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Heterogeneous Expression of α_1-Adrenoceptor Subtypes among Rat Nephron Segments

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SUMMARY

α_1-Adrenoceptor subtypes mediate many of the actions of the renal nerve, but their locations along the nephron are unknown. We investigated the distribution of α_1-adrenoceptor subtype mRNA and protein in rat proximal tubules and medullary thick ascending limbs (MTAL) using reverse transcription combined with polymerase chain reaction (PCR) and radioligand binding methods. Complementary primers were designed to span cDNA sequences in each of the third intracellular loops of the rat α_{1B}- and α_{1D}-adrenoceptors. Expression of the mRNA of α_{1B}- and α_{1D}-adrenoceptors was first detected in total RNA from whole rat kidney, and the PCR product identity was confirmed by sequencing. Endogenous expression of α_{1B}- and/or α_{1D}-adrenoceptor mRNA was then investigated in microdissected segments of the rat proximal convoluted tubule (S_2 segments) and the MTAL. mRNA was reverse-transcribed directly from permeabilized microdissected segments and the resulting cDNA was subjected to PCR with the α_1-adrenoceptor primers. In proximal convoluted tubules, amplification of both α_{1B}- and α_{1D}-adrenoceptor mRNA was observed. In MTAL segments, only α_{1D}-adrenoceptor mRNA was detected. We also measured receptor protein using [^{3}H]prazosin in saturation and competition binding experiments. Proximal tubular membranes contained 3.3-fold more α_1-adrenoceptor than did MTAL membranes (163 ± 21 versus 49 ± 3 fmol/mg of protein). When the alkylating agent chloroethylclonidine (CEC) (10 μM, 10 min) was used to define α_1-adrenoceptor subtypes, proximal tubules were found to contain primarily CEC-insensitive (α_{1A}) sites (68 ± 4%) and MTAL primarily CEC-sensitive sites (75 ± 3%). Most [^{3}H]prazosin binding sites (72 ± 2%) in MTAL segments were also sensitive to the alkylating agent SZL-49, consistent with their identification as α_{1D}-adrenoceptors. In competition studies with the antagonists WB4101, 5-methylurapidil, and (+)-niguldipine, both high and low affinity sites were observed in proximal tubules. WB4101 interacted with only one site in MTAL membranes, intermediate in affinity between those sites found in proximal tubules. We conclude that reverse transcription-PCR is a useful method for demonstrating the expression of α_1-adrenoceptor subtypes in small amounts of tissue. Results from our experiments suggest that α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors are all expressed in proximal tubules and that α_{1D}-adrenoceptors are the primary α_1-adrenoceptor subtype expressed in MTAL. The distinct anatomical distribution of each of these adrenoceptor subtypes suggests that they serve different functions in the kidney.

α_1-Adrenoceptors are densely expressed in the rat kidney (1, 2) and are primary mediators of sympathetic innervation to the kidney (3). For example, low-level renal nerve stimulation (which does not change renal blood flow or glomerular filtration rate) causes α_1-adrenoceptor-mediated increases in Na^+ reabsorption in the proximal tubule (4, 5) and the MTAL (6). In addition to antinatriuresis and antidiuresis, renal tubular α_1-adrenoceptors mediate other important physiological effects of the renal nerve, including decreased urinary calcium, phosphate, and bicarbonate excretion (7) and increased gluconeogenesis (8, 9). At higher stimulation frequencies, renal nerve activation causes renal vasoconstriction, which is primarily mediated by vascular α_1-adrenoceptors (7, 10). We have previously shown that the regional distribution of renal α_1-adrenoceptors is not uniform, with the highest density being in the cortex, followed by the outer stripe of the outer medulla, then the ISOM, and then inner medulla (11).

It has recently been discovered that α_1-adrenoceptors are a heterogeneous class of receptors and can be divided into subtypes based on antagonist affinity and sensitivity to alkylating agents (12–14). In a widely accepted classification scheme, α_1-adrenoceptors that are insensitive to the alkylating agent CEC and have a high affinity for the antagonists WB4101, (+)-niguldipine, and 5-methylurapidil are defined as α_{1A}-adrenoceptors (12, 14–16). Those receptors that are alkylated by CEC and have a low affinity for WB4101, (+)-niguldipine, and 5-methylurapidil are classified as α_{1B}-adrenoceptors (12, 14–16).

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ABBREVIATIONS: MTAL, medullary thick ascending limb of Henle; ISOM, inner stripe of the outer medulla; CEC, chloroethylclonidine; PCR, polymerase chain reaction; RT, reverse transcription; bp, base pair(s).

These subtypes have discrete tissue distributions and distinct signal transduction pathways (13).

The existence of multiple α_1-adrenoceptor subtypes has been confirmed in molecular cloning experiments. The α_{1B}-adrenoceptor was originally identified in hamster DDT$_1$ MF-2 smooth muscle cells (17) and was subsequently cloned from the rat (18). An additional rat α_1-adrenoceptor gene, identified by Lomasney *et al.* (19), encodes a protein of 560 amino acids and contains one intron. This receptor was originally reported to be the α_{1A}-adrenoceptor, based on radioligand binding studies of the transfected gene (19). However, Perez *et al.* (20) cloned a rat α_1-adrenoceptor with a virtually identical sequence and found that the expressed protein did not exhibit binding properties consistent with those of the α_{1A}-adrenoceptor (e.g., it was highly sensitive to CEC). They thus suggested that this gene encodes a novel α_1-adrenoceptor, classified as α_{1D}. A recent report from Schwinn and Lomasney (21) has confirmed that this cloned receptor is not the "classical" α_{1A}-adrenoceptor. Because the combined data suggest that the α_{1A}-adrenoceptor has not been cloned, in this paper we refer to the sequence reported by Lomasney *et al.* (19) and Perez *et al.* (20) as the α_{1D}-adrenoceptor. An additional α_1-adrenoceptor subtype, termed α_{1C}, has also been cloned from a bovine brain cDNA library (22). This gene encodes a protein of 466 amino acids and is intronless. When expressed in COS-7 cells, this receptor also does not display a radioligand binding profile that conforms to the accepted pharmacological classification scheme (22).

Recently we reported that the relative distribution of α_1-adrenoceptor subtypes (as determined by CEC sensitivity and WB4101 affinity in radioligand binding experiments) was heterogeneous among the regions of the rat kidney (11). In the cortex and outer stripe of the outer medulla, CEC-insensitive and CEC-sensitive α_1-adrenoceptor subtypes are expressed in an approximately 1:1 ratio; however, in the ISOM, approximately 85% of the α_1-adrenoceptors are CEC sensitive. We have hypothesized that the distinct regional distribution of α_1-adrenoceptor subtypes results from expression of α_1-adrenoceptor subtypes in discrete nephron segments. In the present study we sought to determine, using [^{3}H]prazosin radioligand binding, the relative expression of α_1-adrenoceptor subtypes in membranes prepared from proximal tubules and MTAL. The recent discovery of the nucleic acid sequences encoding the genes for some of these subtypes has made it possible to identify α_1-adrenoceptors on a molecular basis. We therefore also investigated the expression of mRNA for cloned α_1-adrenoceptor subtypes in the kidney, using RT combined with the PCR.

Materials and Methods

Isolation of proximal tubules and MTAL for radioligand binding studies. Male Sprague-Dawley rats (13–16 weeks of age) were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Proximal tubules were prepared by collagenase digestion of renal cortical slices, with subsequent separation by Percoll gradient centrifugation, as described by Gesek *et al.* (23). The proximal tubules thus isolated were characterized by their gross morphology, their high alkaline phosphatase activity, and their low hexokinase activity (23). Tubule purity was >95% in all cases. MTAL were isolated as described by Trinh-Trang-Tan *et al.* (24). The ISOM was carefully dissected from saggital kidney slices, minced, and then incubated at 37° for three 20-min periods with a Krebs-Henseleit buffer (24) containing collagenase (150 units/ml). The tubule suspension was passed through a 105-μm mesh nylon sieve. The MTAL segments did not pass through the sieve and were collected. To identify the isolated MTAL segments, an immunofluorescence method was used to label the Tamm-Horsfall glycoprotein, a cell surface protein found only on MTAL (25). The isolated tubules were incubated for 35 min at 24° with rabbit anti-Tamm-Horsfall serum (Biomedical Technologies Inc., Stoughton, MA), washed twice, and then incubated for 30 min at 24° with goat anti-rabbit IgG-fluorescein isothiocyanate conjugate (Sigma Chemical Co., St. Louis, MO), followed by two additional washes. Labeling was assessed with a Nikon Diaphot epifluorescence microscope (excitation wavelength, 450–490 nm; emission barrier, 510 nm). Nonspecific fluorescence was determined in MTAL segments incubated for 30 min at 24° with only goat anti-rabbit IgG-fluorescein isothiocyanate conjugate. More than 95% of the isolated tubules were found to be fluorescent.

Membrane preparation and radioligand binding. Tubule suspensions were immediately stored at −70° and were used within 4 weeks. Tubule suspensions were homogenized with a Tekmar Tissuemizer in a 50 mM Tris·HCl buffer (with 2 mM EDTA, pH 7.6). Due to the small amount of MTAL obtained, tissue from seven to nine rats was pooled for each experiment. The homogenates were then centrifuged for 20 min at 45,440 × *g*. The pellets were resuspended and centrifuged as before. The methods for treatment of membranes with CEC, for [^{3}H]prazosin binding, and for WB4101 competition experiments were described in our previous study (11). Briefly, for CEC alkylation, 10 μM CEC was added to one half of the membrane suspensions and incubated for 10 min at 37°, in a shaking water bath. The membranes were then pelleted at 45,440 × *g*, washed twice with 50 mM Tris·HCl buffer (with 2 mM EDTA, pH 7.6), and recentrifuged. For saturation binding studies, tubular membranes were incubated with six concentrations (0.02–2 nM) of [^{3}H]prazosin (87 μCi/mmol; NEN, Boston, MA). In the original classification described by Han *et al.* (26), the amount of specific prazosin binding that remains after CEC treatment (CEC-insensitive sites) in paired experiments is considered to be the α_{1A}-adrenoceptor density and the portion of binding eliminated by CEC treatment (total [^{3}H]prazosin binding minus CEC-insensitive sites) represents the density of α_{1B}-adrenoceptors (12). Because at least one additional CEC-sensitive α_1-adrenoceptor subtype has been identified in the rat (20), the irreversible antagonist SZL-49 was used to alkylate α_1-adrenoceptors in some MTAL membrane preparations, to distinguish α_{1B}- and α_{1D}-adrenoceptors (20). SZL-49 (10 nM) was added to one half of the membrane suspensions and incubated for 10 min at 37°, in a shaking water bath. The membranes were then pelleted, washed twice with 50 mM Tris·HCl buffer, and recentrifuged. MTAL membranes were then used in [^{3}H]prazosin saturation studies as described above for CEC-treated membranes. Competition studies were performed in the presence of 0.2 nM [^{3}H]prazosin, with 16 concentrations of the α_1-adrenoceptor antagonist WB4101. Binding data were analyzed with the LIGAND computer program (27). Other statistical analyses were performed with analysis of variance and the Newman-Keuls test for multiple comparisons. All values presented represent mean ± standard error.

Microdissection of proximal tubule and MTAL segments. The method for microdissection of proximal tubules and MTAL was described previously by Umemura *et al.* (28). Male Sprague Dawley rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). The left kidney was retrogradely perfused with 20 ml of ice-cold Krebs-Ringer bicarbonate buffer, followed by 10 ml of collagenase solution (600 units/ml in Krebs-Ringer bicarbonate buffer). The microdissection of nephron segments was performed under a stereomicroscope, in ice-cold Hanks' solution. Nephron segments were identified based on previously established criteria (28). Proximal tubule or MTAL segments (1–2 mm) were captured with glass microbeads and transferred to tubes with 20 μl of dissection buffer containing >1 unit/μl human placental RNase inhibitor (Promega, Madison, WI). The tubules were then rinsed twice with ice-cold dissection buffer before RT.

RT. RT reactions were performed using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). The protocol used in our

experiments was a modification of that described by Moriyana *et al.* (29). The dissection buffer was removed and 9 μl of 2% Triton X-100 solution containing >1 unit/μl RNase inhibitor and 5 mM dithiothreitol were added. The components of the final 20-μl reaction were as follows: 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris·HCl, pH 8.3, 1 mM levels each of dATP, dCTP, dGTP, and dTTP, 1 unit/μl RNase inhibitor, 2.5 μM random hexamers, and 2.5 units/μl reverse transcriptase. The negative control tubes contained all of the aforementioned components except reverse transcriptase. All reaction tubes were incubated at 42° for 45 min. In some experiments, total RNA from whole rat kidneys was also subjected to RT.

PCR. PCR experiments were performed using the GeneAmp RNA PCR kit. We designed and synthesized specific primers of 21 nucleotides in length, using an Applied Biosystems 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). These primers were designed, by using the OLIGO computer program, to span the nucleotide sequence of the third intracellular loop, specifically amplifying from transmembrane region 5 of the α_{1D}- and α_{1B}-adrenoceptors to transmembrane region 6 of the α_{1D}-adrenoceptor or transmembrane region 7 of the α_{1B}-adrenoceptor. This region was chosen due to the low percentage of identity between α_1-adrenoceptor subtypes (60%). For the α_{1D}-adrenoceptor, the upstream primer was defined by bases 759–779, where base 1 represents the first base of the translation start codon, resulting in a sequence of 5′-CGTGTGCTCCTTCTACCTACC-3′. The corresponding downstream primer represents sequences from the antisense strand and was defined by the complement of bases 1042–1062, with a sequence of 5′-GCACAGGACGAAGACACCCAC-3′. The cDNA sequence for this adrenoceptor was described by Lomasney *et al.* (19) and Perez *et al.* (20). The cDNA amplification product of the α_{1D}-adrenoceptor was predicted to be 304 bp in length. The base sequence for the rat α_{1B}-adrenoceptor cDNA was described by Voigt *et al.* (18). The upstream primer for the α_{1B}-adrenoceptor gene was defined by bases 629–649 on the sense strand, as numbered from the start of the open reading frame, with a sequence of 5′-GCTCCTTCTA-CATCCCGCTCG-3′, and the downstream primer corresponded to the antisense strand or complement of bases 983–1003, with a sequence of 5′-AGAACACCACCTTGAACACGG-3′. The cDNA amplification product of the α_{1B}-adrenoceptor was predicted to be 375 bp in length. After RT, 80 μl of PCR reagent mixture were added to each reaction tube. Thus, total PCR volume was 100 μl, including the original 20 μl from the RT reaction mixture. The PCR mixture contained the following components (final concentrations): 50 mM KCl, 10 mM Tris·HCl, pH 8.3, 2 mM $MgCl_2$, 200 μM levels each of dATP, dCTP, dGTP, and dTTP, 0.25 μM levels of each primer, and 2.5 units/100 μl AmpliTaq DNA polymerase. The reaction tubes were overlaid with 70 μl of mineral oil to prevent evaporation. The PCR was performed in an automated thermal cycler (Ericomp Twinblock system; Ericomp, Inc., San Diego, CA). The reaction mixture was heated initially to 95° for 3 min for the initial denaturation step and was thermocycled 30 or 50 times at 95° for 1 min (denaturation), 58.5° for 1 min (primer annealing), and 72° for 1 min (amplification); a final extension was performed at 72° for 5 min. Total rat kidney RNA was cycled 30 times and microdissected proximal tubule and MTAL segments 50 times. PCR products were separated by horizontal electrophoresis on a 3% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME) and were stained with ethidium bromide. DNA was visualized with an UV transilluminator and photographed.

Cloning of PCR products. The PCR products were cloned and sequenced to confirm their identity. The PCR products were cloned into the pCRII vector using the TA cloning kit from Invitrogen, Inc. The PCR adds single deoxyadenosine residues to the 3′ end of all PCR fragments, thus allowing ligation to single 3′ thymidine overhangs present in the cloning site of the pCRII vector. Standard T4 DNA ligase reactions were performed using 1 μl of the undiluted PCR mixture and 2 μl of the vector, with incubation for 4 hr at 12°. Competent cells were transformed with the ligation mixture and the transformed cells were screened for recombinant plasmids by plating of the transformation mixture on Luria-Bertani agar plates with ampicillin (50 μg/ml) and 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside on the surface of the plates.

Plasmid DNA isolation. Single colony isolates were grown overnight in Luria-Bertani medium with ampicillin (50 μg/ml), and plasmid DNA was isolated according to standard alkaline lysis preparation procedures, which included treatment of the plasmids with RNase. The presence of the correct α_{1B}- and α_{1D}-adrenoceptor PCR products in the recombinant plasmids was confirmed by restriction endonuclease digestion with *Eco*RI and visualization of the vector and excised fragments by horizontal agarose gel electrophoresis. The PCR product was ligated into a cloning site that was flanked on each side by *Eco*RI restriction endonuclease sites.

Sequencing. The Sanger dideoxy chain termination method was used to sequence alkaline-denatured double-stranded plasmids, using [α-^{35}S]dATP (specific activity, >1000 Ci/mmol). The plasmids (3–5 μg) to be sequenced were isolated by the alkaline lysis preparation procedure and then treated with RNase before denaturation. Both strands of the plasmids containing the α_{1B}- and α_{1D}-adrenoceptor PCR products were sequenced using either the M13 (−40) forward (GTTTTCCCAGTCACGA) or M13 reverse (CAGGAAACAGCTAT-GAC) sequencing primers outside the multiple cloning site of pCRII. In addition, the α_{1B}- and α_{1D}-adrenoceptor upstream and downstream primers were used to sequence those bases toward the 3′ ends of the strands. In areas in which GC compressions were a problem, the sequence was resolved by substituting dITP and/or 7-deaza-dGTP. The sequencing products were vertically electrophoresed through a 7 M urea/6% acrylamide denaturing gel in buffer containing 0.089 M Tris base, 0.089 M boric acid, and 0.002 M Na_2EDTA, pH 8.3. The gels were transferred to blotting paper, vacuum dried at 80°, and exposed to Kodak XAR-5 X-ray film for a minimum of 8 hr.

Results

Specific, high affinity [^{3}H]prazosin binding was detected in membranes of both proximal tubules and MTAL (Fig. 1). Specific binding was 66–90% in all saturation experiments. Mean α_1-adrenoceptor density in proximal tubules was 163 ± 21 fmol/mg of protein, 3.3-fold higher than the density in MTAL, which was 49 ± 3 fmol/mg of protein. No difference in the affinity for [^{3}H]prazosin was noted between proximal tubules (K_d = 79 ± 7 pM) and MTAL (K_d = 112 ± 23 pM). Pretreatment with CEC significantly ($p < 0.01$) reduced the α_1-adrenoceptor density (Fig. 1), with no change in affinity, in proximal tubules (K_d = 90 ± 7 pM) and MTAL (K_d = 100 ± 5 pM). The α_1-adrenoceptor density remaining after CEC treatment was 11.2-fold higher in proximal tubular membranes,

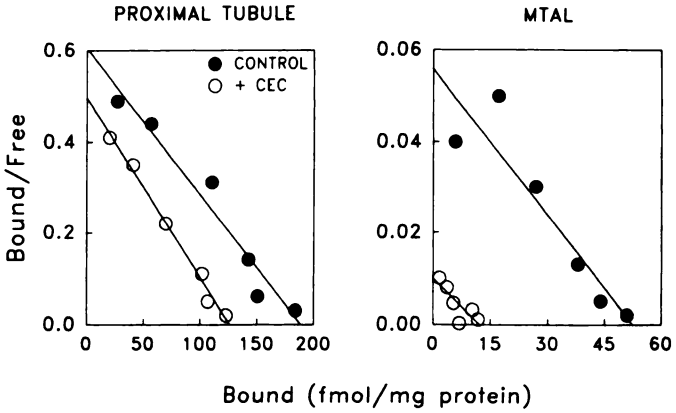

Fig. 1. Typical Rosenthal plots of saturation experiments in membranes from proximal tubules and MTAL. *+CEC*, membranes pretreated for 10 min with CEC (10 μM). Each *curve* is typical of three experiments.

compared with MTAL, but the number of CEC-sensitive sites was similar in proximal tubules and MTAL. Thus, when considered as a percentage of total number of [^{3}H]prazosin binding sites within each segment, CEC-insensitive α_1-adrenoceptors (α_1-like) were found to predominate in proximal tubules but CEC-sensitive sites (α_{1B}- or α_{1D}-adrenoceptors) predominated in MTAL (Table 1).

The competition for [^{3}H]prazosin binding by WB4101, 5-methylurapidil, and (+)-niguldipine was measured as an independent means of determining the relative α_1-adrenoceptor subtype distribution (13). The competition curves for WB4101 in proximal tubules (Fig. 2) were shallow and biphasic. The competition curves for MTAL membranes were steeper and shifted to the right of those obtained for proximal tubules (Fig. 2). Biphasic curves were also obtained in the proximal tubules with 5-methylurapidil and (+)-niguldipine (Fig. 3). Due to the relatively small amounts of tissue obtained with our MTAL separation procedure, competition studies with 5-methylurapidil and (+)-niguldipine were not attempted in MTAL. Analysis of the data with LIGAND (26) revealed that all antagonist competition curves for proximal tubules best fit a two-site model ($p < 0.01$, versus a one-site model) (Table 2), but WB4101 curves for MTAL best fit a one-site model ($p < 0.05$, versus a two-site model), with K_i values intermediate between those of the low affinity sites and the high affinity sites found in proximal tubules (Table 2). The percentages of high and low affinity sites calculated from the antagonist competition studies in proximal tubules were similar to those seen in our studies with CEC treatment (compare Table 1 with Table 2). Thus, the competition data suggest a preponderance of high affinity sites (α_{1A}-adrenoceptor subtype) in the proximal tubules but mostly lower affinity sites (α_{1B}- or α_{1D}-adrenoceptor subtype) in the MTAL segments.

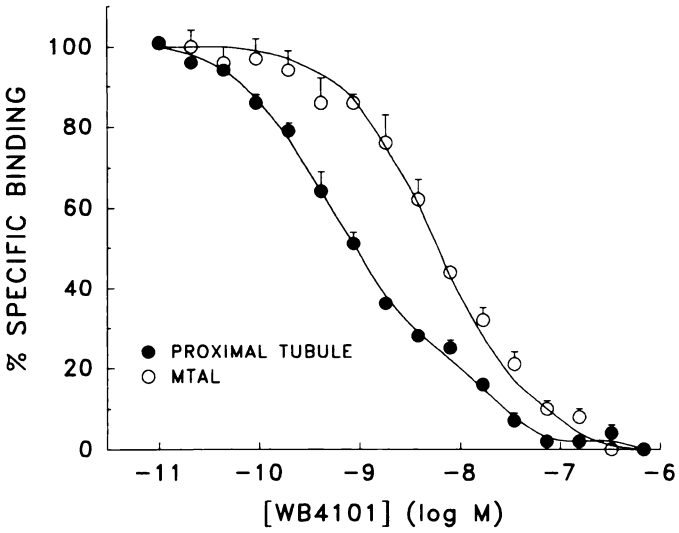

Fig. 2. Competition by WB4101 for [^{3}H]prazosin binding in membranes from rat proximal tubules and MTAL. Each *point* represents the mean of three experiments, each performed in duplicate. The [^{3}H]prazosin concentration was 200 pM. Data for proximal tubules best fit a two-site model (26) and data for MTAL best fit a one-site model. See Table 2 for calculated K_i values.

The expression of mRNA encoding α_1-adrenoceptor subtypes was investigated using RT-PCR methods based on the cDNA sequences obtained from rat brain cDNA libraries (18–20). Fig. 4 shows the amplification of cDNA sequences from the third intracellular loops of α_{1B}- and α_{1D}-adrenoceptors using RT-PCR with rat whole-kidney total RNA. PCR products of the predicted size (304 bp for α_{1D}, 375 bp for α_{1B}) were evident after RT-PCR from whole-kidney total RNA. PCR products were not detected when reverse transcriptase was omitted from the reaction mixture (all other RT-PCR reagents present), either using whole-kidney total RNA or in proximal tubules or MTAL. These results indicate that the products amplified by PCR are from cDNA and not from genomic DNA (Fig. 4). The amplified PCR products were shown to originate from cDNA of α_{1B}- and α_{1D}-adrenoceptors, as determined by cloning and sequencing. The PCR products were cloned into the pCRII vector and four single colonies were isolated for each of the α_{1B}- and α_{1D}-adrenoceptor cDNA recombinant plasmids. When these products were sequenced, all of the plasmids contained inserts with sequences identical to the previously reported sequences (18–20) for either the α_{1B}-adrenoceptor cDNA (bp 629–1003, from the translation start codon) or the α_{1D}-adrenoceptor cDNA (bp 1042–1345) amplified regions. Thus, the combined technique of RT and PCR has been shown to be specific for identifying the presence of both α_{1B}- and α_{1D}-adrenoceptor transcripts in the kidney. These results also indicate that the α_{1D}- and α_{1B}-adrenoceptor subtypes expressed in the rat kidney have similar levels of identity with those found in rat brain (18–20).

When we performed RT-PCR experiments directly on microdissected nephron segments, we observed amplification of PCR products corresponding to both the α_{1B}- and α_{1D}-adrenoceptors in proximal convoluted tubules (Fig. 5). In microdissected MTAL segments, we did not observe any amplification of the PCR product corresponding to the α_{1B}-adrenoceptor (seven experiments) but consistently found the predicted α_{1D}-adrenoceptor product (Fig. 6). To investigate the apparent lack of α_{1B}-adrenoceptor expression in MTAL, we performed additional saturation binding studies with the alkylating agent SZL-49. This irreversible antagonist has been reported (20) to selectively alkylate the α_{1D}-adrenoceptor but not the α_{1B}-adrenoceptor under the conditions used in our study. SZL-49 dramatically reduced the B_{max} for [^{3}H]prazosin in saturation binding experiments. After SZL-49 treatment, α_1-adrenoceptor den-

TABLE 1

[^{3}H]Prazosin binding in membranes from renal tubular segments after CEC pretreatment

Segment	n^a	B_{max}		K_d		Sites	
		Control	+CEC[b]	Control	+CEC	CEC-sensitive	CEC-insensitive
		fmol/mg of protein		*pM*		%	
Proximal tubule	3	163 ± 21	112 ± 19	79 ± 7	90 ± 7	32 ± 4	68 ± 4
MTAL	3	49 ± 3	10 ± 1	112 ± 23	100 ± 5	79 ± 3	21 ± 3

[a] *n*, number of experiments.
[b] Membranes were treated for 10 min with CEC (10 μM).

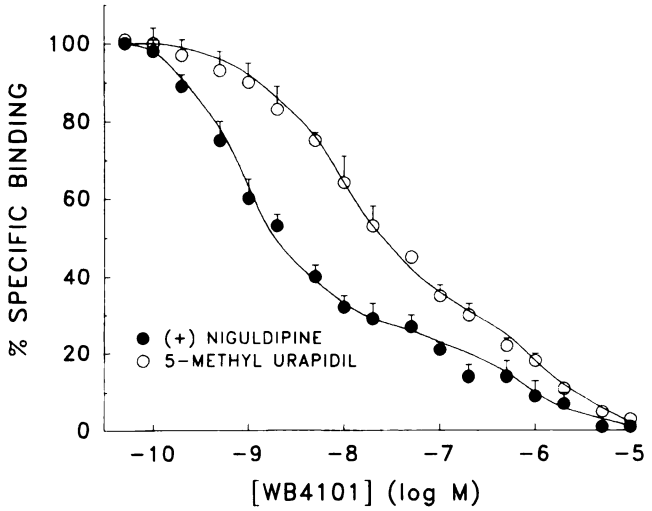

Fig. 3. Competition by (+)-niguldipine and 5-methylurapidil for [^{3}H]prazosin binding in membranes from rat proximal tubules. Each *point* represents the mean of three experiments, each performed in duplicate. The [^{3}H]prazosin concentration was 200 pM. Data from both curves best fit a two-site model (26). See Table 2 for calculated K_i values.

sity was 28 ± 2% of control values in MTAL membranes (46 ± 3 fmol/mg in control group versus 13 ± 1 fmol/mg in SZL-treated group) (Fig. 7). Receptor affinity for [^{3}H]prazosin was decreased somewhat after SZL-49 treatment (K_d values of 54 ± 4 pM in control group versus 95 ± 3 pM in SZL-49-pretreated group). These combined data suggest that the predominant α_1-adrenoceptor expressed in MTAL is of the α_{1D} subtype, whereas the proximal tubules express the α_{1A}, α_{1B}, and α_{1D} subtypes.

Discussion

As of this writing, the existence of at least four rat α_1-adrenoceptors has been suggested, i.e., the CEC-insensitive α_{1A}-adrenoceptor and the CEC-sensitive α_{1B}-, α_{1C}-, and α_{1D}-adrenoceptors (12, 14, 17, 19, 20, 22). The sequence for the α_{1D}-adrenoceptor used for the design of our primers has been reported to be both the α_{1A}-adrenoceptor (19) and the α_{1D}-adrenoceptor (20). This controversy is not fully resolved; however, it does appear that this cloned receptor is sensitive to CEC (19, 20) and has a tissue distribution (19, 20) that is disparate from that of the pharmacologically defined α_{1A}-adrenoceptor that was originally reported by Minneman and colleagues (12, 13). Thus, it appears that a CEC-insensitive α_1 receptor has not been cloned (20, 21), making it likely that the expressed mRNA detected in our experiments represents expression of the α_{1D}-adrenoceptor gene and not the α_{1A}-adrenoceptor gene. Additional research will be necessary to determine the genetic identity of the α_{1A}-adrenoceptor.

In the present study, we have used a combination of molecular biological and radioligand binding techniques to identify for the first time α_1-adrenoceptor subtypes expressed by two highly innervated nephron segments of the rat, the proximal tubule and the MTAL. We limited our study to these two segments because they can be obtained in large enough quantities for saturation and competition binding studies. In proximal tubules, we found in radioligand binding experiments that both CEC-insensitive and CEC-sensitive α_1-adrenoceptors were present, in an approximately 2:1 ratio. Competition binding studies with the competitive antagonists WB4101, 5-methylurapidil, and niguldipine and [^{3}H]prazosin revealed two binding sites, of relatively high and low affinities. The percentages of high and low affinity sites were similar for each antagonist. Thus, the radioligand binding data suggested the existence of at least two types of α_1-adrenoceptors in proximal tubule segments. In RT-PCR experiments, mRNA species corresponding to the α_{1B}- and α_{1D}-adrenoceptors were both expressed within microdissected proximal convoluted tubule segments. Because the α_{1B}- and α_{1D}-adrenoceptor subtypes are both CEC sensitive and have similar affinities for WB4101, 5-methylurapidil, and niguldipine (21), we conclude that at least three different α_1-adrenoceptors are produced by rat proximal tubules, i.e., the CEC-insensitive α_{1A}-adrenoceptor detected in radioligand binding studies and the α_{1B}- and α_{1D}-adrenoceptors detected in radioligand binding and RT-PCR studies.

We recently reported (11) that α_1-adrenoceptor subtypes were heterogeneously distributed in grossly dissected regions of the rat kidney. Of particular interest was the observation that the renal cortex possessed approximately equal densities of CEC-sensitive and CEC-insensitive α_1-adrenoceptors. Our findings in the renal cortex led us to develop two alternative hypotheses. The first was that proximal tubules, which comprise approximately 80% of the renal cortical mass, express both CEC-insensitive and CEC-sensitive α_1-adrenoceptor subtypes. The second was that proximal tubules express only one subtype and other cortical structures, such as glomeruli, blood vessels, and distal segments, express a different subtype. The present study shows for the first time that proximal tubules express both CEC-insensitive and CEC-sensitive α_1-adrenoceptors, in a 2:1 ratio. Because this proportion is 1:1 in the renal cortex (11), our results also imply that the other cortical (distal tubular) structures must express primarily CEC-sensitive α_1-adrenoceptors (α_{1B}- and/or α_{1D}-adrenoceptors).

TABLE 2

Inhibition constants for antagonist competition studies with [^{3}H]prazosin (200 pM)

Drug	Segment	n^a	pK_i^b		Sites	
			$pK_{i(h)}$	$pK_{i(l)}$	High	Low
					%	
WB4101	Proximal tubule[c]	3	9.97 ± 0.01	8.53 ± 0.10	67 ± 1	33 ± 1
WB4101	MTAL[d]	3	8.82 ± 0.06		100	
5-Methylurapidil	Proximal tubule	3	8.50 ± 0.06	6.51 ± 0.15	67 ± 2	33 ± 2
(+)-Niguldipine	Proximal tubule	4	9.53 ± 0.19	7.00 ± 0.15	76 ± 1	24 ± 1

[a] *n*, number of experiments.
[b] $pK_{i(h)}$ is the −log of the inhibition constant for high affinity binding calculated from antagonist competition curves using LIGAND analysis (26), and $pK_{i(l)}$ is the corresponding value for low affinity binding.
[c] A two-site fit was significantly better ($p < 0.01$) than a one-site fit in proximal tubular membranes.
[d] Competition binding data for MTAL conformed to a one-site fit, and the corresponding K_i value is listed as neither high nor low affinity.

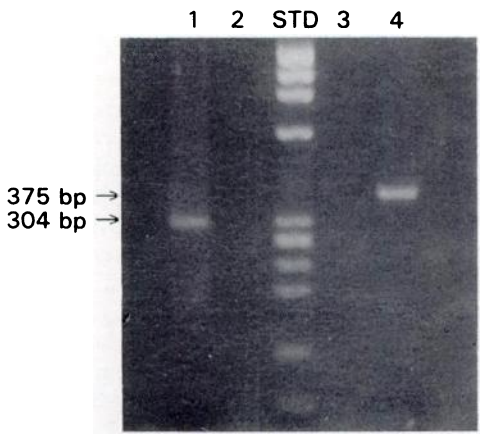

Fig. 4. Gel electrophoresis of RT-PCR products from rat kidney total RNA, obtained using primers for α_{1B}- and α_{1D}-adrenoceptor third intracellular loop sequences. PCR products were electrophoresed on a 3% Nussieve GTG agarose gel and stained with ethidium bromide. *Lane 1*, α_{1D}-adrenoceptor mRNA expression (304-bp predicted product); *lane 2*, negative control for α_{1D}-adrenoceptor primers (rat kidney mRNA and all RT-PCR reagents except reverse transcriptase); *lane 3*, negative control for α_{1B}-adrenoceptor primers; *lane 4*, α_{1B}-adrenoceptor mRNA expression (375-bp predicted product); *STD*, ϕX174 replicative form DNA/*Hae*III molecular size marker [molecular sizes from largest (*top*) to smallest: 1373, 1078, 872, 603, 310, 271, 234, 194, 118, and 72 bp].

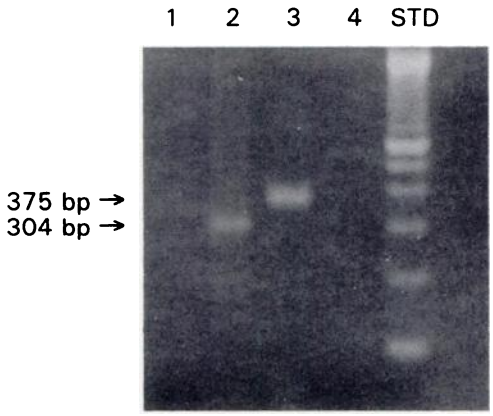

Fig. 5. Gel electrophoresis of RT-PCR products from mRNA from microdissected rat proximal convoluted tubules, obtained using primers for α_{1D}- and α_{1B}-adrenoceptors. *Lane 1*, negative control for α_{1D}-adrenoceptor primers (no reverse transcriptase added); *lane 2*, α_{1D}-adrenoceptor mRNA expression (304-bp predicted product); *lane 3*, α_{1B}-adrenoceptor mRNA expression (375-bp predicted product); *lane 4*, negative control for α_{1B}-adrenoceptor primers; *STD*, 100-bp ladder (*bottom band*, 100 bp).

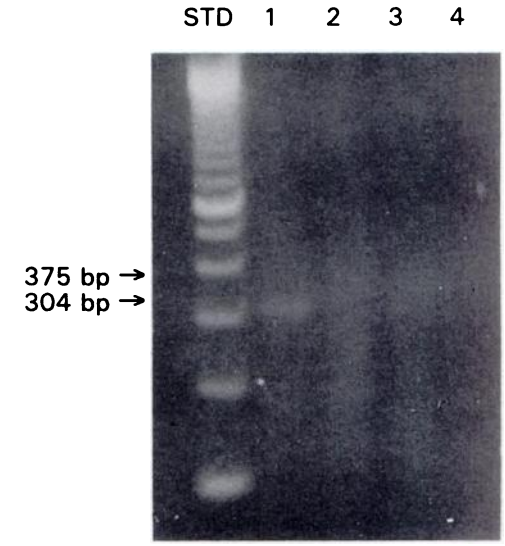

Fig. 6. Gel electrophoresis of RT-PCR products from mRNA from microdissected rat MTAL, obtained using primers for α_{1D}- and α_{1B}-adrenoceptors. *Lane 1*, α_{1D}-adrenoceptor mRNA expression (304-bp predicted product); *lane 2*, negative control for α_{1D}-adrenoceptor primers (no reverse transcriptase added); *lane 3*, α_{1B}-adrenoceptor mRNA expression (375-bp predicted product); *lane 4*, negative control for α_{1B}-adrenoceptor primers; *STD*, 100-bp ladder (*bottom band*, 100 bp).

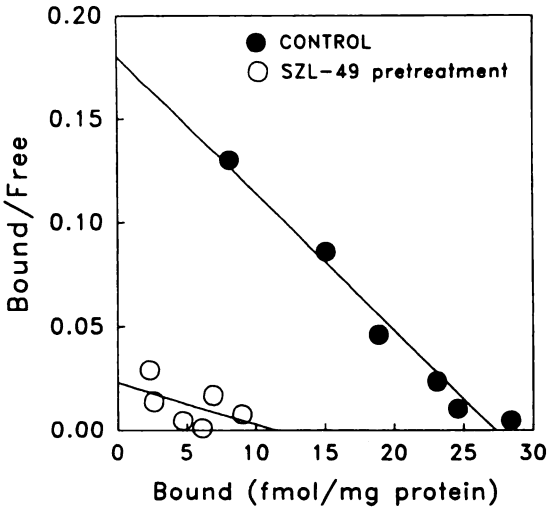

Fig. 7. Typical Rosenthal plots of saturation experiments in membranes from MTAL treated with SZL-49. SZL-49 (10 nM) was added to one half of the membrane suspensions and incubated for 10 min at 37°, in a shaking water bath. Each *curve* is typical of three experiments.

In MTAL, we found that virtually all of the α_1-adrenoceptors were sensitive to alkylation by CEC in [^{3}H]prazosin saturation experiments. Competition binding experiments with WB4101 revealed a single binding site with a relatively low affinity. These data correspond well to our previous findings (11), in which we showed that the ISOM (which contains MTAL, among other segments) contained almost exclusively CEC-sensitive α_1-adrenoceptors that had a low affinity for WB4101. Based on these results, we had concluded in this earlier study that α_1-adrenoceptors in the ISOM were of the α_{1B} subtype (11). Interestingly, when we performed RT-PCR in MTAL segments, we found that the α_{1D}-adrenoceptor gene product was expressed but the α_{1B}-adrenoceptor gene product was not expressed. This lack of α_{1B}-adrenoceptor gene expression was not the result of a technical problem with the RT-PCR procedure, because α_{1B}-adrenoceptor mRNA was detected in whole-kidney total RNA samples and in proximal convoluted tubules from the same rats. The combined results of the CEC alkylation and RT-PCR experiments suggested to us that the CEC-sensitive α_1-adrenoceptors in MTAL segments were of the α_{1D} subtype. To test this hypothesis, we performed additional radioligand binding experiments in MTAL plasma membranes with the irreversible antagonist SZL-49. Under the conditions used in our experiments, SZL-49 has been shown to selectively alkylate α_{1D}- but not α_{1B}-adrenoceptors (20). Our results demonstrated that the CEC and SZL-49 sensitivities were identical to those reported for the cloned α_{1D}-adrenoceptor (20). We thus conclude that the predominant α_1-adrenoceptor expressed by the MTAL is of the α_{1D} subtype. We believe that this is the

first reported example of specific tissue expression of the α_{1D}-adrenoceptor.

We have used RT-PCR to identify α_1-adrenoceptor expression in single nephron segments. We chose this method over other techniques, such as Northern analysis and *in situ* hybridization, because of the high specificity of PCR and the ability to detect the presence of a limited number of mRNA copies in a small amount of tissue. The primers that we chose yield products that span the third intracellular loop, which is a region of low identity among the nucleic acid sequences of adrenoceptors. The nucleotide sequences of the resulting PCR products confirmed that they were identical to the sequences of the corresponding regions of the rat α_{1B}- and α_{1D}-adrenoceptors (18–20). Using Northern blot analysis, Lomasney *et al.* (19) reported that α_{1B}-adrenoceptor but not α_{1D}-adrenoceptor expression could be detected in whole-kidney poly(A)$^+$ RNA, using full length cDNA probes. This is in contrast to our results, because with RT-PCR we observed expression of the α_{1D}-adrenoceptor in whole-kidney rat total RNA and in proximal tubules and MTAL. It is likely that the reason for this discrepancy is the high sensitivity of RT-PCR. Thus, our results demonstrate that RT-PCR can be used for the localization of α_1-adrenoceptor subtypes.

Our combined results indicate that two highly innervated tubular segments mediate nerve signals through different receptors. This is potentially important physiologically and pharmacologically for several reasons. First, the proximal tubule and MTAL play very different roles in the maintenance of renal function. In the proximal tubule, which is the site of bulk reabsorption of filtered water and solute, the renal nerve stimulates α_1-adrenoceptor-mediated sodium and water reabsorption and gluconeogenesis (7, 8). In the MTAL, which is important in urine-concentrating and -diluting ability, stimulation of the renal nerve increases NaCl (but not water) reabsorption (30). Our results suggest that these disparate effects of renal nerve stimulation are actually mediated by at least two different receptor subtypes. Second, because α_1-adrenoceptor subtypes are distinct gene products it is likely that their expression is independently controlled. Further, it is probable that expression of α_1-adrenoceptor subtypes varies according to cell type and location within the kidney. It is possible that the total level of expression or the ratio of subtypes within or among segments varies in different physiological or pathophysiological states. The factors governing expression of α_1-adrenoceptor subtypes have not been determined. Third, the various α_1-adrenoceptor subtypes are reportedly linked to distinct signal transduction mechanisms (26). For example, the α_{1A}-adrenoceptor has been proposed to be coupled to the entry of extracellular Ca^{2+}, whereas the α_{1B}-adrenoceptor is coupled to activation of phospholipase C with release of intracellular Ca^{2+} (26). Thus, the physiological effects subsequent to renal nerve stimulation in proximal tubules and MTAL are probably mediated by discrete biochemical pathways. Fourth, the renal sympathetic nerve may play an important role in the etiology of hypertension and edema-forming states (3). Because the proposed pathogenic role of the renal nerve probably involves α_1-adrenoceptors, specific antagonists of α_1-adrenoceptor subtypes may be useful in treating or preventing these disease states.

The physiological significance of the discrete distribution of α_1-adrenoceptor subtypes within the kidney is not known. However, it may be possible to pharmacologically exploit the specific tissue expression of these unique subtypes for the therapy of renal fluid and electrolyte disorders. For example, one would predict that blockade of renal α_1-adrenoceptors *in vivo* would produce diuresis and natriuresis. However, when nonspecific α_1-adrenoceptor antagonists such as prazosin are used, diuresis and natriuresis do not usually occur because vascular α_1-adrenoceptors are also blocked (31). The resulting reduction in systemic vascular tone reduces renal perfusion and glomerular filtration rate, which counteracts the direct renal effect of prazosin. Interestingly, our results from the present study suggest a prominent expression of CEC-sensitive α_1-adrenoceptors (α_{1B} and α_{1D}) at renal tubular sites. Although cardiovascular changes have been reported with high doses of CEC, the vascular α_1-adrenoceptors that mediate vasoconstriction are predominantly CEC insensitive (32, 33). We recently tested this hypothesis (34) and showed that CEC causes natriuresis and diuresis *in vivo* in rats after volume expansion, with no change in systemic blood pressure or glomerular filtration rate. It remains to be tested whether CEC or another subtype-selective antagonist can be used to diminish or reverse fluid and electrolyte abnormalities such as those found in hypertension or edematous states.

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