Angiotensin II directly stimulates macula densa Na-2Cl-K cotransport via apical AT$_1$ receptors

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Kovács, Gergely, János Peti-Peterdi, László Rosivall, and P. Darwin Bell. Angiotensin II directly stimulates macula densa Na-2CI-K cotransport via apical AT$_1$ receptors. Am J Physiol Renal Physiol 282: F301–F306, 2002—ANG II is a modulator of tubuloglomerular feedback (TGF); however, the site of its action remains unknown. Macula densa (MD) cells sense changes in luminal NaCl concentration ([NaCl]$_l$) via a Na-2Cl-K cotransporter, and these cells do possess ANG II receptors. We tested whether ANG II regulates Na-2Cl-K cotransport in MD cells. MD cell Na$^+$ concentration ([Na$^+$]$_i$) was measured using sodium-binding benzofuran isophthalate with fluorescence microscopy. Resting [Na$^+$]$_i$ in MD cells was 27.7 ± 1.05 mM (n = 138) and increased ([Na$^+$]$_i$) by 18.5 ± 1.14 mM (n = 17) at an initial rate (Δ[Na$^+$]$_i$/Δt) of 5.54 ± 0.53 × 10$^{-4}$ U/s with an increase in [NaCl]$_l$ from 25 to 150 mM. Both Δ[Na$^+$]$_i$ and Δ[Na$^+$]$_i$/Δt were inhibited by 80% with 100 μM luminal furosemide. ANG II (10$^{-9}$ or 10$^{-12}$ M) added to the lumen increased MD resting [Na$^+$]$_i$ and [NaCl]$_l$-dependent Δ[Na$^+$]$_i$ and caused a twofold increase in Δ[Na$^+$]$_i$/Δt. Bath (10$^{-9}$ M) ANG II also stimulated cotransport activity, and there was no additive effect of simultaneous addition of ANG II to bath and lumen. The effects of luminal ANG II were furosemide sensitive and abolished by the AT$_1$ receptor blocker candesartan. ANG II at 10$^{-6}$ M failed to stimulate the cotransporter, whereas increased cotransport activity could be restored by blocking AT$_2$ receptors with PD-123, 319. Thus ANG II may modulate TGF responses via alterations in MD Na-2Cl-K cotransport activity.

furosemide; tubuloglomerular feedback; angiotensin receptor blockade; cytosolic sodium concentration; fluorescent microscopy


The vasoconstrictor hormone ANG II is generally considered to be a specific modulator of TGF (21), because it enhances feedback responses to a given [NaCl]$_l$. It also has been shown to stimulate electrolyte transport processes in many tubular segments, including the thick ascending limb (TAL) and proximal tubule (6, 10, 29). At the present time, the site(s) at which ANG II interacts with the TGF signal transmission process remain(s) unknown. Although there are clearly AT$_1$ receptors located in renal arterioles and mesangial cells (26), recent work established the existence of AT$_1$ receptors in MD cells (11). This has opened up the possibility that ANG II could have effects on MD cell function that might lead to enhanced TGF responses. Indeed, recent work from our laboratory found that ANG II upregulates both MD apical (NHE2) and basolateral (NHE4) Na$^+$/H$^+$ exchanger activities (6, 9, 29, 30).

Because of the central role that NKCC plays in MD cell signaling, we sought to determine whether ANG II might also upregulate apical NKCC in MD cells. Because significant levels of ANG II have been detected in renal tubular fluid and MD cells also possess ANG II receptors at the apical membrane (7), the present study focused on the luminal actions of ANG II. NKCC activity was assessed by measuring intracellular Na$^+$ concentration ([Na$^+$]$_i$) during elevations in [NaCl]$_l$, using the fluorescent dye sodium-binding benzofuran isophthalate (SBFI). We tested the effects of ANG on [NaCl]$_l$-dependent changes in [Na$^+$]$_i$, and also examined the involvement of AT$_1$ and AT$_2$ receptors in this process.

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NaCl, 125 M isosmotic, low-NaCl Ringer solution composed of (in mM) 25 rabbits weighing 0.5 kg. The dissection solution was an iso-osmotic 25 mM NaCl, 125 N-methyl-d-glucamine, 125 cyclic acid, 5 KCl, 1 MgSO4, 1.6 Na2HPO4, 0.4 NaH2PO4, 1.5 CaCl2, 5 D-glucose, and 10 HEPES for buffering, at 4°C. Individual preparations were transferred to a thermostated Lucite chamber mounted on a Leitz Fluorview inverted microscope and bathed in a 25 NaCl, 125 N-methyl-d-glucamine, 125 cyclic acid, 5 KCl, 1 MgSO4, 1.6 Na2HPO4, 0.4 NaH2PO4, 1.5 CaCl2, 5 D-glucose, 10 HEPES for buffering, at 4°C. Individual preparations were transferred to a thermostated Lucite chamber mounted on a Leitz Fluorview inverted microscope and bathed in a modified Ringer solution (composition the same as above, except that 150 mM NaCl replaces N-methyl-d-glucamine, and cyclic acid). The cTALs were cannulated and perfused with the low-NaCl Ringer solution. Both perfusate and bath solutions were bubbled with 100% O2, pH was adjusted to 7.4, and the bath temperature was maintained at 38°C. This preparation contained the otherwise inaccessible MD cells and allowed us to manipulate the composition of the tubular fluid at the apical side (perfusion) independently from the basolateral side (bath).

METHODS

MD preparation. We isolated and perfused cortical TALs (cTALs) with attached glomeruli, dissected from rabbit kidney as described earlier (27–30). Briefly, individual cTALs with attached glomeruli were manually dissected from sagittal slices of kidneys obtained from New Zealand White rabbits weighing 0.5–1 kg. The dissection solution was an iso-osmotic, low-NaCl Ringer solution composed of (in mM) 25 NaCl, 125 N-methyl-d-glucamine, 125 cyclic acid, 5 KCl, 1 MgSO4, 1.6 Na2HPO4, 0.4 NaH2PO4, 1.5 CaCl2, 5 D-glucose, and 10 HEPES for buffering, at 4°C. Individual preparations were transferred to a thermostated Lucite chamber mounted on a Leitz Fluorview inverted microscope and bathed in a modified Ringer solution (composition the same as above, except that 150 mM NaCl replaces N-methyl-d-glucamine, and cyclic acid). The cTALs were cannulated and perfused with the low-NaCl Ringer solution. Both perfusate and bath solutions were bubbled with 100% O2, pH was adjusted to 7.4, and the bath temperature was maintained at 38°C. This preparation contained the otherwise inaccessible MD cells and allowed us to manipulate the composition of the tubular fluid at the apical side (perfusion) independently from the basolateral side (bath).

[Na+]i measurement. [Na+]i of MD cells was measured by fluorescent microscopy and SBFI (TeFlabs, Austin, TX), using techniques similar to that described for Ca2+ and intracellular pH measurements (27, 28, 30). 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-acetoxyethyl ester (AM; 20 μM), dissolved in DMSO, to the luminal perfusate. The nonionic surfactant Pluronic F-127 was added (50 mg/mL) to DMSO to facilitate loading that required ~15 min. Luminal SBFI-AM was removed when counts for each wavelength exceeded 2 × 105 counts/s. Fifteen minutes were then allowed to elapse to ensure conversion to the free SBFI form and to allow fluorescence intensities at both wavelengths to stabilize. SBFI fluorescence ratios (340/380 nm) were converted into [Na+]i, values using an equation that was derived from the calibration procedure. For SBFI calibration, both apical and basolateral membranes of MD cells were exposed to 10 μM nigericin-monomers. The Na+ concentration ([Na+]i) of both bath and lumen was then varied in a stepwise manner between 0 and 150 mM to obtain the relationship between [Na+]i and cell SBFI fluorescence ratio.

Measurements consisted of resting [Na+]i in MD cells under control conditions (isosmotic 25 mM luminal and 150 mM bath [NaCl]) and the magnitude (Δ[Na+]i) and initial rate (Δ[Na+]i/Δt) of increases in [Na+]i, when [NaCl]l was increased from 25 to 150 mM (calculated from a linear fit using PFI software). Experiments were performed in the presence/absence of luminal furosemide; luminal ANG II (both from Sigma, St. Louis, MO) was administered with/without the AT1 and AT2 receptor blockers candesartan (generous gift from P. Morsing, AstraZeneca) and PD-123, 319 (Sigma RBI), respectively, or with/without the AT2 agonist CGP-42112A (Sigma RBI).

Statistical analysis. Data are expressed as means ± SE. Statistical significance was tested using ANOVA. Significance was accepted at P < 0.05.

RESULTS

Basal and [NaCl]l-dependent [Na+]i. Resting MD [Na+]i in the presence of 25 mM NaCl in the perfusate and 150 mM NaCl in the bath, averaged 27.7 ± 1.05 mM (n = 138). As exemplified in a representative recording in Fig. 1 and summarized in Fig. 2, increasing [NaCl]l from 25 to 150 mM caused a rapid and sustained increase in [Na+]i by 18.5 ± 1.14 mM (n = 17), with a Δ[Na+]i/Δt of 5.54 ± 0.53 × 10−4 U/s. This response was highly sensitive to the Na-2Cl-K cotransport blocker furosemide, which was added to the lumi-
nal perfusate. Furosemide reduced baseline $[\text{Na}^+]_i$ by 50% and $\Delta[\text{Na}^+]_i$ by 80% (not shown) and also caused a significant reduction in $\Delta[\text{Na}^+]_i/\Delta t$ after an elevation in $[\text{NaCl}]_L$ from 25 to 150 mM (Fig. 3). These results are all similar to what has recently been reported for MD cell Na$^+$ regulation (28).

**Effects of ANG II.** Similar experiments were performed to determine the effects of luminal ANG II, at concentrations of $10^{-9}$ and $10^{-12}$ M, on changes in MD $[\text{Na}^+]_i$, with increased $[\text{NaCl}]_L$ from 25 to 150 mM. The effects of ANG II on baseline $[\text{Na}^+]_i$, and $\Delta[\text{Na}^+]_i$ and $\Delta[\text{Na}^+]_i/\Delta t$ in response to an increase in $[\text{NaCl}]_L$ are depicted in Fig. 1 and summarized in Figs. 2 and 3. Luminal administration of ANG II ($10^{-9}$ M) in the presence of 25 mM $[\text{NaCl}]_L$ significantly increased resting $[\text{Na}^+]_i$ of MD cells (by $4.6 \pm 1.1$ mM, $n = 14$). In addition, ANG II significantly increased both magnitude and initial rate of $[\text{NaCl}]_L$-induced increases in MD $[\text{Na}^+]_i$. On the basis of $\Delta[\text{Na}^+]_i/\Delta t$ measurements, ANG II at $10^{-9}$ or $10^{-12}$ M caused an approximately twofold stimulation of the MD Na-2Cl-K cotransporter. However, ANG II at a concentration of $10^{-6}$ M failed to stimulate cotransport activity. Finally, as shown in Figs. 2 and 3, the stimulatory effects of 1 nM and 1 pM ANG II were markedly furosemide sensitive.

ANG II also caused a similar stimulation of Na-2Cl-K cotransport activity when added from the basolateral side. ANG II, at $10^{-9}$ M in the bath, increased $\Delta[\text{Na}^+]_i/\Delta t$ from $5.54 \pm 0.53$ ($n = 17$) to $9.8 \pm 1.4 \times 10^{-4}$ U/s ($n = 7$). Simultaneous administration of ANG II to the lumen and bath failed to have an additive effect, and bath addition of ANG II was also inhibited by luminal furosemide (data not shown).

**Effects of AT$_1$ and AT$_2$ receptor blockade.** Candesartan and PD-123,319 were used to determine whether ANG II stimulates $[\text{NaCl}]_L$-induced increases in MD $[\text{Na}^+]_i$ via AT$_1$ or AT$_2$ receptors. As summarized in Fig. 4, both candesartan and PD-123,319 (alone) had no effect on the initial rate of increase in MD $[\text{Na}^+]_i$. However, luminal coadministration of candesartan with ANG II inhibited the stimulatory effects of $10^{-9}$ M ANG II. Interestingly, luminal coadministration of PD-123,319 with $10^{-6}$ M ANG II restored the stimulatory effects of ANG II to the level observed with $10^{-12}$ M ANG II. These findings suggest that, at low, physiological concentrations of ANG II, MD Na-2Cl-K cotransport activity is stimulated via AT$_1$ receptors but that high levels of ANG II may also activate an AT$_2$-mediated inhibitory pathway.

**Effects of AT$_2$ agonist CGP-42112A.** To test our hypothesis that high-dose ANG II ($10^{-6}$ M) activates both AT$_1$ and AT$_2$ receptors and that AT$_2$ receptors antagonize the stimulation of Na-2Cl-K cotransport activity by AT$_1$ receptors, we used CGP-42112A, an AT$_2$-receptor agonist. As shown in Fig. 5, when 10 nM CGP-42112A was administered alone into the lumen, there was no change in the initial rate of increase in MD $[\text{Na}^+]_i$ when $[\text{NaCl}]_L$ was elevated. Coadministration of this AT$_2$-receptor agonist with low-dose ANG II completely abolished the stimulatory effects of ANG II on $\Delta[\text{Na}^+]_i/\Delta t$, whereas there was no effect of CGP-42112A in the presence of a high concentration of ANG II.

**DISCUSSION**

Previous studies have established that ANG II is an important and specific modulator of TGF (13, 21, 35, 36). Mitchell and Navar (21) reported that peritubular capillary infusions of ANG I or ANG II augmented TGF responses as assessed by orthograde microperefusion and stop-flow pressure measurements. Schernmann and Briggs (35) found that ANG II was able to restore TGF feedback responses that were suppressed by prior volume expansion. Recently, Schernmann and coworkers (36) found that feedback responses were markedly suppressed in an AT$_{1A}$ receptor-deficient mouse. Finally, Huang et al. (13) suggested that the effects of modulation of TGF responses by ANG II may be independent of the vasoconstrictive properties of ANG II.
this hormone. This latter finding, coupled with the recent report of AT1 receptors in MD cells (11), makes it attractive to speculate that ANG II may have a modulator role in TGF at the level of the MD.

In this regard, we have recently shown that both NHE2 and NHE4 activities in MD cells are stimulated by physiological concentrations of ANG II via AT1 receptors located at both apical and basolateral cell membranes (6, 29). Whether this stimulation of Na+/H+ exchange by ANG II plays a role in TGF signaling or in modulating TGF responsiveness is, as yet, unknown. What is known, however, is the important role that the Na-2Cl-K cotransporter plays in MD cell signaling. TGF responses are clearly inhibited by physiological concentrations of ANG II via AT1 receptors located at both apical and basolateral cell membranes (6, 29).

These studies used the fluorescent Na-sensitive probe SBFI in the isolated perfused MD preparation. We used [NaCl]L-dependent changes in [Na+]i, as an assay for measuring the activity of the MD apical Na-2Cl-K cotransporter. Elevations in MD [Na+]i in response to increasing [NaCl]L were very sensitive to furosemide, a specific blocker of NKCC. Furosemide reduced baseline [Na+]i as well as [NaCl]L-dependent changes in [Na+]i; both under control conditions and, most importantly, in the presence of ANG II. This is strong support that measurements of [Na+]i can be used to assess NKCC activity.

Previously, we used a NH4+ technique to assess cotransport activity because NH4+ substitutes for K+ on the cotransporter (16, 17, 20). Cotransport activity can then be assessed by the rate of cell acidification as NH4+ is transported into MD cells. However, in the present studies, this technique was not satisfactory because application of luminal ANG II strongly stimulates Na+/H+ exchanger activity and thus opposes NH4+-induced cell acidification. Furthermore, studies cannot simply be done in the presence of Na+/H+ exchange blockade because this alone induces a large cellular acidification. It is possible that other transporters or channels contributed to ANG II stimulation of Na+ entry. However, no Na+ currents have been detected at the apical membrane of MD cells, and there appears to be only a small conductive pathway for Na+ at the basolateral membrane (14, 19). Also, in response to increased luminal [NaCl]L, MD cells depolarize, which clearly would not facilitate the entry of Na+ through a conductive pathway (5). It could be argued that ANG II might be inhibiting a Na+ efflux pathway; however, recent studies of Na+ dynamics in MD cells (28) suggest that there is little Na+-K+-ATPase activity at the basolateral membrane. Thus, although we cannot exclude the effects of ANG II on other transporters and channels, the most straightforward explanation of our results is that [NaCl]L-dependent changes in [Na+]i reflect the activity of NKCC and that ANG II stimulates cotransport activity.

The relatively high resting MD [Na+]i and [NaCl]L-dependent changes in MD [Na+]i are consistent with recent data from our laboratory (28) that demonstrated a high rate of Na-2Cl-K cotransport activity in MD cells but less efficient Na+ efflux. Na+ reabsorption in MD occurs through the apical Na-2Cl-K cotransporter and NHE2 (4, 20). Under steady-state conditions where lumen and bath [NaCl] were constant, luminal ANG II produced a significant increase in MD resting [Na+]i. Similarly, luminal ANG II greatly stimulated the increase in MD [Na+]i in response to elevations in [NaCl]L. These effects were sensitive to luminal furosemide, clearly indicating stimulation of the MD Na-2Cl-K cotransport activity by ANG II. Also, luminal ANG II still tended to have a small effect on MD Na+ dynamics even in the presence of furosemide, which is consistent with the previously published stimulatory effect of ANG II on MD NHE2 activity (29). We estimate that ~80% of Na+ entry occurs via the cotransporter, whereas most of the remaining Na+ entry is through Na+/H+ exchange.

Because the stimulatory effect of ANG II on TGF responses was shown with systemic or peritubular administration of ANG II, we also investigated the effect of basolateral ANG II on apical Na-2Cl-K cotransport activity. We found no difference between apical and basolateral addition of ANG II and no additive effect when ANG II was simultaneously added to both sides of MD cells. These findings, along with previous results from our laboratory (29), suggest a common intracellular signaling pathway of ANG II for either apically or basolaterally located AT receptors.

We found that nanomolar-to-picomolar luminal ANG II stimulated MD NKCC activity, whereas micromolar ANG II failed to alter cotransport activity. This biphasic effect of ANG II appears to be a general phenomenon and has also been described for NKCC activity in
TAL (2) and several NHE activities along the nephron (10, 29, 32). Nearly all previous studies have focused on the effects of ANG II on the other main NKCC isoform (NKCC1), which is found in nonpolar cells and at the basolateral membrane in certain polarized epithelial cells. In mesangial cells (11), vascular smooth muscle cells (1, 25, 37), and endothelial cells (23), there is, generally, a dose- and time-dependent stimulation of NKCC1 by ANG II. We are aware of only one study (2) concerning the regulation of NKCC2 in renal tubular epithelial cells by ANG II. Amlal et al. demonstrated, using the NH4+ technique, that low-dose ANG II inhibited, whereas high-dose ANG II stimulated, the apical Na-2Cl-K cotransporter in medullary TAL via AT1 receptors. However, as indicated previously, the NH4+ technique is problematic in studies of this nature due to the effects of ANG II on Na+/H+ exchange. Thus our studies are consistent with those obtained for the NKCC1 isoform, where low-dose ANG II stimulates cotransport activity.

Co-administration of candesartan with ANG II clearly inhibited the stimulatory effects of 10−8 M luminal ANG II, suggesting an AT1 receptor-mediated response. Interestingly, co-administration of PD-123,319 with high-dose ANG II restored the stimulatory effects of ANG II to the level observed with 10−12 M luminal ANG II. This finding suggests that other, inhibitory ANG II receptor subtypes (AT2) are involved in the overall ANG II response in MD cells. In addition, it would appear that this inhibitory pathway is activated, or its activity is manifested, only at high concentrations of ANG II. At the present time, this is a little puzzling because, as reviewed by Ardaliou (3), both AT1 and AT2 receptors appear to have similar affinities for ANG II (IC50 ~1 nM). Also, we found that administration of an AT2-receptor agonist blocked the effects of 1 nM luminal ANG II on cotransport activity, further supporting an inhibitory role for AT2 receptors in MD cells. On the other hand, it is also possible that there are receptor-independent effects of ANG II on cell function, especially at high micromolar concentrations of this hormone.

The present paradigm has been that, as ANG II levels increase, there is an AT1 receptor-mediated, concentration-dependent activation of various cell-signaling pathways. Recent studies (10, 12, 23, 37) suggest that ANG II receptors can be coupled to a number of signal transduction pathways, including adenylyl cyclase, protein kinases A and C, phospholipase C and A2, cytosolic Ca2+ system, and P-450-arachidonic acid metabolites. This progressive recruitment of signal transduction pathways has been one means of explaining the biphasic effects of ANG II. We speculate that, in MD cells, there may also be a concentration-dependent activation of different AT receptors and that this may also contribute to the biphasic actions of ANG II.

The role of AT2 receptors in the adult has been difficult to ascertain, but it is generally reported (8) that activation of the AT2 receptor opposes the actions of the AT1 receptor. Our results may be another example whereby AT2 receptors function in a manner that is opposite to the actions of the AT1 receptor. Interestingly, Carey et al. (8) have reported that at least some of the effects of AT2 receptor activation may be mediated by the nitric oxide (NO) system. In this regard, MD cells have a high level of calcium dependent-neuronal NO synthase (nNOS) expression (38). Thus it is plausible that high concentrations of ANG II cause MD cell calcium to rise, thereby activating calcium sensitive-nNOS and the generation of NO, although this has not yet been tested experimentally. Recently, Plato et al. (31) reported that NO production inhibits cotransport activity in the TAL. However, extensive studies will be required to further characterize the signaling pathways mediating the effects of AT1 and AT2 receptor activation on MD NKCC activity.

In summary, both luminal and basolateral ANG II, in the nanomolar range, stimulated MD Na-2Cl-K cotransport. This effect of luminal ANG II on cotransport activity occurred via activation of AT1 receptors at the apical membrane. At higher concentrations (10−6 M), ANG II did not stimulate cotransport activity, most likely because of the opposing actions of apical AT2 receptors. Thus ANG II modulation of TGF responses at the MD may occur through AT receptor alterations in Na-2Cl-K cotransport.

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