Angiotensin IV AT4-receptor system in the rat kidney

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Handa, Rajash K., Luke T. Krebs, Joseph W. Harding, and Shelly E. Hanna. Angiotensin IV AT4-receptor system in the rat kidney. Am. J. Physiol. 274 (Renal Physiol. 43): F290–F299, 1998.—Angiotensin IV, [(des-Asp1,Arg2]ANG II (ANG II) or ANG-(3–8), has been shown to preferentially bind to a novel angiotensin binding site (AT4 receptor). The cellular location and function of this receptor in the rat kidney is unknown. Autoradiography localized AT4 receptors to the cell body and apical membrane of convoluted and straight proximal tubules in the cortex and outer stripe of the outer medulla. ANG IV (0.1 pM-1 µM) elicited a concentration-dependent decrease in transcellular Na+ transport (as measured by proximal tubule O2 consumption rates) in fresh suspensions of control or nystatin-stimulated (bypasses rate-limiting step of apical Na+ entry) rat proximal tubules. The inhibitory effect of 1 pM ANG IV was unaltered by either 1 µM losartan (AT1-receptor antagonist) or 1 µM PD-123319 (AT2-receptor antagonist) and yet was abolished by 1 µM divalinal-ANG IV (AT2-receptor antagonist) or ouabain pretreatment. These results demonstrate that the kidney AT4-receptor system is localized to the proximal tubule and suggests that one potential biological role of this system is in the regulation of Na+ transport by inhibiting a ouabain-sensitive component of Na+-K+-adenosinetriphosphatase activity in the rat.

The renin-angiotensin system is composed of a cascade of biochemical reactions involving the generation and processing of the decapetide, angiotensin (ANG) I, and is one of the basic homeostatic mechanisms for maintaining the internal environment of the organism. The general consensus has been that the octapeptide, ANG II, and its shorter fragment [des-Asp1]ANG II (ANG III) were the only biologically active products of the renin-angiotensin system, having a vast range of actions throughout the body, including potent vasoconstrictor and diuretic effects, neuromodulatory activity (central and peripheral nervous system), endocrine actions (e.g., stimulating aldosterone and vasopressin secretion), regulating epithelia transport (most notably increasing renal proximal Na+, Cl-, and HCO3- reabsorption), and influencing cell remodeling (hyperplasia and hypertrophy) (23). Two types of the ANG II receptor have been cloned and termed AT1 (with at least AT1A, AT1B, and AT1C subtypes) and AT2 (proposed subtypes). Most of the functional responses to ANG II and ANG III in the brain and periphery have been ascribed to AT1-receptor activation. However, recent studies (41, 43) suggest that AT2-receptor stimulation may counteract AT1-mediated events in processes, such as blood pressure control, growth, and dipsogenesis.

There is now mounting evidence that processing of ANG I to fragments smaller than ANG II and ANG III [e.g., ANG-(1–7) and ANG IV] can produce a receptor-mediated biological response (17, 38, 46). A new angiotensin binding site, distinct from ANG II type AT1 and AT2 receptors, has been pharmacologically described and demonstrates high specificity and affinity for the hexapeptide, ANG IV (42). This novel binding site has been designated AT4 and, to date, has been shown to be heavily distributed in the brain, spinal cord, aorta, heart, lung, uterus, colon, prostate, adrenals, bladder, kidney, vascular smooth muscle, and endothelial cells of several species (human, monkey, bovine, porcine, horse, sheep, cat, rabbit, rat, and guinea pig) (32, 34, 42, 46). ANG IV can be formed from ANG II and ANG III by the action of aminopeptidases (20, 25) and potentially from ANG I-(3–10) by an angiotensin-converting enzyme-dependent pathway (5, 14). The hexapeptide appears to have weak classic ANG II activities, such as effects on blood pressure and thirst, and yet possesses unique biological attributes potentially important in memory, cell growth, and cardiovascular control (16, 25, 35, 46). Although the kidney contains high-affinity ANG IV binding sites, the functional significance of this receptor system is unknown (8, 10, 11, 21). The hexapeptide has been reported to inhibit (26) or have no effect (31) on renal renin secretion and yet appears to be a potent potentiator of renal cortical blood flow, which was shown to be mediated by a novel ANG IV receptor and linked to the stimulation of nitric oxide (6, 7, 42). The actions of ANG IV on other aspects of renal physiology are unknown. The aim of the present study was 1) to provide evidence for the presence, distribution, and specificity of AT4-receptor sites in the rat kidney, 2) to determine the possible influence of ANG IV on proximal epithelial Na+ transport, 3) to pharmacologically characterize the receptor involved in mediating the tubular action of ANG IV, and 4) to gain some insight into the mechanisms by which ANG IV exerts its action on cellular Na+ transport.

METHODS

Autoradiographic studies. Male rats were anesthetized with an intraperitoneal injection of equithesin or pentobarbital sodium, and the kidneys were perfused with phosphate-buffered saline (PBS, pH 7.4 at room temperature) in vivo. The kidneys were then removed, frozen in isopentane at −20°C, and stored at −70°C until sectioned. Autoradiographic analysis of rat kidney binding was performed using 20-µm tissue sections mounted on gelatin-coated slides. Initially, sections were preincubated for 30 min in isotonic buffer [150 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane, 50 µM Plummer’s inhibitor (carboxypeptidase inhibitor), 20
performed. 1.10-phenanthroline (divalent ion chelators), and 0.1% heat-
treated bovine serum albumin (BSA) at pH 7.4) at room
temperature and then incubated in isotonic buffer containing
0.4 mM labeled ligand with or without 10 μM displacement for 25
min, rinsed with 3 × 2 min isotonic buffer washes, dried, and
exposed to X-ray film (Kodak SB5 in Wolf cassettes, stored at
−70°C for 24–48 h, and then developed with Kodak D19). The
incubation buffer contained an excess of aminopeptidase and
carboxypeptidase inhibitors and ion chelators to prevent the
metabolism of the radiolabeled probe and its binding to ANG
IV-degrading enzyme proteins.

For emulsion-coated autoradiography, kidneys were initially
perfused with PBS followed by 2% paraformaldehyde and
0.5% glutaraldehyde and finally with 20% sucrose in vivo.
The kidneys were frozen, sectioned (12 μm), radiola-
beled (see above procedure), and dried for several days. The
radiolabeled sections were then postfixed with paraformalde-
hyde vapor at 80°C for 2 h, dehydrated by immersion in
graded ethanol (50%-100% ethanol for 3 min), defatted in
xylene (10 min), rehydrated in an inverse series of ethanol
followed by distilled water, and then allowed to air dry. Slides
were then uniformly coated with warm Kodak NTB-2 emul-
sion in a dark room, air dried for 3 h, and stored overnight
at room temperature in desiccant-containing light-proof slide
boxes followed by storage at −70°C for 7–20 days. After
exposure, the slides were developed in Kodak D-19, rinsed in
distilled water, fixed in Ektaflo (Kodak), and counterstained
with hematoxylin and eosin. Sections were examined using
both light- and dark-field microscopy.

Isolation of rat proximal tubules. A suspension of cortical
proximal tubules was obtained by a previously described
method (17). All buffers and solutions used for the isolation
of proximal tubules had a pH of 7.40 and an osmolality of 295
mosmol/kgH2O and were equilibrated with 95% O2-5% CO2.
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In brief, two male rats weighing 350–400 g were anesthetized
with ketamine (50 mg/kg im) and xylazine (10 mg/kg im), and
the cortex was removed, minced, and incubated in the
medium containing a standard cocktail of collagenase, 0.67 mg/ml hyaluronidase, and 0.67 mg/ml BSA. After a complete blood washout, perfusion was
continued with the KHB solution supplemented with 1 mg/ml
collagenase, 0.67 mg/ml hyaluronidase, and 0.67 mg/ml BSA.

A suspension of cortical proximal tubules was placed in a thermoregulated 2-ml
chamber containing 1.85 ml KHB, which was then sealed, and O2 was measured polarographically with a Clarke
oxygen electrode. We have previously reported that receptor-
mediated O2 stimulatory and inhibitory pathways remain
intact after rat proximal tubule isolation procedure (17). The
measurement of O2 can be used as a direct reflection of Na+-K+-adenosinetriphosphatase (ATPase) activity and Na+
transport because of the tight coupling between Na+-K+-
ATPase activity and mitochondrial oxidative phosphorylation
in the proximal tubule (30). In addition, ouabain-suppressible
QO2 provides an index of active transport (30).

In the studies that used angiotensin-receptor antagonists,
the blockers were added to both the proximal tubule suspens-
ion (incubated for 10 min as described above) and the chamber. Other drugs [angiotensin peptides, fenoldapam,
platelet-activating factor (PAF), 5 mM nystatin, and 5 mM ouabain] were added in 25-μl boluses into the tubule-
containing chamber via its injection port. The O2 tension in the
closed chamber was recorded as a function of time, and the
resulting slope indicated the QO2, which was calculated as
a function of tubular protein content as measured by the
method of Lowry et al. (29). To minimize the variability in
basal QO2 from different tubule preparations (range 17–39
nmol O2·min−1·mg protein−1), the effect of ANG IV and other
receptor agonist treatments were expressed as a percent
change from basal or nystatin-stimulated QO2. All drug
treatments were prepared fresh daily, and their molar concen-
trations indicate the final concentrations achieved in the
chamber.

Drugs. We received gifts of fenoldapam (SKF-82526) from
Smith Kline & Beecham, losartan (DuP-753) from Du Pont/
Merck Pharmaceuticals, and PD-123177 and PD-123319 from
Parke-Davis. [Val3]ANG IV, [Nle6]ANG IV, and divalinal-
ANG IV [Val1−3(CH2NH)1–2,3–4]ANG IV previously known as
WSU-1291 were prepared in the laboratory of J. W. Harding.
ANG IV, ANG II, PAF (L-alpha-phosphatidylycholine, beta-acetyl-
O-hexadecyl)), nystatin, and other reagents were purchased from
Sigma.

Statistics. All values are presented as means ± SE. Multi-
ple groups were analyzed by one- or two-way analysis of
variance and the post hoc Student-Newman-Keuls test
(Crunch Interactive Statistical Package or SigmaStat). Differ-
ences between means were taken to be significant at the 5%
level.

RESULTS

Autoradiographic studies. Figure 1 demonstrates the
in vitro autoradiographic localization of AT4-receptor sites in the rat kidney. There was a moderate density of diffuse 125I-ANG IV binding over the entire cortex, with
an especially high density of 125I-ANG IV binding to the
proximal tubule fraction (30). In addition, ouabain-suppressible
QO2 in the proximal tubule (30). In addition, ouabain-suppressible
QO2 in the proximal tubule (30).

The localization of 125I-ANG IV binding sites was
studied using ANG IV (Fig. 1B) and the putative AT4 receptor antagonists,
divalinal-ANG IV (Fig. 1C). To confirm the specificity of
divalinal-ANG IV to bind exclusively to the renal AT4 receptor, we also examined the distribution of 125I-divalinal-ANG IV binding sites. The localization of 125I-divalinal-ANG IV (Fig. 1F) was identical to that of 125I-ANG IV and was displaced by both ANG IV and
divalinal-ANG IV (Fig. 1G and H, respectively). There was no cross displacement of the 125I-divalinal-ANG IV binding with losartan (Fig. 1I) or PD-123177
(Fig. 1J).

Emulsion autoradiography revealed that the AT4
receptor was localized to proximal tubules in the cortex.
and outer stripe of the outer medulla (Figs. 2 and 3). The receptor was present on both the proximal tubule cell body as well as within its lumen. Staining sequential sections with periodic acid-Schiff reagent indicated that the binding observed within the dilated tubule lumen was due to receptors located on the apical brush border (not shown). The highest density of silver grains were observed in straight proximal tubules located in the outer stripe of the outer medulla and extending throughout the medullary rays. The density of binding
sites was less in cortical convoluted proximal tubules and absent in descending thin limbs of Henle, ascending thin and thick limbs of Henle, distal convoluted tubules, and cortical and medullary collecting ducts. Despite the appearance of binding sites in the glomerulus (Fig. 3B), we did not consistently find a difference between binding in superficial, midcortical, juxtamedullary glomeruli, and background (e.g., Fig. 3H, bottom left). A similar distribution of kidney AT₄ binding sites was observed in sections treated with both a combination of losartan and PD-123177.

Tissue O₂ concentration-response curve to ANG IV. Because the results of the autoradiography study were consistent with the localization of AT₄ receptors on proximal tubular structures, we examined its effect on proximal tubule Na⁺ transport. In preliminary studies (Fig. 4), we found that ANG IV inhibited QO₂ in a concentration-dependent fashion in proximal tubules, where the movement of Na⁺ across the apical membrane and into the cell was rate limiting (control group). Treatment of proximal tubules with nystatin (Na⁺ ionophore) bypasses the rate-limiting step of apical Na⁺ entry and permits extracellular Na⁺ to freely enter the cell and intracellular K⁺ to exit the cell, which accelerates basolateral Na⁺-K⁺-ATPase activity, causing ~60% increase in tubule QO₂. Under nystatin-stimulated conditions, ANG IV also concentration dependently inhibited QO₂, suggesting that at least one action of ANG IV was to inhibit Na⁺ transport across the basolateral membrane. The concentration-response curves to ANG IV (10 fM to 1 nM) in control and nystatin-stimulated tubules were superimposable, with a threshold dose for ANG IV biological activity of 100 fM. Although the curves appeared to diverge at higher concentrations of ANG IV (>1 nM), the two concentration-response curves were not significantly different from each other. These results indicate that ANG IV has direct actions on the proximal tubule epithelium to inhibit energy-dependent Na⁺ transport. All future studies were conducted on nystatin-stimulated proximal tubules to allow us to examine changes in QO₂ independent of possible confounding ANG IV actions on apical Na⁺ entry into the cell, and we employed a single ANG IV dose of 1 pM, which was on the linear portion of the concentration-response curve.

Pharmacological characterization of angiotensin-receptor subtype. To demonstrate that the inhibitory action of ANG IV was indeed a receptor-mediated effect, both active and inactive isomers of ANG IV were employed. As shown in Fig. 5, QO₂ in nystatin-treated tubules was inhibited 21% (P < 0.001) by 1 pM ANG IV. Substitution of the L-valine in the NH₂-terminal position 1 of ANG IV with D-valine was without biological activity. This finding indicated the presence of a stereospecific ANG IV binding site on proximal tubules. The subtype of angiotensin receptor involved in the action of ANG IV on QO₂ was examined using selective angiotensin-receptor subtype antagonists. The incubation concentration of the receptor antagonists employed in the present study did not exhibit partial agonist activity, since basal QO₂ measurement of proximal tubules incubated with the angiotensin-receptor antagonists were similar to untreated control rates. In addition, injection of angiotensin-receptor antagonists into chambers containing nystatin-stimulated proximal tubules did not significantly alter QO₂ (vehicle: -0.1 ± 1.4%, n = 7; 1 µM losartan: -1.9 ± 1.2%, n = 6; 1 µM PD-123319: -1.0 ± 3.4%, n = 7; 1 µM PD-123319: -3.2 ± 3.1%, n = 19). The inhibition of Na⁺ transport by ANG IV was not altered by preincubation of tubules with either losartan or PD-123319. In contrast, incubation with divalinal-ANG IV abolished the
inhibitory action of ANG IV on proximal tubule QO2 (Fig. 6).

Specificity of divalinal-ANG IV as an AT4-receptor antagonist. Preincubation of proximal tubules with divalinal-ANG IV did not significantly alter basal QO2 (control: 26.7 ± 1.3 nmol O2·min⁻¹·mg⁻¹, n = 34; divalinal-ANG IV: 24.8 ± 0.9 nmol O2·min⁻¹·mg⁻¹, n = 42) or the effect of nystatin to increase QO2 (control: 69 ± 6%, n = 34; divalinal-ANG IV: 63 ± 5%, n = 42) by enhancing Na⁺-K⁺-ATPase activity. As shown in Fig. 7, divalinal-ANG IV abolished the receptor-mediated inhibition of nystatin-stimulated QO2 by ANG IV or [Nle1]-ANG IV (high-affinity binding analog of ANG IV; Ref. 39) and yet did not interfere with the inhibitory actions of fenoldopam (dopamine D₁-receptor agonist), PAF (lipid-receptor agonist), or ANG II (AT₁- and AT₂-receptor agonist). The reduction in QO2 by dopamine-, lipid-, and ANG II-receptor agonists could be attenuated or abolished by preincubating proximal tubules with the receptor antagonists, SCH-23390, BN-52021, and [Sar¹,Thr⁸]ANG II, respectively (not shown).

Interaction of ANG IV and ANG II: role of Na⁺-K⁺-ATPase. Having determined that both ANG IV and ANG II reduce QO2 of proximal tubules that are maximally transporting Na⁺ and act through different angiotensin-receptor subtypes (AT4 and non-AT4 receptor, respectively), we examined whether their actions were additive and whether the angiotensin peptide congeners inhibited QO2 through a single effector pathway. The reduction in nystatin-stimulated QO2 of 20% by either ANG IV or ANG II (both at submaximal concentrations of 1 pM, which lay in middle of their respective concentration-response curves) was similar to that observed when the angiotensin peptides were administered simultaneously. The magnitude of the ouabain-inhibitable component of proximal tubule QO2 is shown for comparison (Fig. 8). One interpretation of this
finding is that there is cross talk between the two receptor signaling systems that leads to a functional interaction to regulate the reduction in energy-dependent cell Na\(_{\text{i}}\) transport. ANG IV and ANG II were shown not to influence mitochondrial oxidative phosphorylation activity, since they did not alter uncoupled mitochondrial QO\(_2\) induced by 5 µM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, an oxidative phosphorylation uncoupler, not shown). Addition of 5 mM ouabain (Na\(_{\text{i}}\)-K\(_{\text{i}}\)-ATPase inhibitor) to tubules reduced nystatin-stimulated QO\(_2\) by 50%. Under these ouabain-treated conditions, administration of ANG IV or ANG II at 1 pM caused no further reduction in QO\(_2\) (Table 1). Therefore the suppression of QO\(_2\) by ANG IV or ANG II appears to involve a ouabain-inhibitable component of Na\(^{+}\)-K\(^{+}\)-ATPase activity.

**DISCUSSION**

Autoradiograms of kidney 125I-ANG IV binding sites demonstrated a dense labeling of the outer stripe of the outer medulla and diffuse labeling of the entire cortex. The binding was unaffected by specific antagonists...
the ANG II-type AT$_1$ or AT$_2$ receptor and yet was completely displaced by unlabeled ANG IV or the putative AT$_4$-receptor antagonist, divalinal-ANG IV. These results extend our previous study indicating that kidney ANG IV binding protein and AT$_1$ and AT$_2$ receptors are distinct (21). Furthermore, the distribution of $^{125}$I-ANG IV and $^{125}$I-divalinal-ANG IV binding sites were identical and had similar displacement characteristics to selective angiotensin-receptor antagonists, lending support to divalinal-ANG IV being a specific ligand for the ANG IV type AT$_4$ receptor. The pattern of $^{125}$I-peptide labeling was consistent with receptor sites being present in tubular structures. This was confirmed by emulsion autoradiography that localized ANG IV binding sites to both the microvilli and cell bodies of convoluted proximal tubules throughout the cortex, with a higher density and similar distribution of sites present on the straight proximal tubules originating from superficial and midcortical glomeruli (located in medullary rays) and the straight proximal tubules of juxtamedullary glomeruli (located in outer stripe of outer medulla). This distribution of ANG IV binding sites suggests targeting of the rat kidney AT$_4$ receptor to both proximal apical and basolateral membranes, which would be in agreement with the presence of high-affinity ANG IV binding sites in isolated rabbit proximal apical and basolateral membranes (10). Kidney AT$_4$ receptors have also been identified in the monkey and guinea pig (46), rabbit proximal tubule (10, 11), opossum proximal tubule (11), Madin-Darby bovine kidney epithelial cells (19), and gerbil and human proximal tubule (Handa, unpublished observations). Although we found no evidence of AT$_4$ receptors localized to either the glomerulus or the cortical or medullary collecting duct, high levels of ANG IV binding sites have been reported in both rat mesangial cells and human collecting duct cell membranes (2, 8). Together, these results strongly suggest a conserved and presumably functional role of the ANG IV AT$_4$ receptor system in kidney physiology across mammalian species.

We then examined whether ANG IV may influence proximal tubule Na$^+$ transport because of the location of the ANG IV binding sites on proximal tubule cell bodies and microvilli, the critical role of the convoluted and straight proximal tubule in Na$^+$ reabsorption, and the known role of ANG II as a powerful controller of proximal tubule Na$^+$ reabsorption (22). Using primary cultures of rat proximal tubules and measuring tissue QO$_2$ as an on-line, integrated index of transepithelial Na$^+$ transport, we found that ANG IV inhibited energy-dependent Na$^+$ transport in both control proximal tubules and tubules treated with nystatin (Na$^+$ ionophore that allows extracellular Na$^+$ to bypass rate-limiting step of apical Na$^+$ entry into cell and maximally stimulates basolateral Na$^+$-K$^+$-ATPase pump activity). The inhibitory action of ANG IV was not observed in ouabain (Na$^+$-K$^+$-ATPase inhibitor)- or FCCP (mitochondrial oxidative phosphorylation uncoupler)-treated tubules. Together, these results suggest that one site of ANG IV action was at the basolateral membrane to inhibit energy-dependent Na$^+$ transport by reducing ouabain-sensitive Na$^+$-K$^+$-ATPase activity.

ANG IV has been shown to have reasonable efficacy, but low affinity, to a number of known angiotensin-receptor systems, including the ANG II AT$_1$-type receptor to elicit vasoconstriction in the rat aorta, pulmonary, mesenteric, hindlimb, and renal vascular beds (13, 28, 37), feline hindquarter, and mesenteric vascular beds (5, 14), and the ANG II AT$_2$-type receptor to cause kinin-mediated nitric oxide release from isolated canine coronary vessels (40). Our results suggest that ANG IV binds with high affinity to a novel proximal tubule receptor to elicit a biological response because 1) a stereospecific receptor protein for biological activity was present, 2) inhibitory effect of ANG IV on Na$^+$ transport was not mediated by losartan-sensitive AT$_1$-type or PD-123319-sensitive AT$_2$-type receptors, and 3) biological activity was abolished by divalinal-ANG IV, a putative AT$_4$-receptor antagonist. Divalinal-ANG IV is

Table 1. ANG IV and ANG II inhibit a ouabain-suppressible component of proximal tubule O$_2$ consumption

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Change in QO$_2$</th>
<th>Change in O$_2$</th>
<th>% change from nystatin-stimulated PT QO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>nmol O$_2$·min$^{-1}$·mg protein$^{-1}$</td>
<td></td>
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<tr>
<td>Nystatin-stimulated proximal tubules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG IV (1 pM)</td>
<td>12</td>
<td>$-9.4 \pm 1.2$</td>
<td>$-22.3 \pm 2.9$</td>
</tr>
<tr>
<td>Ouabain (5 mM)</td>
<td>8</td>
<td>$-19.4 \pm 1.0$</td>
<td>$-53.7 \pm 1.5$</td>
</tr>
<tr>
<td>Ouabain + ANG IV</td>
<td>8</td>
<td>$-19.4 \pm 0.7$</td>
<td>$-53.8 \pm 1.2$</td>
</tr>
<tr>
<td>ANG II (1 pM)</td>
<td>5</td>
<td>$-11.4 \pm 2.0$</td>
<td>$-23.3 \pm 3.1$</td>
</tr>
<tr>
<td>Ouabain (5 mM)</td>
<td>6</td>
<td>$-20.4 \pm 2.5$</td>
<td>$-51.8 \pm 3.3$</td>
</tr>
<tr>
<td>Ouabain + ANG II</td>
<td>6</td>
<td>$-20.8 \pm 2.9$</td>
<td>$-52.3 \pm 4.6$</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of measurements. QO$_2$, O$_2$ consumption.
a partial nonpeptide of ANG IV with a valine substituted for isoleucine in position 3 and isostere bonds incorporated between the 1–2 and 3–4 amino acids. These modifications provide both stability and metabolic resistance to the peptide as well as receptor antagonist activity (27). The specificity of divalinal-ANG IV as an AT4-receptor antagonist was demonstrated by its ability to displace 125I-ANG IV and 125I-Nle1-ANG IV binding to rat kidney slices, whereas losartan or PD-123319 or both were without effect. On a functional basis, divalinal-ANG IV blocked the response to ANG IV and [Nle1]ANG IV (AT4-receptor agonists) and yet did not influence receptor systems activated by fenoldapam (dopamine D1-receptor antagonist), PAF (lipid-receptor agonist), or ANG II (AT1- and AT2-receptor agonist). The dopaminergic and lipergic receptor systems were selected because both inhibit proximal tubule Na1 reabsorption (12, 18). The ANG II-receptor system was chosen as a result of our finding that low concentrations of ANG II caused a receptor-mediated inhibition of Na1 transport in proximal tubules with maximally elevated Na1-K1-ATPase activity, most likely reflecting a decrease in maximal reaction rate (Vmax) of the enzyme. This differs from reports that ANG II did not change (1, 4) or increased (15) the Vmax of Na1-K1-ATPase in rat proximal tubules, suggesting complex regulation of enzyme activity by ANG II. Similar concentrations of ANG II under non-Vmax conditions can stimulate Na1 reabsorption in proximal tubules primarily by decreasing the Michaelis constant of Na1 for Na1-K1-ATPase (1, 4, 22). Membrane binding studies confirmed that divalinal-ANG IV does not displace 125I-[Sar1,Ile8]ANG II binding to AT1 receptors in the PD-123317-tREATED rat liver or to AT2 receptors in the losartan-treated rat adrenal medulla (not shown). To date, all studies using divalinal-ANG IV have shown it to be a specific antagonist of the AT4-receptor system (6, 25, 27, present study).

The intracellular signaling mechanisms of the ANG IV AT4-receptor system are presently unknown. ANG IV produced a sustained increase in intracellular Ca2+ and inositol phosphates in vascular smooth muscle cells that was distinct from the transient spike evoked by ANG II, suggesting different mechanisms in mobilizing Ca2+ (9). Activation of these vascular AT4 receptors may result in the stimulation of nitric oxide biosynthesis (7). The AT4 receptor has been shown not to be coupled to G proteins, and its affinity is generally unchanged (45, 46) or, at least in one case, increased by sulfhydryl-reducing agents (24). In contrast, Dulin et al. (11) reported that ANG IV caused a transient, dihydropyridine-sensitive, increase in intracellular Ca2+ which did not result from phosphinositol metabolism in the opossum OK7A proximal tubule cell line. The ANG IV binding site appeared to be G protein linked and was strongly inhibited by a sulfhydryl-reducing agent (11). When alternative renal transporting epithelia were used, the ANG IV binding site in human collecting duct cells and bovine kidney epithelial cells did not exhibit classical G protein coupling, unaltered by sulfhydryl-reducing agents (8, 19), and mobilized intracellular Ca2+ only at low concentrations (19). The hexapeptide appears to have no or minimal effect on guanosine 3’,5’-cyclic monophosphate and adenosine 3’,5’-cyclic monophosphate production in renal epithelial cells (8, 11). Some of these apparent contradictions may be due to the existence of AT4 receptor subtypes and the receptor having multiple intracellular signaling pathways that are cell specific.

Our results suggest that one potential role of the renal ANG IV AT4-receptor system may be in the handling of Na+ by the kidney. Supporting this contention is the demonstration that 1) ANG IV binding sites are present in Na+ transporting epithelia, such as the renal convoluted and straight proximal tubule and collecting duct (8, 11, present study), 2) stimulation of proximal tubule AT4 receptors caused a decrease in energy-dependent transcellular Na+ transport (present study), 3) anesthetized rats infused intrarenally with ANG IV can elicit a natriuresis and diuresis in the absence of changes in total renal blood flow or blood pressure (Handa, unpublished observations), and 4) in Wistar-Kyoto rats, a high-salt diet for 2 wk resulted in a 28% increase in the concentration of renal ANG IV binding sites (K. L. Grove and C. F. Deschepper, personal communication). Together, these findings provide a basis for further investigation into the physiological significance of this novel receptor system in tubular function.

The renal actions of ANG IV do not appear to be limited to the nephron. Ardaillou and Chancel (2) have recently found high levels of AT4 receptors in rat mesangial membranes and reported that ANG IV inhibited the rat mesangial cell contractile response to ANG II, suggesting the possibility that the ANG IV AT4-receptor system may influence glomerular function, e.g., permeability, filtration, and growth. Although our results do not support the presence of AT4 receptors in the glomerulus, autoradiographic techniques may not provide the sensitivity necessary to detect a low density of AT4-receptor populations. Alternatively, growing glomerular mesangial cells in vitro may stimulate the expression of AT4 receptors. Infusion of ANG IV into the renal artery of anesthetized rats has been reported to increase cortical blood flow that could be prevented by AT4-receptor blockade or reversed to a cortical vasoconstriction after nitric oxide synthesis inhibition, implying that ANG IV’s predominant effect was an AT4 receptor-mediated stimulation of nitric oxide that masked an underlying renal vasoconstrictor action of ANG IV (6, 7, 42). However, infusing ANG IV into the renal artery of anesthetized rats caused a biphasic decrease in total renal blood flow that was abolished by AT4-receptor blockade without revealing a vasodilatory action of ANG IV (44). Similarly, intravenous bolus injections of ANG IV caused only an AT1 receptor-mediated renal vasoconstriction in the conscious rat (13). Clearly, additional studies are needed to resolve the role of ANG IV in renal vascular function, including the conclusive demonstration of the existence and localization of AT4 receptors in the renal vascular bed, the influence of vasomotor tone in ANG IV’s renal blood
flow response, and whether there is cross-talk between the \( \text{AT}_1 \)- and \( \text{AT}_4 \)-receptor systems.

Studies have also shown the ANG IV receptor system to be present in many central and peripheral tissues (42, 46) and to be potentially involved in many diverse regulatory functions, including memory retrieval (46), growth of cardiac, endothelial and neuronal cells (3, 16, 35), blood flow regulation in the brain (20, 27, 36), angiogenesis, wound healing, and thrombosis (16, 25, 45). The wide distribution and many functions of the angiotensin \( \text{AT}_4 \)-receptor system suggest that it may be an important homeostatic system in the regulation of the internal environment. Despite ANG IV being an agonist with high affinity for the \( \text{AT}_4 \) receptor, it has yet to be determined whether the hexapeptide is the natural endogenous ligand for the receptor. A recent study has suggested that LVV-hemorphin-7, generated by proteolytic processing of globin precursors, may be an endogenous ligand for the \( \text{AT}_4 \) receptor in the brain (33). However, it is clear that the ANG IV molecule provides a tool to elucidate physiological and pharmacological properties of this novel receptor system. The recent development of divalinal-ANG IV as an \( \text{AT}_4 \)-receptor antagonist will also greatly aid in exploring the relative importance of this endogenous \( \text{AT}_4 \)-receptor system.

In summary, the results from autoradiographic and functional studies complement each other and demonstrate that rat proximal tubules contain the \( \text{AT}_4 \) receptor system and that this system can potentially regulate proximal tubule Na\(^+\) transport. ANG IV reduced energy-dependent Na\(^+\) transport by acting exclusively through a non-\( \text{AT}_1 \), non-\( \text{AT}_2 \) angiotensin receptor that could be blocked by the selective \( \text{AT}_4 \)-receptor antagonist, divalinal-ANG IV, and inhibited a ouabain-suppressible component of Na\(^+\)-K\(^+\)-ATPase activity.

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