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Regulatory Mechanisms of Angiotensin II on the Na^+/H^+ Antiport System in Rabbit Renal Proximal Tubule Cells. II. Inhibitory Effects of ANG II on Na^+ Uptake

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Many reports represent that angiotensin II (ANG II) caused a dose dependent biphasic effects on fluid transport in the proximal tubule. However, respective roles of different signaling pathways in mediating these effects remain unsettled. The aim of the present study was to examine signaling pathways at high doses of ANG II on the Na^+ uptake of primary cultured rabbit renal proximal tubule cells(PTCs) in hormonally defined serum-free medium. High concentrations of ANG II ($>10^{-9}$ M) inhibited Na^+ uptake and increased $[Ca^{2+}]_i$ level in the PTCs. However, low concentrations of ($<10^{-11}$ ANG II) stimulated Na^+ uptake and did not affect $[Ca^{2+}]_i$ level. 8-(N, N-diethylamino)-octyl-3,3,5- trimethoxybenzoate (TMB-8), ethylene glycol-bis(β-amino ethyl ether)-N,N,N', N'-tetra acetic acid (EGTA), and nifedifine partially blocked the inhibitory effects of ANG II on Na^+ uptake. When ANG II and bradykinin (BK) were treated together, Na^+ uptake was further reduced (88.47±1.98% of that of ANG II, 81.85±1.84% of that of BK). In addition, W-7 and KN-62 blocked the ANG II-induced inhibition of Na^+ uptake. Arachidonic acid reduced Na^+ uptake in a dose-dependent manner. When ANG II and arachidonic acid were treated together, inhibitory effects on Na^+ uptake significantly exhibited greater reduction than that of each group, respectively. When PTCs were treated by mepacrine (10^{-6} M) and $AACOCF_3$ (10^{-5} M) for 1 hr before the addition of 10^{-9} M ANG II, the inhibitory effect of ANG II was reversed. In addition, econazole (10^{-6} M) blocked ANG II-induced inhibition of Na^+ uptake. In conclusion, the $[Ca^{2+}]_i$ (calcium-calmodulin-dependent kinase) and phospholipase A_2 (PLA_2) metabolites are involved in the inhibitory effects of ANG II on Na^+ uptake in the PTCs.

Key Words: Kidney, Angiotensin II, Na^+/H^+ antiporter, Ca^{2+}

INTRODUCTION

A dose-dependent biphasic effect of ANG II on NaCl, bicarbonate and water reabsorption in the proximal tubule has been repeatly reported (Cogan, 1990; Wang & Chan, 1990). Low concentrations of ANG II increase, whereas high concentrations inhibit the apical Na^+/H^+ antiporter activity in the proximal tubule, but the respective roles of the different signaling pathways in mediating these effects remain unsettled. ANG II has been suggested to activate phospholipase C (PLC), protein kinase C (PKC) and phospholipase A_2 (PLA_2), and release IP_3, and increase intracellular Ca^{2+} and 5,6-epoxyeicosatrienoic acid (EET) production in proximal tubule preparations of different species (Welsh et al, 1988; Liu & Cogan, 1990; Romero et al, 1990; Wang & Chan, 1990; Morduchowicz et al, 1991; Poggioli et al, 1992; Schelling et al, 1992; Karim et al, 1995). The inhibitory effect of high doses of ANG II on HCO_3^-

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reabsorption in the proximal tubule may be mimicked by the calcium ionophore and suppressed by blocker of intracellular Ca^{2+} mobilization (Chatsudthipong & Chan, 1991). In addition, high concentrations of ANG II increased cytosolic Ca^{2+} through opening of calcium channel (Lu et al, 1996; Schnackenberg & Granger, 1997). On the other hand, the inhibitory effect of high doses of ANG II on the apical Na^+/H^+ antiporter is suppressed by P-450 epoxygenase inhibitor in rabbit proximal tubules (Carroll et al, 1996). Other regulatory mechanisms including pH, nitric oxide and K conductance are implicated in regulating sodium reabsorption by the proximal tubules (Coppola & Froter, 1994; McLay et al, 1995; Houillier et al, 1996).

The PTCs have been observed to retain a number of differentiated typical functions of the renal proximal tubule, including Na^+-dependent α-methyl-D-glucoside uptake, Na^+-dependent phosphate uptake, and parathyroid hormone-sensitive cAMP production (Chung et al, 1982; Han et al, 1996). Therefore primary cultured rabbit kidney proximal tubule cells in hormonally defined, serum free culture conditions would be powerful tools for studying regulatory mechanisms of ANG II on the Na^+/H^+ antiport system. The aim of the present study was to examine signaling pathways of inhibitory effects of ANG II on Na^+ uptake in the PTCs.

METHODS

Materials

Male New Zealand white rabbits (1.5~2.0 kg) were used for these experiments. 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), A23187 (Ca^{2+} ionophore), ethylene glycol- bis(β-amino ethyl ether)-N,N,N', N'-tetra acetic acid (EGTA), 8-(N,N-diethylamino)-octyl-3,3,5-trimethoxybenzoate (TMB-8), nifedifine, bradykinin (BK), $AACOCF_3$, KN-62, W-7, arachidonic acid, eicosatetraynoic acid (ETYA), econazole, BSA fraction V, and ouabain were obtained from Sigma Chemical Company (St. Louis, MO). Fluo-3/AM was purchased from Molecular probes. $^{22}Na^+$ was 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 and 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: Primary rabbit renal proximal tubule cell cultures were prepared by a modification of the method of Chung et al (1982). The basal medium, D-MEM/F12, pH 7.4, was a 50 : 50 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12. The basal medium was further supplemented with 15 mM HEPES buffer (pH 7.4) and 20 mM sodium bicarbonate. Immediately prior to the use of the medium, three growth supplements (5 µg/ml insulin, 5 µg/ml transferrin, and 5×10^{-8} M hydrocortisone) were added. Water utilized in medium preparation was purified by means of a Milli Q deionization system. Kidneys of a male New Zealand white rabbit were perfused via the renal artery, first with PBS, and subsequently with D-MEM/F12 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/F12 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/F12 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/F12 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/F12 medium containing the three supplements. Medium was changed one day after plating and every three days thereafter.

Na^+ uptake: The confluent monolayers were incubated with 10^{-9} M ANG II for 4 hrs before Na^+ uptake experiment. Uptake experiment was conducted

as described by Sakhrani et al (1984). 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 uptake buffer (10 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 140 mM Choline chloride) containing 0.25 μCi/ml $^{22}Na^+$ and 5×10^{-5} M ouabain (pH 7.4). At the end of the incubation period, the monolayers were gently washed three times with ice cold 100 mM Tris-HCl buffer, pH 7.4, and the cells were solubilized with 1 ml of 0.1% SDS. To determine the $^{22}Na^+$ incorporated intracellulary, 900 μl of each sample was removed 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.

Measurement of $[Ca^{2+}]_i$ by confocal microscopy: Fluo-3/AM (excitation : 485 nm, emission : 503 nm) was initially dissolved in dimethyl sulfoxide and

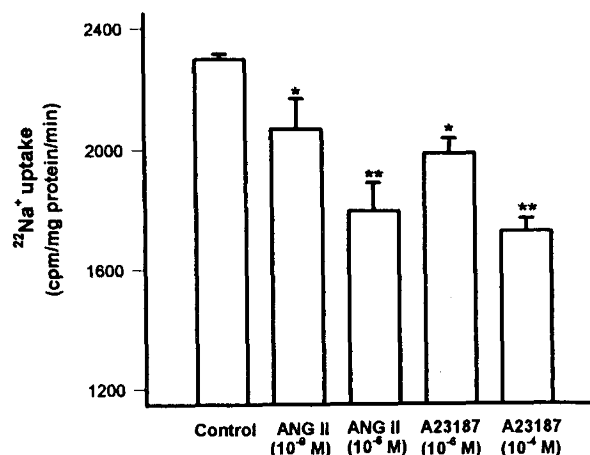


Fig. 1. Comparison of effects of ANG II and Ca^{2+} ionophore, A23187, on Na^+ uptake in primary cultured rabbit renal proximal tubule cells. The PTCs were treated with ANG II and A23187, respectively. Values are the means ±S.E. of 12 separate experiments performed on 4 different cultures. * $P<0.05$ vs. the control. ** $P<0.05$ vs. ANG II (10^{-9} M) or A23187 (10^{-6} M).

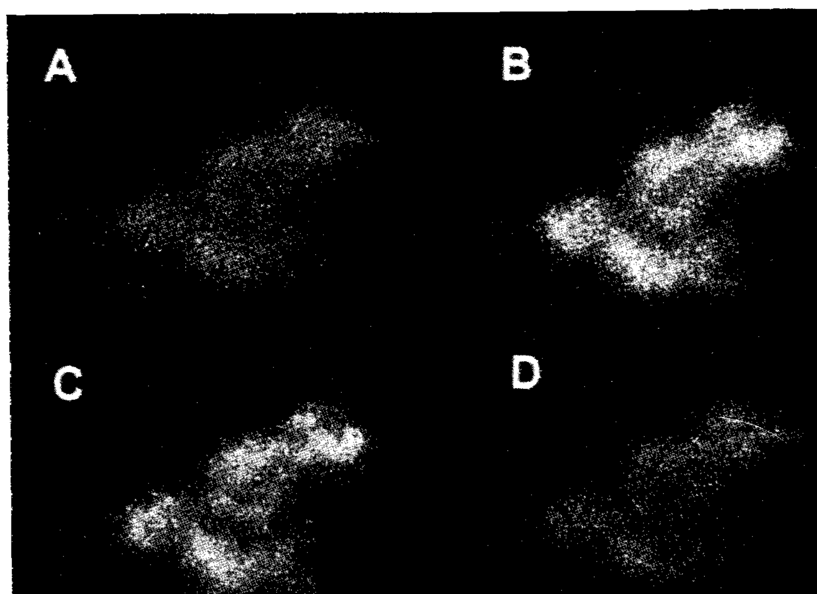


Fig. 2. Angiotensin II-induced variations in fluorescence of PTCs loaded with the Ca^{2+} indicator dye fluo-3/AM. Increased fluorescence intensity indicates increased $[Ca^{2+}]_i$. Images are demonstrated as the sequential process of $[Ca^{2+}]_i$ response induced by ANG II (10^{-9} M): (A) Fluorescence of PTCs before ANG II exposure, (B) $[Ca^{2+}]_i$ peak after the treatment, (C) sustained phase of $[Ca^{2+}]_i$ increment, (D) damped phase of $[Ca^{2+}]_i$ in PTCs, respectively.

stored at $-20^{\circ}C$. The confluent monolayers, grown on the 35 mm culture plate, were rinsed twice with bath solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM HEPES, pH 7.4), incubated in bath solution containing 3 μM fluo-3/AM with 5% CO_2-95% O_2 at $37^{\circ}C$ for 1 hr, rinsed twice again with bath solution, mounted on a perfusion chamber, and scanned by confocal microscopy ($\times 400$) (Leica TCS 4D). During the scanning, cells were treated with different doses of ANG II ($10^{-6} \sim 10^{-12}$ M). In the experiments using TMB-8 and EGTA, after the preincubation of TMB-8 (10^{-4} M) or EGTA (10 mM) for 3 hrs in DMEM/F12 medium, the monolayers were incubated with bath solution containing 3 μM fluo-3/AM with 5% CO_2-95% O_2 at $37^{\circ}C$ for 1 hr and then washed twice with bath solution before scanning. A23187 (2.5 μM) and dimethylsulfoxide (30 μM) were used as positive and negative control, respectively. Data were analyzed by Microsoft Excel.

Statistical analysis

Results were expressed as means ± standard errors (S.E.). The difference between two mean values was analyzed by Student's *t*-test. The difference was considered statistically significant when $P < 0.05$.

RESULTS

Effect of ANG II on cytosolic free calcium concentration

As shown in Fig. 1, calcium ionophore, A23187, on Na^+ uptake in the PTCs mimicked the ANG II-induced inhibitory effect on Na^+ uptake. To determine that any concentration of ANG II evokes elevation of cytosolic free calcium we measured intracellular Ca^{2+} level using confocal microscopy. During the scanning, cells were treated with different doses of ANG

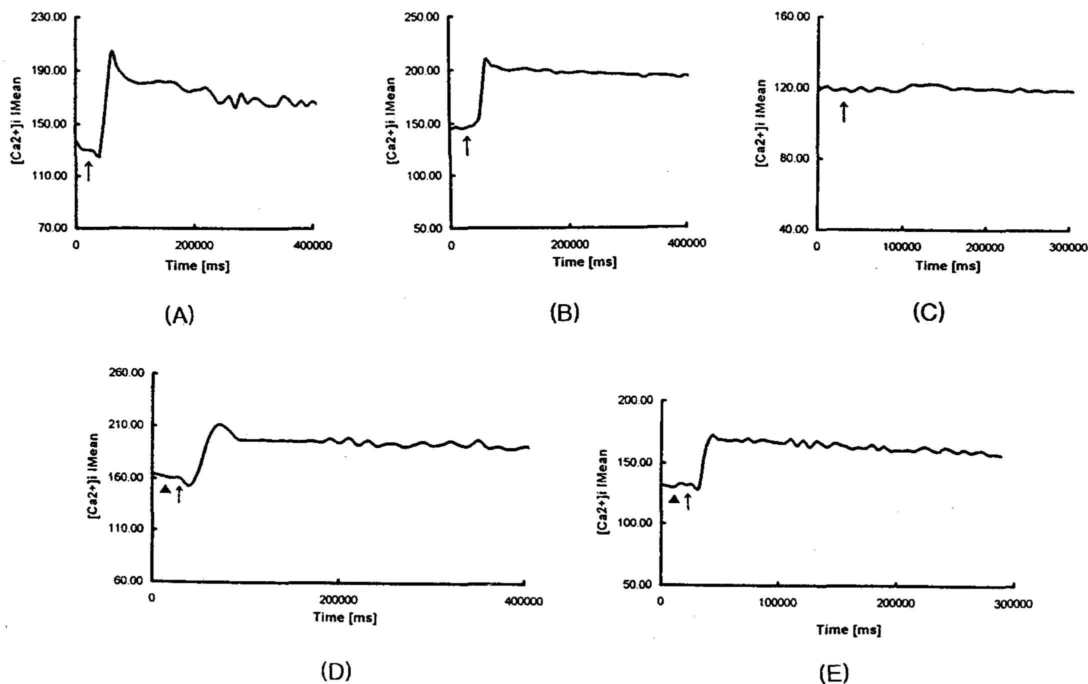


Fig. 3. Representative graphs evaluating effect of ANG II on $[Ca^{2+}]_i$ and the effect of TMB-8 and EGTA on ANG II-induced rise in $[Ca^{2+}]_i$. Cells were loaded with fluo-3/AM by incubation for 1hr at $37^{\circ}C$ in bath solution containing 3μm fluo-3/AM. After the incubation period, the primary cultured rabbit renal proximal tubule cells were scanned by confocal microscopy ($\times 400$). (A) 10^{-6} M ANG II (B) 10^{-9} M ANG II (C) 10^{-11} M ANG II (D) 10^{-9} M ANG II + TMB-8 (10^{-4} M) (E) 10^{-9} M ANG II + EGTA (10 mM). IMean ; absolute value of intensity mean.

Table 1. Calcium transients in response to ANG II and effect of EGTA and TMB-8 on ANG II-induced $[Ca^{2+}]_i$ transients

ANG II, M	$[Ca^{2+}]_i$, %
0	100
10^{-6}	155.78±9.28
10^{-7}	143.37±4.61
10^{-8}	137.14±8.63
10^{-9}	140.72±4.70
10^{-10}	122.28±5.98
10^{-11}	106.62±1.16
10^{-12}	101.99±1.17
10^{-9} + EGTA	124.41±4.41
10^{-9} + TMB-8	122.98±7.00

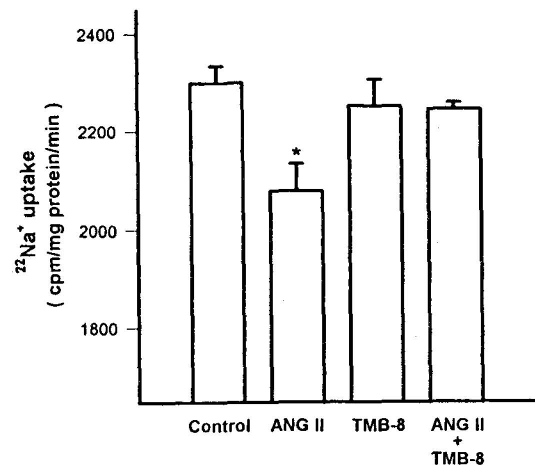
Values are means±S.E. of 30 separate experiments performed on 10 different cultures. Fluo-3/AM loaded proximal tubule cells incubated in bath solution containing 1 mM $CaCl_2$ were challenged with the indicated concentration of ANG II. In the experiments of EGTA (10 mM) and TMB-8 (10^{-4} M) pretreatment (see "Material and Method"), the fluo-3/AM loaded PTCs were scanned by confocal microscopy under the treatment of only 10^{-9} M ANG II. Data are analyzed by Microsoft Excel and expressed as % control of fluorescence base line.

II ($10^{-6} \sim 10^{-12}$ M). In Fig. 2, confocal microscopic images showed that ANG II(10^{-9} M) evoked $[Ca^{2+}]_i$ increment. The present results showed that high concentrations of ANG II ($>10^{-9}$ M) increased $[Ca^{2+}]_i$ level in PTCs (Fig. 3A, B; Table 1). However, low concentrations of ANG II ($<10^{-11}$ M) did not affect $[Ca^{2+}]_i$ level (Fig. 3C; Table 1). In the experiment of treatment of EGTA (extracellular Ca^{2+} chelating reagent, 10 mM) and TMB-8 (10^{-4} M) to know the source of cytosolic free calcium, we could see the increment of $[Ca^{2+}]_i$ level in both groups (Fig. 3D, E; Table 1).

Role of intracellular and extracellular Ca^{2+} in ANG II-induced inhibition of Na^+ uptake

To this purpose, TMB-8 (10^{-6} M) was used in this experiment to prevent intracellular calcium mobilization from the intracellular calcium storage pool. As shown in Fig. 4, ANG II (10^{-9} M) significantly inhibited Na^+ uptake by 92.42±2.42% of that of control.

(A)



(B)

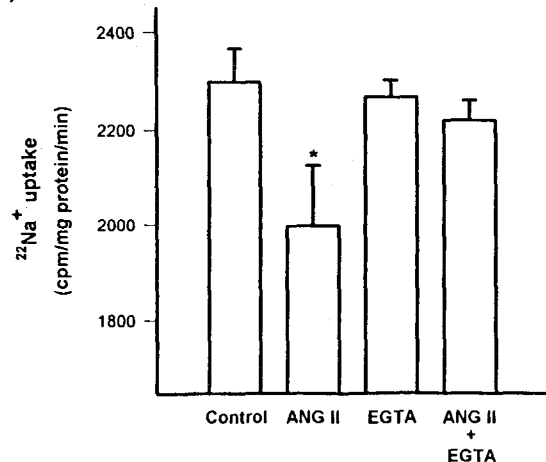


Fig. 4. Effect of 8-(N, N-diethylamino)-octyl-3,3,5-trimethoxybenzoate (TMB-8) (A) or ethylene glycol-bis (β-amino ethyl ether)-N,N,N',N'-tetra acetic acid (EGTA) (B) on ANG II-induced inhibition of Na uptake in the PTCs. TMB-8 (10^{-6} M) in a calcium free medium or EGTA (1 mM) in a medium containing 1mM $CaCl_2$ was added only to PTCs prior to treatment of ANG II (10^{-9} M). Values are the means±S.E. of 21 separate experiments performed on 7 different cultures. * $P<0.05$ vs. the control.

TMB-8 itself had no significant effect on Na^+ uptake; however, it partially prevented the inhibitory effect of ANG II on Na^+ uptake (Fig. 4A). Therefore as a next step in eliminating extracellular Ca^{2+}, EGTA (1 mM) was treated to the PTCs. It also partially blocked the effect of ANG II-induced inhibition of Na^+ uptake (Fig. 4B). We also examined the effect of high dose

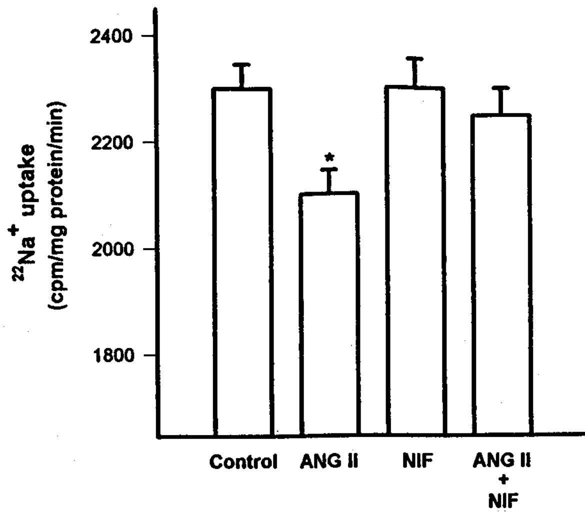


Fig. 5. Effect of nifedifine, L type Ca^{2+} channel blocker, on ANG II-induced inhibition of Na^+ uptake. After the preincubation of nifedifine (10^{-6} M) for 1hr, 10^{-9} M ANG II was added. Values are the means ± S.E. of 15 separate experiments performed on 5 different cultures. * $P<0.05$ vs. the control.

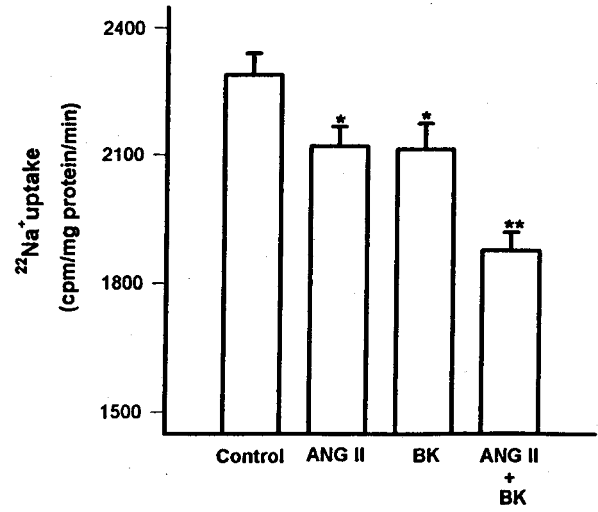


Fig. 6. Effect of bradykinin (BK) on ANG II-induced inhibition of Na^+ uptake in primary cultured proximal tubule cells. BK and ANG II were treated to the PTCs at the concentration of 10^{-7} and 10^{-9} M, respectively. Values are the means ± S.E. of 15 separate experiments performed on 5 different cultures. * $P<0.05$ vs. the control. ** $P<0.05$ vs. ANG II or BK alone.

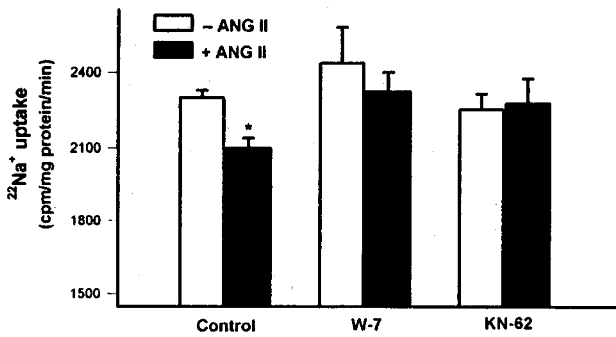


Fig. 7. Effect of W-7 and KN 62 on ANG II-induced inhibition of Na^+ uptake. After the pretreatment of W-7 (10^{-4} M) or KN-62 (10^{-7} M) for 1 hr, the monolayers were incubated with 10^{-9} M ANG II alone or vehicle. Values are the means ± S.E. of 12 separate experiments performed on 4 different cultures. * $P<0.05$ vs. the control.

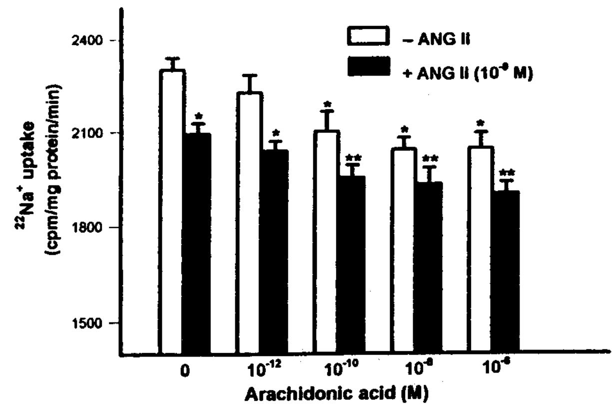


Fig. 8. Effect of arachidonic acid, intermediate metabolite of phospholipase A_2, on ANG II-induced inhibition of Na^+ uptake. Arachidonic acid ($10^{-6}\sim10^{-12}$ M) and ANG II (10^{-9} M) were challenged to the PTCs. Values are the means ± S.E. of 12 separate experiments performed on 4 different cultures. * $P<0.05$ vs. the control. ** $P<0.05$ vs. ANG II alone.

of ANG II (10^{-9} M) under the pretreatment of nifedifine, L type calcium channel blocker. Fig. 5 demonstrated the abolishment of ANG II-induced inhibition on Na^+ uptake. When ANG II and BK were added together, Na^+ uptake was further reduced (88.47 ± 1.98% of that of ANG II, 81.85 ± 1.84% of that of

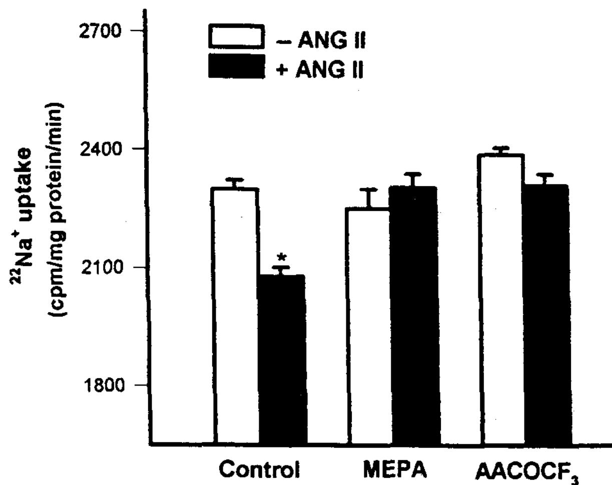


Fig. 9. Effects of mepacrine and $AACOCF_3$, PLA_2 inhibitors, on ANG II-induced inhibition of Na^+ uptake in PTCs. After the preincubation of mepacrine (10^{-6} M) or $AACOCF_3$ (10^{-6} M) for 1hr, the monolayers were treated with 10^{-9} M ANG II. Values are the means±S.E of 12 separate experiments performed on 4 different cultures. * $P<0.05$ vs the control.

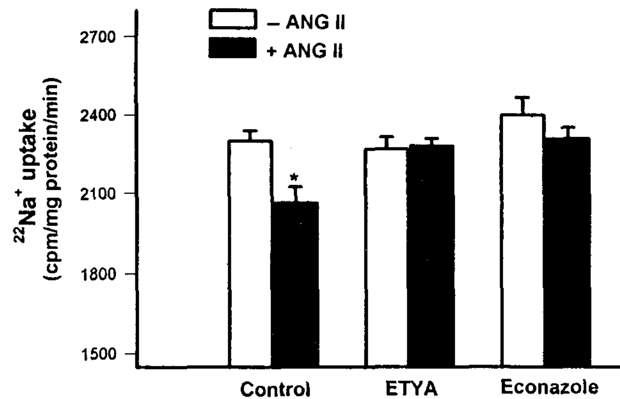


Fig. 10. Effects of ETYA, econazole on ANG II-induced inhibition of Na^+ uptake. After the preincubation of ETYA (10^{-6} M) or econazole (10^{-6} M) for 1 hr, the monolayers were incubated with 10^{-9} M ANG II alone or vehicle. Values are the means±S.E. of 12 separate experiments performed on 4 different cultures. * $P<0.05$ vs. the control.

BK) (Fig. 6).

Effect of calmodulin-dependent kinase inhibitors (W-7 and KN-62) on ANG II-induced inhibition of Na^+ uptake

To examine the effect on ANG II-induced Na^+ uptake of W-7 (10^{-4} M, calmodulin antagonist) and KN-62 (10^{-7} M, Ca^{2+}/calmodulin-dependent protein kinase II inhibitor), W-7 and KN-62 were treated to the PTCs. In this uptake experiment, W-7 and KN-62 blocked the ANG II-induced inhibition of Na^+ uptake (Fig. 7).

Effect of PLA_2 inhibitors (mepacrine and $AACOCF_3$) and arachidonic acid on ANG II-induced inhibition of Na^+ uptake

To test the effect of PLA_2 on ANG II-induced inhibition of Na^+ uptake, arachidonic acid ($10^{-6}\sim10^{-12}$ M) was used Indeed, arachidonic acid decreased Na^+ uptake in a dose dependent manner. But ETYA(nonmetabolizable arachidonic acid, 10^{-6} M) blocked ANG II-induced inhibition on Na^+ uptake (Fig 10). When ANG II and arachidonic acid were treated together, the cotreated group significantly exhibited more reduction than that of ANG II-induced inhibition on Na^+ uptake (Fig. 8). To clarify this result, we studied the effect of two PLA_2 inhibitors [mepacrine (10^{-6} M) and $AACOCF_3$ (10^{-5} M)]. When PTCs were pretreated with mepacrine (10^{-6} M) and $AACOCF_3$ (10^{-5} M) for 1 hr before the addition of 10^{-9} M ANG II, the inhibitory effect of ANG II was reversed (Fig. 9). In addition, when cells were pretreated for 1 hr with econazole (cytochrome P-450 epoxygenase inhibitor, 10^{-6} M), ANG II did not exhibit its inhibitory effect on the Na^+ uptake (Fig. 10).

DISCUSSION

In the present study, 10^{-9} M ANG II inhibited Na^+ uptake. As shown in our result, calcium ionophore, A23187, mimicked the ANG II-induced inhibition effect on Na^+ uptake. It has been proposed that cytosolic free calcium may be involved in inhibition of ANG II on Na^+ uptake. Therefore we measured $[Ca^{2+}]_i$ by concofcal micoroscopy in condition of different doses of ANG II. Our results showed that high doses of ANG II ($>10^{-9}$ M) increased in $[Ca^{2+}]_i$: however low doses of ANG II ($<10^{-11}$ M) did not affect the level of $[Ca^{2+}]_i$. These observations support hypothe-

sis that intracellular Ca^{2+} serves as a second messenger in the inhibitory effect of high concentrations of ANG II on Na^+ uptake in PTCs. Thus we investigated where are the sources of Ca^{2+} in high ANG II action and the role of intracellular or extracellular Ca^{2+} in ANG II-induced inhibition of Na^+ uptake. TMB-8 has been used to inhibit calcium mobilization in the proximal tubule cells (Aboolian et al, 1989). Most of these studies indicated that agonist-mediated calcium mobilization was blocked by TMB-8 in a calcium free medium indicating that TMB-8 specifically acted on intracellular calcium store. The data from our experiments showed that TMB-8 alone had no significant effect on Na^+ uptake. However, the effect of 10^{-9} M ANG II was partially blocked by TMB-8. In addition, since recent study reported the rabbit PTCs have a nifedifine-sensitive calcium channel (Zhang & Roger, 1996), nifedifine was treated to the PTCs. Nifedifine partially blocked the effect of ANG II-induced inhibition of Na^+ uptake. These results indicated that extracellular calcium plays an important role in the actions of a high dose ANG II. The ANG II-induced rise in $[Ca^{2+}]_i$ was also evaluated in the absence of extracellular calcium by chelation with EGTA prior to the treatment of the ANG II. Under this condition, the rise in $[Ca^{2+}]_i$ represents mobilization of calcium from intracellular stores. In the experiment of EGTA pretreatment to observe the effect of extracellular calcium influx, EGTA also partially blocked the decrease of ANG II on Na^+ uptake. To clarify intracellular calcium mobilization, the role of BK, a calcium mobilizing agonist in cells of renal proximal tubule origin, was also measured. Acute exposure of a primary culture of rabbit renal proximal tubule cells to BK resulted in a transient increment in $[Ca^{2+}]_i$ that was independent of extracellular Ca^{2+}. Furthermore, the heterologus desensitization between BK and ANG II, in terms of elevating $[Ca^{2+}]_i$, suggests that these two agonists release Ca^{2+} from a common intracellular store (Aboolian & Nord, 1988). However, in the present study, ANG II and BK have synergistic effect on inhibition of Na^+ uptake. Our data suggest that the rise in $[Ca^{2+}]_i$ which results from internal Ca^{2+} mobilization is not prerequisite for inhibition of the Na^+ uptake by ANG II. The results in this study show that ANG II leads to an increase in intracellular free calcium concentration by a dual mechanism involving both Ca^{2+} entry and Ca^{2+} release from intracellular stores. These results agree with a previous study in proximal tubule cells which also pointed to influx of extracellular Ca^{2+} caused by ANG II (Welsh et al, 1988). A similar mechanism appears to be operational in adrenal glomerulosa cells where Ca^{2+} channel agonists potentiate ANG II-induced Ca^{2+} influx during the more sustained phase of increase $[Ca^{2+}]_i$ (Latshw et al, 1996). Calcium calmodulin-dependent kinase has been shown to inhibit the Na^+/H^+ antiport activity in brush border membranes from proximal tubules and the apical Na^+/H^+ antiporter of the cell line LLC- PK_1 (Weinman et al, 1992). In this uptake experiment, W-7 and KN-62 blocked the ANG II-induced inhibition of Na^+ uptake. These results indicate that the calcium-calmodulin-dependent kinase is involved in the mediation of the 10^{-9} M ANG II. The molecular mechanisms whereby the Na^+ uptake is inhibited through Ca^{2+} dependent and independent pathway need to be characterized.

Arachidonic acid has been implicated in signaling actions in several cell types. The present result showed that mepacrine blocked the effect of ANG II-induced inhibition of Na^+ uptake. Similarly, mepacrine has been reported to abolish the effect of ANG II on receptor-mediated endocytosis and arachidonic acid release in LLC-PK_1 cells transfected with rabbit AT_1 receptor (Becker & Harris, 1996). Mepacrine is believed to interact with PLC as well as PLA_2 in cultured mesangial cell (Schlondorf et al, 1987). Therefore, we cannot attribute the inhibitory effects of mepacrine to PLC alone. Also the product of PLA_2 may be involved in ANG II action. PLA_2 activity is an important signal transduction pathway for proximal tubule AT_1 receptor mediating salt and water flux. Experimental data in proximal tubule (Li et al, 1994) and in perfused tubule preparations (Douglas et al, 1990) suggest that vectorial ion and water flux is a direct consequence of ANG II-mediated PLA_2 activity. The results of the present study with PTCs, strongly support that the inhibitory effect of high dose ANG II is mediated by an increased production of metabolites of arachidonic acid through stimulation of PLA_2. The addition of arachidonic acid inhibited the Na^+ uptake and inhibition of PLA_2 suppressed the inhibitory effect of ANG II on Na^+ uptake. Whether the stimulation of PLA_2 by ANG II is a direct effect of the hormone or an indirect consequence of the activation of the PLC-dependent cascade remains to be determined. Recent studies in cul-

tured rabbit proximal tubular cells show that ANG II at concentations $>10^{-9}$ M raises intracellular calcium through cytochrome P-450-dependent arachidonic acid metabolism (Madhun et al, 1991). Compared with lower concentrations, higher concentrations of luminal ANG II may stimulate cytochrome P-450-dependent arachidonic acid metabolism either secondary to the greater release of arachidonic acid or through other independent mechanisms. In present study, econazole blocked ANG II-induced inhibition of Na^+ uptake. This result could be suggested that arachidonic acid metabolites of cytochrome P-450 epoxygenase is involved in the inhibitory effect of ANG II. Further studies are needed to verify mechanisms that PLA_2 affects ANG II-induced Na^+ uptake. Additional signaling pathways also remain to be elucidated. In summary, the $[Ca^{2+}]_i$ (calcium-calmodulin-dependent kinase) and PLA_2- and cytochrome P-450-dependent metabolites of arachidonic acids are involved in the inhibitory effect of ANG II on Na^+ uptake in the PTCs.

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