Dopamine D₄ receptor isoform mRNA and protein are expressed in the rat cortical collecting duct

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Sun, Duo, Teresa W. Wilborn, and James A. Schafer. Dopamine D₄ receptor isoform mRNA and protein are expressed in the rat cortical collecting duct. Am. J. Physiol. 275 (Renal Physiol. 44): F742–F751, 1998.—We reported previously [Am. J. Physiol. 271 (Renal Fluid Electrolyte Physiol. 40): F391–F400, 1996] that dopamine inhibits vasopressin (AVP)-dependent water permeability and Na⁺ transport in the rat cortical collecting duct (CCD) apparently through a D₄ dopamine receptor. The present experiments used RT-PCR of total RNA extracted from microdissected rat CCD to determine whether the D₂ and D₄A dopamine receptor isoforms are expressed. Specific primers were used to amplify three regions of the D₄ cDNA. All three gave products with 98–100% nucleotide identity to the known rat D₁ sequence; however, there was an extra 6-bp insert at the 3′ end of the second transmembrane region that was identical to the human and mouse sequences but which had not been documented in the rat sequence. D₂ receptor protein was also localized exclusively to the CCD and medullary collecting ducts by immunohistochemistry. Two regions of the D₁A dopamine receptor message were also amplified by RT-PCR of RNA from rat CCD and were verified by sequencing and immunohistochemistry. We conclude that both D₄ and D₁A dopamine receptors are expressed in the rat CCD, but the physiological effects are attributable to a D₄ receptor.

Dopamine receptors; mineralocorticoid; arginine vasopressin; antidiuretic hormone; reverse transcription-polymerase chain reaction; immunohistochemistry; sodium reabsorption; water reabsorption

DOPAMINE HAS A MARKED natriuretic and diuretic action in human, rats, and other species (14), and the hormone is clinically useful for treatment of shock and heart failure because of these properties (18, 27). Although the hemodynamic effects of dopamine may be the major determinant of excretory changes, direct actions of exogenous and endogenous dopamine on the nephron are also important (2, 14, 23, 37). Renal dopamine excretion has been shown to increase in proportion to the natriuresis produced by a salt load (18, 27), and both the natriuresis and dopamine excretion are diminished by inhibition of renal dopamine synthesis (14). It is now widely accepted that the source of this dopamine is not the renal nerves but that the dopamine is synthesized primarily by proximal tubules, which possess the necessary metabolic pathways (14), and that this dopamine acts as an autocrine or paracrine regulator of Na⁺ reabsorption (14, 36).

Most studies of the tubular effects of dopamine have centered on the proximal tubule where dopamine inhibits volume reabsorption by inhibiting both the activity of the Na⁺/H⁺ antiporter and the Na-K-ATPase (7, 12, 13, 14). These effects of dopamine in the proximal tubule are mediated by the D₂A dopamine receptor isoform in rats and mice (equivalent to the human D₁ receptor). This receptor is coupled to the G protein, Gₛ, which stimulates cAMP production and inhibits the Na⁺/H⁺ antiporter (6, 12, 14). The D₁A receptor is also coupled to phospholipase A₂ (PLA₂) and phospholipase C activation, which inhibits Na-K-ATPase activity (14, 30).

Several studies have shown that the normal inhibitory action of dopamine on salt and water reabsorption by the kidney is important in the maintenance of normal blood pressure (14). For example, Albrecht et al. (1) showed that defective coupling of the D₁A receptor to the inhibition of the Na⁺/H⁺ antiporter in renal proximal tubules cosegregated with hypertension in the F₂ generation of crosses between spontaneously hypertensive (SHR) and normotensive Wistar-Kyoto (WKY) rats. The same investigators showed that mice lacking functional D₁A receptors had elevated systolic and diastolic blood pressures that were greater in the mice that were homozygous than in those that were heterozygous for the defective receptor gene (1).

Dopamine also inhibits Na⁺ and water reabsorption in the cortical collecting duct (CCD). In the isolated perfused rabbit CCD, Muto et al. (23) showed that dopamine inhibited the increase in osmotic water permeability (Pₒ) with arginine vasopressin (AVP) and ascribed this effect to a D₂ dopamine receptor, because it could be prevented or reversed by metoclopramide, an inhibitor of members of the D₂ family of dopamine receptors. (At the time, there was no subclassification of D₂ receptors; so any of them, which includes the D₂, D₃, and D₄ isoforms, could have been inhibited.) Binding studies and RT-PCR have demonstrated both D₂-type and D₁-type receptors (D₁A and D₁B in rat and mouse) in glomeruli and medullary tissue as well as in proximal tubules (10, 14); however, it has not been possible to demonstrate the presence of D₂-type receptors by Northern blot of mRNA from renal cortex (11), possibly because of their low relative abundance.

We have recently examined the effects of dopamine in the isolated perfused rat CCD and found that it inhibited AVP-dependent Pₒ, as well as the lumen-to-bath flux of Na⁺ (Jₙa⁺) and transepithelial voltage (Vₑ) (37). When Pₒ and Jₙa⁺ were stimulated by 8-(4-chlorophenylthio)-cAMP, dopamine had no effect, which we interpreted to indicate that the action was mediated by a D₂-type receptor that was coupled to inhibition of adenyl cyclase by the Gₛ-type G-protein.
In contrast, Satoh et al. (30, 31) have shown that dopamine increases cAMP production in isolated but nonperfused rat CCD via a D1-type receptor, which in turn inhibits Na-K-ATPase activity and might be expected to inhibit JNa. In our studies, we found no effect of the D1 agonists fenoldopam or SKF-81297 on JNa, or JNa in the presence or absence of AVP, and the effects of dopamine in the presence of AVP were not reversed by the D1A antagonist Sch-23390 (37). The D2 receptor-specific agonist quinpirole also had no effect on the AVP-dependent VT, and the D2 and D3 antagonists domperidone and pimozide did not reverse the dopamine inhibition of VT. Only clozapine, a D3-specific antagonist, reversed the effects of dopamine on AVP-dependent VT, JNa, and VT (37). In more recent studies in which cAMP generation was measured in intact rat CCD segments, we found that neither dopamine nor fenoldopam stimulated cAMP production in the absence of AVP. On the other hand, dopamine inhibited AVP-dependent cAMP production by ~50%, and this inhibition was reversed by clozapine but not by other D2- and D3-type antagonists (16). Thus we concluded that the inhibition of AVP-dependent transepithelial Na+ and water transport in the rat CCD by dopamine is mediated via a D2 receptor.

The present studies were undertaken to determine, using RT-PCR and immunohistochemistry, whether D1A and D2 dopamine receptors are expressed in the rat CCD and thus, ultimately, to identify the alternate signaling pathways that may be involved in the regulation of Na+ and water transport in this nephron segment.

METHODS

Male Sprague-Dawley rats obtained from Harlan Sprague Dawley (Indianapolis, IN) were maintained on a regular 16% protein rodent diet (diet 8746; Teklad, Madison, WI) containing 0.35% NaCl (measured in our laboratory) for 2–4 wk, at which time they were 5–7 wk of age and weighed 70–170 g. Other rats, used for isolated perfused CCD experiments, were maintained on this diet and also were treated with deoxycorticosterone (DOC) to increase the Na+ reabsorptive rate and thus increase the ability to observe change in VT with luminal dopamine treatment (4). A slow-release (3-wk) pellet containing 2.5 mg of DOC was implanted subcutaneously in the dorsum of these rats. It should be noted that DOC treatment thus increase the ability to observe change in VT with luminal dopamine treatment (4). A slow-release (3-wk) pellet containing 2.5 mg of DOC was implanted subcutaneously in the dorsum of these rats. It should be noted that DOC treatment

In the RT-PCR experiments we initially microdissected CCD segments from slices of kidney that had been digested with collagenase according to standard methods (see Ref. 34) and obtained samples of 2–20 mm total tubule length. Although we have been able to amplify mRNA from ≤2 mm of CCD for several targets, we could not easily obtain sufficient quantities to detect mRNA for the apparently less abundant dopamine D1A and D2 receptors. During this time, we developed a modified collagenase digestion technique for dissecting larger numbers of nephron segments, which has been described in detail (34). Here, we will describe the method in brief. After a kidney was removed and the capsule was stripped away, a Thomas-Stadie-Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ) was used to cut sections 0.5- to 1.0-mm thick tangential to the cortical surface. The slices were then torn into small chunks. These pieces of tissue were incubated in 2 ml of warm MEM containing 0.5 mg/ml of class 2 collagenase, 5 mM glycine, 50 U/ml DNase, and 48 µg/ml soybean trypsin inhibitor in a 20-ml scintillation vial. [All reagents were from Sigma Chemical (St. Louis, MO), with the exception of the collagenase (catalog no. 4176), which was obtained from Worthington Biochemical (Freehold, NJ)]. The cortical pieces were briefly (~5 s) and very gently agitated by hand and incubated without shaking at 37°C. At ~15-min intervals, the suspension was gently agitated, and the cloudy, tubule-rich supernatant was poured off the sedimented tissue into a 5 ml ice-cold test tube. The tubule segments sedimented rapidly in the test tube, and the supernatant was removed with a Pasteur pipette and replaced with 2 ml of ice-cold, enzyme-free MEM containing 1% BSA. CCD segments were manually sorted at 4°C from the resulting suspension of nephron segments. For each experiment, we and obtained samples of 2–20 mm total tubule length.

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D₄ Receptor Isoform

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<tr>
<th>Isoform</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>PCR Product Size, bp</th>
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<tr>
<td>#1 (161 bp)</td>
<td>317</td>
<td>477</td>
<td>865</td>
</tr>
<tr>
<td>#2 (346 bp)</td>
<td>856</td>
<td>1201</td>
<td>3</td>
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<tr>
<td>#3 (213 bp)</td>
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D₁A Receptor Isoform

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</tr>
</thead>
<tbody>
<tr>
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<td>1390</td>
<td>1916</td>
</tr>
<tr>
<td>#2 (256 bp)</td>
<td>232</td>
<td>24</td>
<td>112</td>
</tr>
</tbody>
</table>

Fig. 1. Location of PCR primer pairs for D₄ and D₁A dopaminergic receptor isoform cDNA. Diagrams show the organization of the corresponding receptor mRNA. The seven transmembrane coding regions are shown as solid black, the 3'-untranslated region is shaded in gray, and the location of genomic introns is shown by vertical lines. The coding region of the D₄ gene has a single intron in the 5'-untranslated region (45), which is not shown. Forward primers are shown by --- with a number indicating the 5' end of the amplified region. Reverse primers are shown by --- accompanied by a number indicating the 3' end of the amplified region. The total lengths of the products predicted from the published mRNA sequences are indicated by the numbers in parentheses.

2% agarose gel. For restriction analysis, PCR products were ethanol precipitated, then resuspended in sterile water. Purified products were digested with the restriction endonucleases (Table 1) according to the manufacturer's instructions.

When the presence of an isoform was indicated by restriction enzyme analysis, the PCR product was cloned into the plasmid vector pCR 2.1 by TA cloning (Invitrogen, San Diego, CA) according to manufacturer's instructions. Bidirectional sequence analysis was performed using the T7 and M13 universal primers and dye-termination reactions at the University of Alabama DNA Sequencing Core Facility (Dr. S. Hollingshead, Director). The sequences were aligned with the appropriate GenBank sequences using the GAP program of the Wisconsin Sequence Analysis Package (version 8; Genetics Computer Group, Madison, WI).

Antibodies for immunoblotting and immunohistochemistry of D₄ and D₁A dopamine receptors. We examined the distribution of the D₄ dopamine receptor using an anti-D₄ polyclonal antibody (catalog no. AS-3545G, lot 7685; R & D Antibodies, Berkeley, CA), which had been raised in rabbit against a peptide corresponding to the second extracellular loop (amino acids 176–185) of the human receptor. This corresponds to amino acids 169–178 of the published rat D₄ sequence (accession no. 231977), also in the second extracellular loop. The D₄ antibody was supplied and used as the IgG serum fraction. According to the manufacturer, it was not possible to affinity purify this antibody, because removal from the affinity column results in denaturation. Specificity of antibodies was verified by the supplier (R & D Antibodies) using immunoblotting and immunohistochemical localization to rat brain areas known to express the D₄ receptor. We have further confirmed the specificity of this D₄ receptor antibody by immunoblotting (see below).

The D₁A receptor antobody was a generous gift from Dr. Robert M. Carey (Univ. of Virginia, Charlottesville, VA). Carey and his associates (24) raised this rabbit polyclonal antibody against amino acids 299–307 corresponding to most of the third extracellular loop of the rat receptor. They carefully confirmed the specificity of the antibody (using the IgG serum fraction, without affinity purification) by demonstrating selective labeling in fibroblasts (cell line LTK−) stably transfected with the D₁A message in comparison with nontransfected cells, and they also showed specific localization of binding in rat brain sections (24). We also examined a second antibody (catalog no. D1DR; Santa Cruz Biotechnology, Santa Cruz, CA), which was raised in goat against the COOH-terminal amino acids of the human D₁ receptor, which is identical to the rat D₁A receptor in this region. Using protein extracted from rat kidney, heart, or brain, however, we could not obtain specific immunoblotting with either of two separate lots of this antibody. In these unsuccessful attempts, we first tried the blocking procedure and polyvalent biotinylated rabbit anti-goat IgG as recommended by the manufacturers. We then tried a variety of alternative blocking procedures, all without success. Thus the results reported here are those obtained using the D₁A antibody from Dr. Carey and the D₄ antibody from R & D Antibodies.

Protein extraction and immunoblotting. The heart and the opposite kidney were removed from some of the rats used for CCD microdissection and RT-PCR. The tissues were homogenized with a Tissue Tearor (model 985–370, type 2; Bartlesville, OK) in 3 ml/g of extraction buffer (20 mM Tris·HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, and 25 μg/ml each of aprotinin and leupeptin), and incubated on ice for 20 min. The supernatant was retained after centrifugation at 12,000 rpm for 20 min, and the protein concentration was determined by Micro BCA protein assay (Pierce Chemical, Rockford, IL). Extracted proteins were resolved by electrophoresis on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and stained with Ponceau S for molecular weight marker localization. The membrane was blocked overnight at 4°C in Blotto, consisting of 5% nonfat dried milk (NFDYM) and 0.025% Tween 20 in Tris-buffered saline (TBS, pH 7.4). The membrane was then incubated overnight with the D₄ antibody (see above) diluted 1:2,000 in Blotto. After three 10-min washes with TBS containing 0.025% Tween 20 (T-TBS), the membrane was incubated for 1

Table 1. Predicted sizes and restriction sites of PCR products for dopamine D₁A and D₄ cDNA

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>PCR Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₁A</td>
<td>1390–1412</td>
<td>1895–1916</td>
<td>527</td>
</tr>
<tr>
<td>D₄</td>
<td>317–340</td>
<td>455–477</td>
<td>161</td>
</tr>
<tr>
<td>D₁A</td>
<td>1066–1085</td>
<td>1302–1321</td>
<td>256</td>
</tr>
<tr>
<td>D₄</td>
<td>856–877</td>
<td>1181–1201</td>
<td>346</td>
</tr>
<tr>
<td>D₄</td>
<td>530–550</td>
<td>721–742</td>
<td>213</td>
</tr>
</tbody>
</table>

Sequences used for the analysis were as follows: D₁A (GenBank accession no. M.S3077; Ref. 44) and D₄ (MB4223; Ref. 22). Numbers in parentheses are the sizes of the restriction products predicted for the given isoform sequence. *Because of the presence of a 6-bp insert not reported in the published sequence, the actual product size was 167 and there was an additional Ava II cut site; see text.
h at 25°C in a 1:6,000 dilution in Blotto of donkey anti-rabbit IgG linked to horseradish peroxidase. Following three 10-min washes in T-TBS, the membrane was incubated with enhanced chemiluminescence substrate (ECL; Amersham, Arlington Heights, IL) and exposed to autoradiography X-ray film.

Immunohistochemistry. Rat kidneys were removed quickly, sliced in 1- to 2-mm thick coronal sections, and fixed with 4% ultrapure formaldehyde in PBS (pH 7.4). Slices were embedded in paraffin, and 4-μm sections were cut and mounted on slides. The slides were incubated at 56°C overnight, deparaffinized with xylene, and rehydrated in a graded series of ethanol solutions. Endogenous peroxidase was blocked by 0.3% H2O2 in methanol for 30 min at room temperature. For the D4 antibody, nonspecific staining was blocked with 3% normal goat serum (NGS) in PBS for 20 min at room temperature. For the D1A antibody, we blocked with 3% NGS plus 1% NFDM in Tris buffer (pH 7.2) as described by O’Connell et al. (24). (This procedure was also found to be a satisfactory alternative blocking procedure for D4 immunoblotting.) After removing the blocking solution, the sections were incubated with dilutions of the D4 antibody (1:1500 to 1:2,000 in PBS containing 2% NGS) for 30 min or of the D1A antibody (1:5 to 1:2,000 in PBS with 1% NGS plus 0.5% NFDM) for 2 h. The slides were washed twice with PBS, and the primary antibody was detected with biotinylated goat anti-rabbit IgG using an immunoperoxidase reaction (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) according to the kit instructions. Antibody localization was visualized using diaminobenzidine as the substrate for peroxidase color development and light hematoxylin counterstain. The sections were then dehydrated, and cover slips were permanently mounted. The specificity of the immunolabeling was confirmed by controls: 1) incubation without primary antibody and 2) incubation without secondary antibody.

Measurement of Vt in isolated perfused CCD. For these experiments, CCD segments were dissected from kidneys without collagenase treatment and were perfused in vitro using an isotonic artificial bathing solution similar to rat serum and a hypotonic perfusate resembling early distal tubular fluid. [Methoxy-3H]inulin was added to the perfusate as the volume marker. Perfusion at 10 to 20 nl/min was carried out at 38°C. Vt was measured between Ag/AgCl electrodes in the perfusate and bathing solution and was recorded continuously on a strip chart recorder.

Sources of other biochemicals. Amplification grade DNase I (catalog no. 18068) and SuperScript II reverse transcriptase (catalog no. 188064–014) were from Life Technologies (Gaithersburg, MD). Oligonucleotide primers were from Integrated DNA Technologies (Coralville, IA) and Oligos Etc. (Gulfport, CT). Restriction endonucleases Acc I and Ava I were from New England Biolabs (Beverly, MA) and Ava II, Ban I, Ban II, Nru I, and Pvu II were from Promega. Protein molecular weight standards were from Bio-Rad Laboratories (Hercules, CA; high-range standards, catalog no. 161–0303).

RESULTS

RT-PCR analyses were conducted for dopamine D1A and D4 isoforms in the rat CCD with, respectively, seven and eight separate groups of CCD from which RNA was extracted. Based on previous results in isolated, perfused CCD in our laboratory (37) and in nonperfused CCD segments in the laboratory of Katz and colleagues (30, 31, 32), mRNA for both D1A and D4 dopamine receptors was expected to be present. In our early experiments, in which RNA was extracted from <20 mm of CCD, we detected either isoform only infrequently. In subsequent studies, we extracted RNA from 100–200 mm of CCD, and thus RNA from ~4,000 to 8,000 cells was amplified. As summarized in Table 2, single PCR products of the expected size were readily observed for both isoforms, with a single exception in the case of each.

The first set of D4 primers (161-bp product, Table 1) restricted as anticipated with Acc I and Ban II and was detected in CCDs in seven of eight RNA extractions. The PCR product was dened into the pCR 2.1 plasmid vector, and bidirectional sequence analysis indicated 100% nucleotide identity with the published sequence from rat atria (25); however, there was an additional 6-bp (GTCCAG) insert within our PCR product precisely at the site of the first intron. As shown in Fig. 2, our PCR sequence is identical to the mouse and human D4 receptor sequences in this region, and our 6-bp sequence matches the beginning of exon 2 in those species (9, 40). Our sequence was also confirmed by the fact that the extra six bases introduce an Ava II restriction site in the PCR product, and we obtained products of the size predicted by the human and mouse sequences (9, 40). We used the same primer pair to amplify cDNA from rat atria and brain and confirmed the presence of the same 6-bp insert by Ava II restriction and sequence analysis (data not shown). In the rat sequence published by O’Malley et al. (25), the same nucleotide sequence was included as the ending of the first intron, but subsequent sequencing of the rat D4 cDNA by Dr. O’Malley’s laboratory revealed the presence of the same additional 6 bases (personal communication) in the exon (see DISCUSSION).

The second set of D4 receptor primers produced the expected 346-bp product, corresponding to the region from c3 to tm7, which restricted with Ava I, Ban I and Pst I. Identity of the PCR product was verified by bidirectional sequencing. The third set of primers produced the expected 213-bp product, from the c2 to the tm5 region, which restricted as predicted with Acc I, Nru I, and Pvu II (Fig. 3), and was verified by bidirectional sequencing.

In experiments with the first set of D1A receptor primers, the 527-bp product, corresponding to nucleotides from tm7 to the 3′-untranslated region, restricted with Ava I and Ban II (Fig. 4) as anticipated from the published sequence, and was detected in four of four experiments. The second primer set, corresponding to the third cytoplasmic loop (tm5–tm6) of the D1A recep-

Table 2. Detection of dopamine receptor isoforms in rat CCD by RT-PCR

<table>
<thead>
<tr>
<th>Nephron Segment</th>
<th>D1A Isoform</th>
<th>D4 Isoform</th>
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<tbody>
<tr>
<td>CCD</td>
<td>6/7</td>
<td>7/8</td>
</tr>
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</table>

Results are expressed as number of experiments in which the dopamine receptor isoform was detected compared to the total number of groups of cortical collecting ducts (CCD) from which RNA was extracted. Each group consisted of ~100 mm of microdissected CCD segments, and ~10% of the total cDNA obtained from reverse transcription was amplified (see text).
tor isoform, was 258 bp and restricted as expected with Ban I and Bcl I. It was detected in three of four experiments. Both PCR products were verified by bidirectional sequencing.

We initially attempted to confirm the presence of both receptors at the protein level by immunoblotting of protein extracted from microdissected CCD segments. Extraction of 100 mm of CCD yields slightly more than 10 µg of protein, and we have been successful in using such samples to demonstrate the presence of specific protein kinase C isoform proteins in the rat and rabbit CCD (41). However, despite repeated attempts using a variety of antibody dilutions, and blocking and development procedures, we were unable to obtain acceptable immunoblots of protein extracted from 100–140 mm of CCD segments using the D4 antibody. (We did not examine the D1A antibody from Dr. Carey, because its specificity had already been confirmed.) However, using protein extracted from rat heart, which has a 20-fold higher expression of the D4 receptor mRNA than other tissues (24), and a greater amount of total protein extracted from whole rat kidney cortex than could be obtained from the microdissected CCD segments, the D4 antibody reacted strongly with a band of the expected 41–42 kDa size of the nonglycosylated protein as shown in Fig. 5. There was also a heavy band of 58–59 kDa and a very faint band of ~48 kDa in the heart protein, but only the heavier band was present in the kidney cortex. Labeling of all three bands was blocked by preadsorption of the antibody to its antigenic peptide. Because the D4 receptor protein has a glycosylation site, it is likely that the heavier band represents the glycosylated form of the protein and that this is the primary form found in the kidney cortex.

We then used this D4 antibody and the D1A antibody from Dr. Carey for immunohistochemistry on coronal slices of rat kidney. We found that the latter antibody labeled the tissue as described by O’Connell et al. (24). Labeling was observed primarily in the cortex and not the medulla. The cortical labeling was diffuse and light throughout proximal tubules, distal tubules, and CCD (data not shown). In contrast, the D4 antibody labeled exclusively in the medulla and medullary rays in the cortex, where it is was specific to CCD segments and to

| D4 PCR product | 5’-CTACTCCGAG | GTCCAG | GGTGGGCT | T | GTGGCT |
| Rat D4 sequence | 5’-CTACTCCGAG | ...... | GGTGGGCT | T | GTGGCT |
| Mouse D4 sequence | 5’-CTACTCCGAG | GTCCAG | GGTGGGCT | T | GTGGCT |
| Human D4 sequence | 5’-CTACTCCGAG | GTCCAG | GGTGGGCT | C | GTGGCT |

Fig. 3. Restriction analysis of the PCR product obtained using primers for the 3rd transmembrane region and the 3rd cytoplasmic loop of the D4 receptor isoform. Product was of the correct size and restriction pattern predicted. Values on left are band sizes in bp.

Fig. 4. Restriction analysis of the PCR product obtained using primers for the 7th transmembrane region and the 3’-untranslated region of the D4A receptor isoform. Product was of the correct size and was cut into the predicted fragments by the three restriction enzymes used. Values on left are band sizes in bp.
significant hyperpolarization of five experiments shown in Fig. 7, AVP produced a bathing solution, thus returning to the conditions of the finally, the removal of dopamine and clozapine from the bathing solution significantly depolarized cant effect. Addition of 10 µM dopamine to the bathing solution of isolated perfused rat CCD and not functional effects of dopamine only when added to the surface than on the basolateral membrane. that the immunoreactivity was greater on the luminal whole length of the medullary collecting ducts (Fig. 6, A and B). No staining was seen in any other segments. At high magnification (Fig. 6C), it appeared that the immunoreactivity was greater on the luminal surface than on the basolateral membrane.

In our previous studies (37), we had examined the functional effects of dopamine only when added to the bathing solution of isolated perfused rat CCD and not the lumen. Because of the apparent localization of immunoreactive D_4 protein on the luminal membrane, we conducted a series of experiments in which we examined the effect of luminal dopamine on V_T. In the five experiments shown in Fig. 7, AVP produced a significant hyperpolarization of V_T in the CCD from DOCT-treated rats; however, the further addition of 100 µM dopamine to the luminal perfusate had no significant effect. Addition of 10 µM dopamine to the bathing solution significantly depolarized V_T, and the effect was reversed by the further addition of 10 µM clozapine to the bathing solution as reported previously (37). Finally, the removal of dopamine and clozapine from the bathing solution, thus returning to the conditions of the second period, had no effect on V_T. In summary, the only effect of dopamine on V_T was observed when it was added to the bathing solution, and that effect was reversed by clozapine.

DISCUSSION

The data presented above demonstrate the presence of mRNA for both the D_1A and D_4 dopamine receptor isoforms in the rat CCD (Table 2) and of protein immunoreactive with a D_4 antibody in the CCD and medullary collecting duct (Figs. 5 and 6). We also observed labeling of all cortical nephron segments with the D_1A antibody as described by O’Connell et al. (24). Other investigators have demonstrated the presence of D_1-type and specific D_1A and D_2 receptors by binding studies and RT-PCR of digest of rat renal cortex and medulla (10, 21, 25), as well as in microdissected and sieved rat proximal tubules and juxtaglomerular cells (8, 42, 43). A D_1-type receptor has also been found by binding studies in the rat CCD (39). RT-PCR has also revealed D_3 and D_4 receptor isoform mRNA in rat juxtaglomerular cells (29) and the D_4 isoform in human kidney mRNA (20). The fact that we had to use a relatively large sample of CCD segments to observe expression of either the D_1A or D_4 isoform by RT-PCR suggests that the mRNA is present in low copy number. This would also explain why the D_4 isoform was not observed by Northern blot analysis of RNA from whole kidney, especially considering that the isoform is confined to the CCD and medullary collecting duct (see Fig. 6).

We expected that the age of the rats used in these studies (5–7 wk) was appropriate for detecting at least the D_1A receptor. Kansra et al. (15) reported that there is a ~50% decrease in renal D_1A receptor density in old (23–24 mo) rats compared with adult rats (6 mo), and that there was also decreased expression of receptor-linked G protein in the older rats. Felder et al. (8) reported that there was no age-related difference in the maximum D_2 receptor density in proximal convoluted tubules from SHR and WKY rats examined at 3, 8, and 20 wk of age; however, there was an increase in dopamine-stimulated adenyl cyclase activity in the natriuretic effect of dopamine with age in the WKY rats. If the D_4 receptor density also declines with age, then this may explain why our functional studies with CCD from young rats have indicated the presence of this receptor (37), whereas there is no functional evidence for its presence in other studies in which older animals were used (e.g., Refs. 30, 38).

Sequencing with the first set of D_4 primers showed an extra 6 bp in comparison with the published rat D_4 sequence (25), corresponding to the beginning of the second exon in the mouse and human sequences (Fig. 2). Sequencing of the RT-PCR products from rat atria and brain RNA also showed the same 6-bp insert. We have since learned from Dr. K. O’Malley (personal communication) that her group has examined rat D_4 cDNA, and they also observed this 6-bp insert, which was originally overlooked, probably as the consequence
of a mis-assigned splice acceptor site in the genomic sequence.

Immunohistochemistry supported the presence of the D4 receptor isoform in the CCD and also in the medullary collecting duct (Fig. 6). We verified the specificity of the D4 antibody by immunoblotting (Fig. 5), and we also supplied a sample of the same lot of the antibody to Dr. K. O’Malley (Washington Univ., St. Louis, MO) for additional testing. Dr. O’Malley’s laboratory found (personal communication) that the antibody stained structures in the retina, primarily in the inner nuclear layer, in a pattern which was similar to that which they observed previously for the D4 receptor mRNA by in situ hybridization (5). (The D4 receptor in the retina has been demonstrated to be coupled to the modulation of the dark level of light-sensitive cAMP production in photoreceptors; Ref. 5). O’Malley’s group (personal communication) also found that our D4 antibody labeled a ~41-kDa protein on immunoblot analysis of proteins extracted from a cell line that had been stably transfected with the D4 receptor message, but did not react with proteins from nontransfected cells.

Cortical labeling was seen only in the medullary rays and was associated exclusively with the CCD. Labeling of the medullary collecting duct was, if anything, more dense than in the CCD, and the entire length of the collecting duct showed the presence of the receptor. Maeda et al. (19) have examined the effect of dopamine of AVP-dependent cAMP production in a limited num-

Fig. 6. Immunohistochemistry to localize binding of antibody reactive with the D4 receptor isoform in the rat kidney. A: low-power (×2) magnification of a section from near the cortical surface (top) to the inner medulla (bottom). Note staining of medullary collecting ducts and medullary rays extending into the cortex. B: localization of labeling to cortical collecting duct (CCD) in medullary rays (×12). Region shown is just above the corticomedullary junction. C: high-power (×60) magnification of staining in a CCD within a medullary ray. Section has been rotated 90° from the orientation in A and B.

Fig. 7. Effect of dopamine added to the luminal versus bathing solutions on transepithelial voltage (VT). Voltages are presented as −VT; i.e., all are lumen negative, although they are presented as positive values. Arginine vasopressin (AVP) was added (+) to the bathing solution in the 2nd period through the last period at 22 pM. Luminal dopamine (DA) was present at 100 µM from the third to the last period. Dopamine was added to the bathing solution at 10 µM in the 4th and 5th period, with 10 µM clozapine (cloz.) added in the 5th period. Average values for 5 experiments are presented. *Significantly different from preceding period mean at P < 0.01, determined by ANOVA with Scheffé posthoc test for significance.
number (n = 3) of inner medullary collecting duct (IMCD) segments. Although they found no effect of dopamine at 0.01 or 1 µM, 100 µM produced a significant inhibition of cAMP production in the presence of 100 pM AVP. Maeda et al. (19) suggested that the effect of such a high dose might have been mediated by the α2-adrenergic receptors that they observed in the IMCD in the same study; however, they did not test the effect of concentrations in the range of 1–10 µM that we have found to be the most effective (16, 37).

It was puzzling that in both the cortex and the medulla the labeling with the D4 antibody appeared to be heavier on the luminal surface than on the basolateral surface. For that reason, we undertook experiments with isolated perfused CCD to see whether we could obtain an effect of luminal dopamine. As shown in Fig. 7, we were unable to detect any inhibition of AVP-dependent Vt in these experiments, so the function of the luminal receptors remains unclear.

The effects of dopamine on the distal regions of the nephron have been a matter of controversy. Muto et al. (23) showed that dopamine inhibited AVP-stimulated osmotic water permeability in the isolated perfused rabbit CCD presumably by a D2-type receptor. On the other hand, Satoh et al. (30, 31, 32) have shown that dopamine decreases Na-K-ATPase activity in nonperfused rat CCD segments via a D2-type receptor, which increases the production of cAMP and activates protein kinase A. Despite the fact that dopamine appears to decrease Na-K-ATPase activity in the basolateral membrane, it seems improbable to us that dopamine could inhibit transepithelial Na+ transport by such a mechanism, because AVP is known to stimulate Na+ reabsorption in the rat CCD by increasing intracellular cAMP, which thereby increases the Na+ conductance of the luminal membrane (4, 28, 33, 35). Thus it would seem that any effect of dopamine to decrease Na-K-ATPase activity through a D2 receptor is superseded by the effect of cAMP on the luminal membrane Na+ channels.

As discussed in the introduction, we have demonstrated that dopamine inhibited the Na+ and water transport in isolated perfused rat CCD and the inhibition was mediated by a D2-like receptor (37). In more recent experiments (16), we also examined the effect of dopamine and fenoldopam on cAMP generation in microdissected, intact CCD segments. We found that neither fenoldopam nor dopamine had any significant effect on cAMP generation in the absence of AVP. However, when cAMP generation was increased by AVP, dopamine but not fenoldopam inhibited this increase, and this effect was reversed by 10 µM doxapine (16). Clozapine also has limited affinity for serotonin (5-HT) receptors; however, the 5-HT1 subgroup of these receptors, the subgroup that inhibits cAMP production, is not affected by dopamine.

There are two caveats to our interpretation of these results. First, our studies were conducted in young (5- to 7-wk-old) rats. Although Felder et al. (8) observed no difference in D1A receptor density in proximal tubules from the WKY rat, they did observe diminished cAMP production in response to dopamine in the younger rats, which might explain our inability to demonstrate an increase in cAMP generation with fenoldopam or dopamine. The second caveat is that the CCD used in our previous studies with isolated perfused segments came primarily from DOC-treated rats (37). One set of experiments in that study did examine CCD from rats that were untreated with DOC and showed the same pattern of response to dopamine and doxapine, with no effect of fenoldopam. Our studies on cAMP production were also conducted on CCD from rats receiving no DOC treatment (16), as were all of the experiments in the present study, with the exception of those reported in Fig. 7.

It is also possible that the D4 receptors could also be associated with alternative intracellular coupling mechanisms. Although the D4 receptor has been linked to inhibition of adenylly cyclase (5) as have the other D2-type receptors, when the D2 receptor is expressed in CHO cells, dopamine and the D2 receptor agonist quinpirole stimulate PLA2 (26). It seems unlikely, however, that this is the mechanism of dopamine action in the rat CCD. Quinpirole has no effect on transepithelial transport process stimulated by AVP (37), and we have found that prostaglandin E2 is not inhibitory to either Na+ transport or water permeability in the rat CCD (3).

Based on the present data as well as our past studies (16, 37), we conclude that both the D1A and D4 dopamine receptors are expressed in the rat CCD. The effects mediated by the D1A receptor have been observed only in nonperfused preparations in which transepithelial transport was not measured, and any effects of this receptor do not override the effect of dopamine on transepithelial transport in the presence of AVP. Thus we conclude from the current data and previous functional studies (16, 37) that the diuretic and natriuretic effects of dopamine in the CCD are primarily mediated by the D4 receptor.

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