Urodilatin Binds to and Activates Renal Receptors for Atrial Natriuretic Peptide

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Urodilatin is a recently described member of the atrial natriuretic peptide family, thought possibly to be synthesized in the kidney. To determine if urodilatin binding sites are present in rat and human kidney, we evaluated the effect of urodilatin on iodine-125-labeled atrial natriuretic peptide (ANP) (100 pM) binding to tissue sections using an in situ autoradiographic technique. 125I-ANP binding occurred primarily in glomeruli and medullary structures of both rat and human kidney. Increasing concentrations of urodilatin yielded a monophasic displacement of 125I-ANP binding with an IC50 of 4.2 nM, a value nearly identical to that achieved with unlabeled ANP (7.2 nM). In additional experiments, rat glomeruli and inner medullary collecting duct cells were isolated and incubated in vitro with either ANP or urodilatin (10^-11 to 10^-6 M) and cyclic guanosine-3',5'-monophosphate accumulation measured by radioimmunoassay. Dose-response curves for the two peptides were superimposable in each tissue; at 10^-8 M, ANP generated 613 ± 41 and urodilatin 603 ± 55 fmol cyclic guanosine monophosphate per 10 minutes per milligram protein in inner medullary collecting duct cells (p = NS). Thus, urodilatin is as effective as ANP in displacing 125I-ANP binding to both rat and human renal tissue and in generating cyclic guanosine monophosphate in renal target cells in the rat, suggesting that its physiological effects may occur through the same receptors and signaling pathways that mediate the actions of ANP. (Hypertension 1993;21:432-438)

KEY WORDS • autoradiography • guanosine cyclic monophosphate • kidney glomerulus • kidney tubules, collecting • renal artery • natriuretic peptide, atrial

Three major types of receptor for atrial natriuretic peptide (ANP) have been identified in target tissues: ANP A-receptor and ANP B-receptor contain a membrane-bound guanylate cyclase in their cytoplasmic domains that mediates the production of the intracellular second messenger cyclic guanosine-3',5'-monophosphate (cGMP). ANP has greatest affinity for the ANP A-receptor, whereas the naturally occurring ligand for ANP B-receptor may be the recently identified C-type natriuretic peptide (CNP). ANP C-receptor is a so-called clearance receptor, which to date has no clearly established function but may bind and degrade natriuretic peptides. The C-receptor has been identified because it binds biologically inactive analogues of ANP with high affinity, whereas the guanylate cyclase receptors do not. Ligand binding studies with radioactively labeled peptides have indicated that A-receptors are concentrated in glomeruli, inner medullary collecting duct (IMCD), and vascular structures, whereas C-receptors are more concentrated in glomeruli. Recently, a four-amino acid N-terminal extension of ANP has been identified in renal tissue and urine. Termed urodilatin (URO) or renal natriuretic peptide, its function like that of ANP has not been fully determined, although it has been advanced as a candidate to mediate renal tubular actions previously ascribed to circulating ANP, including diurnal oscillations in urinary sodium excretion and the natriuretic response to an acute intravenous saline infusion. There is as yet little information on the binding properties of this peptide or its ability to stimulate production of cGMP in responsive tissues.

The purpose of our study was therefore to characterize, using the technique of autoradiography, the ability of URO compared with CNP and unlabeled ANP to displace 125I-ANP from its binding sites in renal tissue. In addition, we examined the effectiveness of URO in stimulating production of cGMP by isolated glomeruli and IMCD cells studied in vitro. Our results are consistent with the interpretation that URO binds to the same biologically active receptors as ANP in renal tissue.

Methods

Male Sprague-Dawley rats (Bantin Kingman, Fremont, Calif.) weighing 280–350 g were housed in a
controlled environment with a 12-hour light/dark cycle and provided standard rat chow and water ad libitum.

Receptor Binding Studies

Animals were killed by decapitation, and the kidneys were quickly removed, decapsulated, snap frozen in liquid nitrogen, and stored at -80°C. Human renal tissue was obtained from the normal portion of two neoplastic kidneys removed at nephrectomy and frozen in liquid nitrogen. The distribution of renal ANP receptors was assessed by a modification of an in situ receptor binding assay. Briefly, tissue sections (10 μM) were cut on a cryostat at -15°C, thaw mounted (two per slide) on poly-L-lysine–coated slides, dried in vacuo for 18 hours at -4°C to +4°C over silica gel, and stored in sealed Bakelite boxes at -80°C. Before assay, the slides were brought to room temperature. Endogenous ANP bound to receptors was removed by preincubating the sections for 10 minutes at room temperature in 200 μL of a buffer (buffer A) containing 30 mM sodium phosphate (pH 7.2), 120 mM NaCl, 0.3% bacitracin, and 0.5% receptor grade bovine serum albumin. After preincubation, the buffer was replaced with 200 μL fresh buffer A containing 100 pM 125I-ANP (2,200 μCi/mmol, New England Nuclear, Boston, Mass.), and the sections were placed in a humidified chamber and incubated at room temperature for 15 minutes. After incubation, the slides were rinsed in an ice-cold buffer containing 30 mM sodium phosphate and 120 mM NaCl for 10 seconds, washed in the same buffer for 5 minutes, rinsed in deionized water for 10 seconds, and dried for 2 hours in a stream of cool air. For autoradiography, the slides were exposed to LKB-Ultrofilm for 3–5 days at room temperature. Films were processed with Kodak D-19 developer (Kodak, Rochester, N.Y.) and Kodak rapid fixer, and the amount of radioligand bound to the tissue sections was determined by counting the slides in a gamma counter.

Nonspecific binding was determined on adjacent sections under identical incubation conditions except for the addition of 1 μM unlabeled rat ANP-(1-28) (Peninsula Laboratories, Belmont, Calif.). Clearance receptors were identified by addition of 10 μM CNP-(4-23) (Peninsula Laboratories). Emulsion autoradiography was used to obtain more precise cellular localization of the ANP binding sites. After incubation with radioligand and washes as described above, serial sections were fixed in paraformaldehyde vapors for 2 hours at 80°C, left in air for a minimum of 4 hours, dipped in photographic emulsion (Kodak NTB2), and exposed for 15–20 days at 4°C. The emulsion was developed using Kodak D-19 and Kodak rapid fixer. To assist in the identification of structures associated with the grains, selected sections were stained with hematoxylin-eosin.

The time course of binding was assessed by determining the total amount of radioligand bound after 2.5–45 minutes of incubation. The reversibility of binding was assessed by the addition, after 15 minutes of incubation, of either unlabeled rat ANP (1 μM) or URO (1 μM) (Peninsula Laboratories). To determine the extent to which radioligand binding was inhibited by unlabeled peptides, adjacent sections were incubated with 100 pM 125I-ANP in the presence of increasing concentrations (10 pM–10 μM) of rat ANP or URO. The concentration of unlabeled peptides that resulted in 50% inhibition (IC50) of radioligand binding was calculated using the LIGAND program. Because binding studies of ANP may be influenced by endogenous hormone bound to its receptors, we attempted to establish that our preincubation with buffer A was adequate to remove already bound peptide by comparing the binding of 100 pM 125I-ANP to adjacent kidney slices (n=6) that were preincubated for 10 minutes with buffer A or an acidic wash formed by adding 0.5% acetic acid to buffer A. Total counts per minute (corrected for background) with buffer A were 3,532±437 compared with 4,254±362 with the acid wash (p=NS). These results suggest that prebound ligand did not materially influence our binding results and are in accord with the more extensive studies of Brown et al.

Preparation of Glomeruli and Inner Medullary Collecting Duct Cells

Rats were anesthetized with pentobarbital (50 mg/kg i.p.). Glomeruli were isolated as follows: the kidneys...
were perfused with cold isotonic heparinized saline until blanched (50–60 mL saline in 2 minutes). The renal cortices were dissected and minced to a pastelike consistency. The homogenate was passed successively through a 106-μm sieve that excluded tubules and blood vessels and a 75-μm sieve that retained the glomeruli and allowed cells to pass through. Glomeruli were suspended in ice-cold 20 mM tris(hydroxymethyl)aminomethane hydrochloride (tris HCl) buffer, pH 7.4, containing (mM) NaCl 125, KCl 10, sodium acetate 10, and glucose 5 (buffer B) and were centrifuged at 120g for 2 minutes. The supernatant was discarded, and the pellet was resuspended in the same buffer and recentrifuged. By light microscopy examination, samples of the final pellet consisted of nearly pure isolated decapsulated glomeruli with less than 5% tubular contamination. No afferent or efferent arterioles were observed.

IMCD cells were isolated as follows:13 the kidneys were perfused with 50–60 mL of Joklik's minimal essential medium (Applied Scientific, San Francisco, Calif.) followed by 5 mL of a solution of Joklik's medium containing 0.2% of collagenase (Type II, Sigma Chemical Co., St. Louis, Mo.). The inner medullas were excised, finely minced, and incubated in the same solution for 90 minutes at 37°C. The resulting suspension of inner medullary cells was layered on a 16% Ficoll (Sigma) solution in nonbicarbonate Ringer buffer (buffer C) and centrifuged at 2,300g for 40 minutes. The supernatant was discarded, and the pellet was resuspended in the same buffer, pH 7.4, containing (mM) NaCl 125, KCl 10, sodium acetate 10, and glucose 5 (buffer B). Cells were subsequently washed through buffer C and then a solution of buffer C containing 7.5% albumin to remove any traces of contaminating collagenase. Samples of IMCD cells were then embedded in poly/BED 812 and examined by transmission electron microscopy using a JEM JEOL 100 SC microscope; photographs were magnified x6,900. Cell preparations were homogeneous and exhibited the characteristic ultrastructural appearance of IMCD cells.13

Determination of Cyclic GMP Generation by Glomeruli and Inner Medullary Collecting Duct Cells

Freshly isolated glomeruli were resuspended in buffer B containing 1 mM CaCl₂, and 1 mg/mL bacitracin, and IMCD cells in buffer C containing bacitracin, 7.5 mM glucose, and no pyruvate or acetate and preincubated in room air for 10 minutes at 37°C in a shaking water bath; in one set of experiments using isolated glomeruli, the protease inhibitors aprotinin (500 KIU/mL), pepstatin A (20 μg/mL), phenylmethylsulfonyl fluoride (200 μg/mL), and SQ28,603 (1 mg/mL) were added to the incubation medium. Incubations were carried out for 10 minutes at 37°C in the presence of increasing doses (10⁻¹¹ to 10⁻⁴ M) of ANP or URO, dissolved in the incubation medium in volumes of 10 μL. Incubations took place in the absence of any phosphodiesterase inhibitor. Incubations were terminated by adding 750 μL of ice-cold trichloroacetic acid (TCA) (final concentration 6.6%) and cooling to 4°C. The precipitated protein was sedimented by centrifugation at 4,500 rpm for 15 minutes at 4°C, and the pellets were dissolved in 1N NaOH and assayed for protein content by the method of Lowry et al14 using bovine serum albumin as standard. The supernatant was extracted five times with four volumes of water-saturated ethyl ether to remove the TCA before being evaporated to dryness under a stream of air; it was stored at -70°C until assayed for cGMP content. For the cGMP assay, samples were dissolved in 50 mM sodium acetate buffer, pH 6.2, mixed thoroughly, and 100 μL aliquots acetylated according to the manufacturer's instructions (New England Nuclear). Average results of triplicate determinations are expressed as femtomoles cGMP generated per 10 minutes per milligram protein.

Data Presentation and Statistical Analysis

Data are presented as mean±SEM. Comparisons between groups were done by the use of Student's t test for paired or unpaired variables, and one-way analysis of variance with repeated measures when multiple comparisons were carried out. A value of p<0.05 was used to assign statistical significance.

Results

Receptor Binding Studies

Binding of ¹²⁵I-ANP was found in both cortical and medullary regions of rat kidney (Figure 1). In the cortex, the radioligand binding was localized primarily in glomeruli (Figure 2). In the outer medulla, bound radioligand was found primarily in longitudinal bands in the inner stripe, corresponding to medullary vascular bundles.6 Minimal radioligand binding was found in the interbundle areas, whereas binding to papillae was moderate. An additional area of specific radioligand binding was observed in major branches of the renal artery (Figure 2) and in the muscular layer of the intrarenal pelvis. Nonspecific binding was less than 10% (Figure 1). Incubation with CNP markedly reduced binding of the radioligand to glomeruli (Figure 1). Incubation with URO also inhibited binding of the radioligand (Figure 1) to an extent similar to that seen with unlabeled rat ANP.

Figure 3 shows the time course of binding of radioligand to sections of rat kidney. Total binding increased rapidly, reaching a plateau after 15 minutes of incubation. Both unlabeled rat ANP and URO progressively displaced the labeled ligand by 31% and 33% of the control binding, respectively. Increasing concentrations of both rat ANP and URO resulted in a monophasic displacement of radioligand binding, with an IC₅₀ of 7.2 and 4.2 nM, respectively (Figure 4).

The pattern of radioligand binding to human kidney tissue is illustrated in Figure 5. As in the rat kidney, binding was present primarily in glomeruli. This binding was markedly inhibited by unlabeled ANP and URO, but only slightly by CNP (Figure 5).

Cyclic GMP Generation in Glomeruli and Inner Medullary Collecting Duct Cells

Figure 6 illustrates the stimulation of cGMP generation in glomeruli and IMCD cells in response to increasing concentrations of ANP and URO. ANP induced a
superimposable displacement curves and the nearly identical IC\textsubscript{50} values obtained with URO and ANP offer very strong evidence that each peptide has equal affinity for the same receptor or receptors. This is not an unexpected result in view of the identical structure of the two peptides save for the N-terminal extension of four amino acids possessed by URO. Addition and substitution studies of the binding of ANP to the A-receptor have identified several factors necessary for optimum binding. These include integrity of the ring structure and the three C-terminal amino acids; extension at the N-terminal has relatively little effect on binding.\textsuperscript{23} Since each of these key structural properties is shared by both ANP and URO, it is not surprising that URO should be effective in displacing \textsuperscript{125}I-ANP from these receptors. The fact that the displacement curve and the IC\textsubscript{50} are nearly identical provides evidence that the N-terminal extension of URO neither impedes nor enhances its ability to displace \textsuperscript{125}I-ANP from both isolated glomeruli and IMCD cells. At 10\textsuperscript{-8} M, the values were roughly 10-fold and sixfold higher, respectively, for glomeruli and IMCD cells than basal values. Superimposable dose-response curves were obtained in both glomeruli and IMCD cells with URO. The presence of protease inhibitors in the incubation medium did not alter these results: at 10\textsuperscript{-8} M ANP, cGMP accumulation in glomeruli in the absence of the inhibitors was 638 ± 81 versus 628 ± 103 fmol/mg per 10 minutes in their presence. With 10\textsuperscript{-8} M URO, the corresponding results were 598 ± 105 versus 608 ± 129 fmol/mg per 10 minutes (n=6 for each).

\section*{Discussion}

Our results indicate that URO is as effective as unlabeled ANP in displacing \textsuperscript{125}I-ANP from binding sites in both rat and human kidney. The virtually dose-dependent increase in cGMP generation in both glomeruli and IMCD cells. At 10\textsuperscript{-8} M, the values were roughly 10-fold and sixfold higher, respectively, for glomeruli and IMCD cells than basal values. Superimposable dose–response curves were obtained in both glomeruli and IMCD cells with URO. The presence of protease inhibitors in the incubation medium did not alter these results: at 10\textsuperscript{-8} M ANP, cGMP accumulation in glomeruli in the absence of the inhibitors was 638 ± 81 versus 628 ± 103 fmol/mg per 10 minutes in their presence. With 10\textsuperscript{-8} M URO, the corresponding results were 598 ± 105 versus 608 ± 129 fmol/mg per 10 minutes (n=6 for each).
nous infusion of a saline load.\textsuperscript{5,6,19} The signal (or signals) governing the fluctuations in its excretion, presumably reflecting changes in synthesis and secretion, are unknown. An entirely plausible possibility that is not addressed by our studies is that URO may act through a different set of receptors that recognize the N-terminal extension of the peptide. Receptor binding studies that use \textsuperscript{125}I-URO as the ligand will be necessary to test for this possibility.

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\textbf{References}


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