Molecular and biochemical characterization of prostacyclin receptors in rat kidney

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Nasrallah, Rania, Joseph Zimpelmann, Sonia Singh, and Richard L. Hébert. Molecular and biochemical characterization of prostacyclin receptors in rat kidney. Am J Physiol Renal Physiol 280: F266–F277, 2001.—The prostacyclin (IP) message was detected by RT-PCR in the renal cortex, outer (OM) and inner medulla (IM), and in freshly isolated (IMCD-f) and cultured inner medullary collecting duct (IMCD-c), and also in the E-prostanoid (EP)1,3,4 receptor subtypes, but not EP2. Digoxigenin in situ hybridization localized IP mRNA in the tubules of the OM and IM, and the vasculature, and also in the glomeruli, arteries, and tubules of the cortex. IP splice variants or subtypes could not be detected by RT-PCR followed by TA cloning, though several nonfunctional point mutations or single base pair deletions were observed. Iloprost (ILP), cicaprost (CCP), PGE2, and arginine vasopressin (AVP) stimulated cAMP in both IMCD preparations. In addition, AVP-stimulated cAMP in IMCD-f was inhibited by all three prostanoids, but not in IMCD-c. Calcium experiments were performed on IMCD-c or micro-dissected IMCD (IMCD-m). CCP, ILP, and PGE2 did not alter intracellular calcium concentration ([Ca2+]i) in IMCD-c. However, on IMCD-m, both PGE2 and ILP increased [Ca2+]i levels equipotently and CCP had no effect. Pretreatment with the EP1 antagonist AH-6809 indicates that the response to ILP and PGE2 is mediated via EP1. These results suggest that IP receptors in the rat IMCD mediate the cAMP but not calcium signaling linked to PGL2; to date no subtypes or splice variants have been identified.

adrenosine 3′,5′-cyclic monophosphate measurements; in situ hybridization; intracellular calcium; rat inner medullary collecting duct; reverse transcriptase-polymerase chain reaction; TA cloning

Comparative to PGE2, prostacyclin is a major product of the arachidonic acid cascade within the kidney (3). Its production is dependent on the action of cyclooxygenases, as well as prostacyclin synthase. This enzyme converts the intermediate endoperoxides into prostacyclin in the cyclooxygenase pathway (35). Since its discovery in 1976 (32), it has been implicated in the maintenance of homeostatic functions and the pathogenesis of many diseases and plays various roles in different organ systems. Although initial studies to investigate the role of prostacyclin were limited by the fact that it is quite labile and rapidly metabolized into 6-keto-PGF1α, many pharmacological analogs are now available to facilitate these endeavors (36). The most common of these are iloprost (ILP) and the more selective prostacyclin (IP) agonist cicaprost (CCP). Like other members of the prostanooid family, IP elicits its effects by binding to G-protein-coupled cell surface receptors, IP receptors (6, 16). However, activation of other signaling pathways has been observed in many studies. For instance, in cultured adipose cell lines, prostacyclin was shown to increase free calcium ion concentrations (33). Furthermore, after cloning of the mouse IP cDNA, it was expressed in Chinese hamster ovary cells and found to increase both cAMP and inositol 3,4,5-trisphosphate levels in these cells in response to ILP (20). Moreover, in the rat kidney, multiple signaling is observed in different segments of the nephron. Although in the rat inner medullary collecting duct (IMCD) prostacyclin stimulated cAMP but no inhibition of arginine-vasopressin (AVP)-dependent cAMP stimulation was obtained (34), the reverse occurred in the rat medullary thick ascending limb (mTAL): both prostacyclin analogs, ILP and CCP, inhibited AVP-dependent cAMP stimulation, but no stimulatory response with these compounds was achieved (11).

In 1994, the IP cDNA was cloned from mice (20), humans (2), and rats (25). IP mRNA was detected in several tissues by using various molecular biology techniques. By Northern blot analysis and in situ hybridization in the mouse, the message was detected in the thymus, spleen, heart, lungs, and high levels in the vasculature and the brain (20). Although IP was not detected by Northern blotting in the mouse kidney, it was localized by in situ hybridization (ISH) in the vasculature and glomeruli (22). Further studies also examined the expression of IP receptors in other species (11, 16). However, within the kidney these findings are controversial. For instance, by using ISH on rat tissue sections and RT-PCR, it was shown that high levels of IP mRNA are present in Tamm-Horsfall-positive tubules of the outer medulla. This is a specific protein expressed exclusively in the mTAL and hence

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serves to identify this segment (11). In contrast to findings in the rat, human studies, by using both ISH and immunohistochemistry with an antibody to detect the IP protein, showed that IP receptors, both mRNA and protein, are only found in Tamm-Horsfall-negative tubules (non-mTAL) of the human outer medulla (16). In addition, this study localized the expression to specific structures within the human kidney. There was a great abundance within the vasculature throughout the kidney, mainly in the glomerular regions of the cortex, but very little in the inner medulla (IM). Although extensive work has examined the extra-renal expression of the rat prostacyclin receptor, to date very little is known about the expression of IP receptors in the rodent kidney. Moreover, to date there is no molecular evidence for the presence of IP receptor subtypes or spliced variants. Nonetheless, various biochemical and functional studies indicate that prostacyclin and PGE₂ activate different receptors, even suggesting “IP₁” and “IP₃” subtypes (10) of the prostacyclin receptor (consistent with the E-prostanoid (EP) receptors). Altogether, it remains controversial by which processes IP is evoking these various effects. Whether it is acting through different IP receptor subtypes or splice variants, coupling to different G proteins, or simply acting through EP receptors or other prostanoid receptors remains to be determined. The main hypothesis of this work is that distinct IP receptors mediate the renal effects of prostacyclin in the rat kidney. Therefore, to gain further insight into the role of prostacyclin along the length of the rat nephron, this study examines the renal distribution of the prostacyclin receptor mRNA by using both RT-PCR and ISH. Also, the possibility that prostacyclin receptor subtypes or splice variant can be detected in different regions of the rat kidney was explored. Moreover, the cAMP and calcium signaling in response to prostacyclin analogs in the rat IMCD was assessed.

MATERIALS AND METHODS

Tissue preparation. A bilateral nephrectomy was performed on male Sprague-Dawley rats (175–200 g), and the kidneys were immediately placed in a beaker on ice, containing 300 mosM Krebs-Ringer buffer, pH 7.4, composed of (in mM) 118 NaCl, 14 glucose, 25 NaHCO₃, 4.7 KCl, 1.8 MgSO₄ (7 H₂O), 1.8 KH₂PO₄, and 2.5 CaCl₂. A small superficial incision was then made into each kidney, and the renal capsule was removed. Under a dissecting microscope, on ice, an incision was then made into each kidney, and the renal capsule was removed. Under a dissecting microscope, on ice, the kidney was divided into five transverse slices each 5 mm thick. By using high-intensity light, each region of the kidney, mainly in the glomerular regions of the cortex, but very little in the inner medulla (IM). The tissue was then digested for 1.5–2 h at 37°C, in bubbling 5% CO₂-air. After the digestion, the tube suspension was centrifuged at 800 rpm for 2 min, washed in Krebs buffer (×3), and placed in a hyposmotic solution (1.4, Krebs buffer-H₂O) for 3 min, while the tube was gently, continuously inverted. The combination of collagenase digestion and osmotic shock destroys all the cells in the inner medulla (i.e., medullary interstitial cells, vasa recta) with the exception of the IMCD (15, 37). The suspension was then centrifuged once more as described above, and the IMCD pellet was collected. Previous immunocytochemical analysis of these cells in our laboratory, by using specific antibodies for the detection of principal and α-intercalated cells, and peanut lectin agglutinin β-intercalated cells, revealed that all three collecting duct (CD) cell types were present in this preparation, with the majority (>50%) of cells being of the principal cell type (data not shown).

IMCD culture. After the IMCD pellet was obtained as described above, the pellet was resuspended in the desired volume of DMEM-F-12 culture media (GIBCO), pH 7.4, containing 10% fetal bovine serum (GIBCO-BRL), 1% penicillin-streptomycin-fungizone (GIBCO-BRL), 5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), 5 ng/ml selenium (Sigma), 2.5 mM tridithyronine/sodium salt (Sigma), and 50 nM hydrocortisone (Sigma). The rat IMCD cells were plated on petri dishes and grown for 3 days at 37°C and 5% CO₂. Before the experiments, once the cells reached 90–100% confluence, they were serum deprived overnight.

RNA isolation and RT-PCR. Total RNA was isolated from the various tissues by using the Trizol method, as described by the manufacturer (GIBCO-BRL), and was DNase (Boehringer Mannheim) treated to eliminate genomic DNA. Three microliters containing 1 μg of DNase-treated RNA were used for each sample. The RNA was reverse transcribed into cDNA by using MuLV RT and random hexamers provided in the Gene-AMP RNA PCR core kit (PerkinElmer). Samples were prepared in duplicate for each reaction, the duplicates serving as controls for the reverse transcription because the RT was served as controls. The upstream and downstream primers used for PCR amplification of each cDNA were FF-1 5′-GGCAGAGAAGAGTGAGTATGGTTACCC-3′ and FF-2 5′-GTCAG-GGGACACGAGTCAATGTTG-3′ (IP receptor; 407 bp; nucleotides 856–1263); EP₁,₁ 5′-CGCGGATT-CACCCACAGCA-3′ and EP₁,₂ 5′-CAGCAGGGACAC-TATGC-3′ (EP₁ receptor; 336 bp; nucleotides 865–1201); EP₂,₁ 5′-AGCAGCTGAGTGGCAGAGGAC-3′ and EP₂,₂ 5′-CAGCAGTTTACCTTCTCCAAGT-3′ (EP₂ receptor; 401 bp; nucleotides 757–1158); EP₃,₁ 5′-CCGGCCACGTGTT-GCTTAC-3′ and EP₃,₂ 5′-TAGAACAGGATAAAAACCGG-3′ (EP₃ receptor; 437 bp; nucleotides 538–975); and EP₄,₁ 5′-TTCGCCCTGTTGGCAAGTTGTT-3′ and EP₄,₂ 5′-GAGGGT-GTTGCTGTTGGCCACG-3′ (EP₄ receptor; 423 bp; nucleotides 941–1364) (1). The DNA amplification was performed by using a thermal cycler, the PerkinElmer Gene-AMP PCR System 2400. Depending on the primers used, the PCR reaction consisted of the following parameters: denaturing at 94°C for 2 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 63°C for 45 s, extension at 72°C for 60 s; extension at 72°C for 10 min; and finally cool down to 4°C. The given amplification products were then separated by gel electrophoresis, on a 1.5–2% agarose gel, for size determination with standards, and visualized under ultraviolet light by using ethidium bromide.
**Endonuclease restriction digest.** To verify the identity of the amplified fragment of the prostacyclin receptor, endonuclease digestion was performed. The rat prostacyclin receptor contains a *Nco* I restriction site in its COOH-terminus tail, located within the 407-bp product, amplified by using the IP primers mentioned above. This endonuclease generates two fragments from the IP cDNA fragment: 256 and 151 bp. To purify the amplified PCR product before digestion, the 407-bp band was isolated from an agarose gel by using the BIO 101 GeneClean kit, which consists of separating the DNA from agarose by using NaI, and then binding the DNA to a glassmatrix, followed by an elution with H2O. The restriction digestion was carried out by incubating the restriction enzyme with the sample at 37°C for 90 min. The digested products were then separated on a 3% agarose gel. Because the EP3 receptor PCR product amplified does not contain a restriction site for this enzyme, it was used as a negative control for the digest.

**ISH.** The kidneys were removed from male Sprague-Dawley rats and immediately frozen with CO2 powder on dry ice and then stored at −80°C until needed. To localize the expression of the prostacyclin receptor within the rat kidney, longitudinal rat kidney cryosections, 8–10 μm thick, were fixed in 4% paraformaldehyde and treated with proteinase K and 0.1% active diethyl-pyrocatechone (DEPC). A 40-bp sense and antisense oligonucleotide sequence, 5′-AAGTTCTGGTTGTTGTCGTTGCCCCCTCTCTGATCG-3′, with no homology to any known rat kidney mRNA (Geneblast), was selected. This corresponds to a region following the 7th transmembrane domain in the COOH-terminus of the prostacyclin receptor cDNA. By using a nonradioactive labeling method, the oligoprobe was labeled by using the digoxigenin (DIG) oligonucleotide tailing kit (Boehringer Mannheim). The hybridization consisted of incubating the tissue sections with DIG-labeled sense and antisense probes for 18 h at 14°C with T4 DNA ligase, which ligates the DNA fragment into pCR2.1 vector by using the TA cloning method (original TA cloning kit, Invitrogen). Also, specific features of the pCR2.1 vector facilitate the insert analysis: ampicillin resistance gene for selection and TA cloning and sequencing. Total RNA from different regions of the kidney was obtained as described above. This included the cortex, outer medulla, inner medulla, mTAL, and IMCD. To determine whether IP receptor subtypes or spliced variants could be identified in any of these regions, fragments of the rat IP cDNA were amplified by RT-PCR, purified, then cloned and sequence analyzed. By this method, any sequence with some homology to a region of the IP cDNA can be detected. Briefly, four sets of primers were selected, spanning the entire length of the rat prostacyclin receptor cDNA. The primer map is shown in Fig. 1. Each of these primer sets, and combinations thereof, was used to amplify various fragments along the entire cDNA within each region.

After separation of the fragments by gel electrophoresis, the desired band was excised and purified from the agarose by using glassmilk (as described above). Next, 5–10 ng of the DNA were incubated for 18 h at 14°C with T4 DNA ligase, which ligates the DNA fragment into pCR2.1 vector by using the TA cloning method (original TA cloning kit, Invitrogen). Also, specific features of the pCR2.1 vector facilitate the insert analysis: ampicillin resistance gene for selection and maintenance in *Escherichia coli* (*E. coli*), and T7 promoter and M13 reverse priming sites for sequencing of the insert. By using a heat shock technique, TOP10F™-competent cells (a strain of *E. coli*) were transformed with plasmids containing the given insert. Next, they were placed for 1 h at 37°C, with continuous shaking at 225 rpm. Aliquots were plated on

![Fig. 1. Primer map for amplification of prostacyclin (IP) receptor cDNA fragments. Illustrated are the various sets of primers used for amplification of different fragments spanning the entire length of the IP receptor cDNA. The IP cDNA is represented in black, and a color scheme is utilized to identify individual sets of primers and their location along the cDNA. The various products obtained for each set, as well as combinations thereof, are shown.](http://ajprenal.physiology.org/)

<table>
<thead>
<tr>
<th>Set</th>
<th>Primers</th>
<th>Products obtained for each set, as well as combinations thereof, are shown.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>(10-33)</td>
<td>1.2 (462-483)</td>
</tr>
<tr>
<td>2.1</td>
<td>(448-471)</td>
<td>2.2 (939-960)</td>
</tr>
<tr>
<td>3.1</td>
<td>(917-939)</td>
<td>3.2 (1318-1341)</td>
</tr>
<tr>
<td>FF-1</td>
<td>(856-878)</td>
<td>FF-2 (1241-1341)</td>
</tr>
</tbody>
</table>

**NH3**

1.1

COOH

1.1

2.1

2.2

3.1

3.2

FF-1

FF-2

474 bp

513 bp

425 bp

407 bp

893 bp

950 bp

817 bp

345 bp

3485 bp

1255 bp

1331 bp
ampicillin-coated agar plates for selection of positive colonies by using blue-white screening, after which the plasmid DNA was purified and sequenced by using a dye-sequencing method.

**cAMP radioassays.** For experiments on cultured IMCD, cells were grown to confluence in 24-well plates for 3 days and serum starved for 24 h. The cells were then pretreated for 15 min in DMEM-F-12 containing 0.5 mM IBMX (Sigma) and 10 μM indomethacin (Sigma). At timed intervals, the cells were then stimulated with 1 μM-0.1 nM AVP, PGE₂, ILP, and CCP. The samples were all prepared in duplicate. To stop the reaction, 300 μl of 10% TCA were added to each well. After a 30-min incubation on ice, the samples in TCA were transferred to Eppendorf tubes and centrifuged for 10 min at 4,000 g. Next, 250 μl of each sample were transferred to glass test tubes, and the four ether extractions of TCA were performed by using 4× the volume of H₂O-saturated diethyl ether/extraction. One molar Tris-HCl was used to bring the pH of the samples to 7–8. By using the cAMP radioassay kit (Intermedico), cAMP levels in each sample were then measured in 100 μl of sample according to the manufacturer’s instructions. For experiments on freshly isolated IMCD, the final IMCD pellet obtained was resuspended in an appropriate volume of DMEM-F-12 containing IBMX and indomethacin (as above), ensuring 180 μl/sample in addition to two samples for protein determinations. In the second set of experiments, to verify the ability of the various agonists to inhibit the AVP-dependent increase in cAMP, the cells were pretreated for 15 min with the above-mentioned concentrations of agonists, and then treated with 0.1 μM AVP, in the presence of the agonist, for 7 min.

**Calcium measurements.** IMCD cells, isolated from 175- to 200-g Sprague-Dawley rats, were cultured on round coverslips for 3 days and serum starved overnight. The cells were then loaded for 45 min with the calcium indicator fura 2. The dual-wavelength deltascan 1 spectrophotometry system was used to measure changes in calcium levels within the cells. The tracings were monitored by computer by using Felix software. The technique is based on a shift in the excitation/emission wavelength of fura 2 on binding of the dye to the ionic species (calcium); therefore, the calcium concentration can be determined based on changes in the ratio of the dye’s fluorescence intensity at the two wavelengths: 340 and 380 nm. Different agonists were applied to the cells by using a 450-mosM solution containing 0.1% albumin (wt/vol) and (in mM) 190 NaCl, 25 NaHCO₃, 5 KCl, 1.2 MgCl₂, 8 glucose, 5 HEPES, 10 urea, 1.5 CaCl₂, and 5 NH₄Cl; then, cellular responses were assessed. The compounds tested were 0.1 μM AVP, PGE₂, ILP, CCP, ANG IV, endothelin, and ATP.

The second set of experiments consisted of microdissecting IMCD from the kidneys of 75-g male Sprague-Dawley rats. The single isolated tubule was then inserted at each end into glass pipettes and was perfused luminally with the solution mentioned above (9). Afterward, the tubule was loaded with fura 2 for 30 min before stimulation with the desired agonists. The compounds of interest, namely, 10 nM PGE₂, ILP, CCP, and AVP, were applied to the basolateral surface of the tubule, and the cellular responses were measured.

Finally, to determine whether the calcium effect obtained with ILP is mediated by the IP or EP₁ receptor, the tubules were pretreated for 3 min with AH-6809, an EP₁ receptor antagonist. Next, 10 nM PGE₂ or ILP was added to the tubule in the presence of the antagonist, and the calcium response was assessed.

**Statistics.** Experiments were performed by using duplicate samples and repeated three to six times each. The SigmaPlot software for windows version 4.01 (1986–1997) was used for data analysis. Results are expressed as means ± SE.

**RESULTS**

Detection of IP mRNA by RT-PCR. Because prostacyclin is a major renal product of the arachidonic acid cascade, and very little is known about its role in the kidney, this study localized the expression of prostacyclin receptors within different regions of the kidney, so as to understand the renal effects of prostacyclin. As shown in Fig. 2, IP mRNA was detected in all three regions of the kidney: cortex, outer medulla, and inner medulla. The expression pattern of the four different

![Fig. 2. Detection, by RT-PCR, of E-prostanoid (EP) and IP receptor mRNA in different rat kidney tissue preparations. Gel electrophoresis shows the amplified RT-PCR products from DNase-treated total RNA of different rat kidney tissue preparations: cortex (A), outer medulla (OM; B), and inner medulla (IM)/papilla (C). The sets of primers for the four EP receptor subtypes and IP receptor were used. Lane 1, 100-bp DNA ladder; lane 2, IP (407 bp); lane 3, EP₁ (336 bp); lane 4, EP₂ (401 bp); lane 5, EP₃ (437 bp); and lane 6, EP₄ (423 bp, n = 4–5).](http://aprendal.physiology.org/)
EP receptor subtypes was also examined in these tissue preparations. While EP$_1$, EP$_3$, and EP$_4$ mRNAs were amplified from all three kidney regions, EP$_2$ receptor mRNA was not amplified even by altering PCR conditions: increasing cycle number, decreasing annealing temperatures, and varying the amount of starting RNA. Next, we isolated total mRNA from preparations of cultured IMCD, freshly isolated IMCD tubules, and mTAL. Once again, the IP receptor was detected in all three cellular preparations; and with the exception of EP$_2$, consistent with previous findings (5), the aforementioned EP receptors were also found as shown in Fig. 3.

**Analysis of 407-bp PCR product.** To verify the identity of the IP product amplified by RT-PCR, endonuclease digest and sequencing analysis were performed. Because the 407-bp amplified IP fragment contains a restriction site for the restriction enzyme NcoI, we performed a restriction digest and found that, indeed, the expected digest products were obtained: 256 and 151 bp, as shown in Fig. 4. As expected, the absence of an NcoI restriction site within the EP3 product served as a negative control for the digest. Also, we used TA cloning to isolate and express the 407-bp fragment in a plasmid, which was then transfected into bacteria and grown. Then, the DNA was purified and sequenced. Indeed, the product amplified corresponds to the published sequence for the rat IP receptor cDNA (data not shown).

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**Localization of renal IP mRNA by ISH.** In addition to the RT-PCR experiments, the expression of the IP receptor within the kidney was analyzed by ISH. By using a DIG-labeled oligoprobe, the IP message was detected on rat kidney tissue sections as indicated in Fig. 5. First, the IP mRNA is present throughout the kidney: cortex, outer medulla, and inner medulla. Although the most intense signals were found within the tubules of the outer medulla, diffuse tubular staining was also visible within the cortex and inner medullary regions. In the cortex, glomerular staining is obvious as indicated by the arrow, with intense signals in the vasculature. These results are consistent with previous findings in various species. For instance, in the mouse, ISH revealed intense IP signals within the renal vasculature (22). Also, in the rat, high levels of IP mRNA were detected in the mTAL of the outer medulla (11). And finally, in human kidney, IP expression was found in all regions, with staining in the vasculature, glomeruli, Tamm-Horsfall-negative distal tubules, and the CDs (16).

**Detection of IP receptor subtypes and/or splice variants.** Because there is a great deal of controversy in the literature regarding the mechanisms by which prostacyclin elicits its various effects, RT-PCR and TA clon-
The methods were employed to determine whether IP subtypes or alternatively spliced forms of this receptor can be detected within the different regions of the kidney. By selecting four different sets of primers (Fig. 1) spanning the entire length of the rat prostacyclin receptor cDNA, we first amplified fragments of the cDNA by using these primer sets, and then combinations thereof, to ascertain whether products similar to the published IP sequence exist. Table 1 summarizes the data obtained from the initial experiments by using the four primer sets. As shown, no positive colonies (containing an insert) were obtained out of 15 selected in the cortex; therefore, sequence analysis was not done for this region. In the outer medulla, any product sequenced was either identical to the published sequence of the IP cDNA or contained single mutations or deletions that did not alter the protein itself (open reading frame analysis). Two of the plasmid preparations analyzed did not contain any insert, and no amplification product was obtained by PCR with the IP set of primers regardless of the PCR conditions used, i.e., annealing temperature, number of cycles, starting RNA concentration, and primer concentration. The same results can be seen for the inner medulla, where all the sequences were identical to the published IP cDNA. In contrast, in the mTAL, by using the FF-1/2 primers, a product was amplified corresponding to 300 bp in addition to the expected 407 bp. This same product was also noted in other tissue preparations, inconsistently. However, sequencing analysis revealed that it is a product with very little homology to the IP cDNA, but stronger homology at the primer sequences. Therefore, we conclude that it is a result of nonspecific amplification. Likewise, in the IMCD a product of 600 bp was obtained with IP. But in this case, sequencing of the product unveiled a 400-bp sequence identical to the published IP cDNA, and not a 600-bp product. In the IMCD, a product was amplified by using the IP2 primers, confirming the quality of the primers and the adequacy of the PCR conditions employed.

cAMP assays. With the exception of previous work on cultured rat IMCD (34), very little is known about the signaling pathways linked to the IP receptor in the rat IMCD, and studies in other organ systems remain contradictory. Therefore, these experiments measured the cellular levels of cAMP in response to stimulation of both freshly isolated and cultured IMCD with various prostanoids: PGE2, ILP, and CCP, as well as AVP. The first set of experiments consisted of examining the stimulatory effect of the various agonists on the cAMP pathway. In cultured rat IMCD, as shown in Fig. 6, although all compounds tested stimulated cAMP levels, the greatest stimulation at all concentrations was obtained with AVP at percentages of control ranging from 27 ± 2% at 0.1 nM to 62 ± 4% at 1 μM. The stimulation with the different prostanoids was comparable at agonist concentrations between 1 nM and 10 μM. Despite a greater stimulation with AVP on freshly isolated IMCD, from 61.3 ± 6 to 72 ± 7%, as opposed to the cultured cells, the stimulation obtained with the different prostanoids was similar to, if not less than, that of the cultured IMCD. However, once again all three prostanoids comparably stimulated cAMP production in the rat fresh IMCD: from 6.8 ± 3 to 46 ± 2% with PGE2, from 4 ± 2 to 38 ± 6% with ILP, and from 6 ± 4 to 42 ± 3% with CCP. It is of interest to note that a stimulatory effect was only obtained at concentrations of PGE2 greater than 100 nM. Furthermore, decreasing the concentrations of AVP to 0.01 nM did not decrease the stimulatory response in both fresh and cultured IMCD (data not shown).

In the second set of experiments, we examined the ability of the three prostanoid analogs to inhibit the AVP-stimulated cAMP production in cultured and freshly isolated IMCD. Although all the compounds...
tested did not inhibit the stimulatory effect of 100 μM AVP in cultured cells (data not shown), an inhibitory effect was obtained in response to all three prostanoids in freshly isolated IMCD, as shown in Fig. 7. Although the smallest inhibition was achieved with ILP at all agonist concentrations, there was a comparable inhibition with CCP and PGE2 at higher concentrations, above 10 nM. But at 1 nM, the inhibitory effect on AVP achieved with CDP was much greater than with the other two prostanoid analogs, reaching 70 ± 15%, suggesting that the CCP effect may be mediated by a distinct IP receptor subtype or splice variant. A decrease in agonist concentrations to 0.01 nM did not result in a further inhibitory response with any of the prostanoids above (data not shown).

Calcium measurements. To further clarify the prostanoid signaling mechanisms in the IMCD, calcium responses linked to the IP receptor were examined by using analogs of prostacyclin, ILP, and CCP. First, changes in intracellular calcium concentration ([Ca2+]i) were measured on cultured IMCD cells grown on coverslips. None of the various compounds tested, 100 nM AVP, 100 nM PGE2, 100 nM ILP, 100 nM CCP, 1 μM endothelin, 100 μM ATP, and 10 μM ANG IV, produced a rise in [Ca2+]i levels (data not shown). Some of these compounds have previously been shown to trigger a rise in calcium in other cell preparations. For instance, PGE2 is known to increase calcium via the EP1 receptor in rabbit cortical collecting duct (RCCD) cells (5, 10). Also, ILP has been shown to increase [Ca2+]i levels in isolated perfused RCCD (9). In addition, 100 μM ATP has previously been shown to elicit a calcium response in cultured IMCD (26); however, no effect was detected in our experiments by using any of these compounds.

Because a positive calcium response was not obtained on cultured IMCD cells with any of the compounds tested, calcium levels were measured on microdissected rat IMCD. This is represented in Fig. 8. The mean baseline level of calcium was 25.4 ± 18 nM. When 10 nM ILP was added to the tubule, an increase in calcium to a peak of 1.35 ± 0.4 μM was obtained within 30–60 s. This was followed by a shoulder reaching a plateau at 229 ± 56 nM. This same pattern was obtained when 10 nM PGE2 was added to the rat IMCD. The peak calcium level attained was slightly lower at 560 ± 149 nM, which rapidly decreased to a plateau at 201 ± 68 nM.

Because no calcium signal was obtained with CCP, and ILP is a less selective agonist of the IP receptor with greater affinity for the EP1 receptor (21), it is difficult to conclude from these studies that the calcium response observed was attributable to the IP receptor. Therefore, to determine whether or not the effect obtained with ILP is linked to the EP1 receptor, AH-6809, an EP1-receptor antagonist was used to inhibit the calcium response. Once again, experiments were performed on microdissected rat IMCD, and the calcium responses were noted in the presence of 10 μM AH-6809. Compared with a baseline value of 25 ± 18 nM, the calcium responses to both PGE2 and ILP were completely inhibited in the presence of the EP1-receptor antagonist: 23 ± 2 nM with 10 nM PGE2 + AH-6809, and 52 ± 31 nM with 10 nM ILP + AH-6809. These results are demonstrated in Fig. 8, which is a

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**Table 1. TA cloning analysis of amplified DNA fragments from different kidney tissues**

<table>
<thead>
<tr>
<th>Region</th>
<th>Primers/Predicted Size of Fragment</th>
<th>Size of Fragment Analyzed</th>
<th>Number Sequenced</th>
<th>Published sequence</th>
<th>No result</th>
<th>Difference from published sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>FF1/2 (407 bp) IP1 (474 bp) IP2 (513 bp) IP3 (425 bp)</td>
<td>ND</td>
<td>No positive colonies (of 15 selected)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>FF-1/2</td>
<td>IP1</td>
<td>7</td>
<td>2 (Vector)</td>
<td>4 (del, 2 μ, 1 μ)</td>
<td></td>
</tr>
<tr>
<td>Inner medulla</td>
<td>FF-1/2</td>
<td>IP1</td>
<td>2</td>
<td>1 (Vector)</td>
<td>1 (1 μ)</td>
<td></td>
</tr>
<tr>
<td>mTAL</td>
<td>FF-1/2</td>
<td>IP1</td>
<td>1</td>
<td>1 (DNA)</td>
<td>300 bp</td>
<td></td>
</tr>
<tr>
<td>IMCD</td>
<td>FF-1/2</td>
<td>IP2</td>
<td>0 (PCR)</td>
<td>1 (2 DNA sequences)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IP1</td>
<td>IP2</td>
<td>0 (PCR)</td>
<td>1 (Vector)</td>
<td>4 (1 μ)</td>
<td></td>
</tr>
</tbody>
</table>

Summary of results obtained by dye-chemistry sequencing. For each region of the kidney, 4 different primer sets were used, spanning the entire length of the published cDNA, and the size of the fragment analyzed is indicated only when aberrant from the predicted size. The number of bacterial colonies examined for each region is indicated, as well as the sequencing results obtained, i.e. whether it differs from the entire length of the published cDNA, and the size of the fragment analyzed is indicated only when aberrant from the predicted size. The results were published IP cDNA, and the difference was noted. No result indicates that no insert was present in the vector or that the DNA purity was too poor for sequencing. IP, prostacyclin receptor; mTAL, medullary thick ascending limb; IMCD, inner medullary collecting duct; ND, not done; del, deletion.
representative tracing of the three experiments performed. To assess whether the effect of AH-6809 was reversible at the end of the experiment, 10 nM ILP was added once more, and a peak calcium response of 387 ± 73 nM was obtained, followed by a plateau at 112 ± 32 nM. These results suggest that the rise in \([\text{Ca}^{2+}]_{i}\) in response to ILP is in fact mediated by the EP1 receptor, and not through an alternate receptor such as an IP subtype or splice variant.

**DISCUSSION**

We know that prostaglandins are an important group of biologically active compounds implicated in the maintenance of homeostasis and the pathogenesis of certain diseases, both in the vasculature and in specific organ systems throughout the body. Their significance in the kidney is clearly demonstrated by the undesirable renal effects, associated with the use of nonsteroidal anti-inflammatory drugs, which inhibit the production of prostaglandins. Over the past decade or so, the cell surface receptors that mediate the effects of prostanoids, in particular those of thromboxane receptors and \(\text{PGE}_2\) (EP receptors), have been cloned and characterized (5, 6, 21). Within the kidney, these receptors have been localized to the glomerular region, the renal vasculature, and the distal segments of the nephron, including the mTAL and the CD. This was accomplished by using a variety of techniques: binding studies, immunohistochemistry, ISH, RT-PCR, and Northern blot analysis. The presence of prostanoid receptors within these regions is consistent with the fact that they have been implicated in various renal functions such as regulating glomerular filtration rate, glomerular hemodynamics, renin release, and salt and water transport in the CD. However, to date, there remains some controversy in the literature regarding these findings and also with respect to differences between species. For instance, it was shown by immunohistochemistry that the EP4 receptor is not present in the human CD (17), but several biochemical studies indicate that this receptor subtype, via coupling to \(G_{s}\), mediates the stimulatory effects of \(\text{PGE}_2\) on cAMP production throughout the rodent CD (5, 11). Also, the EP3 mRNA was not detected in the human IMCD (4), whereas in rodents it is expressed in this segment of the tubule (29). Also of interest are the conflicting results found in human kidney between localization of
the mRNA and protein for the EP3 receptor in the outer medulla: mRNA was detected in the mTAL (4), but the protein was only found in non-mTAL (17). The same is true for the IP receptor. Although it is highly expressed in the mouse (22) and human (16) kidney, very little is known regarding its distribution within the rat kidney. Previous work by Hébert et al. (11) gave the first indication of the expression of IP receptors in epithelial cells and localized this message to the mTAL in the outer medullary region of the rat kidney. In contrast, in the human kidney (16), the outer medullary expression of the IP receptor was restricted to non-mTAL.

Therefore, to pursue these findings and enlighten the investigation of EP and IP receptor expression in the rat kidney, this study used RT-PCR to determine whether the four EP receptor subtypes and IP receptor mRNA can be detected within the three regions of the rat kidney (cortex, outer medulla, inner medulla), and in specific tissue preparations of the distal nephron (fresh IMCD, cultured IMCD, mTAL). With the exception of the EP2 receptor, the mRNA for the EP1,3,4 and IP receptors were observed in all tissue preparations examined. The expression pattern of EP receptors within the kidney is consistent with the role of PGE2 along the nephron. For example, in the rodent CD, acting through the EP1 receptor, it is thought to be involved in the inhibition of sodium transport by PGE2; through EP3 it inhibits AVP-dependent water reabsorption, and finally through the EP4 receptor it stimulates water reabsorption (5). The lack of EP2 receptors within the kidney is in accordance with previous findings indicating that a butaprost-insensitive mechanism through the EP2 receptor, and not the EP2 receptor, mediates the stimulatory action of PGE2 on cAMP production in the rodent CD (4, 5), also that no Ga coupled EP2 mRNA was detected in the human kidney by ISH (4). Because our results corroborate previous work showing a lack of EP2 in the rat kidney, we suggest that it is via the EP4 receptor that PGE2 elicits its effects in the renal cortex. With respect to the presence of multiple bands for the EP1 receptor, previous work in our laboratory (data not shown) suggests that the upper band (700 bp) represents a fragment of protein kinase N, which shares a significant homology in the region of the gene for the EP1 receptor, amplified by using the upstream and downstream primers employed in our study. Furthermore, the presence of a second band at 300 bp when the 407-bp IP fragment was amplified led us to believe that spliced variants of the IP receptor may exist. Of further interest was the fact that this band was only detected in certain preparations and not consistently amplified. This suggested that, by varying the RNA pool by utilizing different tissue preparations, we increased the chances of detecting rarer RNA species, depending on their relative abundance within the tissue preparations. Unfortunately, further characterization of this product by sequencing analysis did not reveal anything interesting.

To localize the IP receptor mRNA within the kidney, ISH was employed. Our findings demonstrate that all three regions of the kidney contain the IP message. In the cortex, the signal is observed within the glomerular region, in the vasculature, and in tubular structures. The highest levels are located within the tubules of the outer medulla, and there is diffuse staining in tubules of the inner medullary region. This is in agreement with previous ISH studies in rodents showing, for instance, staining of the renal vasculature and glomeruli in the mouse (22). But it also confirms the finding of rat IP receptor mRNA in Tamm-Horsfall-positive tubules of the outer medulla, i.e., the mTAL (11). This is consistent with the RT-PCR results of this study, localizing the IP message within the mTAL. However, further investigation into the role of the prostacyclin/IP signaling mechanisms in this nephron segment is needed to account for the discrepancies noted between rodent and human kidneys, because in human studies the IP mRNA expression was restricted to the Tamm-Horsfall-negative, i.e., non-mTAL, segments of the outer medullary region (16).
Taken together, these findings substantiate the implication of prostacyclin and the IP receptor in various renal functions. Being a potent vasodilator (6, 16, 20, 21), prostacyclin, acting through vascular IP receptors, can alter renal blood flow and glomerular filtration rate. Also, it is known to be a greater stimulator of renin release than PGE2 (7). Given that there is high expression of IP in the outer medullary tubules, it surely may participate in the modulation of sodium and water transport in these segments. Furthermore, it may play a role at the level of the macula densa, thereby regulating renal function of these cells. In cultured rat IMCD, for instance, PGI2 did not inhibit AVP-dependent cAMP stimulation (34), but in isolated perfused RCCD, ILP did inhibit AVP-stimulated water flow (10). Other putative functions of the prostacyclin-IP system may include the regulation of gene transcription in the kidney; however, whether it is a direct effect, or via an indirect mechanism, is still uncertain. Finally, reports have been made that IP may activate the peroxisome-proliferator-activated-receptor-transduction pathway, thereby altering gene transcription (12).

After having localized the renal expression of the prostacyclin receptor in the rat, the next study examined whether or not IP receptor subtypes or splice variants can be detected in various regions of the kidney, because to date there is no molecular evidence for their existence. Nevertheless, many findings suggest that these forms may exist (10, 23, 24, 30). In this study, TA cloning and sequencing analysis revealed no product homologous to any fragment spanning the length of the published rat IP receptor cDNA. This finding is not surprising, considering that a phylogenetic classification scheme places the IP receptor in cluster 1 with DP, EP2, and EP4 receptors (31), all of which do not have any subtypes or splices; however, no alternative signaling pathways for those receptors have been suggested other than the stimulation of cAMP through coupling to Gs. In contrast, IP is known to activate various intracellular signaling messengers. Both functional and biochemical studies support this argument. In isolated perfused RCCD, ILP inhibits AVP-dependent water flow in a manner independent of the EP3 receptor subtype, possibly acting through an “IP3” subtype of the IP receptor (10, 11). Furthermore, characterization of the IP receptor by binding analysis by using various IP analogs showed conflicting binding affinities in the rat central nervous system for each analog, suggesting that a different IP receptor subtype is located in certain regions of the brain, such as the hippocampus (30). Although these studies support the hypothesis, there remains the possibility that IP and its analogs are acting through other receptors such as the EP receptors, or simply that these effects are mediated by coupling of the existing IP receptor to different G proteins. Evidence for the latter stems from the initial work done to characterize the receptor by Namba et al. (20), showing that expression of the cloned mouse IP receptor cDNA into Chinese hamster ovary cells gave rise to both an increase in cAMP and inositol trisphosphate levels on stimulation with ILP. It is still possible that IP action is mediated by all three mechanisms. Although the current study does not provide evidence for the presence in the rat kidney of IP receptor subtypes or alternatively spliced forms that are homologous to the published IP cDNA, it does not exclude the possibility that sequences with low homology to the cloned IP receptor do exist.

To further examine the possibility that multiple signaling pathways are associated with the binding of IP to the IP receptor in the rat kidney, cAMP assays were performed in the IMCD. Although a stimulatory response to CCP, ILP, PGE2, and AVP was obtained in both fresh and cultured IMCD, an inhibition of AVP-dependent cAMP stimulation by the three prostanoi was only observed in freshly isolated IMCD. This lack of inhibitory response in cultured IMCD cells is consistent with previous work by Sonnenberg and Smith (28) in RCCD cells, suggesting the possibility that the G-protein signaling pathway is aberrant in cultured cells. Whether this defect is due to a lack of receptor coupling to Gs, to an inactivation of one of the subunits, or to an absence of Gi due to a protein downregulation in response to culture conditions is not clear for this system. Another interesting aspect of these results is that the stimulation of cAMP production in response to PGE2 is only seen at higher agonist concentrations. This is also consistent with previous findings showing that at 1 nM PGE1 inhibits AVP-stimulation of cAMP, whereas it increases cAMP levels at 100 nM in RCCD cells (27). The stimulatory effect obtained with the prostacyclin analogs, CCP and ILP, is consistent with the fact that the major signaling pathway linked to the IP receptor is the activation of adenylate cyclase (20), but also with previous work in cultured rat IMCD, showing an increase in cAMP levels in response to PGL2 (34). However, the demonstration in this study of an inhibitory effect of CCP and ILP on AVP-dependent stimulation of cAMP provides further evidence for the existence of IP receptor subtypes or splice variants in the rat freshly isolated IMCD. It is also of interest to note the difference in cellular response to both CCP and ILP; CCP is by far a more potent inhibitor of the AVP-dependent stimulation of cAMP, again supporting the aforementioned argument that these two compounds are activating different receptors within the same cells.

The final part of this work examined the calcium signaling pathways linked to the prostacyclin-IP system in the rat IMCD. Although no [Ca2+]i changes were observed in cultured rat IMCD in response to many compounds, CCP, ILP, PGE2, AVP, endothelin, ANG IV, and ATP, both ILP and PGE2 increased [Ca2+]i in microdissected rat IMCD. Of interest is the fact that CCP did not increase [Ca2+]i in microdissected tubules as well as in cultured IMCD. Very little is known about the calcium signaling mechanisms in the cultured IMCD, but previous work did show an increase in [Ca2+]i on stimulation with 100 μM ATP (26). In addition, it was shown that AVP increases calcium in renal papillary collecting tubule cells in...
culture (13). At this time we cannot account for this discrepancy in cultured cells to explain the lack of response in our preparation. However, several other studies support the use of the above-mentioned compounds as positive controls in our study. For instance, in isolated perfused RCCD, both PGE₂ and ILP increased [Ca²⁺]i (10); whether or not this effect was mediated by the EP₁ receptor only or by two different receptors is uncertain. Nonetheless, the highest levels of EP₁ are found in the CD, and this receptor does couple to Gs, to increase calcium levels (5). Also, previous work in rat terminal IMCD showed that PGE₂ caused a rapid increase in [Ca²⁺]i (18). Furthermore, endothelin is known to increase calcium via the endothelin (ET₁) receptor (19), which is highly expressed in the IMCD (8, 14). An increase in calcium in response to endothelin was observed in rat terminal IMCD (19). To further clarify the calcium signaling in response to prostaglandins in the rat IMCD, we used the EP₁ antagonist AH-6809 to block the increase in calcium obtained in response to PGE₂ and ILP. The calcium spike was in fact abolished in the presence of AH-6809 for both compounds, and this effect was reversible. Although it appears that ILP increases calcium in the rat IMCD via the EP₁ receptor, we cannot yet dismiss the possibility that other receptors also mediate its effect on calcium metabolism.

In conclusion, this study localized the expression of EP receptor subtypes and the IP receptor in rat kidney. The IP receptor is clearly expressed in rat IMCD and mediates the effects on cAMP signaling pathways in response to prostacyclin analogs, but not the calcium response in this segment of the nephron.

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REFERENCES


28. Sonnenburg WK, Zhu J, and Smith WL. A prostaglandin E receptor coupled to a pertussis toxin-sensitive guanine nucleo-


33. Vassaux G, Gaillard D, Alihaus G, and Negrel R. Prostacyclin is a specific effector of adipose cell differentiation. Its dual role as a cAMP- and Ca
2


