 or 10 μM PGF2α. C, images of collagen lattice contraction following pretreatment with vehicle, PDC113.824 (PDC) or C3 exoenzyme (C3) and subsequent PGF2α or oxytocin (OT) stimulation. White dashed lines depict the area used to quantify contraction. D, quantification of contraction from cells with different pretreatments, followed by agonist stimulation. Results are representative of at least three independent experiments. *, p < 0.05, **, p < 0.01 compared with vehicle.
because immunofluorescent signals were not detected in HEK293 cells, which do not express endogenous FP receptor, but were detected at the cell surface in HEK 293 cells transfected with FP receptor. Strong labeling of FP receptor at the cell surface was also detected in myometrial cells (Fig. 3A). We also quantified the levels of endogenous FP receptor at the plasma membrane in myometrial cells using [3H]PGF2α binding studies (Fig. 3B). Both PGF2α and the selective FP receptor antagonist AL-8810 displaced bound [3H]PGF2α from myometrial cells, and FP receptor expression was ~5–10 fmol/mg total protein.

Next, PGF2α and PDC113.824 regulation of myometrial cell contraction was tested in vitro using a cell-induced collagen lattice contraction assay (Fig. 3C). Cells were layered on top of collagen and grown for a period of 16 h, which resulted in a decrease in the diameter of the collagen matrix due to self-contraction of the cells. Addition of PGF2α further increased contraction of hTERT-C3 cells. PDC113.824 alone had no effect on hTERT-C3 contraction per se. On the other hand, it inhibited PGF2α-mediated cell contraction responses (Fig. 3, C and D). Moreover, PDC113.824 exhibited no effect on the myometrial contraction induced by a distinct uterotonic agent, oxytocin (Fig. 3D, OT), demonstrating the specificity of the action of the compound for FP receptor. Because the contractile function of FP receptor on smooth muscle has been shown to involve Rho-kinase activation (25, 26), we also verified its contribution on myometrial cell contraction. As shown in Fig. 3 (C and D), both basal and agonist-induced cell contractions were dependent on Rho GTPase activation, because C3 exoenzyme blocked both responses.

To assess how PGF2α-dependent activation of Rho was regulated by PDC113.824, we next used a biosensor for Rho activation expressed in HEK293 cells, because these cells are considerably easier to transfect than myometrial cells. We first characterized the binding properties of FP receptor in these cells by stably expressing HA-tagged receptors (hereafter referred to as FP receptor cells, see “Experimental Procedures”). No specific [3H]PGF2α binding to untransfected cells was detected (Fig. 4A, inset). However, radiolabeled PGF2α binding on FP receptor-expressing cells was robustly displaced by both unlabeled PGF2α as well as AL-8810 (Fig. 4A, inset). The effect of PDC113.824 on [3H]PGF2α binding was also tested in these cells. PDC113.824, at concentrations that inhibited myometrial contraction (1 μM) or higher (10 μM), displaced no more than 15% of PGF2α binding to the FP receptor.

Rho family GTPases are known FP receptor effectors (13), whose activities can be regulated by Gα_{12/13} (27). Using FP receptor cells, we next tested PGF2α-dependent activation of Rho GTPases by imaging the fluorescent FRET-based biosensor Raichu-RBD, which consists of the RBD of Rhotekin flanked by the FRET pair YFP and CFP (20). Under basal conditions, intramolecular interaction of YFP and CFP in the Rho biosensor generated a detectable FRET signal (Fig. 4B). Binding of endogenous, activated GTP-bound Rho to this biosensor following FP receptor stimulation induced a decrease in FRET signal. Although PDC113.824 alone had no effect on Rho activation (data not shown), the response to PGF2α stimulation was decreased in its presence (Fig. 4B). Quantification of the FRET signal was assessed by measuring changes in the YFP/CFP ratio (20) and revealed a time-dependent, agonist-mediated activation of Rho (Fig. 4C, i.e. decrease in FRET signal). PGF2α-stimulated Rho activity was greatly

![Image](50x410 to 407x734)

FIGURE 4. PGF2α-mediated Rho activation is inhibited by PDC113. 824. A, binding of [3H]PGF2α to FP receptor cells in the presence of 1 μM PGF2α, 10 μM AL-8810, 1 μM or 10 μM PDC113.824. Inset: HEK293 cells transfected with pcDNA3.1 were incubated with [3H]PGF2α alone or with 1 μM unlabeled PGF2α. B and C, FP receptor cells were transfected with Raichu-RBD and pre-treated or not with 1 μM PDC113.824, followed by PGF2α stimulation. FRET signals were recorded as described under “Experimental Procedures” and represented as pseudocolor changes (B), or quantified by calculating the YFP/CFP intensity ratio (C) and D. C, YFP/CFP ratio with or without pre-treatment with PDC113.824 in a time course of stimulation using PGF2α, D, YFP/CFP ratio on FP receptor or AT, R-expressing cells following different treatments. NT, untreated cells; C3 exo or Gα_{12/13}: cells were pre-treated with C3 exoenzyme for 4 h or co-transfected with Gα_{12/13} (Q231L/D299N) followed by stimulation with PGF2α for 25 min. The scale bar represents 10 μm. Results are representative of six (A), eight (B and D, left panel), and five (D, right panel) independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001 compared with not treated.
reduced at all time points in the presence of PDC113.824. PGF2α-mediated activation of Rho, like contraction in myometrial cells was sensitive to C3 exoenzyme (Fig. 4D).

We next assessed the contribution of Gα12 in Rho activation by expressing a dominant negative version of this Gα subunit (Gα12DN, Q231L/D299N, Fig. 4D, left panel). An inhibition of FP receptor-mediated Rho activation was observed in a cell line expressing the Gα12DN. This response was specific to FP receptor, because PDC113.824 had no significant effect on the angiotensin II (AngII) type 1 receptor (AT1R), another GPCR known to activate Rho (29) (Fig. 4D, right panel).

Rho GTPases can engage downstream targets, including the protein kinase ROCK (30), as well as promoting actin cytoskeletal rearrangement (31). We therefore assessed the effect of PGF2α and PDC113.824 on reorganization of the actin cytoskeleton manifested by membrane ruffling using phalloidin staining. Cell ruffling, as characterized by morphological rounding of cell edges, was detected after 5 min of PGF2α stimulation (Fig. 5A) and persisted for more than 60 min (data not shown). This response was dependent on FP receptor-mediated activation of Gα12, because co-expression of Gα12DN strongly inhibited PGF2α-induced cell ruffling (Fig. 5B). Pretreatment of cells with PDC113.824 again had a significant inhibitory effect on PGF2α-mediated cell ruffling (Fig. 5, D and E), while treatment of cells with either selective Rho GTPase (C3 exoenzyme) or ROCK (Y27632) inhibitors, both blocked completely PGF2α-mediated cell ruffling (Fig. 5, C and E).

We also assessed the potential involvement of other signaling pathways downstream of the FP receptor on cell ruffling. Treatment of cells with selective inhibitors for PKC (Gö6983) or phosphatidylinositol 3-kinase (LY294002, data not shown) had no effect on membrane ruffling. The extent to which ERK1/2 MAPKs were involved in regulating cell ruffling was assessed, because they have been proposed to modulate Rho signaling (32). Treatment of cells with the MEK1 inhibitor (PD98059) blocked only weakly PGF2α-mediated cell ruffling (Fig. 5E). Taken together our results suggested that PDC113.824 acts as an allosteric modulator of FP receptor-mediated myometrial contraction and cytoskeletal reorganization through inhibition of the Rho/ROCK signaling pathway.
Figure 6. PDC113.824-mediated increase of PGF2α-dependent ERK1/2 activation. A, effect of PGF2α and PDC113.824 on ERK1/2 MAPK activation. FP receptor cells were serum-starved for 30 min prior to treatment with various times with either PGF2α (1 μM, top panel) or PDC113.824 (2 μM, bottom panel). Cell lysates were analyzed by Western blot using anti-phospho-ERK and anti-total ERK antibodies. “+” denotes a control condition for MAPK activation from cells only stimulated with PGF2α (1 μM, 5 min). B-D, effect of PDC113.824 on MAPK activation induced by PGF2α in FP receptor cells (B), by AngII in AT1R cells (C), by PGF2α in hTERT-C3 myometrial cells. Cells were treated with 2 μM PDC113.824 for 30 min and then stimulated with increasing concentrations of PGF2α (B) or AngII (C) for 2 min or PGF2α for 5 min (D). Signals were quantified by densitometry and plotted in dose-response curves as fold over basal (i.e., not treated) activation versus PGF2α concentration. Results are representative of three (A) six (B), seven (C), and four (D) independent experiments. **, p < 0.01; ***, p < 0.001.

Figure 7. PGF2α-induced cellular contraction and ERK1/2 activation are independently regulated. A, effect of the MEK inhibitor, PD98059, on myometrial cell contraction. Left panel: hTERT-C3 cells were pre-treated with 10 μM PD98059 for 2 h prior to treatment with 1 μM PGF2α for 18 h. Quantification of myometrial cell contraction was calculated as in Fig. 3. Right panel: representative images of collagen lattices used for quantification. B, effect of C3 exoenzyme on PGF2α-mediated ERK1/2 activation. FP receptor cells were serum-starved for 30 min prior to pre-treatment with 1 μg/ml C3 exoenzyme for 4 h. Cells were then treated with PGF2α (1 μM) for indicated times. Cell lysates were analyzed by Western blot using anti-phospho-ERK and anti-total ERK antibodies. Bands were quantified by densitometry. Results are representative of four independent experiments. ***, p < 0.001 compared with DMSO or PD alone.
PDC113.824 prior to stimulation with PGF2α resulted in a 2-fold increase in the efficacy of the response as well as a 2-fold left shift in the potency of PKC recruitment (PDC113.824, EC50 = 1.17 nM; Fig. 8E). The specificity of PDC113.824 for FP receptor was again demonstrated, because it had no effect on AngII-dependent PKC activation (Fig. 8F). Together, these results suggest that PDC113.824 acts as positive allosteric modulator (PAM) on the PKC-MAPK signaling pathway induced by PGF2α.

PGF2α-mediated G Protein Coupling to FP Receptor Is Differentially Regulated by PDC113.824—Our ligand binding experiments suggested that PDC113.824 allosterically regulates FP receptor binding to PGF2α and/or its coupling to G proteins. Allosteric modulators are known to affect the off-rate of ligand binding to the orthosteric site on receptors (34, 35). We first investigated how PDC113.824 affected PGF2α binding to FP receptor by measuring the kinetics of dissociation of PGF2α. Upon exposure to excess cold agonist, the dissociation rate of [3H]PGF2α in the presence of PDC113.824 was increased by >1.5-fold as compared with control treatment with vehicle (Fig. 9A). On the other hand, no significant effect of PDC113.824 was observed on binding off-rates for AT1R (Fig. 9B).

We next assessed the effect of PDC113.824 on FP receptor coupling to G proteins by monitoring [35S]GTPγS loading onto Gαs and Gα12, following PGF2α stimulation. Preincubation of FP receptor cells with PDC113.824 alone increased [35S]GTPγS loading onto Gαs to levels similar to that of PGF2α (i.e., in the absence of PDC113.824). Consistent with the effects on PKC and ERK1/2, treatment with PDC113.824 also significantly

FIGURE 8. PDC113.824 is a positive modulator of PGF2α-induced PKC activation. A, effect of PKC inhibitor G66983 on PGF2α-mediated ERK1/2 activation. FP receptor cells were serum-starved for 30 min prior to pre-treatment with 1 μM of G66983 for 30 min. Cells were then treated with PGF2α (1 μM) for indicated times. Cell lysates were analyzed by Western blot using anti-phospho-ERK and anti-total ERK antibodies. Bands were quantified by densitometry. B and C, quantification of PKC translocation using FP cells transiently transfected with PKCβI-GFP (see “Experimental Procedures”). Fluorescence intensity as a function of the pixel distance (C) is taken from a line crossing the cell (B). D, images obtained by confocal microscopy showing translocation of PKCβI-GFP to the cell membrane following PGF2α and PDC113.824 treatment. E and F, quantification of the PKCβI-GFP translocation in FP receptor cells (E) or AT1R cells (F). Cells were serum-starved for 30 min, treated either with vehicle (water) or PDC113.824 (2 μM) for 30 min, and then stimulated with increasing concentrations of PGF2α or AngII. The scale bar represents 10 μM. Results are representative of four (A), five (B–E), or three (F) independent experiments. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
activation of AT1R, which also resulted in [35S]GTPγS binding to Gα12, half-life of [125I]AngII in presence of vehicle or PDC113.824; **p < 0.05 compared with PDC (Fig. 9, E and F).

DISCUSSION

Here, we describe the design and characterization of PDC113.824 as a new allosteric modulator with biased signaling properties on FP receptor, acting both as potent tocolytic agent in vivo and as an inhibitor of myometrial contraction in vitro and ex vivo. PDC113.824 actions were specific to FP receptor, because no significant effects were observed on two other GPCRs, AT1R and the oxytocin receptor. Functional studies revealed that PDC113.824 increased agonist-mediated activation of MAPK by FP receptor via Gαq, whereas it inhibited cytoskeletal rearrangement and myometrial contraction through uncoupling of the receptor to the Gα12−Rho-ROCK signaling pathway (summarized in Fig. 10). The functional selectivity of PDC113.824 toward two distinct and opposite G protein-dependent events supports the biased nature of this compound on PGF2α-mediated, FP receptor-dependent signaling.

A hallmark of allosteric affinity modulators is their ability to promote conformational changes in the receptor, which mechanistically can translate into alterations in the dissociation kinetics of preformed orthosteric ligand-receptor complexes (36, 37). These effects, however, are not seen if interacting ligands compete for the same orthosteric site. Our findings that PDC113.824 partially decreases [3H]PGF2α binding to the FP receptor (Fig. 4A, albeit no more than 15% of maximum binding) suggested that it can either act as an allosteric modulator of orthosteric site affinity (i.e. through a conformational change in the orthosteric binding site) and/or as a weak (partial) competitive ligand. The increased rate of dissociation of PGF2α in the presence of PDC113.824 is, however, more consistent with a conformational change in the orthosteric binding site of the receptor, which mechanistically can translate into alterations in the dissociation kinetics of preformed orthosteric ligand-receptor complexes (36, 37). These effects, however, are not seen if interacting ligands compete for the same orthosteric site. Our findings that PDC113.824 partially decreases [3H]PGF2α binding to the FP receptor (Fig. 4A, albeit no more than 15% of maximum binding) suggested that it can either act as an allosteric modulator of orthosteric site affinity (i.e. through a conformational change in the orthosteric binding site) and/or as a weak (partial) competitive ligand. The increased rate of dissociation of PGF2α in the presence of PDC113.824 is, however, more consistent with a conformational change in the orthosteric binding site of the receptor, which mechanistically can translate into alterations in the dissociation kinetics of preformed orthosteric ligand-receptor complexes (36, 37).
FIGURE 10. **PDC113.824-mediated biased signaling effects through FP receptor.** PGF2α induces ERK1/2 activation via Goq, and actin reorganization and contraction through Go12. PDC113.824 increases the coupling of the FP receptor to Goq, which increases PGF2α-mediated activation of PKCβ and ERK1/2 (i.e., acts as a positive allosteric modulator, PAM). In contrast, PDC113.824 reduces PGF2α-induced cytoskeletal reorganization, modulation of cell ruffling, and myometrial cell contraction via decreased coupling of FP to Go12 (i.e., acts as a negative allosteric modulator, NAM). FP receptor-mediated activation of phospholipase Cβ (PLCβ) and production of inositol trisphosphate (IP3) promotes intracellular Ca2+ release, which stimulates Ca2+-dependent, calmodulin-mediated activation of myosin light chain kinase and subsequent phosphorylation of myosin light chain to promote smooth muscle cell contraction. Activation of the Rho-ROCK pathway through Go12 facilitates inhibitory regulation of the myosin light chain (MLC) phosphorylation and the increase in myosin light chain phosphorylation, which maintains the cell in the contracted state (50).

The extracellular loops of GPCRs have been demonstrated to partially compete with PGF2α for the orthosteric binding site, our results, showing that PDC113.824 minimally decreases total binding of PGF2α while increasing agonist off-rate kinetics of ligand binding to the receptor, strongly suggest that PDC113.824 primarily acts as a negative affinity allosteric modulator of the FP receptor.

Although a number of studies have characterized the allosteric properties of synthetic compounds on different GPCRs (see Refs. 38 and 39 for review), their potential to bias receptor signaling remains largely unexplored. To our knowledge, PDC113.824 represents the first example of a synthetic allosteric modulator derived from a specific region of a GPCR (e.g., the NH₂-terminal domain of the second extracellular loop of FP receptor), which promotes functional selectivity for two distinct G protein-mediated signaling events. Our study not only provides conceptual insights into FP receptor signaling relevant to myometrial contraction, but also for the potential development of new classes of tocolytic drugs and other allosteric GPCR modulators with biased signaling properties.

The extracellular loops of GPCRs have been demonstrated to be important for both ligand recognition and allosteric modulation of certain receptors (40–43). Transmembrane domains involved in ligand recognition and receptor activation have also been shown to influence extracellular loop conformations (44, 45). For instance, light-dependent activation of rhodopsin has been shown to induce changes in the conformation of the second extracellular loop. Moreover, a recent study on the β₂-adrenergic receptor also revealed that ligands known to differently affect the conformation of transmembrane domains and subsequent receptor activity also stabilize distinct conformation of the second extracellular loop (45). Thus, PDC113.824, which mimics structural features of the second extracellular loop of the FP receptor, could constrain the conformation of agonist-bound receptor into selective coupling configurations, promoting more efficient Goq activation, while reducing coupling to Go12. Interestingly, differential G protein coupling with the FP receptor was modulated by PDC113.824 even in the absence of the orthosteric ligand, suggesting that it primes two distinct pre-existing receptor/G protein complexes of FP receptor/Goq and FP receptor/Go12 but in distinct ways. Alternatively, PDC113.824 may be capable of interacting with receptors that are constitutively active but not necessarily pre-coupled per se. Although PDC113.824 acted as a negative allosteric modulator for agonist binding, it seemed to affect how FP receptor coupled to G protein in the absence of agonist in a biased fashion.

Our study also yields new information regarding FP receptor signaling and the mechanisms underlying myometrial contraction and cytoskeletal remodeling. We observed that blocking MAPK activation had only a marginal effect on cell ruffling, a response dependent on the Go12-Rho-ROCK signaling pathway (46, 47), and no effects on myometrial cell contraction. Inhibiting Rho only affected cytoskeletal rearrangement and not FP receptor-dependent activation of ERK1/2. Our findings suggest an important role of the Go12-Rho-ROCK pathway in regulating myometrial cell contraction and that Go12, which is involved in controlling the PKC-MAPK signaling pathway, is independently regulated. However, we cannot exclude that other signaling events upstream of MAPKs, such as those seen for the PDC113.824-dependent increase in FP receptor-mediated activation of PKC, contribute to blocking functional coupling of FP receptor to Go12/13.

Our findings underscore the importance of FP receptor signaling through the Go12-Rho-ROCK pathway as a pharmacological target in the management of parturition and preterm labor. Our in vitro and in vivo data are consistent with both the observations that RhoA activity is increased in the myometrium during pregnancy, and that inhibition of ROCK blocks both PGF2α- and LPS-induced preterm labor in mice (48–50). However, the extent to which MAPK contributes to myometrial contraction and preterm labor remains an open question (51). That PDC113.824 sensitizes PGF2α-dependent activation...
of ERK1/2 in myometrial cells, while inhibiting myometrial contraction and parturition in mice, suggests that MAPK plays a minor role in uterine contraction. Development of biased FP receptor ligands that selectively engage the Goq-PKC-MAPK signaling pathway, without affecting the Goq2-Rho-ROCK signaling pathway, will be of great value in addressing this issue.

To date, only a very few examples of biased allosteric modulators have been described that direct GPCR signaling toward distinct effector pathways. The metal ion Gd³⁺ allosterically modulates the orthosteric ligand glutamate for the mGluR1 homodimer to promote differential coupling of the receptor to either Goq or Gαi (52). It is unlikely, however, that Gd³⁺ acts in a selective manner, because it could affect other class C GPCRs.

A calcium-sensing receptor autoantibody has also been shown to potentiate the Ca²⁺/Gαq response, while inhibiting the Goαq-dependent activation of MAPK (53). Recently, a drug screen has identified a new allosteric antagonist of NK2 receptor, which biases the receptor toward increased Ca²⁺ signaling, while inhibiting cAMP production (54). PDC113.824 represents a significant addition to this new “repertoire” of allosteric modulators that bias receptors toward distinct G protein-dependent signaling events. Our findings are also distinguishable, because they highlight the possibility of developing GPCR-specific synthetic allosteric and biased modulators by constructing mimics to particular regions of a given receptor. Ligands acting on orthosteric sites have also been shown to bias GPCR signaling (55, 56). However, because allosteric sites on receptors are presumably more diverse than orthosteric sites, it is likely that many allosteric ligands described to act on GPCRs will also turn out to have unsuspected biased signaling properties.

The clinical utility of allosteric compounds has recently attracted more attention (57–59). These modulators can be used at saturating concentrations, because their effects are only revealed in the presence of endogenous ligands (e.g. neutral allosteric ligands), potentially reducing adverse effects (60–62). The design of allosteric ligands with biased signaling properties, as in the case of PDC113.824, offers not only the advantage of specificity for a single GPCR, but also selectivity for a specific subset of signaling pathways, further reducing unwanted side effects. At present, tocolytic drugs used in clinic have significant off target and/or non-selective actions (14, 15). Sympathomimetics (e.g. β-agonists), or non-steroidal anti-inflammatory drugs (e.g. indomethacin) target multiple tissues and organs leading to unwanted responses in both the mother and fetus. Moreover, the benefit of oxytocin receptor blockade using antagonists (e.g. Atosiban) in preventing pre-term labor remains limited, because the oxytocin receptor, in contrast to FP receptor, is not involved in regulating the initial stages of preterm parturition (7, 8). Thus, the future development of biased, allosteric compounds specific for FP receptor will not only help further our understanding of mechanisms underlying parturition, but may also contribute to the design of better and more selective tocolytic drugs.

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