Korean J Physiol Pharmacol
Vol 3: 83−91, February, 1999

The Roles of Arachidonic Acid and Calcium in the Angiotensin II-induced Inhibition of Na^+ Uptake in Renal Proximal Tubule Cells

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Angiotensin II (ANG II) has a biphasic effect on Na^+ transport in proximal tubule: low doses of ANG II increase the Na^+ transport, whereas high doses of ANG II inhibit it. However, the mechanisms of high dose ANG II-induced inhibition on Na^+ uptake are poorly understood. Thus the aim of the present study was to investigate signal transduction pathways involved in the ANG II-induced inhibition of Na^+ uptake in the primary cultured rabbit renal proximal tubule cells (PTCs) in hormonally defined serum-free medium. ANG II (10^{-9} M)-induced inhibition of Na^+ uptake was blocked by losartan (10^{-8} M, AT_1 antagonist), but not by PD123319 (10^{-8} M, AT_2 antagonist) ($P<0.05$). ANG II-induced inhibition of Na^+ uptake was also completely abolished by neomycin (10^{-4} M, PLC inhibitor), W-7 (10^{-4} M, calmodulin antagonist), and $AACOCF_3$ (10^{-6} M, PLA_2 inhibitor) ($P<0.05$). ANG II significantly increased [^{3}H]arachidonic acid (AA) release compared to control. The ANG II-induced [^{3}H]AA release was blocked by losartan, $AACOCF_3$, neomycin, and W-7, but not by PD123319. ANG II-induced [^{3}H]AA release in the presence of extracellular Ca^{2+} was greater than in Ca^{2+}-free medium, and it was partially blocked by TMB-8 (10^{-4} M, intracelluar Ca^{2+} mobilization blocker). However, in the absence of extracellular Ca^{2+}, it was completely blocked by TMB-8. In addition, econazole (10^{-6} M, cytochrome P-450 monooxygenase inhibitor) and indomethacin (10^{-6} M, cyclooxygenase inhibitor) blocked ANG II-induced inhibition of Na^+ uptake, but NGDA (10^{-6} M, lipoxygenase inhibitor) did not affect it. In conclusion, PLA_2-mediated AA release is involved in ANG II-induced inhibition of Na^+ uptake and is modulated by $[Ca^{2+}]_i$ in the PTCs.

Key Words: Angiotensin II, Arachidonic acid, Ca^{2+}, Kidney, Na^+ transport

INTRODUCTION

Angiotensin II (ANG II) exerts effects on renal hemodynamics and also acts directly on tubular epithelium to influence salt and water transport (Zupan et al, 1993; Becker & Harris, 1996). ANG II has been reported to have a biphasic effect on Na^+ transport: at low doses (10^{-12} M$\sim10^{-11}$ M), it stimulates the Na^+ transport, whereas at high doses (10^{-9} M$\sim10^{-6}$ M) it inhibits the transport (Li et al, 1994; Reilly et al, 1995). The signal transduction pathways induced by low doses of ANG II were well known to be involved in the cAMP and PKC pathway (Liu & Cogan, 1989; Houillier et al, 1996). In contrast, the signal transduction pathways involved in ANG II-mediated natriuretic effects are not fully elucidated.

The actions of ANG II are mediated by two pharmacologically and biochemically distinct classes of receptors: AT_1 which includes two subtypes (AT_{1A} and AT_{1B}) and AT_2 (Dulin et al, 1994). ANG II receptors are coupled to a variety of signal transduction pathways which include G protein, adenylate cyclase, PLC, PKC, and PLA_2 (Morduchowicz et al, 1991; Schelling et al, 1992; Karim et al, 1995). The cAMP-dependent protein kinase A is shown to inhibit brush border Na^+/H^+ exchange (Kahn et al, 1985) but it is not yet clear whether this pathway is in-

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volved in the ANG II-induced inhibition of Na^+ uptake. In many tissues, the actions of ANG II are mediated by receptor-coupled activation of PLC, and thereby production of IP_3 and mobilization of intracellular Ca^{2+} from intracellular stores (Marrero et al, 1996; Schnackenberg & Granger, 1997). However, it is uncertain whether ANG II has an ability to generate PLC signals in proximal tubule cells. PKC also mediate the inhibition or stimulation of Na^+/H^+ antiport in the kidney (Helmle-Kolb et al, 1990).

In isolated perfused proximal tubule, luminal ANG II (10^{-8} M) also inhibits J_v via the activation of PLA_2 (Li et al, 1994). In the rabbit renal brush border membrane vesicles, ANG II affects Na^+ transport via pertussis toxin (PTX)-sensitive PLA_2 activation (Morduchowicz et al, 1991). However, in LLC-PK-AT_1R cells, ANG II is coupled to PTX-insensitive and Ca^{2+}-independent PLA_2 (Becker et al, 1995; 1997). PLA_2 catalyzes release of arachidonic acid (AA) esterified in the sn-2 position of the membrane phosholipids. AA can be metabolized by cyclooxygenase, lipoxygenase, or cytochrome P-450 monooxygenase into a wide range of biologically active compounds (Bonventre & Nemenoff, 1991). In rat isolated kidney, the product of the lipoxygenase pathway, arising within the kidney, contribute to the renal hemodynamic effects of ANG II (Bell-Quilley et al, 1993). However, ANG II results in the afferent arteriolar vasoconstriction via lipoxygenase and P-450 epoxygenase pathway but not via cyclooxygenase pathway (Imig & Deichmann, 1997).

Therefore, we examined signal pathways involved in the effect of high dose of ANG II on Na^+ uptake in primary cultured rabbit renal proximal tubular cells (PTCs). In this study, we found that PLA_2-mediated AA release is involved in the inhibitory effect of ANG II on Na^+ uptake. Furthermore, the AA release induced by high dose of ANG II is modulated by $[Ca^{2+}]_i$.

METHODS

Materials

Dulbecco's Modified Eagle's Medium : Nutrient Mixture F-12 (Ham) (DMEM/F-12, 1 : 1), Class IV collagenase and soybean trypsin inhibitor were purchased from Life Technologies (Grand Island, NY). Angiotensin II (ANG II), ethylene glycol-bis (β-amino ethyl ether)-N,N,N',N'-tetra acetic acid (EGTA), ethylenediaminetetraacetate (EDTA), 8-(N, N-diethylamino)-octyl-3,3,5-trimethoxy-benzoate (TMB-8), pertussis toxin (PTX), arachidonyl trifluromethyl ketone ($AACOCF_3$), neomycin, N-(6-Aminohexyl)-5-Chloro-1-Naphthalenesulfonamide (W-7), arachidonic acid (AA), indomethacin, econazole, nordihydroguaiaretic acid (NGDA), BSA fraction V, and ouabain were obtained from Sigma Chemical Company (St. Louis, MO). SQ 22536 was purchased from BIOMOL. PD123319 was purchased from Parke- Davis. Losartan (DuP 753), $^{22}Na^+$, [^{14}C]- α-methyl- D-glucopyranoside, ^{32}P, and [^{3}H]AA were purchased from Dupont/NEN. All other reagents were of the highest purity commercially available. Liquiscint was obtained from National Diagnostics (Parsippany, NY). Iron oxide was prepared by the method of Cook & Pickering (1958). Stock solutions of iron oxide in 0.9% NaCl were sterilized using an autoclave and diluted with phosphate buffered saline (PBS) prior to use.

Methods

Isolation of rabbit renal proximal tubules and culture conditions: Male New Zealand White rabbits (1.5~2.0 kg) were used for these experiments. PTCs were prepared by a modification of the method of Chung et al (1982). The PTCs were grown in D-MEM/F-12 with 15 mM N-[2-Hydroxyethyl]piperazine-N'-[2-ehanesulfonic acid] (HEPES) buffer (pH 7.4) and 20 mM sodium bicarbonate. Immediately before the use of the medium, three growth supplements (5 μg/ml insulin, 5 μg/ml transferrin, and 5×10^{-8} M hydrocortisone) were added. Kidneys were perfused via the renal artery, first with PBS, and subsequently with D-MEM/F-12 containing 0.5% iron oxide (wt/vol) until the kidney turned grey-black in color. Renal cortical slices were prepared by cutting the renal cortex and then homogenized with 4 strokes of a sterile glass homogenizer. The homogenate was poured first through a 253 μm and then a 83 μm mesh filter. Tubules and glomeruli on top of the 83 μm filter were transferred into sterile D-MEM/F-12 medium containing a magnetic stirring bar. Glomeruli (containing iron oxide) were removed with a magnetic stirring bar. The remaining proximal tubules were briefly incubated in D-MEM/F-12 containing 60 μg/ml collagenase (Class IV) and 0.025% soybean trypsin inhibitor. The dissociated tubules were then washed by centrifugation, resuspended in D-MEM/F-12 containing the three supplements, and transferred into tissue culture dishes. PTCs were maintained at 37°C, in a 5% CO_2-humidified environment in D-MEM/F-12 medium containing the three supplements. The medium was changed one day after

plating and every two days thereafter.

Na^+ uptake studies: The confluent monolayers were incubated with 10^{-9} M ANG II for 4 hrs before Na^+ uptake experiments. The uptake experiment was conducted as described by the method of Rindler et al (1979). For Na^+ uptake studies, the medium was removed by aspiration. Before the uptake period, the monolayers were washed twice with 100 mM Tris-HCl buffer, pH 7.4. Na^+ uptake was measured at 37°C for 30 mins in an uptake buffer (10 mM Tris/HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 140 mM choline chloride, pH 7.4) containing 0.25 μCi/ml $^{22}Na^+$ and 5×10^{-5} M ouabain. At the end of the incubation period, the monolayers were gently washed three times with ice cold 100 mM Tris-HCl buffer, and the cells were solubilized with 1 ml of 0.1% SDS. To determine the $^{22}Na^+$ incorporated intracellularly, 900 μl of each sample was taken and counted in a liquid scintillation counter (Beckmann Co.). The remainder of each sample was used for protein determination (Bradford, 1976). The radioactivity counts in each sample were then normalized with respect to protein and were corrected for zero-time uptake per mg protein. All uptake measurements were made in triplicate.

P_i uptake study: P_i uptake experiments were conducted as described by the method of Rabito (1983). After culture medium was removed by aspiration, the monolayers were gently washed twice with the uptake buffer (150 mM NaCl, 1.2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 10 mM 2-[N-Morpholino]ethansulfonic acid (MES)/Tris, pH 7.4). After the washing procedure, the monolayers were incubated at 37°C for 30 mins in an uptake buffer containing 1.5 μCi/ml ^{32}P and 1 mM unlabeled phosphate. At the end of the incubation period, the monolayers were again washed three times with ice-cold uptake buffer, and the cells were solubilized in 1 ml of 0.1% SDS. Next steps were conducted as described in Na^+ uptake.

α-MG uptake study: α-MG uptake experiments were conducted as described by the method of Sakhrani et al (1984). To study α-MG uptake, the culture medium was removed by aspiration, the monolayers were gently washed twice with an uptake buffer (136 mM NaCl, 5.4 KCl, 0.41 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.44 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5 mM HEPES, 2 mM glutamine, and BSA 0.5 μg/ml, pH 7.4). After the washing procedure, the monolayers were incubated at 37°C for 30 mins in the uptake buffer containing 0.5 mM α-MG and ^{14}C-α-MG (0.5 μCi/ml). At the end of the incubation period, the monolayers were again washed three times with the ice-cold uptake buffer, and the cells were solubilized in 1 ml of 0.1% SDS. Next steps were conducted as described in Na^+ uptake.

[^{3}H]AA release experiments: [^{3}H]AA release experiments were performed by the method of Slivka & Insel (1988). The cells were labeled with 0.5 μCi [^{3}H]AA/ml/dish (for 24 hrs in a 35 mm dish), and were washed three times with Na^+ uptake buffer (10 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 140 mM Choline chloride, pH 7.4). Treatment of cells with agents of interest was started by replacing the uptake buffer containing the specified agents at final concentration for indicated times. Stimulation of cells with agonists was stopped by aspirating the incubation medium and transferring it to ice-cold tubes containing 100 μl of 55 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA) and EDTA (final concentration, 5 mM each). The uptake buffer was then subjected to centrifugation ($\times$ 12,000 g) to eliminate the cell debris, and the radioactivity in the supernatant was determined by liquid scintillation counter. Cells left attached to the plate were scraped with 1 ml of 0.1% SDS. Then 900 μl was counted for radioactivity and the remainder for protein determination. In the experiments of Ca^{2+}-free condition, the PTCs were washed four times with Ca^{2+}-free Na^+ uptake buffer (10 mM Tris, 1 mM $MgCl_2$, 140 mM choline chloride, pH 7.4) and incubated with same buffer containing 1 mM EGTA for 30 mins prior to the treatment of agonists. The release of [^{3}H]AA was normalized as a mg protein and percentage of the total prestimulation-incorporated radioactivity (the total of released radioactivity plus the total of cell-associated radioactivity at the end of stimulation) for the different treatment conditions.

Statistical analysis: Results were expressed as means$\pm$standard errors (S.E.). The difference between two mean values was analyzed with ANOVA test. The difference was considered statistically significant when $P<0.05$.

RESULTS

Effects of ANG II on Na^+ uptake

To investigate the effect of ANG II (10^{-9} M) on brush border membrane transporter, Na^+, P_i, or α-MG uptake was measured in the PTCs. As shown in Table 1, ANG II (10^{-9} M) inhibited $^{22}Na^+$ uptake but did not affect [^{14}C]-α-MG and ^{32}Pi uptakes. To determine receptor subtype mediating the effect of ANG II on Na^+ uptake, we examined the effect of losartan (AT_1 specific antagonist), and PD123319

Table 1. Effects of angiotensin II on Na^+/H^+, Na^+/Glucose, and Na^+/P_i transporter activity

	$^{22}Na^+$ (n=12) (nmol/mg protein/30mins)	[^{14}C]-α-MG (n=12) (pmol/mg protein/min)	$^{32}P_i$ (n=9) (pmol/mg protein/min)
Control	432±24	592±17	241±8
Angiotensin II	381±21*	601±24	248±12

Monolayer cells were treated with angiotensin II (10^{-9} M) for 4 hrs and incubated with respective uptake buffer including $^{22}Na^+$ 0.25 μCi/ml, [^{14}C]-α-MG 0.5 μCi/ml, or $^{32}P_i$ 1.5 μCi/ml for 30 mins at 37°C. Then uptake experiment was conducted as described in the section of "methods". *P 0.05 vs. control.

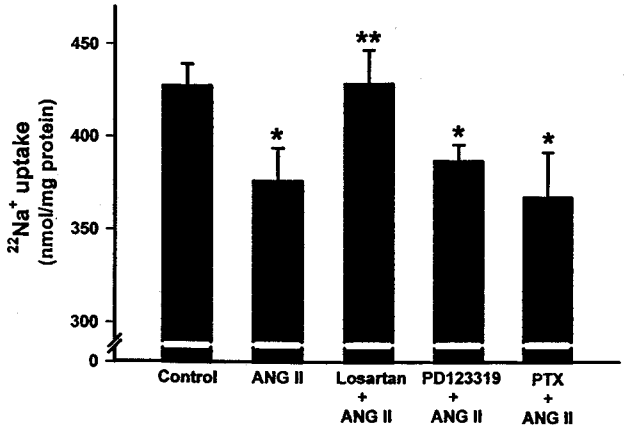

Fig. 1. Effect of losartan, PD123319 and pertussis toxin on angiotensin II-induced inhibition of Na^+ uptake. Losartan (10^{-8} M), PD123319 (10^{-8} M), or PTX (50 ng/ml) was treated to the primary cultured rabbit renal proximal tubule cells for 30 mins before the addition of angiotensin II. Then angiotensin II (10^{-9} M) was treated to the cells for 4 hrs. Na^+ uptake was measured at 37°C for 30 mins. Values are the means±S.E. of 9 experiments performed on 3 different cultures. *P<0.05 vs. control; **P<0.05 vs. angiotensin II alone.

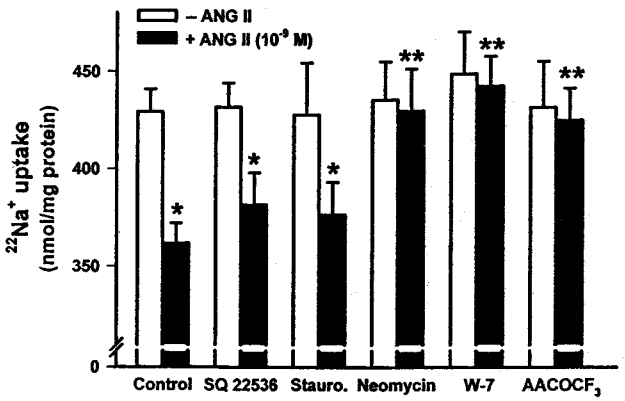

Fig. 2. Effect of SQ 22536, staurosporine, neomycin, W-7, and $AACOCF_3$ on angiotensin II-induced inhibition of Na^+ uptake. Primary cultured rabbit renal proximal tubule cells were treated with SQ 22536 (10^{-7} M), staurosporine (Stauro., 10^{-7} M), neomycin (10^{-4} M), W-7 (10^{-4} M), or $AACOCF_3$ (10^{-6} M) before the addition of angiotensin II (10^{-9} M). Na^+ uptake was measured at 37°C for 30 mins. Values are the means± S.E. of 9 experiments performed on 3 different cultures. *P<0.05 vs. control; **P<0.05 vs. angiotensin II alone.

(AT_2 specific antagonist) on Na^+ uptake. The ANG II-induced inhibition of Na^+ uptake was blocked by the losartan (10^{-6} M), and not by the PD123319 (10^{-6} M) (ANG II: 376±18 vs. losartan+ANG II: 429±19, PD123319+ANG II: 381±9 nmol/mg protein; P<0.05). In order to examine the involvement of G protein, PTX (50 ng/ml) was applied to the PTCs. PTX did not affect the ANG II-induced inhibition of Na^+ uptake (Fig. 1).

Identification of PLC and PLA_2 signal pathways on the ANG II-induced inhibition of Na^+ uptake

To examine the roles of cAMP, PKC, PLC, and PLA_2 signal pathway on ANG II-induced inhibition of Na^+ uptake, SQ 22536 (10^{-7} M, adenylyl cyclase inhibitor), staurosporine (10^{-7} M, protein kinase C inhibitor), neomycin (10^{-4} M, phospholipase C inhibitor), W-7 (10^{-4} M, calmodulin antagonist), or $AACOCF_3$ (10^{-6} M, phospholipase A_2 inhibitor) was applied to the PTCs. Fig. 2 showed that neomycin, W-7, and $AACOCF_3$ blocked the ANG II-induced inhibition of Na^+ uptake. However, SQ 22536 and staurosporine did not block the ANG II-induced inhibition of Na^+ uptake. These results indicate that both PLC (calmodulin) and PLA_2 pathways are involved in the ANG II-induced inhibition of Na^+ uptake.

Effect of ANG II on [^{3}H]AA release

To determine the effect of ANG II on the PLA_2 activity, we performed [^{3}H]AA release experiment. ANG II (10^{-9} M) increased [^{3}H]AA release (35±6%

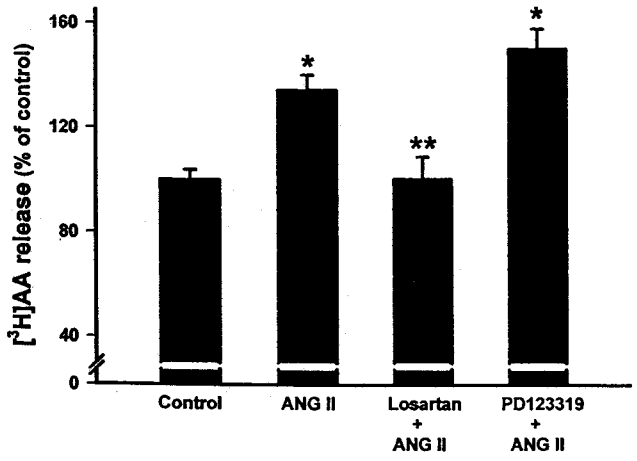

Fig. 3. Effect of angiotensin II on [^{3}H]arachidonic acid release and angiotensin II receptor blockers. After the incorporation of [^{3}H]arachidonic acid (0.5 μCi/ml) into the primary cultured rabbit renal proximal tubule cells for 24 hrs, angiotensin II (10^{-9} M) was treated to the cells for 1 hr. Losartan (10^{-8} M) or PD123319 (10^{-8} M) was treated to the cells for 30 mins prior to the treatment of angiotensin II (10^{-9} M). Values are the means$\pm$S.E. of 9 experiments performed on 3 different cultures. *P$<$0.05 vs. control; **P$<$0.05 vs. angiotensin II alone.

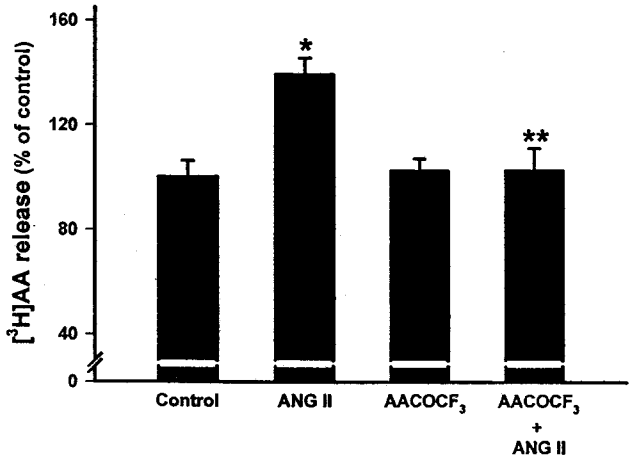

Fig. 4. Effect of phospholipase A_2 inhibitor on angiotensin II-induced [^{3}H]arachidonic acid release. After the incorporation of [^{3}H]arachidonic acid (0.5 μCi/ml) into the primary cultured rabbit renal proximal tubule cells for 24 hrs, angiotensin II (10^{-9} M) was treated to the cells for 1 hr. $AACOCF_3$ (10^{-6} M) was treated to the cells for 30 mins prior to the treatment of angiotensin II (10^{-9} M). Values are the means$\pm$S.E. of 9 experiments performed on 3 different cultures. *P$<$0.05 vs. control; **P$<$0.05 vs. angiotensin II alone.

increase vs. control; P$<$0.01). This increase in [^{3}H]AA release could be due to the direct activation of PLA_2 coupled to the ANG II receptor. The ANG II-induced PLA_2 stimulation is mediated by the AT_1 receptor in PTCs, since it was completely inhibited by losartan but not by PD123319 (Fig. 3) (ANG II: 135$\pm$6% vs. losartan+ANG II: 100$\pm$9%; PD123319 +ANG II: 150$\pm$8% of control; P$<$0.05). This ANG II-induced [^{3}H]AA release was also completely blocked by $AACOCF_3$ (Fig. 4) (ANG II: 139$\pm$6% vs. $AACOCF_3$+ANG II: 103$\pm$9% of control; P$<$0.05).

Role of Ca^{2+} on ANG II-induced [^{3}H]AA release

Since both PLA_2 and PLC pathway are involved in the effect of high dose of ANG II on Na^+ uptake, we examined whether ANG II-induced [^{3}H]AA release is related to the PLC/PKC or not. PTCs were pretreated with neomycin, W-7, or staurosporine prior to the application of ANG II. Fig. 5 shows neomycin and W-7 blocked the ANG II-induced [^{3}H]AA release. However, staurosporine did not block it. Furthermore, we also examined the role of extracellular Ca^{2+} and intracellular mobilized Ca^{2+} (Fig. 6). The results show that ANG II-induced [^{3}H]AA release in the presence of extracellular Ca^{2+} was greater than that in Ca^{2+}-free condition (Ca^{2+}

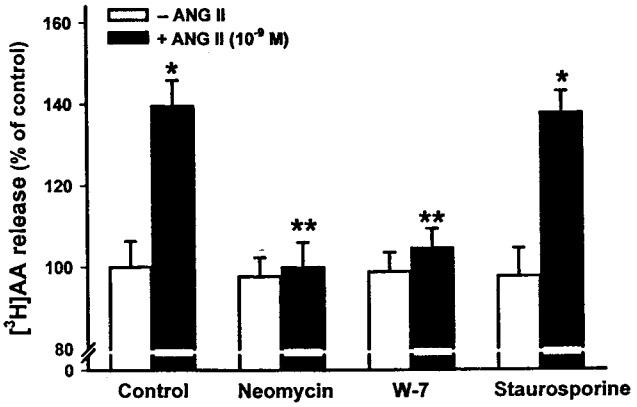

Fig. 5. Effect of phospholipase C inhibitor, calmodulin antagonist and protein kinase C inhibitor. After the incorporation of [^{3}H]arachidonic acid (0.5 μCi/ml) into the primary cultured rabbit renal proximal tubule cells for 24 hrs, angiotensin II (10^{-9} M) was treated to the cells for 1hr. Primary cultured rabbit renal proximal tubule cells were treated with neomycin (10^{-4} M), W-7 (10^{-4} M), or staurosporine (10^{-7} M) for 30 mins before the treatment of angiotensin II (10^{-9} M). Values are the means $\pm$S.E. of 9 experiments performed on 3 different cultures. *P$<$0.05 vs. control; **P$<$0.05 vs. angiotensin II alone.

free: 114$\pm$5% vs. 1 mM $CaCl_2$: 138$\pm$7% of control; P$<$0.05). The application of TMB-8 in the presence of extracellular Ca^{2+} (1 mM $CaCl_2$) partially blocked ANG II-induced [^{3}H]AA release, but the application

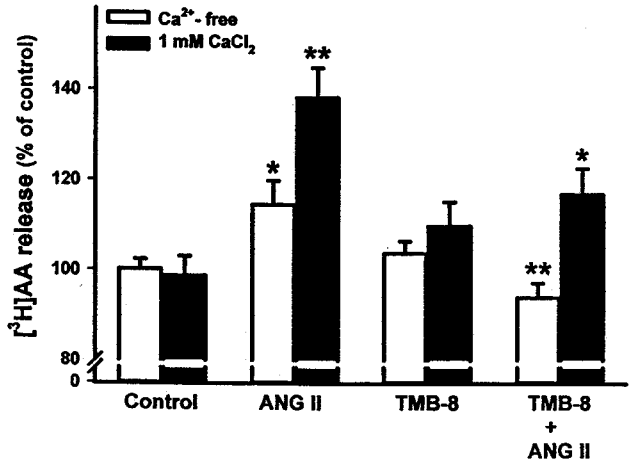

Fig. 6. Effect of Ca^{2+} on angiotensin II-induced [^{3}H] arachidonic acid release. Primary cultured rabbit renal proximal tubular cells were treated with TMB-8 (10^{-4} M) for 30 mins before the treatment of angiotein II (10^{-9} M) in a Ca^{2+}-free or 1 mM $CaCl_2$ buffer condition. Then angiotensin II (10^{-9} M) was treated to the cells for 1 hr. Values are the means±S.E. of 9 experiments performed on 3 different cultures. *$P<0.05$ vs. the control with Ca^{2+}-free condition; **$P<0.05$ vs. angiotensin II with Ca^{2+}-free condition.

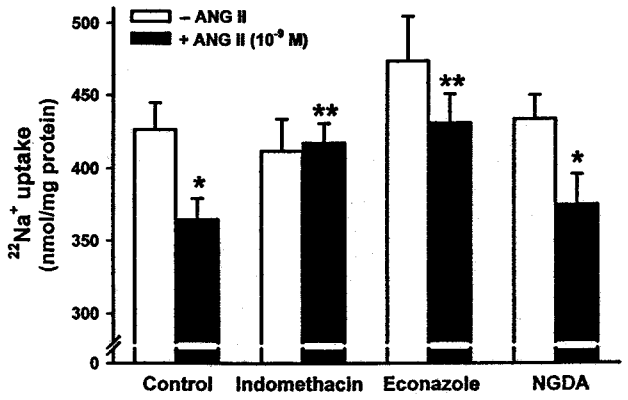

Fig. 7. Effect of arachidonic acid metabolite pathway inhibitors on the angiotensin II-induced inhibition of Na^+ uptake. Indomethacin (10^{-6} M), econazole (10^{-6} M) or NGDA (10^{-6} M) was treated to the primary cultured rabbit renal proximal tubular cells for 30 mins before the treatment of angiotensin II (10^{-9} M). Then angiotensin II (10^{-9} M) was treated to the cells for 4 hrs. Values are the means±S.E. of 12 experiments performed on 4 different cultures. *$P<0.05$ vs. control; **$P<0.05$ vs. angiotensin II alone.

of TMB-8 in the absence of extracellular Ca^{2+} completely blocked it. These results suggest that both extracellular and intracellular mobilized-Ca^{2+} are involved in ANG II-induced [^{3}H]AA release.

Identification of major metabolic pathways of AA on ANG II-induced inhibition of Na^+ uptake

To examine which AA metabolic pathway is related to the ANG II-induced inhibition of Na^+ uptake, PTCs were treated by indomethacin (10^{-6} M, cyclooxygenase inhibitor), NGDA (10^{-6} M, lipoxygenase inhibitor), and econazole (10^{-6} M, cytochrome P-450 epoxygenase inhibitor) for 30 mins prior to the addition of 10^{-9} M ANG II. The inhibitory effect of ANG II on Na^+ uptake was blocked by indomethacin and econazole, but not by NGDA ($P<0.05$) (Fig. 7). These results indicate that cyclooxygenase and cytochrome P-450 dependent epoxygenase metabolites are related to the effects of ANG II on Na^+ transport in the PTCs.

DISCUSSION

The intracellular mechanisms underlying the effect of ANG II on Na^+ transport have not been yet clearly identified. In this study we found that both the AA and Ca^{2+} were involved in ANG II-induced inhibition of Na^+ uptake. Furthermore, ANG II-induced AA release was modulated by $[Ca^{2+}]_i$.

In the present study, ANG II inhibited Na^+ uptake but did not affect α-MG and P_i uptakes. These results suggest that ANG II specifically regulate Na^+ transport. A dose-dependent biphasic effect of ANG II on Na^+ transport in PTCs was found in the previous study by Han et al (1997). Both stimulatory and inhibitory effects on Na^+ transport are mediated by ANG II receptor. In our previous study, ANG II-induced stimulation of Na^+ uptake in the PTCs was mediated by AT_1 (Han et al, 1997). However, it is obscure which receptor subtypes mediate the ANG II-induced inhibition of Na^+ uptake. The inhibitory effects of ANG II on Na^+ uptake in the PTCs were blocked by AT_1 antagonists. These results indicate that AT_1 receptors also mediate the inhibitory effect of Na^+ uptake in the PTCs as well as the stimulatory effect. These findings were supported by the report that approximately 95% of renal ANG II receptors are of the AT_1 subtype and that 5% are of AT_2 receptors (Harris et al, 1996). AT_1 receptors are characterized by losartan-inhibitable angiotensin II binding (Timmerman et al, 1991) and G-protein coupling (Sasaki et al, 1991). The ANG II-induced Na^+ transport across confluent monolayers of cultured rabbit proximal tubule cells was inhibited by PTX, indicating the involvement of a G_i regulatory protein coupling receptor and adenylyl cyclase activity (Han et al,

1997). However, in the present study, PTX and SQ 22536 did not affect the effect of high dose of ANG II. These results suggest that G_i protein and adenylyl cyclase are not involved in the effect of high dose of ANG II on Na^+ transport in PTCs.

Although there is no direct evidence of PLC signal pathway on ANG II-induced inhibition of Na^+ uptake, apical AT_1 ANG II receptor-mediated PLC induces cytoskeleton-dependent endocytosis in cultured rat proximal tubule cells (Schelling et al, 1992), and PLC is coupled to AT_1 ANG II receptors in LLC-$PK_{1/Cl4}$-AT_1R cells (Burns & Harris, 1995). In rabbit proximal tubule, KN-62 (calmodulin dependent protein kinase II) inhibited Na^+/H^+ antiport, but PMA stimulated Na^+/H^+ antiport (Yamada et al, 1996). In the present study, neomycin and W-7 blocked the ANG II-induced inhibition of Na^+ uptake. However, staurosporine did not block it. These results suggest that PLC and calmodulin are involved in the effect of ANG II.

Several lines of evidence suggest that ANG II-induced proximal tubular fluid transport and natriuresis are mediated by apical PLA_2 activity (Morduchowicz et al, 1991; Li et al, 1994). In the present study, ANG II-induced inhibition of Na^+ uptake was also blocked by $AACOCF_3$. These results suggest that the effect of ANG II is mediated by AA released by PLA_2. Indeed, ANG II stimulated the [^{3}H]AA release. This ANG II-induced AA release is linked to the AT_1R (Fig. 3), which is in keeping with those obtained by others using LLCPK_1-AT_1R cells (Becker et al, 1997) and rat aorta endothelial cells (Pueyo et al, 1996). However, there is a report that ANG II-mediated AA release is linked to AT_2 receptor in rabbit proximal tubule epithelial cells (Jacobs & Douglas, 1996). Although the explanation for this difference is not readily apparent, differences in culture condition and in the growth state of the cells may be one factor. Indeed, it has been shown that growth factors down-regulate the expression of AT_2 but not that of AT_1 (Timmermans et al, 1993). It remains to be determined whether renal ANG II receptors are linked to PLA_2 by a G protein. Morduchowicz et al (1991) reported that PLA_2-mediated AA release was PTX-sensitive in the rabbit proximal brush border vesicle. This result was not observed in the PTCs (data not shown). However, this result is in agreement with the report that PTX did not block the ANG II-mediated AA release in LLC-PK-AT_1 cells and in rabbit proximal tubular epithelial cells (Jacobs & Douglas, 1996; Becker et al, 1997). Therofore, although our data suggest that ANG II receptors are not linked to PLA_2 by a PTX-sensitive, coupling to a PTX-insensitive G protein is not precluded.

Mammalian cells contain structurally diverse forms of PLA_2 including Ca^{2+}-independent PLA_2 and Ca^{2+}-dependent 85-kDa cytosolic PLA_2 ($cPLA_2$) (Leslie, 1997). $cPLA_2$ plays an important role in mediating AA release. In rabbit kidney $cPLA_2$ has been reported to exist in the proximal tubule (Sheu et al, 1997). However to date there is no report about the involvement between ANG II and $cPLA_2$ in the proximal tubule. On the other hand, haloenol lactone suicide substrate (HELSS, Ca^{2+}-independent PLA_2 inhibitor) and palmytoyl trifluromethyl ketone (Ca^{2+}-independent PLA_2 inhibitor) inhibited apical ANG II-stimulated [^{3}H]AA release in LLCPK-AT_1R cells, suggesting the potential involvement of Ca^{2+}-independent PLA_2 activity (Becker et al, 1997). In the present study, ANG II-stimulated [^{3}H]AA release in the presence of extracellular Ca^{2+} was greater than in the absence of extracellular Ca^{2+}. TMB-8 in the absence of extracellular Ca^{2+} completely blocked the ANG II-induced [^{3}H]AA release in the PTCs. This result suggests that both intra- and extracellular Ca^{2+} are involved in the ANG II-induced [^{3}H]AA release. Free AA has also been shown to be involved in intracellular signalling, including PKC (Qui & Leslie, 1994), PLC (Takenawa, 1981) and Ca^{2+}-calmodulin dependent protein kinase (Piomelli et al, 1989). In the present experiment, we investigated the relationship between PLC and PLA_2 signal pathway. It is of note that ANG II-stimulated [^{3}H]AA release was blocked by PLC inhibitor and calmodulin antagonist, which is in agreement with the result of ANG II-induced inhibition of Na^+ uptake. These data suggest that ANG II-induced AA release is involved in the inhibition of Na^+ uptake and its effect is modulated by $[Ca^{2+}]_i$. Satoh et al (1993) also proposed that PLC/PKC activated the PLA_2 and thus inhibited Na^+ transport in rat proximal tubule. However, PKC-mediated inhibition of Na^+ uptake and release of [^{3}H]AA in the ANG II-induced signaling pathways was not observed. Wang & Chan's report (1991) also support our results in the report that intracellular-mobilized Ca^{2+} but not PKC mediates the ANG II-induced inhibitory effect of Na^+ transport in the proximal tubule. It seems that $cPLA_2$ may be involved in the [^{3}H]AA release and mediate the inhibition of Na^+ uptake in the ANG II signal pathway. In contrast, Jacobs & Douglas (1996) reported that Ca^{2+}-independent PLA_2 might be involved in the ANG II-stimulated AA release in rabbit renal proximal epithelial cell. Thus, we could not rule out the possibility that Ca^{2+}-independent PLA_2 might be

involved in the effect of ANG II.

AA can be metabolized by cyclooxygenase, lipoxygenase, or cytochrome P-450 monooxygenase into a wide range of biologically active compounds. In the present study, indomethacin and econazole prevented the ANG II-induced inhibition of Na^+ uptake but NGDA did not. These results suggest that ANG II-induced inhibition of Na^+ uptake was mediated by cyclooxygenase and cytochrome P-450 epoxygenase pathway. The mechanism underlying these results has been explained by the several reports (Romero et al, 1991; Carroll et al, 1993) that ANG II stimulates cytochrome P450 AA epoxygenase-dependent formation of epoxy-eicosatrienoic acid (EET) in proximal tubules and that 5,6-EET is the most potent vasodilator and is dependent on transformation by endothelial cyclooxygenase for expression of its vascular activity. A similar result was observed in rat cortical collecting duct, where cytochrome P-450 monooxygenase and cyclooxygenase but not lipoxygenase pathway mediate the inhibition of Na^+ transport (Satoh et al, 1993). Further studies are required to examine which metabolite products play a central role in ANG II-induced inhibition of Na^+ transport in PTCs. It is assumed that the binding of ANG II to AT_1 receptor activates a G protein-regulated PLC which induces the hydrolysis of phosphatidyl inositol. $[Ca^{2+}]_i$ may activate $cPLA_2$, which releases AA. AA may be converted to a specific form by cyclooxygenase and cytochrome P-450 monooxygenase, which mediate the inhibition of Na^+ uptake in PTCs.

In summary, ANG II-induced [^{3}H]AA release was involved in the inhibitory effects of ANG II (10^{-9} M) on Na^+ uptake and was modulated by $[Ca^{2+}]_i$.

ACKNOWLEDGEMENT

This study was supported by grants awarded to Dr. H. J. Han from Korea Science and Engineering Foundation (KOSEF 961-0606-057-1, HRC 96-0401-0401)

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