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Angiotensin II Directly Stimulates ENaC Activity in the Cortical Collecting Duct via AT_1 Receptors

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Abstract. Angiotensin II (AngII) helps to regulate overall renal tubular reabsorption of salt and water, yet its effects in the distal nephron have not been well studied. The purpose of these studies was to determine whether AngII stimulates luminal Na^+ transport in the cortical collecting duct (CCD). Intracellular Na^+ concentration ($[Na^+]_i$), as a reflection of Na^+ transport across the apical membrane, was measured with fluorescence microscopy using sodium-binding benzofuran isophthalate (SBFI) in isolated, perfused CCD segments dissected from rabbit kidneys. Control $[Na^+]_i$, during perfusion with 25 mM NaCl and a Na^+-free solution in the bath containing the Na^+-ionophore monensin (10 μM, to eliminate basolateral membrane Na^+ transport) averaged 19.3 ± 5.2 mM ($n = 16$). Increasing luminal [NaCl] to 150 mM elevated $[Na^+]_i$ by 9.87 ± 1.5 mM ($n = 7$; $P < 0.05$). AngII (10^{-9} M) added to the lumen significantly elevated baseline $[Na^+]_i$ by 6.3 ± 1.0 mM and increased the magnitude ($\Delta = 25.2 \pm 3.7$ mM) and initial rate (≈5 fold) of change in $[Na^+]_i$ to increased luminal [NaCl]. AngII when added to the bath had similar stimulatory effects; however, AngII was much more effective from the lumen. Thus, AngII significantly increased the apical entry of Na^+ in the CCD. To determine if this apical entry step occurred via the epithelial Na^+ channel (ENaC), studies were performed using the specific ENaC blocker, benzamil hydrochloride (10^{-6} M). When added to the perfusate, benzamil almost completely inhibited the elevations in $[Na^+]_i$ to increased luminal [NaCl] in both the presence and absence of AngII. These results suggest that AngII directly stimulates Na^+ channel activity in the CCD. AT_1 receptor blockade with candesartan or losartan (10^{-6} M) prevented the stimulatory effects of AngII. Regulation of ENaC activity by AngII may play an important role in distal Na^+ reabsorption in health and disease.

The renin-angiotensin system (RAS) is a major regulator of body fluid and sodium balance (1), predominantly through the actions of its main effector angiotensin II (AngII). Besides its well-known role as a vasoconstrictor, AngII directly affects the overall renal transepithelial transport of salt and water (2). Sustained low-dose infusion of AngII leads to a progressive long-term rise in arterial pressure due to cumulative sodium retention primarily mediated by the direct intrarenal effects of AngII on sodium reabsorption (1). In previous work, the effects of AngII on tubular transport processes have been mostly studied in the proximal tubule (3–6). In this segment, low concentrations of AngII added to either the tubular lumen or at the basolateral membrane augmented both apical Na:H exchange and basolateral $Na:HCO_3$ cotransport via AT_1 receptors. Stimulation of both of these Na-dependent transport processes resulted in enhanced proximal tubular salt and water reabsorption.

Although the effects of AngII on proximal tubular transport have been extensively examined, much less is known about the effects of AngII on tubular transport by more distal nephron segments. Studies have reported that AngII can alter transport in distal tubular segments (7,8), cortical collecting ducts (9), and macula densa cells (10,11). In the distal tubule and cortical collecting duct, AngII may stimulate Na:H exchange (7–9). Also of potential importance is the findings by Wang and Giebisch (12) that the effects of AngII to stimulate volume reabsorption in the late distal tubule not only involves the acid base transporters (Na:H exchange and $NaHCO_3$ cotransport) but may act via Na^+ channels. They found that the effects of AngII on volume reabsorption were inhibited by amiloride, an agent that blocks Na^+ channels. Although amiloride also blocks Na:H exchangers, the isoform expressed at the apical membranes is quite amiloride-resistant. Thus, it is important to establish if AngII directly acts on Na^+ channels in the distal nephron/collecting duct segment.

Why is it important to study the effects of AngII and its blockade in distal nephron segments? Although the proximal tubule is responsible for reabsorbing the bulk of the glomerular filtrate, it is the distal nephron segments that are ultimately responsible for the rate of urine formation and its composition. This is also the nephron site that is responsible for BP-induced changes in urine flow and Na^+ excretion (pressure natriuresis and diuresis). In older studies by Hall *et al.* (13,14), they demonstrated in a one-kidney dog model that intrarenal infusions of AngII can cause hypertension by shifting the pressure natriuresis curve to the right, *i.e.*, it required a higher BP to excrete Na^+ compared with control non-AngII-infused kid-

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neys. This was despite equivalent rates of glomerular filtration in the control and AngII-infused groups. Thus, AngII can cause hypertension through its effects to promote Na^+ reabsorption most likely in the collecting duct.

Elements of a paracrine RAS along the distal nephron have recently been described (15). Angiotensinogen is synthesized by proximal tubule cells and secreted into tubular fluid, and then uncleaved angiotensinogen transits through the entire nephron and can be found in final urine (15,16). Renin is synthesized and secreted by connecting tubule cells (15). The existence of endopeptidases and carboxypeptidase in distal tubular fluid (17) may also have a potential role in the generation of angiotensins from AngI. Immunohistochemical studies (18) have additionally established the existence of AT_1 receptors at both the apical and basolateral membranes in distal nephron segments including the collecting duct. It is also well established that a significant amount of AngII is secreted into the tubular fluid (19–21). These elements of a tubular RAS may be involved in the coordinated regulation of sodium reabsorption by the distal nephron.

The purpose of these studies was to develop a model in which to study ENaC activity in the collecting duct. When this was accomplished, we sought to determine if AngII could alter Na^+ transport via this apical channel, and finally we evaluated the effects of AT_1 receptor blockade on ENaC activity.

Materials and Methods

Tubule Preparation

We isolated and perfused individual CCD segments dissected from rabbit and mouse kidney as described earlier for the cortical thick ascending limb (22). This preparation allowed us to manipulate the composition of the tubular fluid at the apical side (perfusate) independently from the basolateral interstitium (bath). The dissection solution was a modified Ringer's solution composed of 148 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 5 mM D-glucose, and 5 mM HEPES and adjusted to a pH of 7.4. Tubules were cannulated and perfused with this same Ringer's solution, except that [NaCl] was lowered to 25 mM using N-methyl-D-glucamine cyclamate to maintain osmolality at 300 mOsm/kg. The preparation was bathed in the bathing Ringer solution continuously aerated with 100% O_2 and exchanged at a rate of 1 ml/min. Temperature was maintained at 37°C. For the experiments, the preparations were bathed in a similarly modified Ringer's solution in that NaCl was isosmotically replaced with N-methyl-D-glucamine cyclamate to achieve a [NaCl] of 0 mM.

$[Na^+]_i$ Measurement

$[Na^+]_i$ of CCD cells was measured using photometry-based fluorescence microscopy (Photon Technology International, Lawrenceville, NJ) and sodium-binding benzofuran isophthalate (SBFI) (Teflabs, Austin, TX) with similar techniques as described for Ca^{2+} and pH_i measurements (11,22). SBFI fluorescence was measured at an emission wavelength of 510 nm in response to excitation wavelengths of 340 and 380 nm. Cells were loaded with the dye by adding SBFI-AM (20 μM) dissolved in dimethyl sulfoxide (DMSO) to the luminal perfusate. The nonionic surfactant, Pluronic F-127 was added (1 mg/ml) to DMSO to facilitate loading that required approximately 15 min, then the SBFI-AM in the lumen was removed. After approximately 15-min incubation of the tubule with the control perfusion solution, fluorescence intensities for both wavelengths stabilized at constant level. SBFI fluorescence ratios (340/380 nm) were converted into $[Na^+]_i$ values after permeabilizing cell membranes on both sides of the tubules with 10 μM nigericin + monensin and equilibrating $[Na^+]_i$ with ambient $[Na^+]$ in a stepwise manner between 0 to 150 mM.

We used measurements of $[Na^+]_i$ as a reflection of Na^+ transport across the apical membrane. To assess the activity of ENaC alone, the basolateral membrane was permeabilized to Na^+ with monensin, and bath [NaCl] was reduced to zero. Luminal Na^+ ($[NaCl]_L$) was then increased from 25 to 150 mM. EnaC-specific activity was assessed by applying 10^{-6} M of the ENaC blocker benzamil, a concentration that does not affect Na^+:H^+ exchange. Measurements consisted of the resting $[Na^+]_i$ under control conditions (isosmotic 25 mM luminal and 0 mM bath [NaCl]), and the magnitude (ΔNa^+_i) and initial rate ($\Delta Na^+_i/\Delta t$) of increases in $[Na^+]_i$ when $[NaCl]_L$ was increased from 25 to 150 mM using a PTI software. Experiments were performed in the presence/absence of luminal benzamil or the Na:H exchanger blocker HOE694 (from Hans-Jochen Lang, Aventis Pharma, Frankfurt, Germany), luminal or basolateral AngII (both from Sigma Chemical Co., St. Louis, MO) administered with/without the AT_1 receptor blocker candesartan (generous gift from Peter Morsing, AstraZeneca, Molndal, Sweden) or losartan (generous gift from Leslie Koch, Merck, Rahway, NJ). Final DMSO concentrations were below 0.1%.

Statistical Analyses

Data are expressed as mean ± SE. Statistical significance was tested using ANOVA. Significance was defined as $P < 0.05$.

Results

Basal and $[NaCl]_L$-Dependent $[Na^+]_i$

After removing bath Na^+ and permeabilizing the basolateral membrane with the Na^+-ionophore monensin (10 μM), $[Na^+]_i$ stabilized at a constant level (19.3 ± 5.2 mM; $n = 16$) when perfusing with 25 mM $[NaCl]_L$. Representative recordings in Figure 1A illustrate that increasing $[NaCl]_L$ from 25 to 150 mM caused rapid, sustained, and reversible elevations in $[Na^+]_I$, which averaged 9.87 ± 1.5 mM ($n = 7$). In addition to reducing the baseline, this increase was almost completely blocked by 10 to 6 M benzamil, a specific inhibitor of ENaC added to the luminal perfusate ($\Delta Na^+_i = 0.7 \pm 0.4$ mM; $n = 5$; $P < 0.05$). The specific Na:H exchange blocker HOE694 added to the luminal perfusate (1 mM) caused no significant changes in control Na^+_i dynamics ($\Delta Na^+_i = 7.72 \pm 2.6$ mM; $n = 7$) (Figure 1A). These findings strongly suggest that, in our experimental model, $[NaCl]_L$-dependent increases in CCD Na^+_i are due to apical ENaC activity rather than Na:H exchange.

Effects of AngII

Figure 1B exemplifies and Figure 2 summarizes the effects of 10^{-9} M AngII added to the bath or the luminal perfusate. Luminal AngII, which was more effective than from the bath, increased baseline $[Na^+]_i$ by 6.3 ± 1.0 mM ($n = 12$; $P < 0.05$) and significantly increased the magnitude ($\Delta Na^+_i = 25.2 \pm 3.7$ mM) and initial rate ($\Delta Na^+_i/\Delta t \approx$5-fold) of increases in $[Na^+]_i$ when $[NaCl]_L$ was increased from 25 to 150 mM. In some experiments, low doses of luminal AngII had similar stimulatory effects on the rabbit CCD $[Na^+]_i$ dynamics. Added

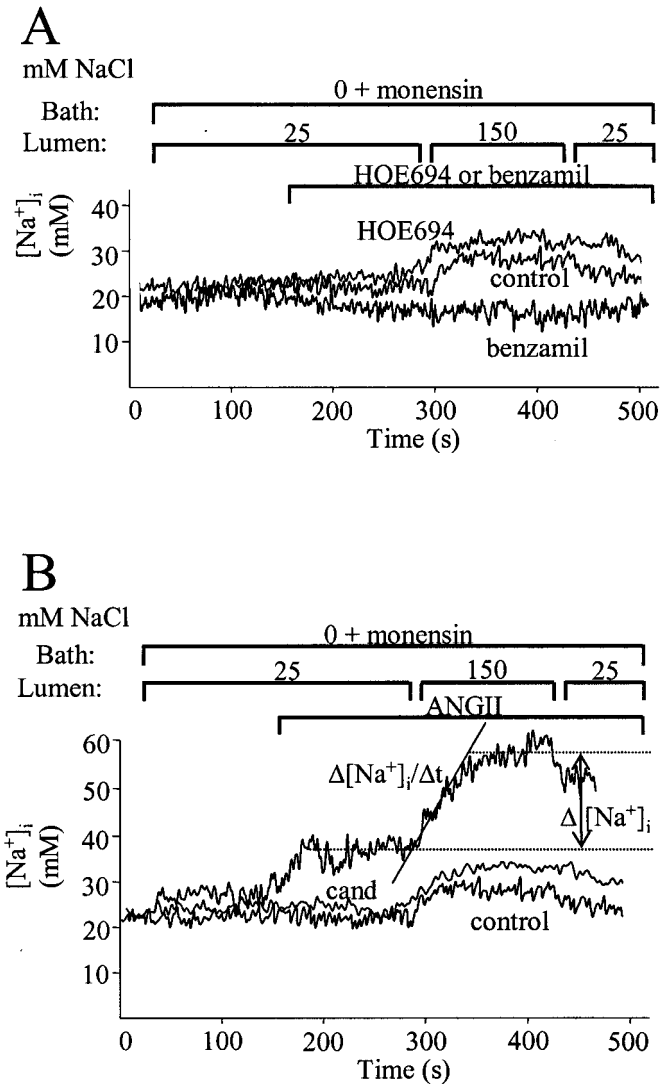

Figure 1. Representative recordings of the effects of increasing luminal [NaCl] from 25 to 150 mM on intracellular Na^+ concentration ($[Na^+]_i$) in the rabbit cortical collecting duct (CCD). (A) Effects of the specific epithelial Na^+ channel (ENaC) blocker benzamil (10^{-6} M) or Na:H inhibitor HOE694 (1 mM) added to the luminal perfusate. (B) $[Na^+]_i$ dynamics in the absence or presence of luminal angiotensin II (ANGII) (10^{-9} M) alone and coadministered with candesartan (10^{-6} M). Measured parameters of the $[Na^+]_i$ dynamics were the baseline $[Na^+]_i$ under control conditions (isosmotic 25 mM luminal and 0 mM bath [NaCl]) and the magnitude (ΔNa^+_i) and initial rate ($\Delta Na^+_i/\Delta t$) of increases in $[Na^+]_i$ when $[NaCl]_L$ was increased from 25 to 150 mM.

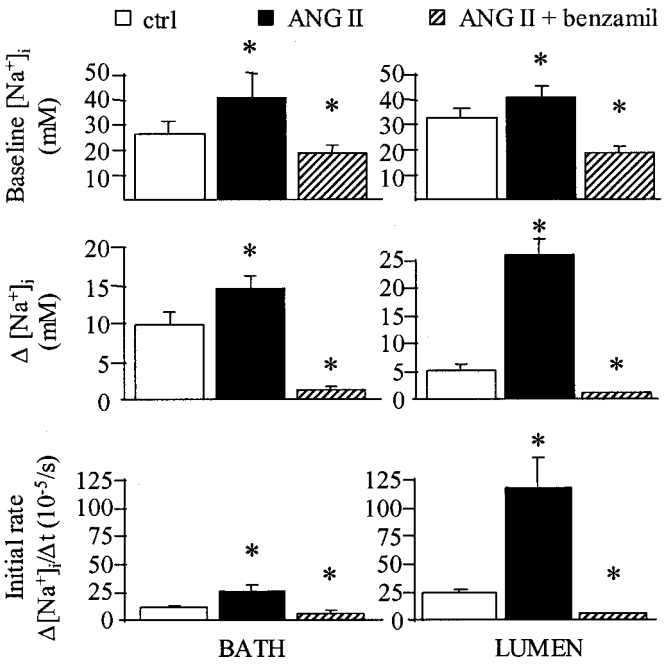

Figure 2. Effects of luminal/bath ANGII (10^{-9} M) on the 150 mM luminal [NaCl]-induced elevation in $[Na^+]_i$ of the rabbit CCD in presence or absence of the specific ENaC blocker benzamil (10^{-6} M). ($n = 7$; * $P < 0.05$ compared with control).

to the luminal perfusate, 10^{-12} M AngII caused ≈2.5-fold increase in the initial rate of increases in $[Na^+]_i$ ($\Delta Na^+_i/\Delta t$) when $[NaCl]_L$ was increased from 25 to 150 mM. AngII, added to the bath was also stimulatory but smaller in scale (Figure 2, left panels). The specific ENaC blocker benzamil added to the luminal perfusate (10^{-6} M) significantly decreased baseline $[Na^+]_i$ by 19.2 mM ($n = 5$) and almost completely inhibited the $[NaCl]_L$–dependent increase in $[Na^+]_i$ when coadministered with luminal AngII. Similar effects were observed with bath AngII (Figure 2). These findings clearly suggest that the effects of AngII on $[Na^+]_i$ dynamics were due to the stimulation of ENaC at the apical membrane.

Because of transgenic knockout mouse models, it was of interest to determine if AngII had similar effects in mouse CCD. Additional studies were performed in mouse CCD using similar techniques and experimental maneuvers (Figure 3). Similar to its stimulatory effect in rabbit, AngII, added to the

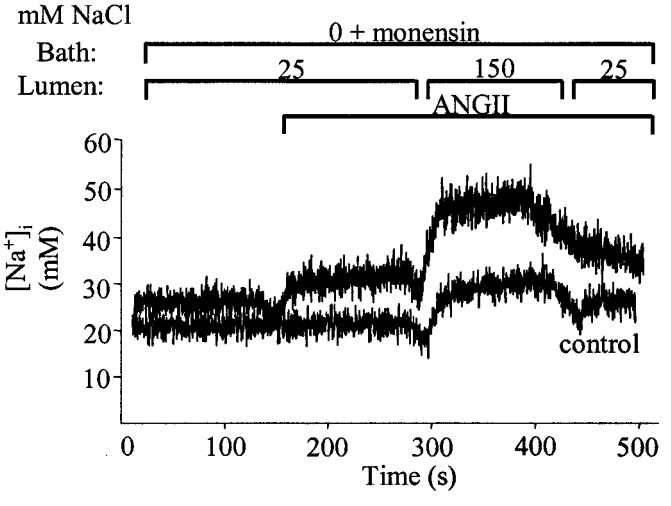

Figure 3. Representative recordings of the effects of increasing luminal [NaCl] from 25 to 150 mM on $[Na^+]_i$ in the mouse CCD in the absence or presence of low concentrations of luminal angiotensin II (10^{-12} M).

luminal perfusate in low concentration (10^{-12} M), significantly increased baseline Na^+_i by 9.05 ± 1.53 ($n = 4$) and caused an ≈2.5-fold increase in both ΔNa^+_i and the initial rate of increases in $[Na^+]_i$ ($\Delta Na^+_i/\Delta t$) when $[NaCl]_L$ was increased from 25 to 150 mM, compared with control. Thus luminal AngII, in the 10^{-9} to 10^{-12} M range, increases the apical membrane permeability to Na^+ in the CCD in both mouse and rabbit.

Effects of AT_1 Blockade

Coadministration of AngII with the AT_1 receptor blocker candesartan or losartan (both 10^{-6} M; Figure 4) to the luminal perfusate prevented all stimulatory effects of AngII on $[Na^+]_i$ dynamics (baseline Na^+_i, ΔNa^+_i, and $\Delta Na^+_i/\Delta t$). Luminal candesartan or losartan alone had no effect on any of these parameters. These data suggest that the stimulatory effects of AngII were mediated via AT_1 receptors.

Discussion

We have established a method to specifically assess the activity of the apical Na^+ channel (ENaC) in the CCD. Developing an electrochemical gradient for Na^+ across the apical membrane by removing bath Na^+ and permeabilizing the basolateral membrane with the Na^+-ionophore monensin, greatly improved the detection of an amiloride-sensitive apical Na^+-influx in CCD cells in our experimental model. This method is of general utility and can quantify the rate of conductive or electrically neutral ion transport for which appropriate fluorescence probes and specific inhibitors are available. In the present studies, we used the specific amiloride-analog benzamil in a concentration (10^{-6} M) that has no effect on either intercalated cell Na^+/H^+ exchange or basolateral Na^+,K^+-ATPase activity (23). Thus, the benzamil-sensitive effects of high $[NaCl]_L$ and AngII on $[Na^+]_i$ dynamics (Figure 2) were due to ENaC activity at the apical membrane of principal cells in the CCD. Also support for an effect of AngII on ENaC is that addition of the specific Na:H exchange blocker HOE694 to the luminal perfusate did not influence $[Na^+]_i$ dynamics (Figure 1A). To assess ENaC activity, we measured parameters of $[Na^+]_i$ dynamics in the CCD (baseline, magnitude, and initial rate of the 150 mM $[NaCl]_L$–induced change in $[Na^+]_i$), as described for other ion transporters in our previous work (10,11).

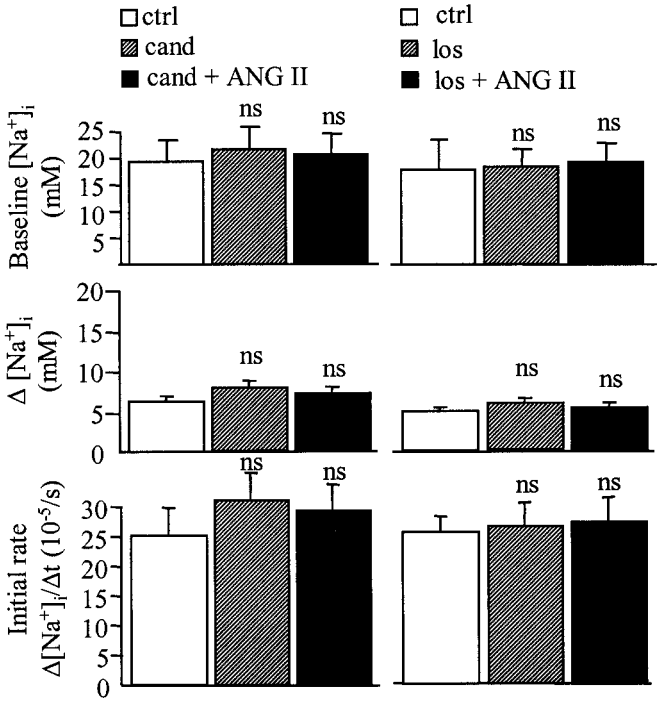

Figure 4. Effects of luminal AT_1 receptor blockade with candesartan (cand) or losartan (los) (both 10^{-6} M) on the 150 mM luminal [NaCl]-induced elevation in $[Na^+]_i$ of the rabbit CCD in presence of 10^{-9} M luminal ANGII. ($n = 6$; ns, not significant).

Baseline $[Na^+]_i$ under control conditions was stable around 20 to 25 mM and was sensitive to benzamil in both the presence and absence of AngII (Figure 2). This suggests a significant contribution of ENaC activity and hence apical Na^+-transport in maintaining baseline $[Na^+]_i$. More importantly, the 150 mM $[NaCl]_L$–induced increase in both ΔNa^+_i and $\Delta Na^+_i/\Delta t$ were almost completely abolished in the presence of benzamil, indicating that the majority of apical Na^+-entry during this maneuver was mediated by ENaC.

AngII significantly increased benzamil-sensitive elevations in $[Na^+]_i$ in response to 150 mM $[NaCl]_L$, suggesting stimulation of ENaC activity. Addition of AngII to either tubular perfusate or bath produced stimulatory effects, indicating that AngII acts through luminal and basolateral AngII receptors to stimulate the apically located ENaC in CCD segments. However, AngII was much more effective from the lumen. By measuring the initial rate of the high $[NaCl]_L$-induced increase in $[Na^+]_i$, luminal AngII caused an approximately fivefold stimulation of ENaC activity (Figure 2). Of particular importance, AngII was also effective in low concentrations (10^{-12} M). This suggests that AngII, in the low physiologic concentration range of 10^{-9} to 10^{-12} M, is capable of increasing the apical membrane permeability to Na^+ in the CCD. In terms of the physiologic importance of this paracrine loop, the mouse model will in the long run offer many more opportunities in genetically engineered animals.

Stimulatory effects of AngII were prevented by the coadministration of the AT_1 receptor antagonist candesartan or losartan, suggesting AT_1 receptor mediation. The reason that we performed additional experiments with losartan was that candesartan, particularly from the bath (data not shown), produced a slight increase in parameters of $[Na^+]_i$ dynamics. However, this slight stimulation by candesartan did not occur with luminal administration (Figure 4) and was completely absent with losartan.

We found that AngII significantly enhanced apical Na^+ entry in CCD obtained from mice on a normal Na^+ diet. These preliminary studies open the possibilities for future studies in genetically engineered mice in which components of the RAS system have been either eliminated or augmented.

In summary, these studies measured $[Na^+]_i$ as a reflection of Na^+ transport across the apical membrane of CCD. Whether

AngII stimulates net transepithelial Na^+ reabsorption in this important nephron segment needs to be further investigated. Direct stimulation of ENaC by AngII is consistent with the presence of a paracrine RAS in the distal tubule. Elements of this system are regulated by dietary sodium intake (15); therefore, locally produced AngII through ENaC activity may tonically enhance Na^+-reabsorption in the CCD depending on Na^+ intake. This distal tubular RAS-AngII-ENaC system may play an important role in disease mechanisms leading to hypertension, and this site may be an important therapeutic target for RAS inhibitors and receptor blockers.

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