Prostanoid signaling, localization, and expression of IP receptors in rat thick ascending limb cells

RICHARD L. HÉBERT,1,2 TIM O’CONNOR,1 CHRIS NEVILLE,1 KEVIN D. BURNS,1,2 ODETTE LANEVILLE,3 AND LINDA N. PETERSON1,4

Departments of 1Cellular and Molecular Medicine, 2Medicine, 3Biochemistry, Microbiology, and Immunology, and 4Paediatrics, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

Hébert, Richard L., Tim O’Connor, Chris Neville, Kevin D. Burns, Odette Laneville, and Linda N. Peterson. Prostanoid signaling, localization, and expression of IP receptors in rat thick ascending limb cells. Am. J. Physiol. 275 (Renal Physiol. 44): F904–F914, 1998.—It is widely held that only one prostacyclin (IP) receptor exists that can couple to guanine stimulatory nucleotide binding proteins (G\textsubscript{s}) leading to activation of adenyl cyclase. Although IP receptor mRNA is expressed in vascular arterial smooth muscle cells and platelets, with lower level expression in mature thymocytes, splenic lymphocytes, and megakaryocytes, there is no molecular evidence for IP receptor expression in renal epithelial cells. The purpose of the present study was to obtain molecular evidence for the expression and localization of the IP receptor and to study the signaling pathways of IP receptor in rat medullary thick ascending limb (MTAL). Biochemical studies showed that IP prostanoids do not increase cAMP in rat MTAL. However, in the presence of vasopressin, inhibition of cAMP formation by prostacyclin (PGI\textsubscript{2}) analogs is pertussis toxin sensitive and does not activate protein kinase C. In situ hybridization studies localized IP receptor mRNA expression to MTAL in the rat kidney outer medulla. The results of RT-PCR of freshly isolated RNA from MTAL, with primers specific for the mouse IP receptor cDNA, produced an amplification product of the correct predicted size that contained an expected Nco I endonuclease restriction site. We conclude that rat renal epithelial cells express the IP receptor, coupled to inhibition of cAMP production.

G\textsubscript{i} protein; in situ hybridization; prostacyclin IP receptor; rat medullary thick ascending limb; reverse transcriptase-polymerase chain reaction

UNTIL 1993, ONLY ONE TYPE of prostacyclin (PGI\textsubscript{2}) receptor (i.e., IP receptor) linked to activation of adenyl cyclase had been identified in platelets, adipocytes, and vascular smooth muscle (3, 22). Recent functional studies demonstrated that iloprost (ILP) and PGE\textsubscript{2} inhibit vasopressin (AVP)-stimulated water flow and increase cell calcium by binding and activating different receptors (IP\textsubscript{3} and IP\textsubscript{1} for ILP; EP\textsubscript{3} and EP\textsubscript{1} for PGE\textsubscript{2}) in rabbit collecting duct (13). The existence of two types of IP receptors was also inferred from biochemical studies in cultured mast cells, with one receptor coupled to the guanine stimulatory nucleotide protein (G\textsubscript{s}) stimulating adenyl cyclase and the other one linked to phospholipase C activation via G\textsubscript{i} (17). Since PGI\textsubscript{2} at pH 7.4 is an unstable compound, the PGI\textsubscript{2} analog ILP is often used experimentally (7, 13, 29). The other PGI\textsubscript{2} analog cicaprost (CCP) is slightly more potent than PGI\textsubscript{2} and does not appear to possess any agonist activity to activate other prostanoid receptors (7). ILP, however, possesses agonist activity by binding to the EP\textsubscript{1} receptor, known to activate phospholipase C (7).

Nakagawa et al. (17) isolated a cDNA clone encoding the human IP receptor from a lung cDNA library. It has been demonstrated that the IP receptor could couple to both G\textsubscript{s} and G\textsubscript{i}, although the EC\textsubscript{50} required for coupling to phospholipase C was 1,000-fold greater than for activation of adenylate cyclase (22). Namba et al. (19) found a functional cDNA for a prostacyclin receptor (IP) isolated from a mouse cDNA library by reverse transcription followed by polymerase chain reaction and hybridization screening, encoding a 417-amino acid protein. The amino acid sequence for the mouse receptor was 30–40% identical in the transmembrane domains to those in the mouse PGE\textsubscript{2} receptor subtypes (19). Finally, a cDNA clone for rat prostacyclin receptor has been isolated by Sasaki et al. (27). The cDNA encodes a protein of 416 amino acid residues with seven transmembrane domains and belongs to the G protein-coupled receptor superfamily (7).

More recently, Schwaner et al. (28) showed that ILP and CCP could phosphorylate guanine inhibitory nucleotide protein (G\textsubscript{i}) as well as G\textsubscript{s} in cultured human erythroleukemia cells. However, the relevance of cell signaling systems in these tumor cells to normal cells is unknown (28). Binding studies using prostacycin analogs suggest the existence of IP receptors in specific areas of the central nervous system and dorsal root ganglia (31). Northern analysis and in situ hybridization studies show widespread expression of the IP receptor in vascular arterial smooth muscle cells, platelets, mature thymocytes, splenic lymphocytes, and megakaryocytes (7). Northern blot analysis demonstrated that human prostacyclin receptor mRNA was expressed in different tissues such as lung, spleen, heart, pancreas, thymus, stomach, and the kidney (17).

Previous studies with microdissected rat medullary thick ascending limb (MTAL) have shown that PGE\textsubscript{2} can inhibit AVP-induced cAMP formation (36). Culpepper and Andreoli (9) have demonstrated that by a cAMP-dependent process, PGE\textsubscript{2} inhibits, by a pertussis toxin (PTX)-sensitive pathway, AVP-induced NaCl reabsorption in the mouse medullary thick limb (9). More recently, Peterson et al. (23) showed that increased endogenous PGE\textsubscript{2} by hypercalcemia mediates inhibi-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

F904 0363-6127/98 $5.00 Copyright © 1998 the American Physiological Society
tion of rat MTAL chloride reabsorption at a step proximal to the generation of cAMP, and this does not involve activation of protein kinase C (PKC). Nakao et al. (18) suggested that PGE2 and a PGE2 analog, sulprostone (SLP), can function via an EP receptor linked to G1 to attenuate AVP-, calcium-, glucagon-, and parathyroid hormone-induced cAMP formation in both cortical and medullary rabbit TAL cells. Finally, to assess whether IP receptors share the same receptor with EP, functional studies performed by Hébert et al. (12, 13) demonstrated that PGI2 analogs inhibit AVP-induced hydraulic conductivity and increase intracellular calcium liberation by a receptor different from PGE2 in the isolated perfused rabbit cortical collecting duct (CCD). They concluded that these two putative “IP3 and IP,” receptors were functionally distinct from EP3 and EP1 receptors in rabbit CCD (12, 13).

However, from the previous studies, nothing is known about the coupling of IP receptors to signal transduction systems in rat MTAL. More importantly, there is no molecular evidence of the localization and expression of IP receptor in any renal epithelial cells. Therefore, the purpose of the present study was to determine whether the IP receptor is expressed in rat MTAL and to characterize the signaling pathways of IP prostanoids in rat MTAL cells.

MATERIALS AND METHODS

Rat MTAL Dissection and Isolation

Male Sprague-Dawley rats, weighing between 175 and 225 g, were anesthetized with a 60 mg/kg intraperitoneal injection of Somnitol. The kidneys were then placed in dissecting medium on ice. Under a dissecting microscope, the inner medulla was dissected and removed. The cortex and the outer stripe of the outer medulla were separated from the inner stripe of the outer medulla. The cortical tissue was drawn off, and the medullas were resuspended in 1.5 ml of 225 g Krebs bicarbonate buffer. The kidneys were then placed in 200 µl of Krebs bicarbonate buffer at pH 6.2. As previously described in our laboratory, the Bio-Rad protein assay, a modification of the Bradford method, was used to measure protein content in these studies (6, 24, 25).

Prostanoid Stimulation of cAMP and Inhibition of cAMP-Dependent cAMP

The cAMP concentration-response relationship for each prostanoid at concentrations ranging from 10-10 to 10-5 M was assessed for ILP, carbaprostacyclin (cPGI2), and CCP, and for PGE2, SLP, and butaprost (BTP). The preincubation period for each prostanoid was the same duration as was employed in the AVP concentration-response experiments in the presence of indomethacin and Ro-20-1724. The attenuation of the AVP-dependent cAMP generation in the presence of each prostanoid was also tested. At the end of this 10-min incubation period, 10-7 M AVP was added along with a concentration of the prostanoid being tested. At the end of the experimental time course, the reaction was terminated in dry ice, and cAMP was measured.

Pertussis Toxin and Bisindolylmaleimide I Experiments

The mechanism underlying prostanoid receptor-mediated inhibition of cAMP-dependent cAMP accumulation was examined in two sets of experiments. Rat MTAL were pretreated with 1 µg/ml PTX for 60 min. At the end of this preincubation period in the presence of indomethacin and Ro-20-1724, the tubules were treated with 10-8 M of each prostanoid for 10 min. AVP, 10-7 M, was added in the presence of each prostanoid for a further 10 min. In the second set of experiments, rat MTAL were pretreated with 2 µM of the PKC inhibitor bisindolylmaleimide I (Bis I) for 30 min. At the end of this preincubation period in the presence of indomethacin and Ro-20-1724, the tubules were treated with 10-8 M of each prostanoid for 10 min. AVP, 10-7 M, was added in the presence of each prostanoid for a further 10 min.

Immunoperoxidase Staining

The immunoperoxidase staining procedure was done using commercially available goat antiserum to human uromucoid (Organon Teknika and the Vectastain ABC kit from Vector Laboratories). The frozen kidney sections (10 µM) were fixed with 10% buffered Formalin for 5 min at 4°C, followed by two PBS washes. At this point, the slides were immersed in 0.3% H2O2 and MeOH for 30 min in the dark to inhibit endogenous peroxidase activity in the tissue sections (24, 25). Prior to incubation with the Tamm-Horsfall glycoprotein, the tissue sections were incubated with 100 µl of 0.15% rabbit serum in PBS to prevent nonspecific binding between the secondary antiserum (rabbit anti-goat IgG, Vector Laboratories) and rat kidney tissue. The tissue sections were then incubated with 100 µl of a 1:200 dilution of primary antibody. The slides were washed in PBS for 10 min, and the tissue sections were incubated with 100 µl of 2 µg/ml biotinylated secondary antibody for 30 min. The unbound secondary antibody was
were removed and rapidly frozen in CO2 powder on dry ice, kidney prior to cryosectioning. The hybridization was carried Reference marks were made on the surface of the frozen hydrolyzed salmon sperm DNA, 0.1 mg/ml polyadenylic acid, oligoprobes were end labeled with [35S]dATP (2 h, 37°C) and vices and purified by HPLC. Sense and antisense DNA

8

ing sequence was used: 5

3

nucleotidyl transferase (TdT). The nucleotide transfer reaction mixture was composed of 5× tailing buffer, 100 ng oligo probe, 125 µCi [35S]dATP, TdT, and diethyl pyrocarbonate in distilled H2O to a total volume of 25 µl. For the hybridization reaction, the oligos were diluted with hybridization buffer, and dithiothreitol was added to a final concentration of 100 mM. The hybridization buffer consisted of 50% formamide, 25 mM sodium phosphate, 1 mM sodium pyrophosphate, 10% of 50× Denhardt's solution, 0.2 mg/ml acid-alkali hydrolyzed salmon sperm DNA, 0.1 mg/ml polyadenylic acid, 0.12 mg/ml heparin, and 0.1 g/ml dextran sulfate sodium salt. Reference marks were made on the surface of the frozen kidney prior to cryosectioning. The hybridization was carried out on serial cryosections of 12 µm for 18 h at 45°C. Kidneys were removed and rapidly frozen in CO2 powder on dry ice, and stored at −80°C until sectioning. Prior to hybridization, frozen sections were fixed in 10% buffered Formalin (5 min, 4°C). High-resolution silver emulsion autoradiography was used to localize message expression. The slides were covered in the gel emulsion (NTB2, Interscience) for 1–3 wk at 4°C and then viewed under dark-field microscopy. Tamm-Horsfall glycoprotein was identified by peroxidase immunostaining (Vedastain ABC elite, Vector Laboratories) on a serial section (15, 17, 18). The primary antibody was applied at 1:200 dilution (Ori gon Teknika). The section was counterstained with eosin, washed, and mounted to coverslips using Permount (Sigma).

In Situ Hybridization

A 50-bp nucleotide sequence in the IP receptor with no sequence homology to any known mRNA in rat kidney (GeneBlast) was selected for in situ hybridization. The following sequence was used: 5′-CCACAGGTCAGGTGCTGTGTGACATTTCCCCTCCTCGATGACTTTC-3′. Oligoprobes were synthesized by University of Calgary Core DNA Services and purified by HPLC. Sense and antisense DNA oligoprobes were end labeled with [35S]dATP (2 h, 37°C) and purified by column chromatography (NenSorb) (2, 24, 25). Probe labeling involved enzymatic transfer of radiolabeled nucleotide to the oligonucleotide probe using terminal deoxynucleotidyl transferase (TdT). The nucleotide transfer reaction mixture was composed of 5× tailing buffer, 100 ng oligo probe, 125 µCi [35S]dATP, TdT, and diethyl pyrocarbonate in distilled H2O to a total volume of 25 µl. For the hybridization reaction, the oligos were diluted with hybridization buffer, and dithiothreitol was added to a final concentration of 100 mM. The hybridization buffer consisted of 50% formamide, 25 mM sodium phosphate, 1 mM sodium pyrophosphate, 10% of 50× Denhardt’s solution, 0.2 mg/ml acid-alkali hydrolyzed salmon sperm DNA, 0.1 mg/ml polyadenylic acid, 0.12 mg/ml heparin, and 0.1 g/ml dextran sulfate sodium salt. Reference marks were made on the surface of the frozen kidney prior to cryosectioning. The hybridization was carried out on serial cryosections of 12 µm for 18 h at 45°C. Kidneys were removed and rapidly frozen in CO2 powder on dry ice, and stored at −80°C until sectioning. Prior to hybridization, frozen sections were fixed in 10% buffered Formalin (5 min, 4°C). High-resolution silver emulsion autoradiography was used to localize message expression. The slides were covered in the gel emulsion (NTB2, Interscience) for 1–3 wk at 4°C and then viewed under dark-field microscopy. Tamm-Horsfall glycoprotein was identified by peroxidase immunostaining (Vedastain ABC elite, Vector Laboratories) on a serial section (15, 17, 18). The primary antibody was applied at 1:200 dilution (Organon Teknika). The section was counterstained with eosin, washed, and photographed under light microscopy.

RNA Isolation and RT-PCR

Total RNA was prepared from freshly isolated MTAL and mouse spleen using a commercial kit (Trizol, GIBCO). Reverse transcription with random primers was used to generate cDNAs from 5 µg of total RNA extracted from MTAL using reverse transcriptase (Gene-Amp RNA PCR, Perkin-Elmer) (2, 24, 25). The following primers were used for PCR amplification of the resulting cDNA: OL-4, 5′-GGACAGGATGAGTTACC-3′ (nucleotides 777–799) (2); and OL-5, 5′-GTACAGGACAGCTAGCTATG-3′ (nucleotides 1162–1184) (2). Primers were synthesized by the Biotechnology Research Institute at the University of Ottawa and were purified using HPLC. Gene amplification was performed in a Perkin-Elmer Gene-AMP PCR System (model 2400) using the following protocol: 94°C for 120 s and then 40 cycles of 95°C for 30 s, 60°C for 45 s and 72°C for 60 s followed by 240 s at 72°C (2, 24, 25). The amplification products of 407 bp, with and without endonuclease digestion NcoI, were run on 1.8% agarose gels for size determination with standards. The NcoI restriction site generates two fragments (256 and 151 bp) from the IP receptor cDNA fragment. The gel was treated with ethidium bromide, visualized under ultraviolet light, and photographed. The experiment was performed in three separate preparations.

Statistics

Student’s t-test for paired data was used when only two related treatment groups were compared. To determine the statistical significance of differences between more than two groups, ANOVA and the Bonferroni multiple comparison test were used. Differences of P < 0.05 were considered statistically significant. Data are presented as means ± SE.

Reagents

AVP, PGE2, and PTX were purchased from Sigma Chemical, St. Louis, MO. BTP was a gift from Miles Pharmaceutical Division, West Haven, CT. ILP and CCP were gifts of S. B. Rondeau of Berlex Canada. SLp was a gift from Dr. Rubanie from Berlex Laboratories, Cedar Knolls, NJ. bisI and Ro-20-1724 were purchased from Calbiochem-Novabiochem, San Diego, CA. pGlu was purchased from Cayman Chemical, Ann Arbor, MI.

RESULTS

Biochemical Studies

Effect of AVP on cAMP accumulation in rat MTAL. It is well documented that AVP increases cAMP formation in rat MTAL (10, 11, 32) through activation of the V2 receptor. To ensure the functional integrity of the MTAL preparation, the AVP concentration response was initially assessed. This study established a reproducible AVP concentration-response relationship. The maximal response was 367 ± 39 fmol/mg at a concentration of 10−6 M AVP, 4.5-fold greater than the basal value of 52 ± 9 fmol/mg (P < 0.05, Table 1). These cAMP measurements are consistent with previous studies (24, 25, 33) and demonstrate the utility of the rat MTAL preparation to assess this signal transduction system in vitro.

Effect of PGE2, SLP, and BTP on cAMP accumulation in rat MTAL. We studied the signaling pathways of EP prostanoids in rat MTAL. The concentration curve for PGE2 was sigmoidal with a threshold response occurring above 10−8 M. Maximal cAMP levels of 275 ± 48 fmol/µg were 11-fold greater than basal levels and were achieved at 10−6 M PGE2 (P < 0.05, Table 1). These results suggest the involvement of a Gs-coupled EP4 receptor. No significant difference was detected between basal cAMP production and the levels generated by 10−5 and 10−10 M [P = not significant (NS), Table 1]. Therefore, SLP by itself has no effect on cAMP production. It is known that BTP signals in rabbit collecting duct through an EP2 receptor (4). We therefore assessed BTP signaling in rat MTAL. A stimulatory response to CAMP was observed at concentrations above 10−7 M BTP;
however, the maximal response could not be ascertained. The cAMP values showed no signs of a plateau at $10^{-5}$ M BTP where these values were eightfold greater than basal levels at $398 \pm 77$ fmol/µg ($P < 0.05$, Table 1). The stimulatory effect of BTP on cAMP production is greater than the maximal response observed for PGE$_2$. This finding is in contrast to the report that potency of BTP for the EP$_2$ receptor was 10-fold lower than that of PGE$_2$ (7, 8).

Effect of PGE$_2$ and SLP on AVP-dependent cAMP in rat MTAL. Previous work has demonstrated inhibition of PGE$_2$ on AVP-dependent cAMP production in immunodissected cells and microdissected segments of rat MTAL (6, 10, 24, 25, 30, 34–36). Significant inhibition of AVP-stimulated cAMP production was assessed at PGE$_2$ concentrations between $10^{-9}$ and $10^{-8}$ M ($P < 0.05$, Fig. 1). Maximal inhibition was achieved in the presence of $10^{-8}$ M PGE$_2$, where AVP-dependent cAMP levels dropped from $281 \pm 38$ to $117 \pm 33$ fmol/µg ($P < 0.05$, Fig. 1). The effect of PGE$_2$ on AVP-dependent cAMP was biphasic at $10^{-7}$ M. These results clearly show that PGE$_2$ can inhibit AVP-dependent cAMP in freshly isolated rat MTAL. Since it has been shown that SLP inhibits cAMP in the presence of AVP in fresh isolated rabbit CCD cells (20), the effect of SLP on AVP-induced cAMP inhibition in rat MTAL was assessed. In the presence of AVP, $10^{-10}$ M SLP decreased cAMP production from $283 \pm 21$ to $123 \pm 20$ fmol/mg ($P < 0.05$, Fig. 1). Inhibition of AVP-dependent cAMP was present at all concentrations of SLP ($P < 0.05$, Fig. 1). Taken together, these results show that PGE$_2$ and SLP bind and activate an EP$_3$ receptor that inhibits AVP-stimulated cAMP production in rat MTAL.

Effects of ILP, cPGI$_2$, and CCP on cAMP accumulation in rat MTAL. Although it has been shown that PG1$_2$ can increase cAMP levels in rat cultured inner medullary collecting duct cells (36), no studies have investigated the effect of PG1$_2$ on cAMP production in rat MTAL. To evaluate the effect of PG1$_2$ on cAMP production, prostacyclin analogs (ILP, cPGI$_2$, and CCP) were used at concentrations between $10^{-10}$ and $10^{-5}$ M. No significant difference was detected between basal cAMP production rates and cAMP generated in the presence of ILP, cPGI$_2$, and CCP ($P =$ NS, Table 1). These experiments demonstrate that PG1$_2$ analogs do not stimulate cAMP production in these rat renal epithelial cells.

Effect of ILP, cPGI$_2$, and CCP on AVP-dependent cAMP in rat MTAL. Functional studies provide evidence that rabbit CCD expresses two types of IP receptors, a putative IP$_1$ receptor that increases intracellular calcium and a putative IP$_3$ receptor that inhib-

---

**Table 1. Stimulation of cAMP produced by EP and IP prostanoids**

<table>
<thead>
<tr>
<th>Substance</th>
<th>n</th>
<th>Basal</th>
<th>$10^{-10}$ M</th>
<th>$10^{-9}$ M</th>
<th>$10^{-8}$ M</th>
<th>$10^{-7}$ M</th>
<th>$10^{-6}$ M</th>
<th>$10^{-5}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVP</td>
<td>4</td>
<td>52 ± 9</td>
<td>81 ± 13</td>
<td>135 ± 13</td>
<td>259 ± 22*</td>
<td>299 ± 28*</td>
<td>367 ± 39*</td>
<td></td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>4</td>
<td>25 ± 12</td>
<td>24 ± 11</td>
<td>28 ± 21</td>
<td>24 ± 12</td>
<td>117 ± 22</td>
<td>275 ± 48</td>
<td>272 ± 50</td>
</tr>
<tr>
<td>SLP</td>
<td>3</td>
<td>59 ± 38</td>
<td>33 ± 33</td>
<td>37 ± 37</td>
<td>44 ± 30</td>
<td>37 ± 27</td>
<td>47 ± 24</td>
<td>32 ± 30</td>
</tr>
<tr>
<td>BTP</td>
<td>4</td>
<td>48 ± 18</td>
<td>36 ± 19</td>
<td>42 ± 12</td>
<td>47 ± 13</td>
<td>51 ± 15</td>
<td>167 ± 38*</td>
<td>398 ± 77*</td>
</tr>
<tr>
<td>ILP</td>
<td>4</td>
<td>46 ± 15</td>
<td>26 ± 9</td>
<td>46 ± 21</td>
<td>40 ± 14</td>
<td>33 ± 12</td>
<td>30 ± 17</td>
<td>38 ± 17</td>
</tr>
<tr>
<td>cPGI$_2$</td>
<td>3</td>
<td>29 ± 22</td>
<td>36 ± 18</td>
<td>58 ± 24</td>
<td>50 ± 22</td>
<td>46 ± 18</td>
<td>89 ± 34</td>
<td>54 ± 36</td>
</tr>
<tr>
<td>CCP</td>
<td>3</td>
<td>39 ± 14</td>
<td>24 ± 12</td>
<td>30 ± 21</td>
<td>33 ± 14</td>
<td>41 ± 11</td>
<td>24 ± 12</td>
<td>17 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SE in fmol/µg for stimulation of cAMP; n = no. of experiments. Freshly isolated rat medullary thick ascending limbs (MTAL) were incubated for 10 min in the presence of the indicated concentration of arginine vasopressin (AVP), prostaglandin E$_2$ (PGE$_2$), sulprostone (SLP), butaprost (BTP), iloprost (ILP), carbaprostacyclin (cPGI$_2$), and cicaprost (CCP). *P < 0.05 compared with AVP alone (ANOVA, Bonferroni).
To determine whether MTAL express an IP₃ receptor coupled to inhibition of AVP-stimulated cAMP production, ILP, cPGI₂, and CCP were used at concentrations ranging from 10⁻¹⁰ to 10⁻⁶ M. Maximal inhibition for ILP was achieved at 10⁻⁷ M where cAMP levels decreased from 218 ± 47 to 58 ± 14 fmol/µg (P < 0.05, Fig. 2). In the same study, the action of cPGI₂ was also examined. AVP, 10⁻⁷ M, increased cAMP production to 321 ± 22 fmol/µg, but in the presence of 10⁻⁷ M cPGI₂, cAMP production significantly decreased to 82 ± 29 fmol/µg (P < 0.05, Fig. 2). Finally, 10⁻⁶ M CCP significantly blunted cAMP from 225 ± 23 to 103 ± 21 fmol/µg (P < 0.05, Fig. 2). Since the MTAL suspension was treated with Ro-20-1724, a phosphodiesterase inhibitor, inhibition of AVP-dependent cAMP by the IP prostanoids is most likely due to the inhibition of cAMP synthesis, rather than its degradation.

**Signaling Studies**

**Effect of PTX on the AVP-dependent cAMP inhibition by PGE₂ and SLP.** To test the hypothesis that the EP receptor mediating inhibition of AVP-dependent cAMP production is coupled through a Gᵢ protein, the MTAL suspension was treated with PTX. PTX reversed the inhibition of AVP-dependent cAMP in the presence of 10⁻⁸ M PGE₂ and 10⁻⁸ M SLP by 76% and 78% (P > NS, compared with control; Fig. 3), respectively. As in Fig. 1, inhibition of AVP-dependent cAMP by PGE₂ and SLP was statistically significant compared with the amount of cAMP produced in the presence of PTX + AVP, and each prostanoid was divided by the amount of cAMP produced in the presence of PTX + AVP. Results are means ± SE of 4 independent experiments. *P < 0.05 compared with AVP alone (ANOVA, Bonferroni).
in PTX-treated tubules were not significantly different from those in MTAL incubated without PTX.

Effect of Bis I on the AVP-dependent cAMP inhibition by PGE2 and SLP. Previous biochemical studies in rabbit CCD cells demonstrated that PGE2-mediated inhibition of AVP-dependent cAMP was sensitive to both PTX and staurosporine, a PKC inhibitor (1, 12, 20). To determine whether the EP prostanoids activate a PKC-dependent component in MTAL, inhibition of AVP-dependent cAMP was examined using 2 µM Bis I. PGE2- and SLP-mediated inhibition were all insensitive to 30-min pretreatment of the PKC inhibitor, Bis I (Ki = 10 nM) (P < 0.05, compared with experimental; Fig. 4). In the presence of AVP, the levels of cAMP measured in Bis I-pretreated tubules were not significantly different from the levels of cAMP measured in MTAL without Bis I. Thus the mechanism of action by which PGE2 and SLP attenuate AVP-dependent cAMP stimulation does not have a Bis I-sensitive component.

We have recently demonstrated in our laboratory that preincubation of MDCK cells with 2 µM of Bis I for 30 min followed by 10-min stimulation with 100 nM phorbol myristate acetate and A-23187 was fully capable of blocking the synergistic effect on arachidonic acid release (15).

Effect of PTX on the AVP-dependent cAMP inhibition by PGI2 analogs. To test the hypothesis that the IP receptor-mediated inhibition of AVP-dependent cAMP production is coupled through a G protein, the MTAL preparation was treated with PTX. PTX is known to inhibit the α-subunit of the G protein (1, 23). PTX reversed the inhibition of AVP-dependent cAMP induced by 10⁻⁸ M of ILP, cPGI2, or CCP by 76%, 84%, and 82%, respectively (P = NS, compared with control; Fig. 3). As in Fig. 2, inhibition of AVP-dependent cAMP by ILP, cPGI2, or CCP was statistically significant compared with the amount of cAMP generated in the presence of 10⁻⁷ M AVP alone (P < 0.05, Fig. 3). In the presence of AVP, the levels of cAMP produced in PTX-treated tubules were not significantly different from those in MTAL incubated without PTX.

Effect of Bis I on the AVP-dependent cAMP inhibition by PGI2 analogs. Previous functional studies in rabbit CCD demonstrated that ILP and cPGI2-mediated inhibition of AVP-stimulated water flow was sensitive to both PTX and staurosporine, a PKC inhibitor (12). This type of interaction between signal transduction pathways constitutes a form of cell signaling cross talk or feedback inhibition (8, 13, 14). We tested the hypothesis that the inhibitory effect of ILP, cPGI2, and CCP on AVP-dependent cAMP was mediated through an IP1 receptor coupled to Gα protein, which is known to activate PKC. The inhibitory effect of ILP, cPGI2, or CCP on AVP-dependent cAMP production could not be reversed in rat MTAL pretreated during 30 min with 2 µM of the PKC inhibitor, Bis I. Thus the mechanism of action by which ILP, cPGI2, or CCP attenuate AVP-dependent cAMP (P < 0.05, compared with experimental; Fig. 4) does not have a Bis I-sensitive component.

**Molecular Studies**

In situ hybridization. The results of the biochemical studies above suggested the existence of IP receptors in tubule suspensions enriched in rat MTAL. To confirm IP receptor expression and to determine the identity of nephron segments, in situ hybridization studies were performed on serial sections of the outer medulla. A reference mark was made in the tissue to permit precise tubule location within the serial sections (Fig. 5, top left corner of A–C). TAL segments in the outer medulla were identified by immunoperoxidase staining for Tamm-Horsfall glycoprotein, which is known to be expressed almost exclusively in medullary and cortical thick ascending loops of Henle (24, 25) (Fig. 5A). In an adjacent serial section, IP receptor mRNA was detected using a 35S-labeled antisense 50-bp oligoprobe from a region of the IP receptor. The medullary regions labeled by the probe observed under dark-field microscopy (Fig. 5B) are identical to the tubule segments labeled by immunoperoxidase staining with the Tamm-Horsfall
antibody. Tissue binding of the $^{35}$S-labeled sense oligoprobe in the next serial section produced a low background (Fig. 5C).

Reverse transcriptase-polymerase chain reaction. RT-PCR was performed on total RNA isolated from freshly isolated MTAL and spleen using primers specific for the IP receptor sequence. The RT-PCR reaction produced a 407-bp fragment as predicted (Fig. 6). The identification of the 407-bp fragment was performed by digestion of the PCR product with the endonuclease NcoI, which yielded two bands of 256 and 151 bp. The sizes of the 256-bp and 151-bp bands correspond to the location of NcoI restriction site in the 407-bp IP receptor PCR fragment (2, 24, 25). RT-PCR of RNA isolated from rat
spleen produced the same PCR product size and location of the Nco I restriction site as found in the rat MTAL (Fig. 6).

DISCUSSION

The major finding of this study is that rat renal epithelial MTAL cells express an IP receptor, coupled to the inhibition of cAMP formation via a PTX-sensitive pathway. Although the IP receptor coupled through G_s to adenyl cyclase is widely expressed and characterized in vascular tissue (17, 22, 28), our work shows that MTAL cells do not express G_s protein coupled to the putative IP_3 or IP_4 receptor. In contrast, MTAL do express the novel IP_3 receptor. Although it has been shown that PG_l_2 can increase cAMP levels in rat inner medullary collecting duct (36), no studies have investigated the effect of PG_l_2 analogs on cAMP production in rat MTAL. ILP, cPG_l_2, and CCP did not increase cAMP production in these rat renal epithelial cells. The results of our biochemical studies provide strong evidence that IP prostanooids do not activate a putative IP_3 receptor in rat renal epithelial cells. To determine whether the IP prostanooids activate a PKC-dependent component in rat MTAL, inhibition of AVP-dependent cAMP was examined using a PKC inhibitor Bis I. Thus the mechanism of action by which ILP, cPG_l_2, and CCP attenuate AVP-dependent cAMP does not have a Bis I-sensitive component. These results suggest that the inhibitory action of ILP, cPG_l_2, and CCP is mediated exclusively by the putative IP_3 and not the IP_1 receptor. Functional studies in rabbit CCD have shown that the inhibitory effects of ILP and cPG_l_2 on the AVP-induced hydraulic conductivity were PTX and staurosporine sensitive (12). These results suggest that ILP and cPG_l_2 bind and activate the putative IP_3 and IP_4 receptors in rabbit CCD. Interestingly, IP and EP prostanooids signal in a similar manner in rat MTAL; previous functional studies from us (12, 13) and others (26) in rabbit CCD have shown that EP signal both on the apical and basolateral side and IP signal only from the basolateral membrane. Further studies remain to be done to determine specifically the physical orientations, i.e., apical vs. basolateral localizations of IP and EP prostanooid receptors in rat MTAL.

To assess the presence of the IP receptor in these rat renal epithelial cells, molecular studies were required. The results of the present study provide evidence for expression of the IP receptor in renal epithelial cells. It is well recognized that MTAL are the most abundant nephron segments in the outer medulla and have the highest expression of Tamm-Horsfall glycoprotein in the kidney (14, 24, 25). The in situ hybridization studies show clearly that the pattern of IP mRNA expression in the outer medulla was identical to that of TAL identified by Tamm-Horsfall immunostaining. To obtain specificity, the sequences of the primers were designed based on two regions of the IP receptor cDNA, which have no homology with any other known prostanooid receptors. The primers used to detect IP receptor transcripts are specific for the mouse IP receptor and do not recognize other prostanooid receptors as reported earlier (2). The amplification product of RT-PCR of RNA from MTAL segments using primers for a 407-bp in rat MTAL, which is consistent with the existence of a G_s protein coupled to an IP_3 receptor. Previous functional studies in rabbit CCD have shown the existence of two types of novel IP receptors: IP_3, which increases intracellular calcium, and IP_1, which inhibits AVP-stimulated water reabsorption (12, 13). To test the hypothesis that the putative IP_3 receptor is indeed coupled through G_s protein, experiments were performed to examine the sensitivity of this pathway. In our studies, PTX reversed the inhibition of AVP-dependent cAMP induced by ILP, cPG_l_2, and CCP. Thus the receptors mediating inhibition of AVP-dependent cAMP production by ILP, cPG_l_2, and CCP are indeed coupled through a G_s protein that inhibits adenylyl cyclase through the putative IP_3 receptor. Therefore, inhibition of AVP-dependent cAMP by IP prostanooids is mediated by a PTX-sensitive component, and our data certainly provide biochemical evidence for the existence of a putative IP_3 receptor in rat renal epithelial cells. To determine whether IP prostanooids activate a PKC-dependent component in rat MTAL, inhibition of AVP-dependent cAMP was examined using a PKC inhibitor Bis I. Thus the mechanism of action by which ILP, cPG_l_2, and CCP attenuate AVP-dependent cAMP does not have a Bis I-sensitive component. These results suggest that the inhibitory action of ILP, cPG_l_2, and CCP is mediated exclusively by the putative IP_3 and not the IP_1 receptor. Functional studies in rabbit CCD have shown that the inhibitory effects of ILP and cPG_l_2 on the AVP-induced hydraulic conductivity were PTX and staurosporine sensitive (12). These results suggest that ILP and cPG_l_2 bind and activate the putative IP_3 and IP_4 receptors in rabbit CCD. Interestingly, IP and EP prostanooids signal in a similar manner in rat MTAL; previous functional studies from us (12, 13) and others (26) in rabbit CCD have shown that EP signal both on the apical and basolateral side and IP signal only from the basolateral membrane. Further studies remain to be done to determine specifically the physical orientations, i.e., apical vs. basolateral localizations of IP and EP prostanooid receptors in rat MTAL.
sequence of the IP receptor was the same size in base pairs and contained the NcoI endonuclease restriction site that is present in rat spleen. Thus the results of two different experimental approaches demonstrate that the IP receptor is expressed in MTAL. Figure 7 shows the location of the primers used by Namba et al. (19) and those employed by us for in situ hybridization and RT-PCR in the present study. There is considerable sequence conservation between the rat (Fig. 7) and human IP receptor, i.e., 89% in the transmembrane domains and 72% in the extracellular and intracellular regions. It has been shown that the similarity with other prostanoid receptors can be significant when the receptors are coupled to adenyl cyclase through $G_s$ protein such as EP$_2$ and EP$_4$ receptors (3–5).

Elevations in cAMP generated in response to PGE$_2$ have been reported previously in rat MTAL (9, 18, 36). However, little is known about which EP receptors mediate this response. BTP stimulates smooth muscle relaxation by the EP$_2$ receptor (7, 8, 29). It has been shown that BTP activates the $G_s$ protein coupled to an EP$_2$ receptor (7, 8, 29). Although the EP$_4$ receptor is also associated with smooth muscle relaxation, it is not activated by BTP. The EP$_4$ receptor was first found in piglet saphenous vein, where it inhibits smooth muscle contraction (7). At the protein level, there is 38% amino acid homology between EP$_2$ and EP$_4$ receptors (4, 5). Both receptors have been shown to mediate PGE$_2$-dependent increases in cAMP. However, the EP$_2$ receptor can be pharmacologically differentiated from the EP$_4$ receptor through its activation by BTP. The fact that BTP mimics the effect of PGE$_2$ to stimulate cAMP production provides evidence that the EP$_2$ receptor mediates this effect. Our results provide biochemical evidence for the expression of an EP$_2$ receptor in rat MTAL. However, at the present time, localization of mRNA for the EP$_2$ receptor has only been demonstrated in glomeruli in mouse and human kidneys (4, 5).

There is evidence that the inhibitory effect of PGE$_2$ on AVP-stimulated cAMP production is due to activation of the $G_i$ protein coupled to EP$_3$ receptor by PGE$_2$ (12, 13). Previous work has demonstrated inhibition of PGE$_2$ on AVP-dependent cAMP production in immunodissociated cells and microdissected nephron segments of rat MTAL (10, 11, 18, 30). Taken together, these results show that PGE$_2$ and SLP bind to a $G_i$ protein coupled to an EP$_3$ receptor, which inhibits AVP-stimulated cAMP production in freshly isolated rat MTAL. Therefore, inhibition of AVP-dependent cAMP by EP prostanooids is mediated by a PTX-sensitive component; our data certainly provide biochemical evidence for the existence of an EP$_3$ receptor in rat renal epithelial cells. In contrast, the experiments performed with PGE$_2$ and SLP confirmed the hypothesis that the inhibitory effect of PGE$_2$ and SLP on AVP-dependent cAMP was not mediated through an EP$_1$ receptor coupled to $G_i$ protein, which activates PKC in rat MTAL. In cultured rabbit CCD cells, PTX and staurosporine partially reversed the effect of exogenous PGE$_2$ (20). In contrast, PTX completely reversed the inhibition of AVP-dependent cAMP production by SLP (20). This suggests the existence of distinct signaling pathways in rabbit CCD, i.e., one that increases cAMP formation with PGE$_2$, and another one that decreases AVP-induced cAMP formation with SLP (20). Interestingly, it has been demonstrated in Chinese hamster ovary cells that many IP ligands (ILP, carbaprostacyclin, and isocarbaprostacyclin) can bind to the EP$_3$ receptor with $K_i$ values ranging between 20 and 500 nM (16). Also, in freshly isolated rabbit CCD cells, carbaprostacyclin can also bind to the...
As an argument against cross-talk signaling between IP and EP receptors, our results clearly indicate that PGE₂ and BTP increase cAMP through EP₄ and/or EP₂ receptor; similarly ILP, cPGI₂, and CCP do not increase cAMP in rat MTAL. Since there is no specific receptor antagonist known for either IP or EP₃ receptors, it remains to be determined that IP binds and activates its own receptor independent of EP₃ in any renal epithelial cells. Further studies will have to be done to characterize the IP signaling pathways, although this study represents the first step.

In summary, we have shown that IP prostanoids inhibit cAMP formation and do not increase cAMP production in rat MTAL. Inhibition of cAMP by IP prostanoids is PTX sensitive and does not activate PKC. This strongly suggests that IP receptors are coupled to G protein in rat MTAL. However, from our biochemical studies, cross-talk signaling between the IP and the EP₃ receptors remains a possibility. Molecular studies were performed and identified a 407-bp fragment by RT-PCR that represents the IP receptor in freshly isolated rat MTAL. This fragment contained the predicted Nco I restriction site. This is the first demonstration of IP receptor expression and localization in rat renal epithelial cells by in situ hybridization and RT-PCR. These results provide evidence for the existence of a novel IP receptor coupled to G protein and not G₁ protein or G₃ protein in rat MTAL. The receptor can be referred to as an "IP₃" receptor to be consistent with EP receptor nomenclature.

This work was funded in part by Medical Research Council of Canada Grant MT-14103 and by the Kidney Foundation of Canada. Portions of this work have been published in abstract form (J. Am. Soc. Nephrol. 7: 1649, 1996; J. Am. Soc. Nephrol. 7: 978, 1997). Address for reprints requests: R. L. Hébert, Dept. of Cellular and Molecular Medicine, Faculty of Medicine, Univ. of Ottawa, 451 Smyth Road, Room-1337, Ottawa, ON, Canada K1H 8M5.

Received 2 June 1998; accepted in final form 3 September 1998.

REFERENCES

27. Sasaki, Y., T. Usei, I. Tanaka, O. Nakagawa, T. Dando, T. Takayuki, T. Namba, S. Narumiya, and K. Nakao. Cloning...


