

Characterization of a Rabbit Kidney Prostaglandin F_{2α} Receptor Exhibiting G_i-restricted Signaling That Inhibits Water Absorption in the Collecting Duct*

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PGF_{2α} is the most abundant prostaglandin detected in urine; however, its renal effects are poorly characterized. The present study cloned a PGF-prostanoid receptor (FP) from the rabbit kidney and determined the functional consequences of its activation. Nuclease protection assay showed that FP mRNA expression predominates in rabbit ovary and kidney. *In situ* hybridization revealed that renal FP expression predominates in the cortical collecting duct (CCD). Although FP receptor activation failed to increase intracellular Ca²⁺, it potently inhibited vasopressin-stimulated osmotic water permeability (*L*_p, 10⁻⁷ cm/(atm·s)) in *in vitro* microperfused rabbit CCDs. Inhibition of *L*_p by the FP selective agonist latanoprost was additive to inhibition of vasopressin action by the EP selective agonist sulprostone. Inhibition of *L*_p by latanoprost was completely blocked by pertussis toxin, consistent with a G_i-coupled mechanism. Heterologous transfection of the rabbit FP into HEK293 cells also showed that latanoprost inhibited cAMP generation via a pertussis toxin-sensitive mechanism but did not increase cell Ca²⁺. These studies demonstrate a functional FP receptor on the basolateral membrane of rabbit CCDs. In contrast to the Ca²⁺ signal transduced by other FP receptors, this renal FP receptor signals via a PT-sensitive mechanism that is not coupled to cell Ca²⁺.

Prostaglandin F_{2α} is a major prostaglandin excreted in urine (1) and is known to exert potent biological effects via its G protein-coupled cell membrane receptor designated the F-prostanoid (FP)² receptor (2–6). FP receptor mRNA is highly expressed along the genitourinary tract including ovary > uterus and kidney (7–9). Recent studies in mouse kidneys have shown that FP receptor expression is particularly abundant in the distal convoluted tubules and cortical collecting duct (10). The cellular and functional consequences of renal FP receptor activa-

tion in these renal epithelia have not been determined. Like PGE₂ (11), intravenous infusion of PGF_{2α} causes both a natriuresis and diuresis (4); however, because high concentrations of PGF_{2α} can cross-activate prostaglandin E₂-EP3 receptors (12, 13), the molecular basis of PGF_{2α}-associated diuresis remains uncertain. The functional activity of PGE₂ in the rabbit collecting duct epithelium has been elucidated through use of the *in vitro* microperfused tubule (9, 14–16); however, the effect of selective FP receptor activation in this segment has not previously been examined. The aim of the present study was to clone the rabbit FP receptor, determine its intrarenal distribution, and elucidate its functional activity in the kidney.

MATERIALS AND METHODS

cDNA Cloning of a Full-length Rabbit FP Receptor—Total RNA was purified from rabbit ovary using TRIzol reagent (Invitrogen), treated with RNase-free DNase I (Promega), and reverse-transcribed to single-stranded cDNA using Molony murine leukemia virus reverse transcriptase and 2.5 μM of random hexamers according to the manufacturer's protocol (GeneAmp RNA PCR kit; PerkinElmer Life Sciences). The synthesized cDNA was then used to amplify a portion of rabbit FP cDNA using a pair of selective primers designed from conserved sequences of mouse and human FP. The upstream sense primer sequence was 5'-CTG TGC CCA CTT CTT CTA GGC-3', and the downstream antisense primer sequence used was 5'-GTA GAG ATT CTT CAG GAC AGC-3'. PCRs were carried out in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTPs, and 1 μM primers at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min for 30 cycles using a PerkinElmer Life Sciences 2400 thermal cycler. The predicted 591-bp fragment was ligated into pCRTM II 2.1 vector (Invitrogen) and sequenced. BLAST and CLUSTAL were used to determine homology to the coding region of human and mouse FP receptors.

5'-RACE and 3'-RACE were used to obtain the 5' ends of rabbit FP cDNA using the above cloned fragment of rabbit FP. Two gene-specific antisense primers were designed for 5'-RACE: primer 1 (5'-GCA CAA GAA TGA AAC ACC AAG-3') and nested primer 2 (5'-TCT ATC TTC CCA GTC TTC AAC-3'). Two gene-specific sense primers for 3'-RACE were synthesized for obtaining the sequence downstream of the known FP cDNA: a 3' primer (primer 3, a 21-mer, 5'-AGC AAC ACA GAC AAG GGA GAT-3') and a nested primer (primer 4, a 21-mer, 5'-CGC TTT TTG CTC TTC GTA TGG-3'). 1 μg of total RNA from rabbit kidney (see above) was used as a template for the first strand cDNA synthesis, with primer 1 (for 5'-RACE) or the adapter primer (for 3'-RACE) and 200U SuperScriptTM II reverse transcriptase according to the manufacturer's description (Invitrogen). The first

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² The abbreviations used are: FP, F-prostanoid receptor; CCD, cortical collecting duct; PGF_{2α}, prostaglandin F_{2α}; AVP, arginine vasopressin; RACE, rapid amplification of cDNA ends; RT, reverse transcription; UTR, untranslated region; MES, 4-morpholineethanesulfonic acid; EP3, E prostanoid receptor, subtype 3.

strand cDNA was purified, and amplification of the 5'-terminal and 3'-terminal cDNA sequences was performed using primer 2 (for 5'-RACE) or primer 3 and the abridged anchor primer. For 3'-RACE, the PCR product was then used in a second nested PCR with the internal primer 4 and the abridged universal amplification primer. The PCR conditions were as follows: 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 2 min for 35 cycles. PCR products were subcloned into pCR II vector (Invitrogen) and then sequenced.

Construction of an Expression Vector Containing a Full-length FP cDNA—A full-length rabbit FP was obtained by RT-PCR using a pair of primers located in the regions of 5'-UTR and 5' coding region (5'-ACG ACA ATG TCC ATG AAC AAT TCC-3') and 3'-UTR and 3' coding region (5'-GTA TTA CTG TCC TGT TAA GCT CGG-3'). A 1,107-bp full-length rabbit FP cDNA was subcloned into pRC/CMV2 vector (FP/CMV) and sequenced (Invitrogen).

Expression of Rabbit FP Receptor in HEK293 Cells—HEK293 cells were maintained at 37 °C in humidified air containing 5.5% CO₂ in Dulbecco's modified Eagle's medium high glucose supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, and 100 units/ml neomycin. The cells were transfected with rabbit FP receptor tagged at the N terminus with c-Myc using Lipofectamine 2000 (Invitrogen). Clonal cell lines were selected by the addition of medium containing 400 µg/ml G418 at 48 h post-transfection. The cells expressing Myc-tagged rabbit FP receptors were isolated by fluorescence-activated cell sorting using fluorescein isothiocyanate-conjugated anti-c-Myc antibody and cultured in G418-containing medium.

[³H]PGF_{2α} Binding in FP-transfected HEK293 Cells—The cells were rinsed once with ice-cold phosphate-buffered saline containing 1 mM EDTA and lysed by scraping in lysis buffer (15 mM HEPES, pH 7.6, 5 mM EDTA, 5 mM EGTA, 40 mM indomethacin, 2 mM phenylmethylsulfonyl fluoride) and passage through a 21-gauge needle five times. The cell lysate was layered on a 60% sucrose cushion and centrifuged at 150,000 × g for 1 h at 4 °C. The membrane fraction was passed through a 26-gauge needle five times and frozen at -80 °C.

Membranes (30 µg of protein) were incubated with [³H]PGF_{2α} (214 Ci/mmol; Amersham Biosciences) in the absence or presence of unlabeled ligands for 1 h at 30 °C in binding buffer (10 mM MES/KOH, pH 6.0, 1 mM EDTA, 10 mM MnCl₂). The binding reaction was terminated by the addition of 3 ml of ice-cold binding buffer and rapidly filtered under vacuum over Whatman GF/F filters. The filters were washed three times with 3 ml of ice-cold binding buffer, dried, and counted in 4 ml of Ultima Gold scintillation fluid (Packard Biosciences, Groningen, The Netherlands). For saturation binding experiments, nonspecific binding was determined in the presence of 10 nM PGF_{2α} (Cayman). Competition binding experiments were performed in the presence of 2 nM [³H]PGF_{2α}.

Intracellular Calcium Assay—HEK-FP cells were plated on poly-D-lysine coated 96-well black wall clear bottom plates at a density of 80,000 cells/well and used the next day for calcium assay by the FLEX station system (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. Briefly, growth medium was removed by aspiration, and 200 µl of Calcium-3 reagent (Molecular Devices) reconstituted in Hanks' buffered saline solution supplemented with 20 mM HEPES and 10 µM probenecid was added to each well. The cells were then loaded for 60 min at 37 °C. Subsequently, the plates were transferred to a FLEX station for acquisition of fluorometric data. Twenty µl of agonists were added in parallel wells, whereas the [Ca²⁺]_i-sensitive fluorescence was monitored (λ_{ex} = 485 nm, λ_{em} = 525) at 2-s intervals.

Intracellular cAMP Assay—HEK-FP cells were seeded (20,000 cells/well) overnight in complete medium. Thirty minutes before agonist addition, the medium was replaced with Opti-MEM I containing 500

µM isobutylmethylxanthine. The cells were preincubated with 10 µM latanoprost (free acid) for 20 min and then stimulated by 1 µM forskolin for 5 min. [cAMP]_i levels were estimated using an enzyme fragment complementation immunoassay according to the manufacturer's instructions (DiscoverX Corporation, Fremont, CA).

Activation of EP3 Receptor Signaling by Prostanoids via pCRE/lacZ—When the rabbit EP3 receptor is transfected into HEK293 cells, it signals via a cyclic AMP response element (17). HEK293 cells were plated at ~50% confluence were co-transfected with 4 µg of the rabbit EP3 splice variant 77A (17) cDNA and 4 µg of pCRE/lacZ plasmid using Lipofectamine 2000, following the manufacturer's instructions. Six hours after the addition of DNA-Lipofectamine complex, the medium was aspirated and replaced with Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum. Twenty four hours after transfection, the cells were plated in 96-well plates at a density of 5 × 10⁴ cells/well in 100 µl of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 20 µM indomethacin containing 5 mM sodium butyrate, and the cells were incubated an additional 16–18 h, at which point the cells had reached confluence. Increasing concentrations of EP3 agonist sulprostone and FP agonist latanoprost free acid were made at 2× concentration in 100 µl of volume to appropriately meet the final concentration in serum-free OPTI-MEM medium and incubated for another 6 h. After 6 h, the medium was aspirated and gently washed once with phosphate-buffered saline. The plates were developed using 4 mg/ml of substrate chlorophenol red β-D-galactopyranoside and read at 570 nm absorbance (17). The cells transfected with pRC/CMV empty vector were treated as control for this experiment. The data were analyzed using Prism 4.0 and the equation: sigmoidal dose response $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{\text{LogEC}_{50} - X})$.

Solution Hybridization/RNase Protection Assays—To determine the tissue distribution of the FP receptor mRNA expression in rabbit, the 591 bp of rabbit FP cDNA fragment amplified as described above was subcloned into pCRII, and a specific riboprobe was synthesized. RNase protection assays were performed as previously described (16). Briefly, the plasmids containing the rabbit FP (591 bp) and glyceraldehyde-3-phosphate dehydrogenase (174 bp) were linearized, and radiolabeled were riboprobes synthesized in vitro from 1 µg of linearized plasmids using a MAXIscript™ kit (Ambion) for 1 h at 37 °C in a total volume of 20 µl. The reaction buffer contained 10 mM dithiothreitol, 0.5 mM each ATP, CTP, and GTP, 2.5 mM of UTP, and 5 µl of 800 Ci/mmol [³²P]UTP at 10 mCi/ml (PerkinElmer Life Sciences). Hybridization buffer included 80% deionized formamide, 100 mM sodium citrate (pH 6.4), and 1 mM EDTA (RPA II; Ambion). Twenty µg of total RNA from various rabbit tissues were incubated at 45 °C for 12 h in hybridization buffer with 5 × 10⁴ cpm labeled riboprobes. Following hybridization, ribonuclease digestion was performed (37 °C for 30 min), and protected fragments were precipitated and separated on a 6% polyacrylamide gel at 200 V for 4 h. The gel was exposed to Kodak XAR-5 film overnight at -80 °C with intensifying screens.

In Situ Hybridization—Kidneys from New Zealand White rabbits were utilized. A ³⁵S-labeled antisense riboprobe generated from the 591-bp PCR fragment of rabbit FP (see above) was hybridized to the tissue sections and then washed as previously described (18). The slides were dehydrated with graded ethanol containing 300 mM ammonium acetate, dipped in emulsion (Ilford K5; Knutsford, Cheshire, UK) and exposed for 4–5 days at 4 °C. After developing in Kodak D-19, the slides were counterstained with hematoxylin. Photomicrographs were taken using a Zeiss Axioskop microscope with either dark field or bright field optics.

FP mRNA Expression in Microdissected Nephron Segments—An alternative used was to map receptor mRNA by the more sensitive tech-

nique of RT-PCR. As a first step in this procedure, amplification of a fragment of the rabbit FP receptor from whole kidney was performed. Total RNA was purified using TRIzol reagent, treated with RNase-free DNase I, and transformed to single-stranded cDNA. cDNA was amplified using FP receptor selective primers derived from homology to human FP sequence: (nucleotides 780–2146 of the human cDNA sequence yielding a 1365-bp product): sense, 5'-TCTTAGCCCTTG-GTGTTC A-3'; antisense, 5'-TGGCTCAAGGTAAAGCACCT-3'. Tubule segments were microdissected from collagenase-digested kidneys prepared as previously described (19), and FP receptor was amplified by RT-PCR. PCRs contained 100 nM primers, 1 μM Mg²⁺, 40 nM deoxynucleotide triphosphates, reaction buffer, and 10 units of *Pfu* Turbo[®] DNA polymerase (Stratagene) in a final volume of 50 μl. PCR was performed by hot start, using 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 68 °C for 3 min. Thirty-five PCR cycles were also performed with the rabbit glyceraldehyde-3-phosphate dehydrogenase primers sets to normalize for RNA loading.

In Vitro Microperfusion of Collecting Ducts—Individual freehand microdissected cortical collecting ducts were perfused *in vitro* as previously described (15, 20). Briefly, rabbits weighing 1.5–2.5 kg were sacrificed by using an intramuscular injection of ketamine (44 mg/kg) and xylazine (11 mg/kg) for anesthesia followed by decapitation. The left kidney was quickly removed, and 1–2-mm coronal slices were placed in chilled dissection dishes for freehand dissection. Tubules were transferred to a thermostatically controlled chamber of 1-cm³ volume and cannulated using concentric micropipettes. Bath solution was continuously exchanged at 0.5 ml/min by infusion pump (Sage; Orion Research Incorporated, Cambridge, MA) and was maintained at 37 °C. The composition of standard bath medium, dissection medium, and isotonic perfusate were as follows: 105 mM NaCl, 25 mM NaHCO₃, 10 mM sodium acetate, 2.3 mM NaHPO₄, 5 mM KCL, 1.8 mM CaCl₂, 1.0 mM MgSO₄, 8.3 glucose, and 5 mM alanine (osmolality, 300 mOsmol/kg). The composition of hypotonic perfusate was identical to that of isotonic perfusate except for a reduction in NaCl concentration to 30 mM, yielding 150 mOsmol/kg, and the addition of [³H]inulin to the perfusate as a volume marker. Both isotonic and hypotonic perfusate also contained 0.2 mg/ml Food, Drug, and Cosmetic dye No. 3 (Aniline and Chemical Co., Chicago, IL) to detect cell damage and perfusate leakage (15, 20).

Measurement of Water Flux (Hydraulic Conductivity, L_p) in Microperfused Tubules—Perfusate containing [³H]inulin (75 μCi/ml) was collected into a constriction pipette of known volume (between 90 and 130 nl) for determination of [³H]inulin counts/nl (PerkinElmer Life Sciences). The luminal perfusion rate was maintained between 12 and 20 nl/min by adjusting the hydrostatic pressure. At this perfusion rate osmotic equilibration between bath and lumen did not occur.

During the initial 45 min after cannulation, all of the tubules were perfused with an isotonic solution similar to the bath. Subsequently, the perfusate was changed the hypotonic solution. Base-line hydraulic conductivity was determined following an additional 30 min of further equilibration from three timed collections. AVP (46 pM) was then added to the bath, and after a 15-min equilibration period three or four additional timed collections were performed for hydraulic conductivity (L_p). A stable L_p was usually observed 20–50 min after the addition of AVP. Subsequently, latanoprost or sulprostone (0.1 μM) was added (latanoprost/sulprostone + AVP). Six more timed collections were made for L_p determination. In each period the three collections with the greatest calculated L_p were averaged to calculate the mean L_p for this period. Data from tubules with a negative basal L_p (reflecting an inulin leak) were discarded.

In other experiments latanoprost was added prior to the addition of vasopressin. Three timed collections of luminal perfusate were made in

to a pipette of known volume for calculation of basal hydraulic conductivity (L_p). Subsequently, the PGF_{2α} analogue, latanoprost-free acid (1 μM), was added to the bath, and after a 15–30-min equilibration period, three additional timed collections were made to determine the effects of latanoprost on the control L_p. AVP (46 pM) was added in the continued presence of latanoprost, and after 10 min of equilibration, six timed collections were made. The peak L_p was defined as the mean of the three largest contiguous L_p values. Peak L_p was usually observed 15–25 min after the addition of AVP.

Calculation of Hydraulic Conductivity (L_p)—Net volume reabsorption (J_v) was calculated as: J_v = (V_i - V_o)/L, where V_i is the perfusion rate (nl/min), V_o represents the collection rate (nl/min), and L is the tubule length. V_o was measured directly, and V_i was calculated as: V_i = V_o (cpm_o/cpm_i), where cpm_o and cpm_i are perfusate and collected fluid ³H counts·min⁻¹·nl⁻¹, respectively. Hydraulic conductivity (L_p; 10⁻⁷ cm³/atm·s) was calculated according to Dubois *et al.* (21).

$$L_p = (1/RTS) \cdot (1/O_b)^2 [O_b \cdot (V_i - V_o) + O_i \cdot V_i \cdot \ln\{(O_b - O_i) \cdot V_1 / (O_b \cdot V_o - O_i \cdot V_i)\}] \quad (\text{Eq. 1})$$

where R is the gas constant, T is temperature (K), S is the CCD lumen surface area (assumed luminal diameter of 20 μm), and O_b and O_i represent the osmolality of the bath and perfusate, respectively.

Measurement of Intracellular Calcium Concentration [Ca²⁺]_i in Isolated Perfused CCDs—Tubules were perfused *in vitro* as previously described (20). The methods were similar to those described above with the following exceptions: 1) the bath perfusion chamber was a special low volume (0.150 ml) chamber to allow for rapid fluid exchange and 2) bath solution was preheated in using a heated water-jacketed line, and flow rate was between 0.5 and 2.5 ml/min. Tubules were bathed in 2.5 μM acetoxymethyl ester of fura-2 (Molecular Probes, Eugene, OR) (22) for 45 min at 30 °C (flow rate, 0.5 ml/min). After loading, the flow rate was increased to 2.5 ml/min, and the bath temperature was increased to 37 °C, and CCDs were equilibrated for 20–30 min. Intracellular fura-2 fluorescence intensity was measured using continuous rapidly alternating excitation (20 ms/reading) from dual monochrometers set at 340 and 380 nm, respectively (Deltascan, Photon Technology International, New Brunswick, NJ). The monochromator output was coupled to the inverted microscope using a 400-nm dichroic mirror and a 100× lens (Nikon fluor oil immersion). Fluorescent emission of light greater than 435 nm was measured by photon counting. Before loading with fura-2, CCD autofluorescence and background light were measured; this value was continuously subtracted from all measurements. The corrected emission intensity ratio, using 340- and 380-nm excitation (340/380 ratio, R), was continuously recorded by a computer interface. Changes in intracellular calcium concentration ([Ca²⁺]_i) were estimated from the change in the 340/380 ratios.

Reagents—AVP, EGTA, PGF_{2α}, and PGE₂ were purchased from Sigma. ³H-Labeled inulin was purchased from PerkinElmer Life Sciences. Acetoxymethyl ester of fura-2 and 4Br-A23187 were purchased from Molecular Probes. Latanoprost, cloprostenol, fluprostenol, and sulprostone were purchased from Cayman Chemical (Ann Arbor, MI). Indomethacin was purchased from Sigma. Chlorophenol red β-D-galactopyranoside was purchased from Roche Applied Sciences. Lipofectamine 2000 and OPTI-MEM were purchased from Invitrogen.

Statistics—Student's t tests for unpaired data were used when only two unrelated treatment groups were compared. To determine the statistical significance of differences between more than two groups, analysis of variance and the Student-Newman-Kuel multiple comparison

1 gggggatagggggcgcgggggcggggtgcccagtcggcgcaccgagcagcgggctcctctgctcagcagggcgcccgagatgtccagatgtctggactgca 100
 101 agcctgcaccgttctgagagggagatgactggagtgccagcatttatctccacgaca ATG TCC ATG AAC AAT TCC AAA CAG CCA GTA 188
 1 M S M N N S K Q P V 10

189 TCT CCT GCA GCT GGG CTC CTT TCA AAC ACA ACC TGC CAG ACG GAA AAC CGG ATT TCT GTA TTT TTT TCA ATA ATC 263
 11 S P A A G L L S N T T C Q T E N R I S V F F S I I 35

264 TTC ATG ACA GTG GGA ATC CTA TCA AAC AGC CTT GCC ATT GCC ATT CTC ATG AAG GCC TAT CAG AGG TTT AGA CAG 338
 36 F M T V G I L S N S L A I A I L M K A Y Q R F R Q 60

339 AAG TCC AAA GCA TCG TTT CTG CTT TTA GCC AGT GGC CTG GTA ATC ACA GAC TTC TTC GGA CAT CTC ATC AAT GGA 413
 61 K S K A S F L L L A S G L V I T D F F G H L I N G 85

414 GCA ATA GCT GTG TTT GTA TAT GCA TCT AAT AAG GAT TGG ATC CGC TTT GAT CAA TCA AAT GTC CTG TGC AGT ATT 488
 86 A I A V F V Y A S N K D W I R F D Q S N V L C S I 110

489 TTT GGT ATC TGC ATG GTA TTC TCT GGC CTG TGC CCA CTT TTT CTA GGC AGT GTG ATG GCC ATT GAG CGA TGT ATT 563
 111 F G I C M V F S G L C P L F L G S V M A I E R C I 135

564 GGA GTC ACC AAA CCC ATA TTT CAT TCC ACG AAA ATT ACA TCC AAG CAT GTG AAA ATC GTG TTG AGT GGT GTG TGC 638
 136 G V T K P I F H S T K I T S K H V K I V L S G V C 160

639 TTG TTT GCC ATT TTC ATA GCT TTG TTG CCC ATC CTT GGG CAC CGA GAC TAT AAA ATT CAA GCG TCA AGG ACC TGG 713
 161 L F A I F I A L L P I L G H R D Y K I Q A S R T W 185

714 TGT TTC TAC AAC ACT GAG CAC GTT GAA GAC TGG GAA GAT AGA TTT TAT CTT CTG CTT TTT TCT TTT CTG GGG TTC 788
 186 C F Y N T E H V E D W E D R F Y L L L F S F L G F 210

789 TTA GCC CTT GGT GTT TCA TTC TTG TGC AAT GCC ATC ACA GGA ATT ATA CTT TTA AGG GTT AAA TTT AAG AGT CAG 863
 211 L A L G V S F L C N A I T G I I L L R V K F K S Q 235

864 CAA CAC AGA CAA GGG AGA TCT CAT CAT TTA GAA ATG GTC ATC CAA CTC CTG GCT ATA ATG TGT GTC TCC TGC ATT 938
 236 Q H R Q G R S H H L E M V I Q L L A I M C V S C I 260

939 TGC TGG AGT CCA TTT CTG GTA ACG ATG GCC AAC ATT GGA ATA AAT GGA AAT GAT TCT ATG GAA ACC TGT GAA ACA 1013
 261 C W S P F L V T M A N I G I N G N D S M E T C E T 285

1014 ACG CTT TTT GCT CTT CGT ATG GCA ACA TGG AAT CAA ATA TTA GAT CCT TGG GTG TAT ATT CTT CTG CGG AAG GCT 1088
 286 T L F A L R M A T W N Q I L D P W V Y I L L R K A 310

1089 GTC CTG AAG AAT CTC TAC AAG CTT GCC AGG CGC TGC TGT GGA GCA CAT GTC ATC AGC CTA CAC ATT TGG GAG CTT 1163
 311 V L K N L Y K L A R R C C G A H V I S L H I W E L 335

1164 AGC TCC ATT AAA AAC TCC TTA AAG GTT GCT GCC ATT TCT GAG TCA CCA GTT CCA GAG AAA ATA AAT CAG GAA GCA 1238
 336 S S I K N S L K V A A I S E S P V P E K I N Q E A 360

1239 CCG AGC TTA ACA GGA CAG TAA tacagtgtagggctagagcaacattaggggaagattttggcaagatatcagtttaattagtaaatatataca 1331
 361 P S L T G Q * 367

1332 catatataatagcctaactggaaaattcaggcctcggtatcacagtttgggaagtatttctgctcagttcagttcttggattttgttcgattaactgtat 1431
 1432 aattgcacatttcaactgttcttctgcaactggagatagacacataaaaataaggctgtgggagtcacaacaggtaccagttttgagcttatctgtgttatt 1531
 1532 tgtgcttttgaaatgagtgaaatctcttatgttaaagcctaattgtattacctggccttttggctagagaatattcttaagctacatgcacagtgaaatgg 1631
 1632 ctattttgtgaaaagtgttctgcagctattacttagagactaggccttaaggagatagggcaccctcattatttgatgtgatctgttttattcctcattat 1731
 1732 gtgacctcagtcfaatataatccatggtcatgacacctggagtttactgtggtttatatagtgactaccacatttggcaggctctgcttgagcctgatcttgt 1831
 1832 aagcctgttttagaatgggctcattcttgcacatatttacaacacagattgcttgcatttattatgatgaaggacaaggattttttgaaggggaaatttttt 1931
 1932 tgggtggaaaaaatggattttagtatttccagtaattcctcctcctgtgaaaatgagtgatgcacactaatttgtggataagcaaggctataatatttgc 2031
 2032 atatatccatgcaacctgggttatagcagttcccaagctccactagggataatgaaaacaagtaggatccctgcccacatgataggtgcaagaacattg 2131
 2132 aaggcttttaccttgagcca

FIGURE 1. cDNA of Rabbit FP receptor. The underlined sequence represents the 591-bp fragment originally amplified from ovary and used for nuclease protection assays. The double underlined sequence represents primers used to amplify products from kidney, used to obtain 3' coding and UTR sequence and in RT-PCR on microdissected nephron segments.

test was used. The differences of $p < 0.05$ were considered statistically significant. The data are presented as the means \pm S.E.

RESULTS

cDNA Cloning of Full-length Rabbit FP Receptor—A 591-bp fragment of rabbit FP was amplified by RT-PCR from rabbit ovary using a pair of primers designed from conserved sequences of mouse and human FP. 5'-RACE yielded a single 755-bp fragment, sharing high sequence homology to human and mouse FP sequences. 3'-RACE yielded an

additional single 1,137-bp fragment. This product overlaps the original 591-bp PCR product by 92 bp and extended the known rabbit FP 3' sequence by 1,045 bp. The sequence includes a termination codon, TAA, in the open reading frame, as well as a 892-bp 3'-UTR with no poly(A) tail or putative polyadenylation signal, suggesting there may be an additional 3'-UTR sequence missing.

Assembly of the RT-PCR and RACE product sequences yielded a putative full-length rabbit FP open reading frame consisting of 1,101 bp (Fig. 1) encoding a polypeptide of 366 amino acids with an estimated

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                                     TM I
>rabbit      MSMNNSKQPVSPAAGLLSNTTCQTENRISVFFSII FMTV GILSNLSLAIAILMKAYQRFQ
>human      MSMNNSKQLVSPAALLSNTTCQTENRLSVFFSVIFMTV GILSNLSLAIAILMKAYQRFQ
>mouse      MSMNNSKQPVSPAAGLIANTTCQTENRLSVFFSII FMTV GILSNLSLAIAILMKAYQRFQ
***.*** *****. *; *****; *****; *****; *****; *****; *****; *****; *****; *****; *****

                                     TM II
>rabbit      KSKASFLLLASGLVITDFFGH LINGAIAV FVYASNKDWIRFDQSNVLC SI F G I C M V F S G L
>human      KSKASFLLLASGLVITDFFGH LINGAIAV FVYASDKEWIRFDQSNVLC SI F G I C M V F S G L
>mouse      KSKASFLLLASGLVITDFFGH LINGGIAV FVYASDKDWIRFDQSNILC SI F G I S M V F S G L
*****.*****.*****. *; *****; *****; *****.*****

                                     TM III
                                     TM IV
>rabbit      CPLFLGSVMAIERCIGVTKPIFHSTKITSKHVKIVLSGVCLFAIFIALLPILGHRDYKIQ
>human      CPLLLGSVMAIERCIGVTKPIFHSTKITSKHVKMMLSGVCLFAVFIALLPILGHRDYKIQ
>mouse      CPLFLGSAMAIERCIGVTNPIFHSTKITSKHVKMILSGVCMFAVFAVLPILGHRDYQIQ
***.***.*****.*****.*****.*****.*****.*****.*****.*****.*****.*****.*****.*****

                                     TM V
>rabbit      ASRTWCFYNTTEHVEDWEDRFYLL LFSFLGFLALGV SFLCNAITG I ILLRVKFKSQQHRQG
>human      ASRTWCFYNTEDIKDWEDRFYLL LFSFLGFLALGV SLLCNAITG I ILLRVKFKSQQHRQG
>mouse      ASRTWCFYNTTEHIEDWEDRFYLL LFSFLGFLALGV SFCNAVTVGVTLLRVKFRSQQHRQG
*****. :; *****; *****; *****; *****; *****; *****; *****; *****; *****

                                     TM VI
                                     TM VII
>rabbit      RSHHLEMVIQLLAIMCVSVCICWSPFLV TMANIGINGNDSMETCETT L FALR MATW NQ I L D
>human      RSHHLEMVIQLLAIMCVSVCICWSPFLV TMANIGINGNHSLET CETT L FALR MATW NQ I L D
>mouse      RSHHLEMVIQLLAIMCVSVCWSPFLV TMANIAINGNNSPVCETT L FALR MATW NQ I L D
*****;*****;*****;*****.***.* *****

>rabbit      PWVYILLRKAVLKNLYKLARRCCGAHVISLHIWELSSIKNSLKVA AIS E S P V P E K I N Q E A
>human      PWVYILLRKAVLKNLYKLASQCCGVHVISLHIWELSSIKNSLKVA AIS E S P V A E K S A S T
>mouse      PWVYILLRKAVLRNLYKLASRCCGVNIISLHIWELSSIKNSLKVA AIS E S P A E K E S Q Q A
*****;***** :***. :;*****;*****;*****;*****;*****;*****;*****;*****

>rabbit      PSLTGQ
>human      -----
>mouse      SSEAGL

```

FIGURE 2. Alignment of predicted amino acid sequences for rabbit, human and mouse FP receptors. Putative transmembrane domains 1–7 are indicated by the solid line above the amino acid sequence and numbered with *TM* followed by the appropriate Roman numeral.

molecular mass of 40,995 Daltons (Fig. 1). Comparison of the amino acid sequence of rabbit FP with human and mouse homologs revealed 92.76 and 87.70% identity, respectively (Fig. 2). A NCBI Conserved Domain Search revealed that the cloned rabbit FP sequence encodes a protein belonging to the rhodopsin family of seven transmembrane receptor (www.ncbi.nlm.nih.gov/Structure/cdd/).

Tissue Distribution of Rabbit FP mRNA—RNase protection assays on mRNA harvested from different rabbit tissues ($n = 3-4$ for each tissue) revealed that FP mRNA was highly expressed in the ovary as seen in other species including sheep and humans (8, 23–26). In addition, lower levels of FP mRNA expression were detected in the kidney with barely detectable expression in other tissues tested including heart, liver, spleen, lung, stomach, large intestine, and aorta (Fig. 3).

Localization of FP Receptor mRNA in Rabbit Kidney—In situ hybridization confirmed that FP receptor exhibits localized expression along the nephron in rabbit kidney (Fig. 4A). As shown in Fig. 4A, specific FP mRNA signals were detected in the kidney using an antisense riboprobe, with no signal detected using the sense riboprobe. Microscopic examination showed that expression of FP receptor mRNA was especially abundant in Tamm Horsfall negative distal tubules that on high magnification were determined to include cortical collecting ducts (Fig. 4C). Expression of FP receptor was confirmed by RT-PCR of microdissected nephron segments.

Effects of FP Receptor Agonists in Microperfused Rabbit Collecting Ducts—Vasopressin increases osmotic water permeability of the collecting duct by binding to basolateral V2 receptors, activating a G_s -cAMP-coupled mechanism that inserts aquaporin-2 water channels in

the apical membrane of the collecting duct (27). PGE_2 antagonizes vasopressin-stimulated water absorption via both an EP3-coupled pertussis toxin-sensitive mechanism and an EP1-coupled pertussis toxin-insensitive/ Ca^{2+} -dependent mechanism (16, 28, 29). The FP protein sequence exhibits significant sequence homology to both PGE_2 EP1 and EP3 receptors. We therefore tested whether like EP1/3 active agonists, the FP selective agonist latanoprost (free acid form) inhibits AVP-stimulated water flow.

AVP (46 pM) increased water absorption (L_p) in the microperfused CCD roughly 30-fold, from basal levels of 9 ± 2 to 325.9 ± 16.8 $10^{-7} \cdot \text{cm}/(\text{atm} \cdot \text{s})$ ($n = 6$). Subsequent addition of $1 \mu\text{M}$ latanoprost dramatically reduced AVP-stimulated water permeability to 190 ± 17.6 $10^{-7} \cdot \text{cm}/(\text{atm} \cdot \text{s})$, representing a reduction in L_p of 42% compared with AVP-treated time controls ($n = 6$; Fig. 5). To examine whether latanoprost activates a separate pathway, distinct from EP1 and EP3 receptors, we tested whether effects of the FP selective agonist latanoprost and the EP1/EP3 agonist sulprostone (12, 15, 30) were additive. A combination of maximally effective concentrations of latanoprost (1 μM) plus sulprostone (0.1 μM) inhibited AVP-stimulated water absorption by $75 \pm 2.5\%$ from 325.9 ± 16.8 to 73 ± 9 $10^{-7} \cdot \text{cm}/(\text{atm} \cdot \text{s})$ ($n = 6$; Fig. 5C). This inhibition was significantly greater than the percentage of inhibition achieved with supramaximal concentrations of either latanoprost alone (10 μM , $41 \pm 3\%$ inhibition, $p < 0.001$) or sulprostone alone (1 μM , $47 \pm 4\%$ inhibition, $p < 0.001$; Fig. 5C). These additive effects are consistent with latanoprost and sulprostone inhibiting vasopressin action via distinct receptors.

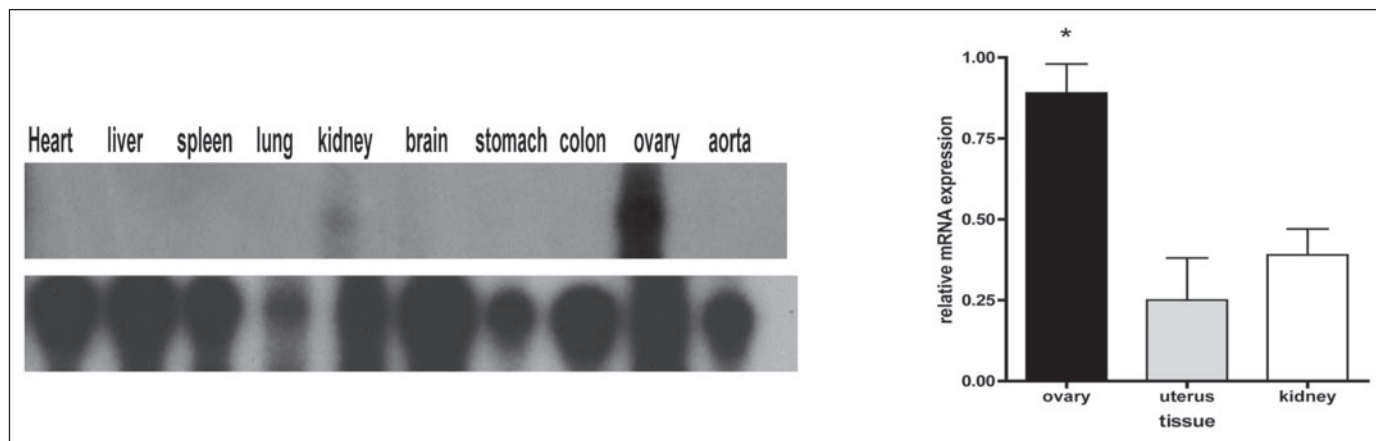


FIGURE 3. **A representative nuclease protection assay for FP receptor mRNAs in rabbit tissues.** Assays repeated on different tissue samples three to four times (depending on the tissue) yielded similar results. Relative expression levels in those tissues in which FP mRNA was detected was measured by densitometry and shown in the right-hand panel. *, $p < 0.05$ versus other tissues, analysis of variance.

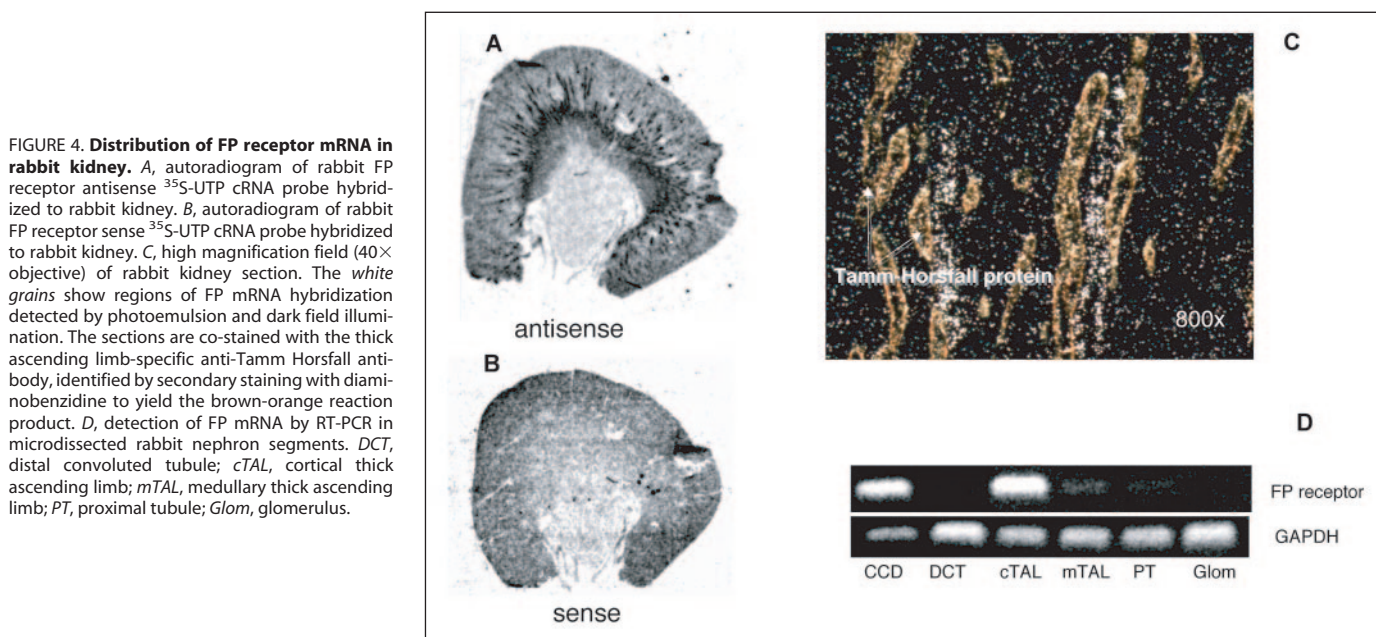


FIGURE 4. **Distribution of FP receptor mRNA in rabbit kidney.** *A*, autoradiogram of rabbit FP receptor antisense ^{35}S -UTP cRNA probe hybridized to rabbit kidney. *B*, autoradiogram of rabbit FP receptor sense ^{35}S -UTP cRNA probe hybridized to rabbit kidney. *C*, high magnification field ($40\times$ objective) of rabbit kidney section. The white grains show regions of FP mRNA hybridization detected by photoemulsion and dark field illumination. The sections are co-stained with the thick ascending limb-specific anti-Tamm Horsfall antibody, identified by secondary staining with diaminobenzidine to yield the brown-orange reaction product. *D*, detection of FP mRNA by RT-PCR in microdissected rabbit nephron segments. *DCT*, distal convoluted tubule; *cTAL*, cortical thick ascending limb; *mTAL*, medullary thick ascending limb; *PT*, proximal tubule; *Glom*, glomerulus.

Pertussis Toxin Blocks Latanoprost Action—Rabbit CCDs were pretreated with pertussis toxin (500 ng/ml, 1 h, 37°C), a selective irreversible inactivator of the inhibitory guanine nucleotide-binding regulatory protein (G_i/G_o). This protocol has been previously shown to prevent PGE_2 inhibition of AVP-induced L_p in the rabbit CCD (20). Pretreatment with pertussis toxin blocked the ability of latanoprost to inhibit vasopressin-stimulated water flow (Fig. 5B). This indicates that latanoprost inhibits AVP action in rabbit CCD via a G_i -coupled pathway.

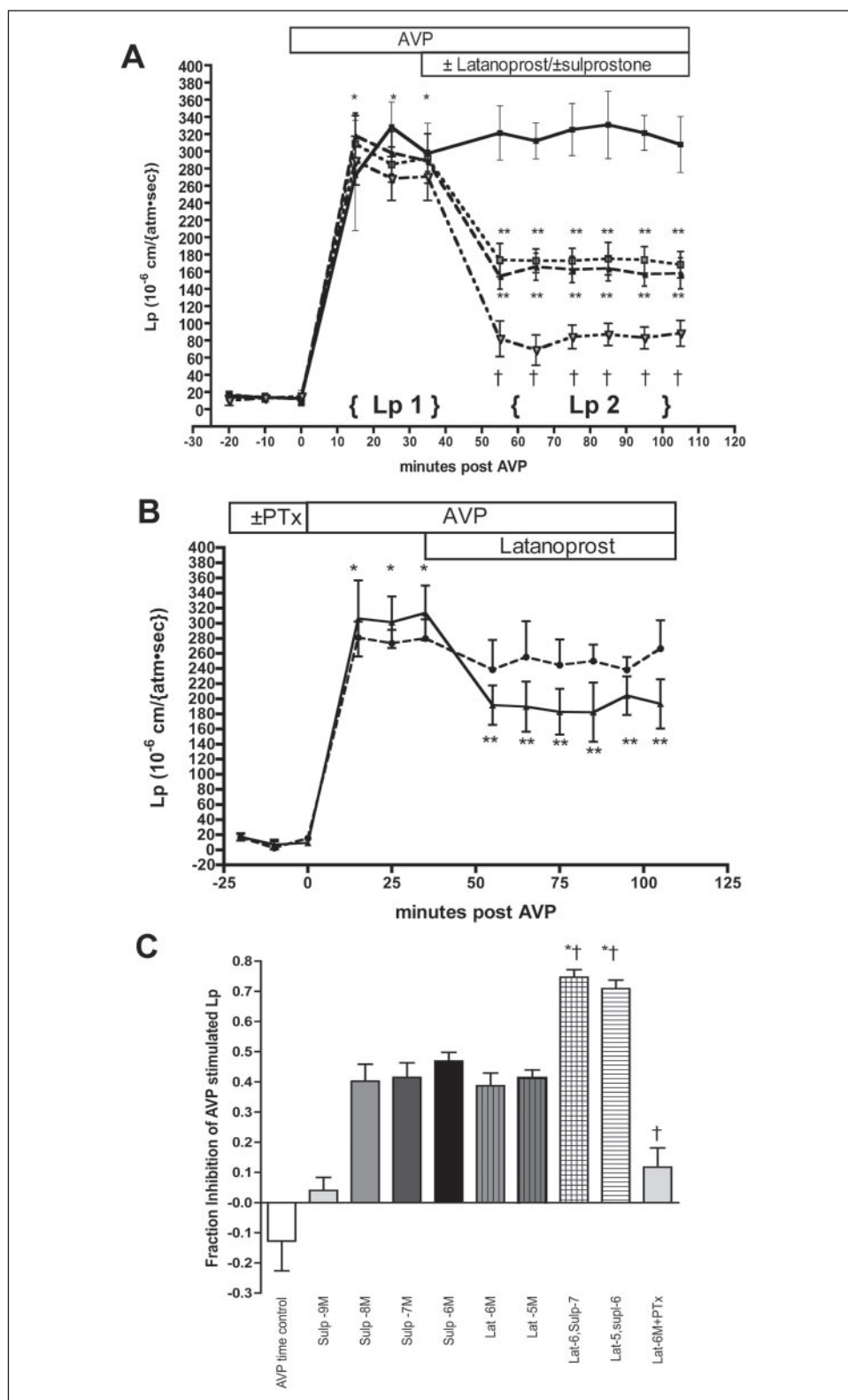
Effect of FP Receptor Agonists on Basal L_p in Cortical Collecting Duct—When administered, prior to vasopressin, $1\ \mu\text{M}$ latanoprost-FA did not alter water permeability over basal value (data not shown). Despite its lack of effect on basal water flow, latanoprost potently inhibited water permeability induced by the subsequent addition of AVP to the same CCDs to 139 ± 15 , a value significantly less than AVP controls at 330 ± 4 ($n = 6$, $p < 0.005$). Thus the FP receptor selective agonist latanoprost significantly inhibits AVP-stimulated L_p but does not increase basal L_p in rabbit CCD.

Effects of FP Agonists on $[\text{Ca}^{2+}]_i$ in Rabbit Collecting Duct—In cultured cells, both $\text{PGE}_{2\alpha}$ and latanoprost have been reported to signal via increased cell calcium associated with inositol phosphate synthesis (31–

34). We therefore measured the effects of several FP selective agonists on $[\text{Ca}^{2+}]_i$ in microperfused rabbit CCDs. Neither latanoprost free acid, cloprostenol, or fluprostenol significantly increased 340/380 emission ratio above basal values; however PGE_2 dramatically increased the 340/380 ratio above base line in these same fura-2-loaded microperfused CCDs. Although the endogenous ligand $\text{PGF}_{2\alpha}$ ($1\ \mu\text{M}$) also increased 340/380 ratios in rabbit CCDs, it was less potent than PGE_2 (Fig. 6). Because $\text{PGF}_{2\alpha}$ can bind to and activate EP1 and EP3 receptors (12, 30), the modest effects of $\text{PGF}_{2\alpha}$ on cell calcium may be mediated by an E-prostanoid receptor, known to be present in the collecting duct. The present results are consistent with the possibility that selective FP activation does not increase cell calcium despite the fact that the Ca^{2+} signaling pathway is intact and activated by EP receptors in the same preparation.

Signaling of the Cloned Rabbit FP in HEK Cells—To directly determine whether the cloned rabbit FP receptor couples to a G_i -dependent signal rather than Ca^{2+} in HEK293 cells as observed in microperfused native rabbit collecting ducts, we stably transfected HEK293 cell line with a c-Myc-tagged-rabbit FP receptor (Myc-RabFP-HEK; Fig. 7). Immunohistochemical localization of the c-Myc-tagged

FIGURE 5. A, effects of latanoprost free acid (dashed line, squares, $10 \mu\text{M}$), sulprostone (long dashed line, triangles, $1 \mu\text{M}$), and their combination (inverted triangles, short and long dashed line) on vasopressin-stimulated osmotic water permeability (control, solid line) in the microperfused rabbit cortical collecting duct. Periods in brackets represent values for L_{p1} and L_{p2} used to calculate the data in C. *, $p < 0.01$ versus basal L_p ; **, $p < 0.01$ versus AVP alone; †, $p < 0.01$ versus AVP versus latanoprost or AVP alone. B, effect of pertussis toxin (Ptx, $500 \text{ ng/ml} \times 90 \text{ min}$) pretreatment on latanoprost ($1 \mu\text{M}$) inhibition of AVP-stimulated water permeability in the collecting duct (dashed line). Effect of $1 \mu\text{M}$ latanoprost on AVP action without pertussis toxin pretreatment (solid line). *, $p < 0.01$ versus basal L_p ; **, $p < 0.01$ versus AVP alone. C, fractional inhibition of AVP stimulated L_p by increasing concentrations of sulprostone and latanoprost separately and in combination. Fractional inhibition is calculated as $(L_{p1} - L_{p2})/L_{p1}$ (see "Materials and Methods" and A). *, $p < 0.01$ versus sulprostone alone; †, $p < 0.05$ versus latanoprost alone.



receptor demonstrates localization primarily to the cell membrane (Fig. 7A). Specific [^3H]PGF $_{2\alpha}$ binding yielded a B_{max} of 0.7 ± 0.1 pmol/mg and a K_d of 7 ± 2 nM. Competition binding studies confirmed that latanoprost FA is a ligand for the cloned rabbit FP receptor with K_i of 0.7 ± 0.3 nM (Fig. 7B), equivalent to the K_i for PGF $_{2\alpha}$ (0.7 ± 0.1 nM).

Latanoprost (free acid, $1 \mu\text{M}$) inhibited [cAMP] $_i$ generation in forskolin-stimulated HEK cells. This effect was blocked by pretreatment with pertussis toxin (500 ng/ml), demonstrating that the cloned rabbit FP receptor couples to a G_i type G protein in HEK cells (Fig. 7). Untransfected HEK cells showed no response to latanoprost (data not shown).

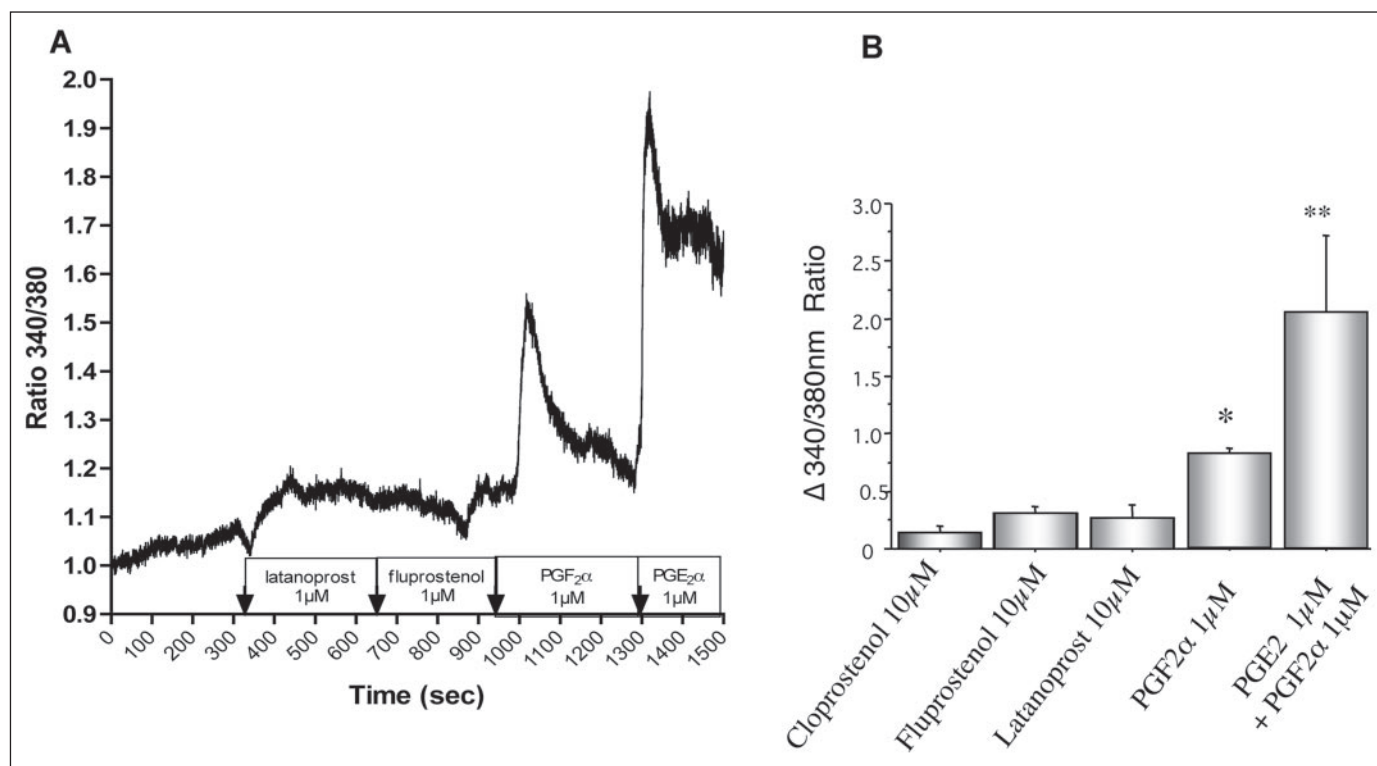


FIGURE 6. *A*, representative experiment showing the effects of three different FP ligands, 1 μM latanoprost free acid, fluprostenol, and $\text{PGF}_{2\alpha}$ versus the effect of PGE_2 on 340/380 emission ratio in a fura-2-loaded isolated perfused rabbit CCD. *B*, average change from base line in 340/380 ratios following basolateral treatment of isolated perfused CCDs with different FP agonists and PGE_2 . *, $p < 0.01$ versus base line.

We further examined whether latanoprost increases intracellular calcium in stable HEK293-FP transfectants. As shown in Fig. 7*B*, 10 μM latanoprost (free acid) failed to increase intracellular calcium in HEK-FP cells, whereas the calcium ionophore A23187 increased Ca^{2+} in these same cells. Furthermore transfection of HEK cells with the mouse EP1 receptor and treatment with an EP1 agonist 17 phenyl trinor PGE_2 induced a robust calcium increase, demonstrating that a prostanoid receptor-mediated calcium increase can be detected by this assay.

Finally, using HEK293 cells transfected with the rabbit EP3 receptor (the 77 A isoform) (17), we demonstrated that latanoprost is not a rabbit EP3 agonist, whereas sulprostone is a full agonist (data not shown). These findings confirm that latanoprost free acid does not signal via this other G_i -coupled prostanoid receptor.

DISCUSSION

The present studies characterized the functional effects of FP receptor activation in transfected HEK293 cells and the rabbit CCD using latanoprost free acid, a highly selective FP agonist (35, 36). Like PGE_2 , latanoprost significantly blunts vasopressin-stimulated osmotic water permeability in the rabbit CCD; however, unlike PGE_2 , latanoprost does not increase intracellular calcium in this segment (15, 20). This latter result was unanticipated because previous studies in cultured smooth muscle cells and fibroblasts demonstrate that FP receptor activation increases intracellular calcium and inositol trisphosphate production (32, 34, 37). Nevertheless, significant differences exist between the present studies and those previous studies, including the cell type tested (*e.g.* NIH3T3 fibroblasts and A7r5 vascular smooth muscle cells) and the agonist used.

To our knowledge the present studies are the first to examine the effects of FP agonists on cell calcium in differentiated epithelial cells. Although FP agonists did not increase calcium, the fact that PGE_2 sig-

nificantly increases $[\text{Ca}^{2+}]_i$ in the collecting duct (via the EP1 receptor, (15, 16)) demonstrates that the Ca^{2+} signaling pathway is intact in the CCD. Nevertheless it is not engaged by FP receptor activation in this epithelium. Nor is this inactivity limited to latanoprost, because neither fluprostenol nor cloprostenol, two distinct FP selective agonists, increased Ca^{2+} in the microperfused CCD. Taken together these findings demonstrate that latanoprost inhibits vasopressin-stimulated water absorption in the collecting duct via a mechanism independent of increased cell calcium.

PGE_2 antagonizes vasopressin-stimulated osmotic water permeability in the collecting duct through at least two pathways: an EP3 G_i -linked, pertussis toxin-sensitive pathway coupled to inhibition of cAMP generation (15, 27, 28, 38, 39) and an EP1 Ca^{2+} /protein kinase C-coupled pertussis toxin-insensitive pathway that inhibits cAMP-dependent protein kinase mediated events (15, 16, 29). Surprisingly, rather than coupling to increased $[\text{Ca}^{2+}]_i$, FP agonists appear to inhibit vasopressin action in the CCD primarily by coupling to a pertussis toxin-sensitive pathway. This unique coupling of the FP receptor could be a consequence of the specific G proteins expressed in the collecting duct or their relative expression levels. At present there is little known regarding the identity of the G proteins that mediate FP receptor action. To our knowledge there are no previous reports of FP receptor activity being mediated via a pertussis toxin-sensitive pathway (41, 42). Of the pertussis-sensitive G proteins, the collecting duct primarily expresses $G\alpha_{12}$ and less $G\alpha_{13}$ (43–45). Although the collecting duct also expresses $G\alpha_{q/11}$ (46), which has been implicated in Ca^{2+} -coupled EP1 signaling (47), this pathway does not appear to be engaged by the FP receptor in collecting duct.

Similar conclusions can be drawn from studies of the rabbit FP receptor signaling in HEK293 cells. These studies demonstrate latanoprost free acid is a high affinity ligand for the rabbit FP receptor with an IC_{50}

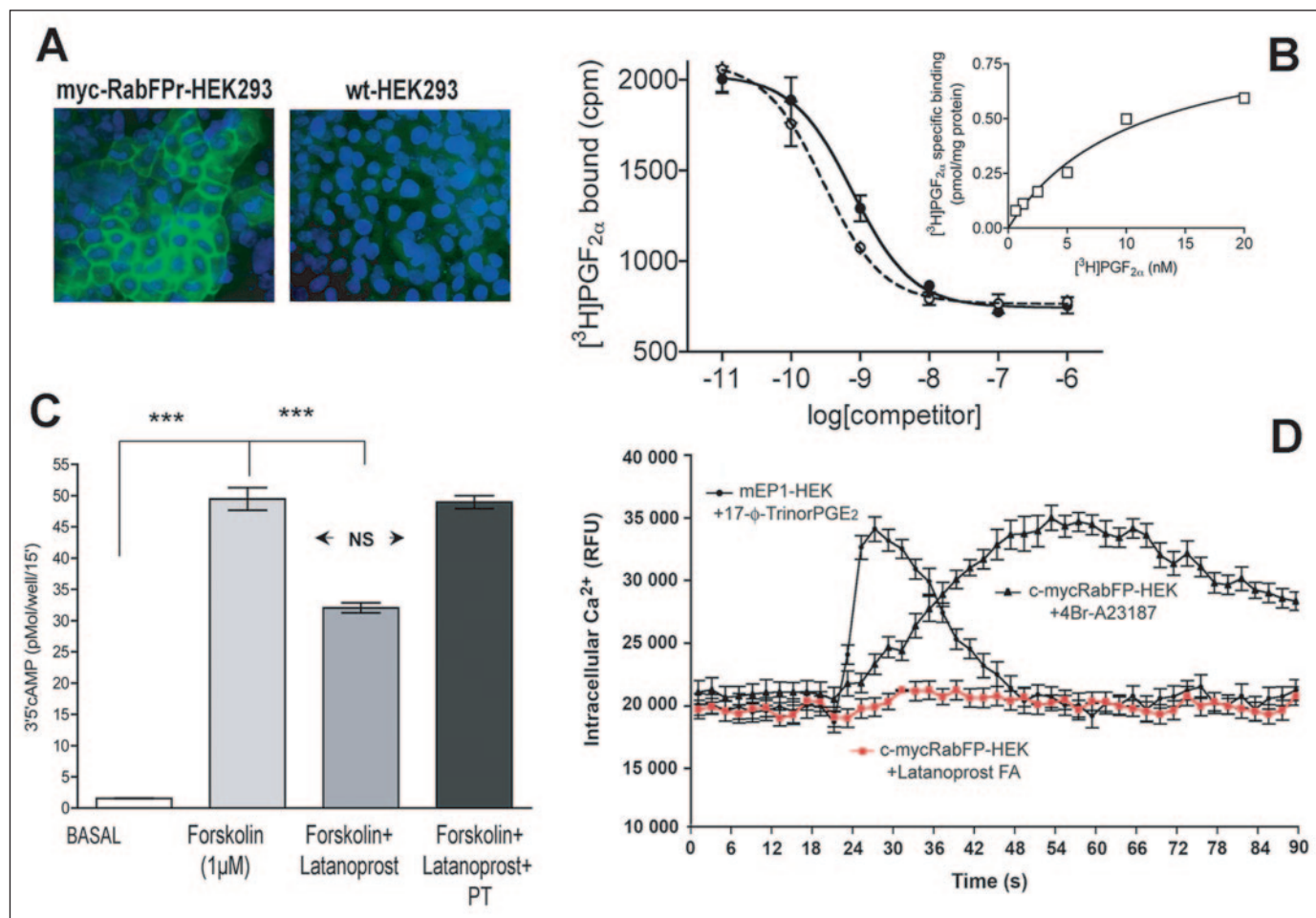


FIGURE 7. A, immunostain of c-Myc-tagged rabbit FP receptor in stable HEK293-FP transfectants. Green shows distribution of the c-Myc-tagged rabbit FP receptor (left panel) and its absence in Hoechst-stained nontransfected cells (right panel). B, competition for [³H]PGF_{2α} binding to c-Myc-RabFP HEK cells with increasing concentrations of unlabeled latanoprost free acid (dashed line) and PGF_{2α} (solid line, ± S.E.). The inset shows saturation isotherm for specific binding [³H]PGF_{2α} binding to c-Myc-RabFP HEK membranes. C, latanoprost-induced inhibition of [cAMP]_i. HEK-FP cells were preincubated with latanoprost 10 μM and then stimulated with 1 μM forskolin, and [cAMP]_i levels were measured (see "Material and Methods"). Where indicated, the cells were treated with 500 ng/ml pertussis toxin for 3 h before latanoprost and forskolin treatment (bars represent the means ± S.E. of three independent experiments. ***, *p* < 0.001, one-way analysis of variance Bonferonni post-test. D, [Ca²⁺]_i response in HEK-FP cells. [Ca²⁺]_i, expressed as fluorescence units, was monitored (described under "Material and Methods") in c-Myc-RabFP HEK cells before and after the addition of 10 μM latanoprost (free acid) or 10 μM 4Br-A23187. Mouse EP1 transfected HEK cells (mEP1-HEK) were responsive to 17-phenyl trinor PGE₂ as an agonist-stimulated positive control. The data points are given as the means ± S.E. (three replicates/data point).

in the range of ~1 nM, in agreement with its relatively high affinity for FP receptors in other species (35, 36). As in the native collecting duct, latanoprost free acid inhibits forskolin-stimulated cAMP generation via a pertussis toxin-sensitive mechanism. Despite binding to transfected HEK293 cells and inhibition cAMP generation, latanoprost does not increase cell calcium in these cells. Based on the present findings, the functional profile of FP receptor in appears to be more similar to the G_i-coupled EP3 receptor than the Ca²⁺-coupled EP1 receptor. Nevertheless in the rabbit collecting duct the FP pathway is distinct from the EP pathway, with the action of latanoprost and sulprostone additively inhibiting vasopressin action in this nephron segment.

Although the predicted amino acid sequence of the FP receptor exhibits the greatest sequence homology to EP1 and TP receptors, it is also quite similar to the EP3 receptor (48, 49). It is notable that the FP and EP3 receptors are syntenic, residing in close proximity on chromosome 1 in humans (50) and chromosome 3 in mice (51). In addition, multiple C-terminal splice variants exist for both FP and EP3 receptors (52–55). This is consistent with the possibility that FP and EP3 receptors might have arisen as a result of duplication of a single ancestral gene. In contrast TP and EP1 receptors are syntenic on human chromosome 19

(50). Within this syntenic pair, both receptors signal via pertussis toxin-insensitive Ca²⁺-coupled pathways (20, 47). Similarly, it is not inconceivable that, as for the EP3 receptor, a pertussis-sensitive signaling mechanism comprises a major pathway downstream of FP receptor activation (28, 56).

Despite differences in FP signaling identified in the present studies versus previous reports, the predicted rabbit FP receptor protein is highly homologous to FP receptors cloned from other species including cat, human, sheep, cow, pig, rat, and mouse (57–60). The cDNA encodes a predicted protein of 366 amino acid residues, and BLAST analysis shows that the rabbit FP receptor is most similar to feline and human FP receptors. Importantly these particular FP receptors have been shown to couple to inositol phosphate turnover and increased cell calcium (33, 61), so differences in FP receptor structure *per se* may not account for the observed differences in signal transduction. Rather the cellular context in which the FP receptor is expressed may determine its major downstream signaling mechanism.

As in humans and other species, nuclease protection showed that rabbit FP receptor mRNA expression is particularly abundant in ovary with lower expression levels in kidney (8, 25, 61). In mouse kidney FP

receptor mRNA is particularly abundant in the distal convoluted tubule and the initial cortical collecting duct, with little expression elsewhere (10). In contrast to the mouse, in the rabbit, renal FP receptor expression appears to be shifted to slightly more distal segments including late cortical and outer medullary collecting duct, but with little expression in microdissected distal convoluted tubule. In both rabbit kidney and mouse (10) there are also diffuse low levels of FP mRNA in the outer medulla that may include other less anatomically distinct structures such as interstitial cells or vasa rectae. The effects of FP receptor activation in these cells remain uncharacterized.

Previous reports demonstrate that like PGE₂ intravenous infusion of PGF_{2α} into rats is natriuretic and diuretic (4, 62). Because high concentrations of PGF_{2α} can activate EP1 and EP3 receptors as well as FP receptors (12, 30), the mechanisms contributing to these effects of PGF_{2α} remain unclear. The present findings now define the cortical collecting duct as a distinct target for FP receptor agonists. The renal cortex is a major source of endogenous PGF_{2α} production (40). By antagonizing vasopressin-stimulated water absorption in the CCD, endogenous renal PGF_{2α} production is likely to promote diuresis.

In summary using FP selective agonists, we have provided evidence for a direct effect of FP receptor activation to inhibit renal epithelial water absorption. Despite being homologous to FP receptors cloned from other species, the signaling of the FP receptor in the rabbit collecting duct differs from other reports in that it does not couple to increased cell calcium. Rather the major action of FP receptor activation appears to be linked to a pertussis toxin-mediated pathway. Whether similar calcium-independent effects of FP receptor activation also occur in other tissues remains to be determined.

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