Korean J Physiol Pharmacol
Vol 1: 413−423, August, 1997

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

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The importance of the kidney in the development of hypertension was first demonstrated by Goldblatt and his colleagues more than fifty years ago. Many hormones and other regulatory factors have been proposed to play a major role in the development of hypertension. Among these factors angiotensin II (ANG II) is closely involved in renal hypertension development since it directly regulates Na^+ reabsorption in the renal proximal tubule. Thus the aim of the present study was to examine signaling pathways of low dose of ANG II on the Na^+ uptake of primary cultured rabbit renal proximal tubule cells (PTCs) in hormonally defined serum-free medium. The results were as follows: 1) 10^{-11} M ANG II has a significant stimulatory effect on growth as compared with control. Alkaline phosphatase exhibited significantly increased activity. However, leucine aminopeptidase and γ-glutamyl transpeptidase activity were not significant as compared with control. In contrast to 10^{-11} M ANG II stimulated Na^+ uptake (108.03±2.16% of that of control), 10^{-9} M ANG II inhibited (92.42±2.23% of that of control). The stimulatory effect of ANG II on Na^+ uptake was amiloride-sensitive and inhibited by losartan (ANG II receptor subtype 1 antagonist) and not by PD123319 (ANG II receptor subtype 2 antagonist). 2) Pertussis toxin (PTX) alone inhibited Na^+ uptake by 85.52±3.52% of that of control. In addition, PTX pretreatment prevented the ANG II-induced stimulation of Na^+ uptake. 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), forskolin, and isobutylmethylxanthine (IBMX) alone inhibited Na^+ uptake by 88.79±2.56, 80.63±4.38, and 84.47±4.74% of that of control, respectively, and prevented the ANG II-induced stimulation of Na^+ uptake. However, 10^{-11} M ANG II did not stimulate cAMP production. 3) The addition of 12-O-tetradecanoylphorbol-13-acetate (TPA, 0.01 ng/ml) to the PTCs produced significant increase in Na^+ uptake (114.43±4.05% of that of control). When ANG II and TPA were added together to the PTCs, there was no additive effect on Na^+ uptake. Staurosporine alone had no effect on Na^+ uptake, but led to a complete inhibition of ANG II- or TPA-induced stimulation of Na^+ uptake. ANG II treatment resulted in a 111.83±4.51% increase in total protein kinase C (PKC) activity. In conclusion, the PTX-sensitive PKC pathway is the main signaling cascade involved in the stimulatory effects of ANG II on Na^+ uptake in the PTCs.

Key Words: Kidney, Angiotensin II, Na^+/H^+ antiporter, cAMP, PKC

INTRODUCTION

The critical importance of the kidney in the development of hypertension was first demonstrated by Goldblatt and his colleagues more than fifty years ago (Smith & Dunn, 1986). Although hormones and other regulatory factors have been proposed to play a major role in the development of hypertension, their effects on the kidney are poorly understood. The complexity of the hormonal milieu, and of the kidney

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in vivo have been major impediments to mechanistic studies. The renal proximal tubule has been proposed to be a likely site which is ultimately responsible for initiating the events which lead to salt-sensitive hypertensive states (Woolfson & Wardener, 1996). One possible explanation for such forms of hypertension is that they originate as a result of a restricted ability of the kidney to excrete sodium. The canine two-kidney, one-clip model of hypertension has been shown to result in an elevation in blood pressure through the actions of the renin-angiotensin system. Indeed the ANG II antagonist (Sar-Thr-angiotensin II) has been shown to reduce blood pressure in this model of hypertension (Cogan, 1990). The role of ANG II in the renal involvement for initiating or maintaining hypertension is certainly important. ANG II has been reported to stimulate the activity of the Na^+/H^+ antiport system in proximal tubule cells (Cano & Miller, 1994). The mechanisms involved in ANG II-mediated Na^+ flux of proximal tubule are still being defined (Ito et al, 1995). However the mechanism by which ANG II regulates the activity of the Na^+/H^+ antiport system is poorly understood.

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. Inhibition of adenylate cyclase by a G_i-coupled mechanism has been proposed to mediate ANG II-induced stimulation of the Na^+/H^+ antiporter (Liu & Cogan, 1989), but adenylate cyclase-independent mechanisms also have been proposed (Cano et al, 1994). In *in vivo* experiments in rat proximal tubule, the increment in solute absorption induced by low dose of ANG II is partly or completely inhibited by PKC inhibitors, which suggests that PKC may be involved in the effects of ANG II (Wang & Chan, 1991). Moreover in *in vivo* microperfusion experiments in rat proximal tubule, luminal perfusion with cAMP to clamp intracellular cAMP abolishes the effect of ANG II (Liu & Cogan, 1989). This result suggests that the effect observed may occur via activating protein kinase A (PKA). However, in opossum kidney (OKP) cell lines, the ANG II-induced increase in Na^+/H^+ antiport activity is cAMP independent (Cano et al, 1994). These conflicting observations may result from differences in animal species or experimental model examined.

A convenient means to evaluate the effects of hormones on renal tubule epithelial cells is to use *in vitro* cell culture systems. The use of established kidney cell lines for ion transport studies is well exemplified by previous studies conducted with the porcine kidney epithelial cell line $LLC\text{-}PK_1$. The $LLC\text{-}PK_1$ does possess a Na^+/glucose cotransport system typical of proximal tubule cells (Lever, 1986). However $LLC\text{-}PK_1$ possesses an arginine vasopressin-sensitive adenylate cyclase typical of distal tubule, rather than a parathyroid hormone-sensitive adenylate cyclase typical of the proximal tubule cells (Goldring et al, 1978). As fructose bisphosphatase activity is absent in $LLC\text{-}PK_1$, this cell line also lacks an intact gluconeogenic pathway (which is typical of the renal proximal tubule) (Gstraunthaler & Handler, 1987). Recently, our laboratory has established a primary rabbit kidney proximal tubule cell culture system, utilizing hormonally defined, serum-free medium (Han et al, 1996). The primary cells have been observed to retain a number of differentiated functions typical 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). The membrane transport studies conducted with such PTCs have the particular advantage that the results can be directly compared with the original renal tissue. Therefore PTCs 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 stimulatory effects of ANG II on Na^+ uptake of 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), insulin, hydrocortisone, transferrin, enzyme

assay substrates (*p*-nitrophenyl phosphate, L-leucine-*p*-nitroanilide, γ-glutamyl-*p*-nitroanilide), amiloride, 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), isobutylmethylxanthine (IBMX), forskolin, pertussis toxin (PTX), 12-O-tetradecanoylphorbol-13-acetate (TPA), staurosporine, BSA fraction V, and ouabain were obtained from Sigma Chemical Company (St. Louis, MO). PD123319 was purchased from Parke-Davis. $^{22}Na^+$ and losartan (DuP 753) were purchased from Dupont/NEN. Protein kinase C (PKC) enzyme assay system (code RPN 77) and Cyclic AMP [^{3}H] assay system (code TRK 432) were purchased from Amersham International plc.

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.

Cell growth study: To determine effects of ANG II, primary proximal tubule cells in culture were initiated in 35 mm dishes for cell growth studies. Briefly, tubules were inoculated at one-fourth the normal inoculum (the normal inoculum being 0.3 mg protein/dish measured by the method of Bradford, 1976; Chung et al, 1982). Cells were maintained in a 37°C, 95% air/5% CO_2 humidified environment in D-MEM/F12 medium supplemented with three supplements (control) or supplemented with ANG II (10^{-11} M) together. During this time, cell counts were determined on day 5, 9, and 13 from triplicate culture plate using a Coulter Model ZF particle counter. The cells were dislodged by incubation with PBS containing 0.05% trypsin and 0.5 mM EDTA. The proteolytic action was then inhibited by soybean trypsin inhibitors (0.05 mg/ml). The cell suspensions were diluted with PBS.

Marker enzymes assay: The confluent monolayers were incubated with 10^{-11} M ANG II for 4 hrs before marker enzymes assay. Alkaline phosphatase activity was assayed using *p*-nitrophenylphosphate as a substrate (Linhardt & Walter, 1963). The culture medium was removed and the cultures were washed three times with PBS (pH 8.0). The cultures were incubated with PBS containing 2 mg *p*-nitrophenyl phosphate/ml. Absorbance of *p*-nitrophenol released was assayed at 420 nm (E420 for *p*-nitrophenol = 18,300) after 15 mins of incubation. The samples were solubilized with 0.1 N NaOH. Leucine aminopeptidase activity was assayed using L-leucine-*p*-nitroanilide as a substrate. The culture medium was removed and the cultures were washed three times with PBS. The cultures were incubated with PBS containing 0.5 mg L-leucine-*p*-nitroanilide/2 ml for 15 mins at room temperature. Absorbance of *p*-nitroanilide released was assayed at 405 nm (E405 for *p*-nitroanilide = 9,600). For γ-glutamyl transpeptidase activity determined by the method using γ-glutamyl-*p*-nitroanilide as substrate and glycylglycine as the amino acid

acceptor (Tate & Meister, 1974). Reagents were added to a Eppendorf tube as follows: 0.2 mg of γ-glutamyl-p-nitroanilide (final concentration, 1 mM), 0.2 ml of glycylglycine (final concentration, 20 mM) and 0.6 ml of 0.1 M Tris-HCl buffer, pH 8.0. The solution was brought to 37°C in a water bath. Cells in culture were washed in ice cold PBS (pH 7.3) and then lysed by a 50 mM imidazole-HCl buffer (pH 7.2 at 25°C) containing 1%(V/V) Triton X-100. The reaction was initiated by adding a suitable amount of cell lysate and the rate of release of p-nitroanilide was measured at 410 nm (E410 for p-nitroanilide = 9,600) after 10 mins of incubation. Each determination was made using triplicate dishes and was standardized with respect to protein. Protein determination was performed by the methods of Bradford (1976) using bovine serum albumin as a standard.

Na^+ uptake: The confluent monolayers were incubated with 10^{-11} 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 an uptake buffer (10 mM Tris, 1mM $CaCl_2$, 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. 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.

PKC assay: For PKC assay, PTCs grown in 35 mm plates were scraped off and suspended into 2 ml of ice-cold buffer A [10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 0.2 mM $CaCl_2$, 1 mM phenylmethylsufonyl fluoride(PMSF), 10 μg/ml leupeptin and 10 mM benzamidine]. For the preparation of cytosolic and membrane fractions, cells suspended in 2 ml of buffer A were sonicated twice for 15 secs using an ultrasonic disintegrator. EDTA was added to 1 mM final concentration, and nuclei and unbroken cells were removed by centrifugation (1,000 $\times$ g for 10 mins). The supernatant was centrifuged at 100,000 $\times$ g for 60 mins to obtain a cytosolic fraction and a membrane pellet. The pellet was suspended by gentle pipetting in 1 ml of 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 1 mM PMSF, 1 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 1% (w/v) Triton X-100. After 15 mins on ice, insoluble material was removed by centrifugation (100,000 $\times$ g for 60 mins) to obtain a solubilized membrane fraction. Aliquots (5 µg of protein) of cytosolic and membrane fractions were assayed for PKC activity by using the PKC enzyme assay system (RPN 77), which is based on ^{32}P transfer to a peptide specific for PKC.

cAMP assay: The confluent monolayers were preincubated with 100 μM IBMX for 30 mins at 37°C to inhibit degradation of cAMP. The sample was extracted by homogenization in buffer containing 4 mM EDTA to prevent enzymatic degradation of cAMP, followed by heating for 5 mins in a boiling water bath to coagulate protein. After centrifugation at 3,000 rpm for 5 mins, the cAMP in the supernatants was transferred into the new tube and stored at 4°C. This samples were used for cAMP assay by using the cAMP [^{3}H] assay system (TRK 432).

Statistical analysis

Results were expressed as means $\pm$ 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

Growth and brush border membrane enzyme activity studies

ANG II has been implicated in modulating PTCs growth and function *in vivo*. In the first set of experiments, we examined the growth responsiveness of PTCs to 10^{-11} M ANG II. Fig. 1 depicts the time course for growth of renal proximal tubule cells in culture. PTCs were treated with ANG II (10^{-11} M) during medium exchange every 3 days. Cell counts were determined on day 5, 9 and 13 using a coulter Model ZF particle counter. Cell numbers increased

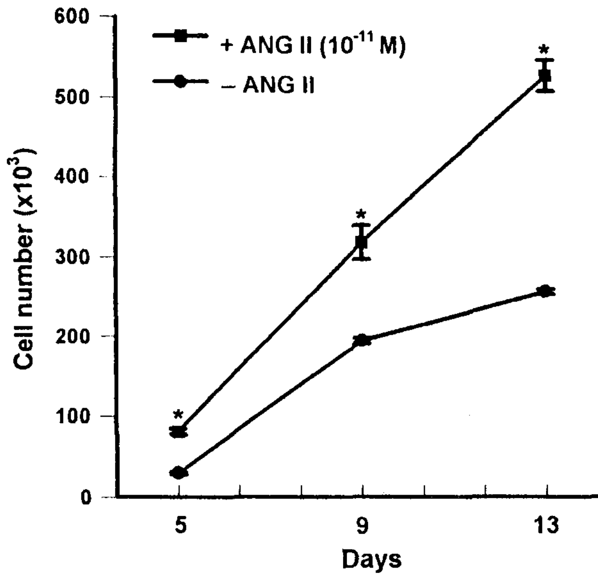

Fig. 1. Effect of ANG II on primary cultured renal proximal tubule cell growth. PTCs were grown in D-MEM/F-12 supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and hydrocortisone (5×10^{-8} M). PTCs were treated with ANG II (10^{-11} M) during medium exchange every 3 days. Cell counts were determined on day 5, 9, and 13 using a coulter Model ZF particle counter. Values are the means ± S.E. of 9 separate experiments performed on 3 different cultures. * $P<0.05$ vs. the control.

Table 1. Effects of ANG II on the brush border membrane enzyme activity of the primary cultured renal proximal tubule cells

Enzyme	Control	ANG II(10^{-11}M)
Alkaline phosphatase (nmoles *p*-nitrophenyl phosphate released/mg protein/min)	10.80±0.39	13.99±1.54*
Leucine aminopeptidase (nmoles *p*-nitrophenyl released/mg protein/min)	70.96±2.67	71.24±3.64
γ-Glutamyl transpeptidase (nmoles *p*-nitrophenyl released/mg protein/min)	11.27±0.19	10.98±0.36

logarithmically from day 5 to 13. On day 9, ANG II (163.69±10.82%) has a significant stimulatory effect on growth compared with control. Alkaline phosphatase exhibited significantly increased activity. However, leucine aminopeptidase and γ-glutamyl transpeptidase activity were not significant as compared with control (Table 1).

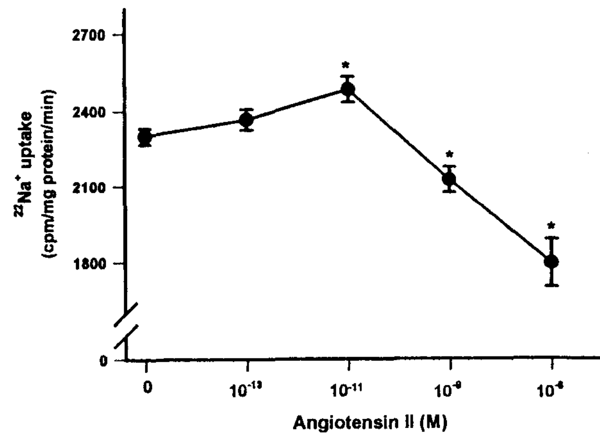

Fig. 2. Dose-dependent effect of ANG II on Na^+ uptake. ANG II was treated to PTCs at concentration of 10^{-13} to 10^{-6} M for 4 hrs. Values are the means ± S.E. of 24 separate experiments performed on 8 different cultures. * $P<0.05$ vs. the control.

Dose response effects of ANG II on Na^+ uptake

The effect of ANG II on Na^+ uptake was examined. ANG II at concentrations of $10^{-13} \sim 10^{-6}$ M or vehicle was applied to PTCs for 4 hrs. The dose-response curve is shown in Fig. 2. ANG II caused a dose dependent biphasic effect on Na^+ uptake. While 10^{-11} M ANG II stimulated Na^+ uptake (108.03 ±2.16% of that of control), 10^{-9} M ANG II inhibited (92.42±2.23% of that of control).

It is legitimate to ask whether Na^+ uptake is the target mechanism for ANG II action because ANG II stimulates or inhibits Na^+ uptake. Thus, we studied the effect of amiloride, an inhibitor of Na^+/H^+ antiporter. Data from this experiment is shown in Fig. 3. Amiloride (10^{-3} M) significantly decreased Na^+ uptake by 66.21±2.71% of that of control. When ANG II (10^{-11} M) and amiloride (10^{-3} M) were added together to the PTCs, Na^+ uptake was similar to that of 10^{-3} M amiloride alone.

Effect of ANG II receptor antagonist on Na^+ uptake

To determine the receptor subtype mediating the

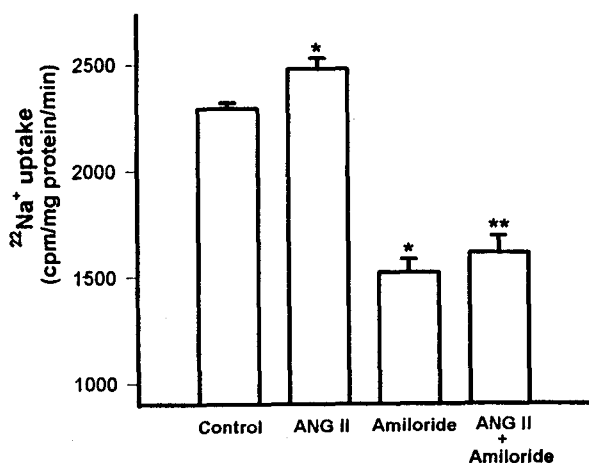

Fig. 3. Effect of amiloride on ANG II-induced stimulation of Na^+ uptake in the proximal tubule cells. ANG II and amiloride were added to PTCs at concentrations of 10^{-11} and 10^{-3} M, respectively. Amiloride was pretreated for 1 hr prior to incubation with ANG II. Values are the means±S.E. of 15 separate experiments performed on 5 different cultures. * $P<0.05$ vs. the control. ** $P<0.05$ vs. ANG II alone.

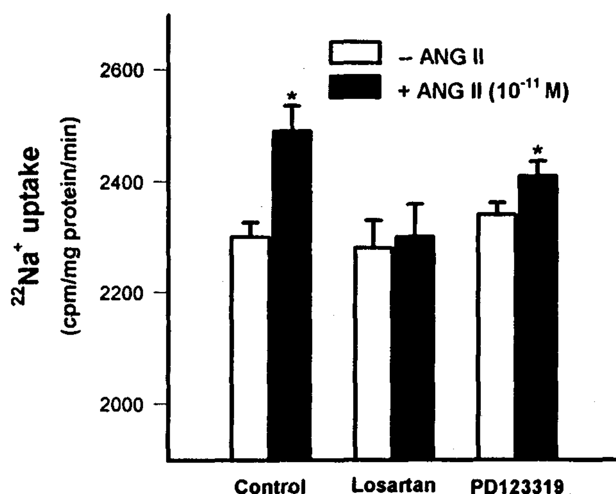

Fig. 4. Effect of losartan and PD123319 on ANG II-induced stimulation of Na^+ uptake. ANG II, losartan, and PD123319 were added to PTCs at concentration of 10^{-11} M, 10^{-8} M, and 10^{-8} M, respectively. Inhibitors were added for 1 hr before addition of ANG II. Values are the means±S.E. of 18 separate experiments performed on 6 different cultures for losartan and of 15 separate experiments performed on 5 different cultures for PD123319. * $P<0.05$ vs. the control.

ANG II-induced stimulation of Na^+ uptake, cells were exposed to the specific receptor antagonist losartan [ANG II receptor subtype 1 (AT_1) antagonist, 10^{-8} M] or PD123319 [ANG II receptor subtype 2 (AT_2) antagonist, 10^{-8} M] in the presence of and absence of 10^{-11} M ANG II. As shown in Fig. 4, ANG II-induced stimulation of Na^+ uptake was inhibited by the losartan and not by the PD123319. This dose of

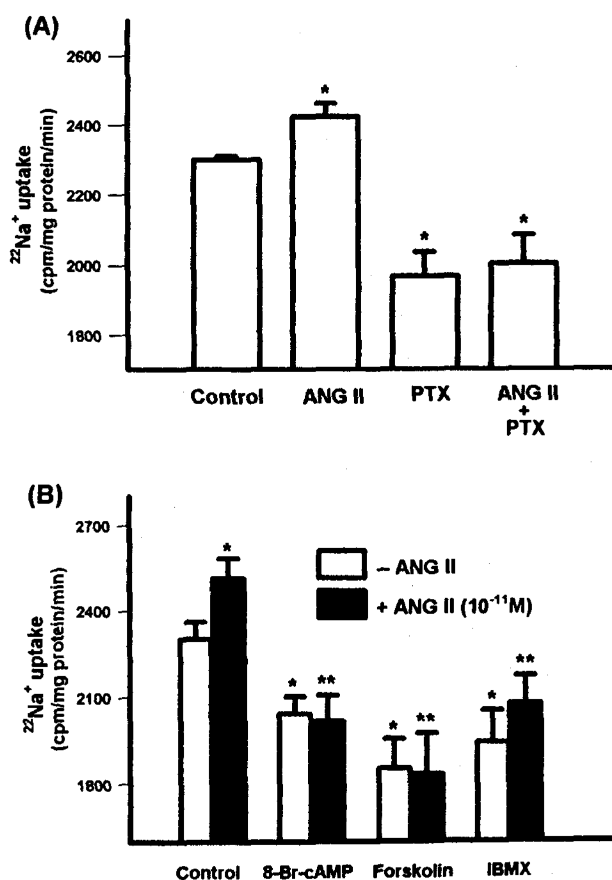

Fig. 5. (A) Effect of pertussis toxin (PTX) on ANG II-induced stimulation of Na^+ uptake. PTCs were pretreated to PTX (50 ng/ml) for 3 hrs before exposure to ANG II (10^{-11} M). Values are the means±S.E. of 6 separate experiments performed on 2 different cultures. (B) Effect of 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), forskolin, and isobutylmethylxanthine (IBMX) on ANG II-induced stimulation of Na^+ uptake. PTCs were treated to 8-Br-cAMP (10^{-4} M), forskolin (10^{-5} M), and IBMX (5×10^{-5} M) alone or together with 10^{-11} M ANG II for 4 hrs. 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.

losartan and PD123319 alone had no effect on Na^+ uptake.

Effect of PTX on ANG II-induced stimulation of Na^+ uptake

To determine if ANG II-induced stimulation was mediated by a PTX-sensitive G protein, the effect of PTX was examined. For this study, PTCs were exposed to 50 ng/ml PTX for 3 hrs before exposure to ANG II (10^{-11} M). As shown in Fig. 5-A, PTX alone inhibited Na^+ uptake by 85.52±3.52% of that of control. In addition, PTX pretreatment prevented the ANG II-induced stimulation of Na^+ uptake.

Effect of cAMP on ANG II-induced stimulation of Na^+ uptake and effect of ANG II on intracellular cAMP level

We evaluated whether changes in cAMP content were involved in the effect of ANG II on Na^+ uptake. For these studies, cells were exposed to 8-Br-cAMP (10^{-4} M), forskolin (adenylate cyclase activator, 10^{-5} M), and IBMX (phosphodiesterase inhibitor, 5×10^{-5} M) alone or together with 10^{-11} M ANG II. As shown in Fig. 5-B, 8-Br-cAMP, forskolin, and IBMX alone inhibited Na^+ uptake by 88.79±2.56, 80.63±4.38, and 84.47±4.74% of that of control, respectively, and prevented the ANG II-induced stimulation of Na^+ uptake. Therefore intracellular cAMP content was measured under basal and ANG II-stimulated conditions. However, 10^{-11} M ANG II did not stimulate cAMP production (Fig. 6).

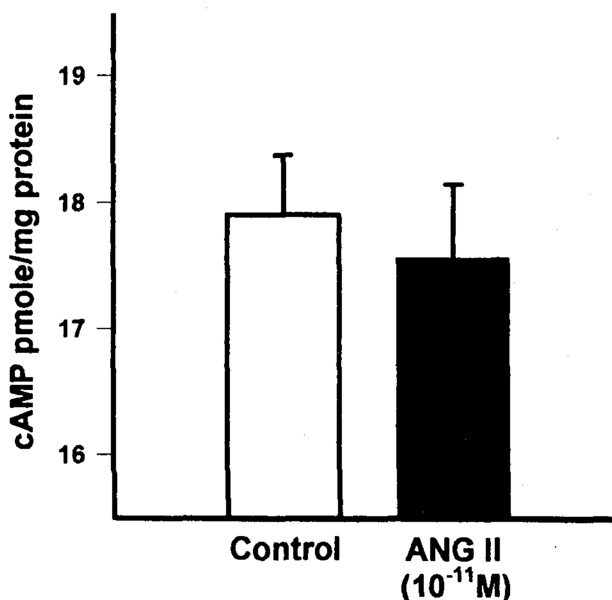

Fig. 6. Effect of 10^{-11} M ANG II on cAMP production. PTCs were preincubated to IBMX (100 μM) for 30 mins before exposure to ANG II (10^{-11} M). Values are the means±S.E. of 9 separate experiments performed on 3 different cultures.

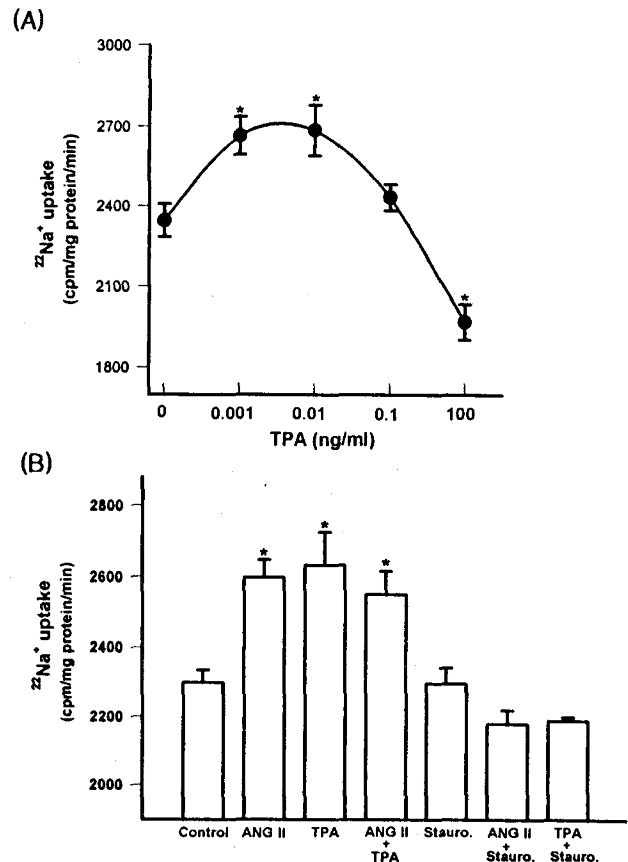

Fig. 7. (A) Dose-dependent effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on Na^+ uptake. PTCs were incubated with four different dose of TPA from 10^{-3} to 10^{2} ng/ml for 4 hrs prior to Na^+ uptake. Values are the means±S.E. of 12 separate experiments performed on 4 different cultures. (B) Effect of TPA and staurosporine on ANG II-induced stimulation of Na^+ uptake. PTCs were exposed to staurosporine (10^{-7} M) for 1 hr before exposure to ANG II (10^{-11} M). TPA (0.01 ng/ml) and ANG II were added together to PTCs for 4 hrs. Values are the means±S.E. of 15 separate experiments performed on 5 different cultures for TPA and of 9 separate experiments performed on 3 different cultures for staurosporine. * $P<0.05$ vs. the control.

Effect of TPA on ANG II-induced stimulation of Na^+ uptake and effect of ANG II on PKC activity

Because PKC has been shown to stimulate Na^+ uptake in the brush border membrane, we wanted to investigate the role of PKC in the ANG II action on the PTCs. First we looked at whether activator of PKC itself could cause stimulation of Na^+ uptake in the PTCs. TPA, a well-known activator of PKC, was used in this experiment. The addition of 0.01 ng/ml TPA to the PTCs produced significant increase in Na^+ uptake (114.43±4.05% of that of control) (Fig. 7-A). Next experiment was designed to investigate if ANG II and TPA could act by a similar mechanism. ANG II and TPA were added together to the cultured cells at concentrations of 10^{-11} M and 0.01 ng/ml, respectively, which are doses that cause maximal stimulation at Na^+ uptake. Since the data from this experiment indicated that there was no additive effect on Na^+ uptake, effect of PKC inhibitor staurosporine on ANG II-induced stimulation of Na^+ uptake was studied and shown in Fig. 7-B. Staurosporine alone had no effect on Na^+ uptake, but led to a complete inhibition of ANG II- or TPA-induced stimulation of Na^+ uptake. Therefore, PKC activity was measured in cytosolic and membrane fraction of control and ANG II-treated cells. As shown in Fig. 8, ANG II treatment resulted in a 111.83±4.51% increase in total PKC activity. Furthermore, this increase in PKC activity was accompanied by a modification of the subcellular distribution of PKC activity. The fraction of membrane-bound PKC was increased from 2.00±0.13 to 2.46±0.18 pmole/mg protein/min (123.00±9.00%) of total PKC activity, indicating PKC activation in ANG II-treated cells.

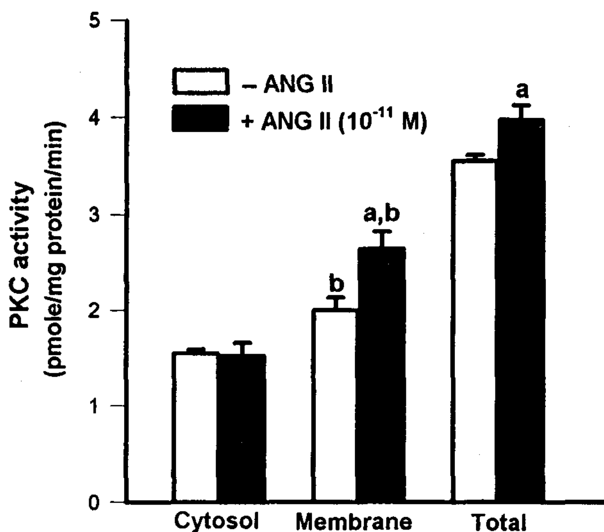

Fig. 8. Effect of ANG II on total and subcellular PKC activity. Cytosolic and membranous fractions from PTCs were prepared and assayed for PKC activity as described under "Materials and Methods". Values are the means± S.E. of 9 separate experiments performed on 3 different cultures. [a] $P<0.05$ vs. each control, [b] $P<0.05$ vs. cytosol.

DISCUSSION

ANG II is recognized as having growth promoting properties in a number of tissues including renal mesangial cells (Wolf & Neilson, 1993). Although several studies support a role for ANG II as a hypertrophic agent (Norman, 1991), some investigators have reported that ANG II causes hyperplasia in adult rat and fetal mesangial cells under certain culture conditions (Wolthuis et al, 1992). In the present study, 10^{-11} M ANG II was observed to stimulate growth of PTCs. Although the mechanism of the proliferative action of ANG II on proximal tubule cells remains to be elucidated, proliferation in other kidney cells is initiated by binding of ANG II to cell surface receptors that are linked to GTP binding proteins. Activation of this signaling pathway further initiates a common cascade of serine/threonine protein kinases that ultimately results in activation of mitogen-activated protein kinases (Pfeilschifter et al, 1995). The mitogen-activated protein kinases, in turn, are responsible for gene regulation and ultimately in regulation of cell mitogenesis. Other studies suggest that autocrine activation of growth factors are necessary for ANG II-induced mitogenesis (Weber et al, 1994). ANG II-stimulated protein synthesis is PTX-sensitive and partly depends on the activation of the Na^+/H^+ antiporter. ANG II increases the expression of several early growth-related genes, including c-*fos* and c-*jun* (Naftilan et al, 1990). In addition, there have been several reports that ANG II promotes extracellular matrix synthesis (Ray et al, 1994).

Recently, Na^+/H^+ exchanger (NHE) has been cloned (Sardet et al, 1989), and four isoforms have been identified (NHE-1~NHE-4). NHE-1 is amiloride-sensitive and seems to be expressed in all cell types with

basolateral localization in polarized cells (Rosskopf et al, 1993). NHE-2 has reduced susceptibility to inhibition by amiloride and seems to be expressed in polarized cells, in an apical localization. NHE-3 and NHE-4 are localized in intestine and stomach, respectively, and their functional properties are currently being investigated. Because amiloride has been found to inhibit Na^+/H^+ exchange in the brush border membrane of the proximal tubule (Kinsella & Aronson, 1981), we investigated the effect of amiloride on ANG II-induced stimulation of Na^+ uptake. Data from our study indicates that 10^{-3} M amiloride inhibits Na^+ uptake by 33.79%. This result is similar to the previous studies in which $J_{HCO_3^-}$ was inhibited 29% by 0.9 mM amiloride in the proximal tubule (Preisig et al, 1987). A similar result was also reported by an *in vitro* study, where ANG II was found to stimulate Na^+/H^+ exchange in renal proximal tubule cells and in renal brush border membranes (Saccomani et al, 1990). This result suggests that the transporter responsible for the Na^+ uptake under our experimental conditions is amiloride-sensitive Na^+/H^+ antiporter. The present study showed that 10^{-11} M ANG II increased Na^+ uptake that the Na^+ uptake transients were blocked by losartan, but not by PD123319. These results suggest that 10^{-11} M ANG II increases Na^+ uptake via AT_1 receptors in the PTCs. The previous results indicate that proximal tubule AT_1 receptor expression is regulated by ambient ANG II levels, and ANG II increases AT_1 receptor mRNA at least in part by decreasing proximal tubule cAMP generation through a PTX-sensitive mechanism. Upregulation of proximal tubule AT_1 receptor by ANG II may be important in mediating enhanced proximal tubule sodium reabsorption in states of elevated systemic or intrarenal ANG II (Cheng et al, 1995).

The present study confirms that 10^{-11} M ANG II stimulates, whereas 10^{-9} M ANG II inhibits Na^+ uptake in the PTCs. 10^{-11} M ANG II stimulated Na^+/H^+ antiporter activity in proximal tubule through activating PKA and PKC. The role of ANG II in regulating proximal tubule adenylate cyclase activity is incompletely understood. *In vivo* studies in rat revealed that infusion of ANG II decreased tubular fluid cAMP delivery and that this was reversed with PTX pretreatment (Liu & Cogan, 1989), suggesting that proximal tubule ANG II receptors are coupled to G protein. In PTCs grown on tissue culture dishes, low concentrations of ANG II (10^{-11} M) significantly reduced levels of cAMP, presumably by inhibition of adenylate cyclase (Douglas et al, 1990). However, studies in rat proximal tubule basolateral membranes and in isolated rabbit proximal tubules found no effect of ANG II on cAMP levels (Eitle et al, 1994). Of interest, a recent study revealed that ANG II had no effect on cAMP levels in the OKP cell, proximal tubule-like cell line, although effects of ANG II on Na^+/H^+ exchange were PTX-sensitive (Cano et al, 1994). In the present study, we found that the effect of ANG II was blocked by PTX pretreatment. This implies involvement of a G_i or G_o protein. Exogenous 8-Br-cAMP also prevented ANG II-induced stimulation of Na^+ uptake. This finding agrees with that of Liu and Cogan (1989) in the micorperfused proximal tubule. Therefore, we next measured the effect of ANG II on cAMP production. In this experiment, we found no effect of ANG II on cAMP production. Based on the above results, we conclude that 10^{-11} M ANG II stimulates Na^+ uptake in PTCs via a cAMP-independent mechanism mediated by an AT_1 and a PTX-sensitive G protein.

The second signaling pathway possibly involved in the low dose ANG II-induced stimulation of Na^+ uptake is the PLC (phospholipase C)-PKC cascade. It has been demonstrated that ANG II increases phosphoinositide turnover and phosphatidylinositol 4,5-bisphosphate hydrolysis in kidney proximal tubules (Wirthensohn & Guder, 1985) and many other cell types (Farese et al, 1984). The stimulation of phosphoinositide turnover is associated with an increase in the formation of two messengers: diacylglycerol (DAG) and inositoltriphosphate (IP_3). DAG activates PKC, and IP_3 acts as a second messenger for Ca^{2+} mobilization (Berridge, 1984). Therefore, it is interesting to investigate the physiological significance of these two second messengers involved in biphasic effect of ANG II. However, its functional role is controversial in the PTCs. Although phorbol ester has been found to stimulate the Na^+/H^+ antiporter in brush-border vesicles (Talor & Mejicano, 1988) and to alkalinize proximal tubule cells *in vitro* (Mellas and Hammerman, 1986), it also reduces Na^+,K^+-ATPase activity (Bertorello & Aperia, 1989). In the single transepithelial transport study available, phorbol ester administration diminished net bicarbonate and volume absorption in the rabbit S_2 proximal convoluted tubule perfused *in vitro* (Baum & Hays, 1988). In additon, previous studies showing phos-

phatidylinositol turnover or a rise in intracellular calcium concentration in the PTCs utilized ANG II concentrations (10^{-8} to 10^{-5} M) that can be associated with inhibition of transport (Welsh et al, 1988). In *in vivo* experiments in rat proximal tubule, PKC inhibition either reduces or abolishes the effects of low dose ANG II. In the present study, there was no additive effect on Na^+ uptake when a stimulatory dose of ANG II and TPA were added together to cultured cells suggesting that ANG II and TPA act by a similar mechanism. We also found that the stimulatory effect of ANG II (10^{-11} M) on Na^+ uptake was blocked by a PKC inhibitor staurosporine. In addition, 10^{-11} M ANG II treatment increased total PKC activity. These results suggest that PKC mediates a significant portion of the ANG II stimulation of Na^+ uptake. However, the part of stimulatory response, the PKC-independent component, was presumably due to decrease in adenylate cyclase activity. Other components of the phosphatidylinositol pathway, calcium, or different signaling mechanism(s) could be involved as well.

In conclusion, the PTX-sensitive PKC pathway is the main signaling cascade involved in the stimulatory effects of ANG II on Na^+ uptake in the PTCs.

ACKNOWLEDGEMENTS

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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