Role of PKC and calcium in modulation of effects of angiotensin II on sodium transport in proximal tubule

Zhaopeng Du, William Ferguson, and Tong Wang

Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520-8026

Submitted 19 July 2002; accepted in final form 6 December 2002

Du, Zhaopeng, William Ferguson, and Tong Wang. Role of PKC and calcium in modulation of effects of angiotensin II on sodium transport in proximal tubule. Am J Physiol Renal Physiol 284: F688–F692, 2003. First published January 14, 2003; 10.1152/ajprenal.00261.2002.—It has been well documented that low concentrations of ANG II (10⁻¹¹ to 10⁻¹⁰ M) stimulate, whereas high concentrations of ANG II (10⁻⁸ to 10⁻⁵ M) inhibit Na⁺ transport in proximal tubules of rat and rabbit kidneys. Measured ANG II concentrations (10⁻⁶ to 10⁻⁵ M) stimulate, whereas high concentrations of ANG II (10⁻³ to 10⁻² M) inhibit cell Ca²⁺ and cellular Na⁺ absorption. When 10⁻⁹ M ANG II and 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester (TMB-8), a blocker of intracellular calcium mobilization, were added together. In contrast, ANG II significantly decreased Jv when PKC was inhibited. When 10⁻⁹ M ANG II was present together with 1-(5-isooquinolinesulfonyl)-2-methylpiperazine and TMB-8, no significant change of Jv occurred. Inhibition of endogenous cAMP activity (no increase in cell Ca²⁺) and that nanomolar concentrations of ANG II inhibit Na⁺ transport in proximal tubules of rabbit kidney in vitro. Our results show that Na⁺ transport is not inhibited by endogenous cAMP, whereas both PKC and cytosolic Ca²⁺ modulate the effects of the nanomolar concentration of ANG II. Dual effects of ANG II of either stimulation or inhibition of transport may be the result of different levels of Ca²⁺ and PKC activation in proximal tubule cells.

MATERIALS AND METHODS

Superficial proximal convoluted tubules (S₂ segments) were dissected and perfused in vitro by using conventional methods (9). Briefly, kidneys from adult female New Zealand white rabbits weighing 2–3 kg were removed and cut into coronal slices. Individual tubules were dissected in cooled (4°C) Hanks’ solution containing (in mM) 137 NaCl, 5 KCl, 0.8 MgSO₄, 0.33 Na₂HPO₄, 1 MgCl₂, 10 Tris-HCl, 0.25 CaCl₂, 2 glutamine, and 2 l- lactate acid. The solution was bubbled with 100% O₂ and had a pH of 7.4. S₂ segments of the proximal tubules were perfused with an ultrafiltrate-like solution containing (in mM) 125 NaCl, 22 NaHCO₃, 1 CaCl₂, 1.2 MgSO₄, 2 glutamine, 2 lactic acid, 10.5 glucose, 5 KCl, and 1.2 phosphoric acid. A similar solution containing (in mM) 101 NaCl, 22 NaHCO₃, 1 CaCl₂, 1.2 MgSO₄, 2 glutamic acid, 2 lactic acid, 10.5 glucose, 5 KCl, 1.2 phosphoric acid, and 32.5 HEPES was used as bath medium. Perfusate and bath solutions were bubbled with 95% O₂-5% CO₂.

Address for reprint requests and other correspondence: T. Wang, Dept. of Cellular and Molecular Physiology, Yale School of Medicine, 335 Cedar St., P.O. Box 208026, New Haven, CT 06520-8026 (E-mail: tong.wang@yale.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

F688 0363-6127/03 $5.00 Copyright © 2003 the American Physiological Society http://www.ajprenal.org
CO₂ and had a pH of 7.4. Osmolalities of the bath and perfusate were adjusted to 300 mosmol/kg H₂O by the addition of either H₂O or NaCl. The bath solution also contained 3 g/dl albumin. Bath fluid was continuously changed at a rate of at least 0.5 ml/min to maintain the constancy of pH and bath osmolality.

ANG II and several inhibitors were added to the luminal solutions. All tubules were perfused at a rate of 10–20 nl/min at 37–38°C in a 1.2-ml temperature-controlled bath. The first period of collection began after an equilibration time of 30–60 min. Net fluid volume absorption (Jᵥ) was measured with [methoxy-3H]-inulin. The extensively dialyzed (methoxy-3H)-inulin was added to the perfusate at a concentration of 30 μCi/ml as a volume marker. For each experimental period, three timed collections of tubular fluid were made, the volume of the perfusate and collected samples were measured in a constant-bore glass capillary, and [3H]inulin concentrations in those samples were determined in a liquid scintillation counter (model LS5801; Beckman).

The rate of net fluid reabsorption was calculated according to the following equation: Jᵥ = Vᵥ – Vᴸ, where Vᵥ is the measured rate of fluid collection and Vᴸ = Vᴸ (IN₀/INᵢ). IN₀/INᵢ is the ratio of radioactive inulin of collected and original perfusion fluid. The rates of fluid absorption were expressed per millimeter of the proximal tubule.

ANG II was purchased from Peninsula Laboratories (Belmont, CA), and [3H]inulin was purchased from DuPont-New England Nuclear (Boston, MA). KT-5720 was purchased from Calbiochem (La Jolla, CA) and 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester (TMB-8), 1-(5-isouquinolinesulfonyl)-2-methylpipеразине (H-7), and all other chemicals were obtained from Sigma (St. Louis, MO).

All data were presented as means ± SE. Student’s t-test was used to compare control and experimental groups. The ANOVA test was used for comparison of several experimental groups with a control group. The difference between the mean values of an experimental group and a control group was considered significant if P < 0.05.

RESULTS

Effect of luminal ANG II on fluid absorption. In the first series of experiments, we confirmed that a low concentration of ANG II stimulates fluid absorption in the S₂ segment of the proximal tubule in vitro, as previously observed in the rat in vivo (12, 21). Proximal tubules were microperfused with an ultrafiltrate-like solution containing 125 mM NaCl and 22 mM NaHCO₃. Under these conditions, volume reabsorption (Jᵥ) results predominantly from net Na⁺ absorption.

As shown in Table 1 and Fig. 1, the addition of 10⁻¹¹ M ANG II to the luminal perfusion solution resulted in significant stimulation of Jᵥ. Jᵥ increased by 127.8%, from 0.36 ± 0.02 to 0.82 ± 0.12 nl·min⁻¹·mm⁻¹, P < 0.001. In contrast, the addition of 10⁻⁹ M ANG II had no effect on fluid absorption. Jᵥ was 0.36 ± 0.02 nl·min⁻¹·mm⁻¹ in control and was 0.32 ± 0.03 nl·min⁻¹·mm⁻¹ in the presence of ANG II (10⁻⁹ M). These findings are in agreement with previous results that 10⁻¹¹ M ANG II stimulates Na⁺ absorption, whereas 10⁻⁹ M ANG II fails to affect transport of fluid (12, 21).

Table 1. Effects of ANG II on fluid absorption in proximal tubules

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Vᵥ, nl/min</th>
<th>Vᴸ, nl/min</th>
<th>L, mm</th>
<th>Jᵥ, nl-min⁻¹-mm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>13.12 ± 1.07</td>
<td>12.71 ± 1.05</td>
<td>1.19 ± 0.07</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td>ANG II (10⁻¹¹ M)</td>
<td>6</td>
<td>13.93 ± 0.58</td>
<td>13.06 ± 0.57</td>
<td>1.05 ± 0.03</td>
<td>0.82 ± 0.12*</td>
</tr>
<tr>
<td>ANG II (10⁻⁹ M)</td>
<td>8</td>
<td>13.43 ± 1.93</td>
<td>13.12 ± 1.93</td>
<td>1.24 ± 0.06</td>
<td>0.33 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE from each group; n, number of perfused tubules; Vᵥ, perfusion rates; Vᴸ, collection rates; L, tubular length; Jᵥ, fluid absorption rate. *Significant difference from control (P < 0.05).

Effect of PKA inhibitor and ANG II on proximal tubule fluid absorption. On the basis of the observation that ANG II decreases cAMP production and stimulates Na⁺/H⁺ exchange (NHE), it was reported that ANG II increases the Na⁺ and HCO₃⁻ reabsorption rate by inhibition of cAMP, which inhibits NHE (15). It was also demonstrated that ANG II stimulates proximal tubule transport by a cAMP-independent mechanism (4). Although a decrease in cAMP production would reduce its inhibitory effect on NHE, resulting in increased Na⁺ and HCO₃⁻ absorption, it is not known whether NHE activity is inhibited by endogenous cAMP under physiological conditions. Therefore, a membrane-permeant PKA specific inhibitor, KT-5720, was used to examine the endogenous cAMP activity in the absence and presence of ANG II (1). As shown in Fig. 2 and Table 2, the addition of the PKA inhibitor KT-5720 and ANG II on proximal tubule transport by a cAMP-independent mechanism (4). Although a decrease in cAMP production would reduce its inhibitory effect on NHE, resulting in increased Na⁺ and HCO₃⁻ absorption, it is not known whether NHE activity is inhibited by endogenous cAMP under physiological conditions. Therefore, a membrane-permeant PKA specific inhibitor, KT-5720, was used to examine the endogenous cAMP activity in the absence and presence of ANG II (1). As shown in Fig. 2 and Table 2, the addition of the PKA inhibitor KT-5720 at concentrations of 500 nM had no significant effect on Jᵥ compared with the control group (0.36 ± 0.05 vs. 0.38 ± 0.04 nl·min⁻¹·mm⁻¹). A similar concentration has been reported by other laboratories (8, 16) to inhibit cAMP in proximal tubule cells. This result indicates the net Na⁺ absorption is not inhibited by endogenous cAMP in the proximal tubule. Additional data shown in Fig. 2 and Table 2 are the effects of both KT-5720 and ANG II on fluid absorption. Two concentrations of ANG II 10⁻¹¹ M and 10⁻⁹ M
were examined. $J_v$ was significantly increased (0.69 ± 0.06 nl/min) when ANG II 10^{-11} M and KT-5720 were added together and was 0.32 ± 0.02 nl·min^{-1}·mm^{-1} when ANG II 10^{-9} M and KT-5720 were added together. There were no significant differences between ANG II alone and ANG II plus PKA inhibitor. This result indicates that ANG II stimulates Na^- absorption in the proximal tubule by cAMP-independent mechanisms.

**Effect of TMB-8 and H-7 on PCT fluid absorption.** Because stimulation of proximal NHE by PKC has been demonstrated (25), we investigated whether this kinase modulates the effects of ANG II at concentrations that do not alter fluid and Na/HCO_3 reabsorption. A similar concentration of the cell-permeable PKC inhibitor H-7 was used (22), and the results of these microperfusion experiments are summarized in Table 3. It should be noted that the addition of either ANG II (10^{-9}) or of H-7 alone did not change $J_v$. However, $J_v$ decreased significantly when both H-7 and ANG II (10^{-9}) were present in the perfusion solutions. These data suggest that ANG II stimulates $J_v$ through PKC activation.

We further investigated the effects of ANG II by exploring the possibility that it could inhibit $J_v$ by increasing intracellular Ca^{2+}. To address this possibility, we assessed the effects of TMB-8 (2 × 10^{-4} M), a blocker of intracellular calcium mobilization, on fluid absorption. This concentration was similar to that used in previous studies (23). As shown in Table 3, exposure of proximal tubules to TMB-8 alone had no effect on $J_v$, whereas perfusion of proximal tubules with ANG II (10^{-9} M) and TMB-8 (2 × 10^{-4} M) resulted in significant stimulation of $J_v$. These findings support the hypothesis that in addition to activation of PKC, ANG II also increases Ca^{2+} mobilization and produces an inhibition of proximal tubule transport. To further examine this hypothesis, we assessed the effects of ANG II on $J_v$ when both PKC and Ca^{2+} mobilization are blocked. As shown in Fig. 3 and Table 3, the addition of ANG II to the lumen in the presence of 2 × 10^{-4} M TMB-8 and 10^{-4} M H-7 did not change $J_v$ significantly compared with results obtained in control conditions or in the presence of ANG II (10^{-9} M) alone. These results confirmed that both PKC and Ca^{2+} modulate the effects of ANG II.

**DISCUSSION**

In this study, we first confirmed previous observations that ANG II stimulates Na^- absorption at a concentration of 10^{-11} M but not at 10^{-9} M in the proximal tubule (21). Second, we have shown that inhibition of endogenous cAMP activity by blocking of PKA did not increase Na^- absorption. Third, we have demonstrated that ANG II at a concentration of 10^{-9} M produces stimulation or inhibition of Na^- transport, depending on whether cytosolic Ca^{2+} mobilization or PKC activity is inhibited. Our data are consistent with the conclusion that both PKC and cytosolic Ca^{2+} modulate the effects of nanomolar concentration of ANG II. These results also suggested that the luminal effects of ANG II are modulated by second messengers, such as Ca^{2+} and PKC. Because the physiological concentration of ANG II in tubule fluid is within the nanomolar range, the modulation of the effects of ANG II may be important for regulating absorption of Na^- in proximal tubules. It should be noted that under our experimental condition, ANG II was only added to the luminal side. It is not clear whether the addition of different concentrations of ANG II in the bath will modulate luminal ANG II effects.

Previous studies (11, 19, 20) in rat and rabbit kidneys had shown that ANG II has a biphasic effect on Na^- and HCO_3 transport in proximal tubules. Several investigations used split-droplet micropuncture procedures and isolated perfused tubules involving application of ANG II to the capillary by intravenous infusion or added it to the basolateral side of the isolated tubules. In previous experiments, we used in vivo continuous microperfusion and applied ANG II directly to the...
lumen of the proximal tubule. We thus obtained a similar dose response of ANG II on volume absorption to that reported previously by Harris and Young (11). Our studies showed that low doses of ANG II (10^{-12} to 10^{-11} M) stimulate and high doses of ANG II (10^{-8} to 10^{-5} M) inhibit HCO_3 transport by modulation of NHE. Maximal stimulation occurs at 10^{-11} M, whereas maximal inhibition was observed at 10^{-6} M (21). Similar results were also reported by Liu and Cogan (14) by using microperfusion of early proximal tubule in vivo. With the use of in vitro microperfusion of rabbit proximal tubule, it was reported that the effects of ANG II differed, depending on the site of application; maximal stimulation resulted from application of 10^{-11} M in the lumen and 10^{-10} M induced maximal stimulation on the basolateral side (12). However, the effects of nanomolar concentrations of ANG II in modulating proximal tubule transport have not been explored.

It was demonstrated (5, 26) that cAMP inhibits NHE and decreases Na^+ and HCO_3 absorption in the proximal tubules. On the basis of observation that ANG II decreases cAMP production and stimulates NHE, it was reported (15) that ANG II stimulates Na^+ and HCO_3 transport by decreasing cAMP in the proximal tubule. A decrease in cAMP production would reduce its inhibitory effect on NHE, resulting in increased Na^+ and HCO_3 absorption. However, it is not certain whether NHE activity is inhibited by endogenous cAMP under the physiological conditions. If NHE were inhibited by endogenous cAMP under basal conditions, inhibition of PKA by KT-5720 would increase NHE activity and increase J_v. KT-5720 had no significant effect on J_v when it was added to the luminal perfusate, indicating NHE is not inhibited by cAMP under basal conditions. In addition, if the stimulatory effect of ANG II on Na^+ transport is due to the reduction of cAMP production, KT-5720 would have a similar incremental effect on J_v. However, as shown in Figs. 1 and 2, the addition of ANG II 10^{-11} M and KT-5720 significantly increased J_v compared with both control and KT-5720 groups. The addition of ANG II 10^{-9} M and KT-5720 did not change J_v, which was similar to ANG II 10^{-9} M alone. These results indicate that ANG II modulates proximal tubule transport independently in cAMP and the PKA signaling pathway.

Experimental data (6, 24, 25) indicate that both activation of PKC and intracellular Ca^{2+} modulate the proximal tubule transport of Na^+ and HCO_3 via regulation of the NHE mechanism. Relevant examples include the activation of PKC resulting in a dose- and time-dependent stimulation of Na^+ and HCO_3 absorption in rat proximal tubules (22). PKC also increased NHE activity in brush-border membrane of rabbit kidneys (25). Moreover, high concentrations (10^{-8} to 10^{-6} M) of ANG II increased cell Ca^{2+} and reduced Na^+ absorption in rabbit proximal tubule (6, 10). It has also been shown that ANG II activates the phospholipase C signaling pathway (27); thus increased release of both inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol elevates cytosolic Ca^{2+} and active PKC, respectively. Experimental data (10, 23) show that PKC inhibitors abolish the elevation of fluid and HCO_3 absorption and Na^+ uptake in microperfused proximal tubules and cultured proximal tubule cells, respectively. This indicates that stimulation of proximal tubule transport by ANG II is mediated by activation of PKC. It was suggested that ANG II acts to stimulate J_v by decreasing cAMP (15), but the decrease of cAMP is at nanomolar range (EC_{50} = 4.4 nM), which is much higher than the stimulation dose of ANG II (18). Therefore, it is uncertain whether the reduction of cAMP by ANG II is the major mechanism of tubule transport stimulation. Our data show ANG II has no effect on J_v when both PKC and Ca^{2+} mobilizations were inhibited (Table 3), suggesting that a decrease of cAMP by ANG II is not the main mechanism of increased proximal tubule transport. The present studies provide strong

| Table 3. Effects of ANG II, TMB-8, and H-7 on fluid absorption in proximal tubules |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| ANG II (10^{-9} M) | n | V_v, nl/min | V_L, nl/min | L_v, mm | J_v, nl/min \(1^{-1}\) mm^{-1} |
| 8 | 13.43 ± 1.93 | 13.12 ± 1.93 | 1.24 ± 0.06 | 0.33 ± 0.04 |
| ANG II (10^{-9} M + TMB-8) | 6 | 17.43 ± 0.59 | 16.38 ± 0.76 | 1.23 ± 0.06 | 0.81 ± 0.15* |
| ANG II (10^{-9} M + H-7) | 9 | 16.38 ± 0.86 | 16.26 ± 0.87 | 1.32 ± 0.06 | 0.08 ± 0.03* |
| ANG II + TMB-8 + H-7 | 6 | 12.08 ± 0.67 | 11.29 ± 0.70 | 1.23 ± 0.06 | 0.42 ± 0.04 |
| TMB-8 (2 × 10^{-4} M) | 7 | 15.71 ± 1.80 | 15.27 ± 1.76 | 1.25 ± 0.04 | 0.36 ± 0.05 |
| H-7 (10^{-4} M) | 12 | 19.98 ± 1.04 | 19.47 ± 1.07 | 1.21 ± 0.05 | 0.39 ± 0.06 |

Values are means ± SE from each group; n, number of perfused tubules; TMB-8, 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester.

*Significant difference from control (P < 0.05).
BIPHASIC EFFECTS OF ANG II ON PROXIMAL TUBULE TRANSPORT

Evidence supporting the view that stimulatory effects of ANG II are due to the activation of PKC, because ANG II significantly decreased the transport after inhibition of PKC. Thus nanomolars of ANG II stimulate PKC activity and upregulate transport. The fact that transport is not affected by 10⁻⁹ ANG II alone is consistent with the documented additional action of ANG II of increasing Ca²⁺ mobilization. The latter inhibits transport and thus opposes stimulation by PKC.

Nanomolar concentrations of ANG II increase Ins(1,4,5)IP₃ (EC₅₀ = 2.9 nM) and intracellular Ca (EC₅₀ = 5.5 nM) in the proximal tubule (18). This suggests that an increase in cell Ca²⁺ is the cellular mechanism of ANG II inhibition. Previous studies (10, 23) have also demonstrated that ANG II >10 nM increases cell Ca²⁺ and reduces Na⁺ and HCO₃⁻ absorption and that blocking Ca²⁺ release from the intracellular pool abolishes the inhibitory effects of ANG II on proximal tubule transport. Nevertheless, this evidence supports the view that ANG II has the dual effect of activating PKC and increasing intracellular Ca²⁺. Our data show that at a dose that does not alter transport, ANG II effects stimulation of tubule transport in the presence of TMB-8, indicating that ANG II (10⁻⁹ M) increases calcium mobilization and decreases proximal tubule transport. Our studies show that opposite effects, simultaneous stimulation and inhibition of tubule transport by activation of PKC and by increased Ca²⁺ mobilization, cancel each other, so that ANG II has no net effect on Jₑ. Our results support the view that ANG II activation of PKC and increased cell Ca²⁺ are major mechanisms mediating its biphasic effects on proximal tubule Na⁺ transport.

We thank Drs. Gerhard Giebisch, Steven Hebert, and Yuehan Zhou for providing assistance and constructive comments.
This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-17433.
Portions of this study were previously published in abstract form (7).

REFERENCES