Effect of Angiotensin II on the Apical K^+ Channel in the Thick Ascending Limb of the Rat Kidney

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ABSTRACT We have used the patch-clamp technique to study the effect of angiotensin II (AII) on the activity of the apical 70 pS K^+ channel and used Na^+-sensitive fluorescent dye (SBFI) to investigate the effect of AII on intracellular Na^+ concentration (Na^+_i) in the thick ascending limb (TAL) of the rat kidney. Addition of 50 pM AII reversibly reduced NP_o, a product of channel open probability (P_o) and channel number (N), to 40% of the control value and reduced the Na^+_i by 26%. The AII (50 pM)-induced decrease in channel activity defined by NP_o was partially reversed by addition of 5 μM 17-octadecynoic acid (17-ODYA), an agent which blocks the cytochrome P450 monooxygenase. The notion that P450 metabolites of arachidonic acid (AA) may mediate the inhibitory effect of AII was further suggested by experiments in which addition of 10 nM of 20-hydroxyeicosatetraenoic acid (20-HETE) blocked the channel activity in cell-attached patches in the presence of 17-ODYA. We have used gas chromatography mass spectrometry (GC/MS) to measure the production of 20-HETE, a major AA metabolite of the P450-dependent pathway in the TAL of the rat. Addition of 50 pM AII increased the production of 20-HETE to 260% of the control value, indicating that 20-HETE may be involved in mediating the effect of AII (50 pM). In contrast to the inhibitory effect of 50 pM AII, addition of 50–100 nM AII increased the channel activity to 270% of the control value and elevated the Na^+_i by 45%. The effect of AII on the activity of the 70 pS K^+ channel was also observed in the presence of 5 μM 17-ODYA and 5 μM calphostin C, an inhibitor of protein kinase C. However, addition of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, abolished completely the AII (50–100 nM)-induced increase in channel activity and addition of an exogenous nitric oxide (NO) donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP), increased channel activity in the presence of L-NAME. These data suggest that the stimulatory effect of AII is mediated by NO. We conclude that AII has dual effects on the activity of the apical 70 pS K^+ channel. The inhibitory effect of AII is mediated by P450-dependent metabolites whereas the stimulatory effect may be mediated via NO.

KEY WORDS: cytochrome p450 monooxygenase • 20-HETE • nitric oxide • K recycling • Na reabsorption

INTRODUCTION

The thick ascending limb (TAL)[1] is responsible for the reabsorption of 20–25% of the filtered sodium load and plays a key role in the urinary concentrating ability (Hebert and Andreoli, 1984; Greger, 1985; Stanton and Giebisch, 1992). The reabsorption of NaCl is a two-step process. First, Na^+ and Cl^- enter the cell via the $Na^+/2Cl^-/K^+$ cotransporter energized by electrochemical Na^+ gradient. Second, Na^+ is pumped out of the cell through Na^+-K^+-ATPase and Cl^- diffuses across the basolateral membrane along its electrochemical gradient (Hebert and Andreoli, 1984; Greger, 1985). Apical K^+ channels play an important role in maintaining reabsorption of NaCl in the TAL where it has been demonstrated that inhibition of the apical K^+ channels decreased the reabsorption rate of NaCl (Hebert et al., 1984).

Three types of K^+ channels, low conductance (30 pS), intermediate conductance (70 pS), and large conductance (100–200 pS), have been identified in the apical membrane of the TAL (Taniguchi and Guggino, 1989; Bleich et al., 1990; Wang et al., 1990; Wang, 1994). Previous studies have demonstrated that the 70 pS K^+ channels contribute ~80% of the apical K^+ conductance under control conditions (Wang and Lu, 1995). The 70 pS K^+ channel has been shown to be sensitive to ATP and pH (Bleich et al., 1990). In addition, previous studies have shown that arachidonic acid (AA) blocked the activity of the 70 pS K^+ channel (Wang and Lu, 1995). The effect of AA on the channel activity is mediated by cytochrome P450-dependent metabolites, since the effect of AA can be blocked by inhibition of the P450-dependent pathway. Furthermore, the AA effect can be mimicked by 20-hydroxyeicosatetraenoic acid (20-HETE), a main product of the P450 metabolic pathway of AA in the TAL (McGiff, 1991).

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[1]*Abbreviations used in this paper:* AA, arachidonic acid; AII, angiotensin II; EET, epoxyeicosatrienoic acid; GC/MS, gas chromatography mass spectrometry; NOS, nitric oxide synthase; TAL, think ascending limb.

A large body of evidence indicates that angiotensin II (AII) plays an important role in the regulation of tubule transport in the kidney (Wang and Chan, 1989; Geibel et al., 1990; Ichikawa and Harris, 1991; Weiner et al., 1995). Geibel et al. (1990) have shown that AII stimulates the Na^+/H^+ exchanger and Na^+/HCO_3^- cotransporter in the proximal tubule. AII has been shown to have biphasic effects on HCO_3^- transport in the proximal tubule: picomolar concentrations of AII stimulated whereas nanomolar concentration inhibited the reabsorption of HCO_3^- (Wang and Chan, 1989). The effect of AII on ion transport in the TAL is largely unknown. Recently, it was shown that systemic infusion of AII enhanced the reabsorption of HCO_3^- in Henle's loop (Capasso et al., 1994). This study is designed to examine the effects of AII on the activity of the 70 pS K^+ channel in the TAL.

METHODS

Preparation of Rat TAL

The TAL was isolated from kidneys of pathogen-free Sprague-Dawley rats which were purchased from Taconic Inc. (Germantown, NY). The weight of the animals was 40–50 g at the time of purchase and increased to 80–120 g before use. Animals were killed by decapitation, and the kidneys were immediately removed. Several thin coronal sections were cut with a razor blade. The TAL was dissected using watch-making forceps in HEPES-buffered NaCl Ringer solution containing (in mM) NaCl 140; KCl 5; $MgCl_2$ 1.5; $CaCl_2$ 1.8; glucose 5; and HEPES 10, pH 7.4 with NaOH, at 22°C and transferred onto a 5 × 5 mm cover glass coated with Cell-Tak (Collaborative Research Inc., Bedford, MA) to immobilize the tubules. The cover glass was placed in a chamber mounted on an inverted microscope (Nikon Inc., Melville, NY), and the tubules were superfused with HEPES-buffered NaCl solution. The TAL was cut open with a sharpened micropipette to expose the apical membrane. The temperature of the chamber (1,000 μl) was maintained at 37 ± 1°C by circulating warm water surrounding the chamber.

Patch-clamp Technique

The methods for patch-clamp experiments have been previously described (Wang and Lu, 1995). An Axon200A patch-clamp amplifier (Axon Instruments, Burlingame, CA) was used to record channel activity. The output of the amplifier was low-pass filtered at 1 kHz using an eight pole Bessel filter (902LPF; Frequency Devices, Haverhill, MA) and was digitized at a sampling rate of 44 kHz using a modified Sony PCM-501ES pulse code modulator and stored on videotape (Sony SL-2700). For analysis, the data stored on the tape were replayed and transferred to an IBM-compatible 486 computer (Gateway 2000) at a rate of 5 kHz and analyzed using the pClamp software system 6.02 (Axon Instruments).

We defined NP_o as an index of channel activity, and no efforts were made to examine whether alterations of channel activity were due to changes in channel number (N) or channel open probability (P_o). The NP_o was calculated from data samples of 30–60 s duration under the steady state as follows:

$$NP_o = \Sigma (t_1 + t_2 + \ldots t_n) , \quad (1)$$

where N is the number of channels, i.e., the maximum number of superpositions of current level seen in the patch, and t_n is the fractional open time spent at each of the observed current levels.

Measurement of 20-HETE and EET (Epoxyeicosatrienoic Acid)

The medullary TALs were dissected and suspended in Ringer's solution (1 ml). The tubules were incubated for 10 min in the presence or in the absence of 50 pM or 50 nM AII. Then, the tubule suspensions were acidified with 10% formic acid (final pH 4) and supplemented with 20,20-dideutero-20-hydroxyarachidonic acid (20-HETE-d_2) as internal standard (1 ng per ml). Lipids were extracted twice with 1 ml of ethyl acetate. After extraction, the protein concentration was measured using the Bio-Rad protein microassay (Bio-Rad Laboratories, Richmond, CA). The organic extracts were taken to dryness under vacuum and dissolved in acetonitrile and purified by reverse phase HPLC using an ultrasphere C_{18} column (250 × 4.6 mm; Beckman Instruments, Inc., Fullerton, CA). Samples were chromatographed using linear solvent gradient of water in acetonitrile (37.5–0%, containing 0.01% acetic acid) at 1.875%/min and at a solvent flow rate of 1 ml/min. Fractions were collected every minute and separate fractions containing 20-HETE, which eluted at 9.6–9.8 min, were taken to dryness and dissolved in 100 μl of acetonitrile. For gas chromatography mass spectrometry (GC/MS) analyses the samples were derivatized as pentafluorobenzyl esters trimethylsilyl ethers. Briefly, samples were treated with 10 μl of pentafluorobenzylbromide (10% solution in acetonitrile) in the presence of catalytic amounts of *N,N*-diisopropylethylamine (Aldrich Chemical Co., Milwaukee, WI) for 20 min at room temperature. The reagents were evaporated to dryness under a flow of nitrogen and the residue was dissolved in 80 μl *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 20 μl of pyridine and reacted for 20 min at room temperature. The samples were finally dissolved in 50 μl of isooctane and analyzed by GC/MS as described (Balazy, 1991). Briefly, 1-μl aliquots were injected into a fused silica capillary column (15 m, 0.25 mm i.d., 0.2 μm film thickness, DB-1; J & W Scientific, Folsom, CA) and eluted with a temperature gradient from 150–300°C at 25°C /min and a flow of helium (linear velocity, 40 cm/s). 20-HETE was detected by the mass spectrometer (HP 5989A; Hewlett Packard, Palo Alto CA) operating in chemical ionization mode with methane (1.7 torr source pressure) as a moderating gas and with detection of negative ions (electron capture ionization). Selected ion monitoring was used to record ion abundances at *m/z* 391 and *m/z* 393 which corresponded to endogenous 20-HETE and the internal standard, respectively. 20-HETE eluted at 4.98 min and was separated from other HETEs. Standard curve was prepared by mixing various amounts of 20-HETE (0.1–2 ng) and 20-HETE-d_2 (1 ng). The ratio of areas under chromatographic peaks for 20-HETE and 20-HETE-d_2 was plotted against the amount of 20-HETE which resulted in linear correlation. The amount of 20-HETE generated by rat kidney tubules was calculated from a regression line ($r > 0.998$). We have measured EET following the method described previously (Balazy, 1991, 1994)

Measurement of Intracellular Na^+ Concentrations

We followed the method described previously by Negulescu and Mechen (1990) to measure intracellular Na^+ concentration. Briefly, the split-open cortical or medullary TAL was loaded with the fluorescent dye SBFI-AM (7 μM) and 0.001% pluronic acid (Molecular Probes, Eugene, OR) at room temperature (22°C) for 60 min. At the end of the incubation period, the tubules were washed with the Ringer solution and transferred to a new cover glass coated with Cell-Tak. The cover glass was transferred to a

chamber mounted to an inverted microscope (Nikon) and the tubules were incubated for an additional 15 min before experiments. Three to five principal cells were selected for each experiment. Fluorescence was imaged digitally with an intensified video imaging system including a SIT 68 camera, controller, and HR 1000 video monitor (Long Island Instruments, North Bellmore, NY). The exciting and emitted light through a ×40 Nikon fluorite objective (NA = 1.30). The microscope (Nikon) was coupled to an alternating wavelength illumination system (Ionoptix, MA). Digital images were collected at rate of 10 ratio pairs/min and analyzed with an Ionoptix software. Dye in the specimen was excited with light of 340 and 380 nM wavelengths using 75-W xenon source, and emission was recorded at 510 nM. Intracellular Na^+ was measured from the ratio of 340 and 380 nM. Fluorescent ratio were calibrated in situ by permeabilizing cells with gramicidin D (10 μM) or 10 μM ionophore lasalocid (Sigma Chemical Co., St. Louis, MO) and altering the Na^+ concentration of the bath. Since results were identical between tubules treated with lasalocid and gramicidin D, we pooled data. Fig. 1 shows a normalized calibration curve for SBFI obtained from four in situ experiments.

Experimental Solution and Statistics

The pipette solution contained (in mM): 140 KCl, 1.8 $MgCl_2$, 1 EGTA, and 10 HEPES, pH 7.40. The bath solution for cell-attached patches were composed of (in mM) 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 1.8 $MgCl_2$, and 10 HEPES, pH = 7.40. Angiotensin II and N^G-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma Chemical Co. Calphostin C was purchased from LC Laboratories (Woburn, MA); 17-octadecynoic acid (17-ODYA) and *S*-nitroso-*N*-acetyl-penicillamine (SNAP) was obtained from Calbiochem Corp. (La Jolla, CA). Calphostin C, 17-ODYA, and SNAP were dissolved in pure ethanol. The final concentration of ethanol in the bath was 0.1%, and it had no effect on channel activity. The turnover time for the bath is ~1–2 s. However, perfusion is discontinued after high resistance seals were formed, and the chemicals were directly added to the bath to achieve the final concentration.

Data are shown as mean ± SEM, and paired Student's *t* test was used to determine the significance of differences between control and experimental periods. Statistical significance was taken as $P < 0.05$.

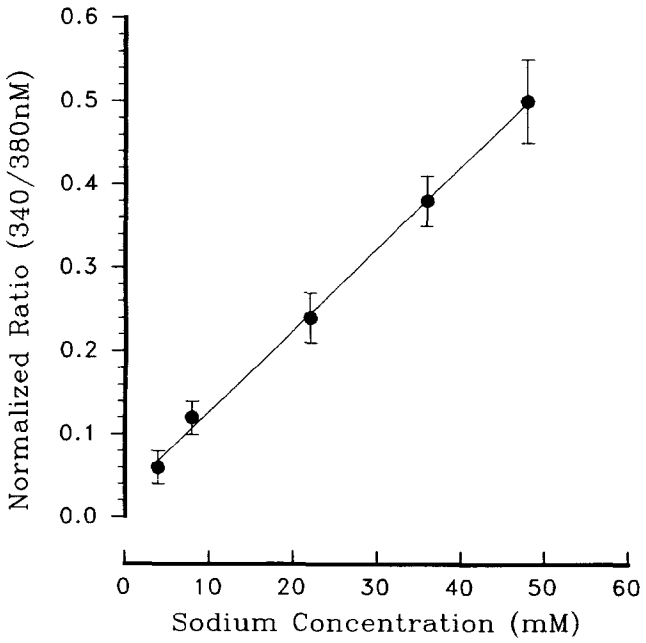

FIGURE 1. A normalized calibration curve for SBFI shows the relationship between Na^+ concentration and fluorescent ratio measured at 340/380 nM ($n = 4$). Calibration was carried out in situ after each experiment.

RESULTS

We have confirmed the previous observations that two types of K^+ channels, 30 pS and 70 pS, are present and that the 70 pS K^+ channel is predominant in the apical membrane of the TAL under control conditions. This study is focused on investigating the effect of AII on the 70 pS K^+ channel. Thus, we carried out the study on those patches with only the 70 pS K^+ channel which was confirmed by measuring the current-voltage relationship over three different holding potentials. Fig. 2 is a representative recording to show the effect of 50 pM AII on channel activity in a cell-attached patch. It is apparent that addition of 50 pM AII reversibly decreased channel activity. In nine experiments, addition of 25–50 pM AII reduced the channel activity to 40 ± 9% of the control value. Since the response to AII of the 70 pS K^+ channel is identical between the cTAL and the mTAL, we pooled data. In previous studies it was demonstrated that cytochrome P450-dependent metabolites of AA blocked the activity of the 70 pS K^+ channel (Wang and Lu, 1995). Thus, we examined whether the inhibitory effect of AII was mediated by the P450-dependent metabolic pathway of AA. Fig. 3 is a recording to show the effect of AII on channel activity in the presence of 17-ODYA, an agent which blocks the P450 monooxygenase. When addition of 50 pM AII had reduced channel activity in a cell-attached patch to a steady state, application of 5 μM 17-ODYA gradually restored the channel activity to 75 ± 8% of the control value in the continued presence of AII ($n = 6$, Table I). The notion that P450 metabolites of AA may be involved in mediating the effect of AA is further suggested by experiments in which addition of 10 nM 20 HETE decreased channel activity to 5 ± 1% of the control value in the presence of 17-ODYA ($n = 4$).

TABLE I

Effect of AII on Channel Activity in the Absence or in the Presence of 5 μM 17-ODYA and 100 μM L-NAME

AII (low)	AII(low) + 5 μM 17-ODYA	AII (high)	AII(high) + L-NAME
40 ± 9%*	75 ± 8%*	270 ± 15*	49 ± 16%*
$n = 9$	$n = 6$	$n = 17$	$n = 10$

Results are presented as percentage of the normalized channel activity of the control value. Data is mean ± SEM, and *n* is observation numbers. * Indicates that data is significantly different from the control value. The low concentration of AII is 50 pM whereas the high concentration of AII is 50–100 nM.

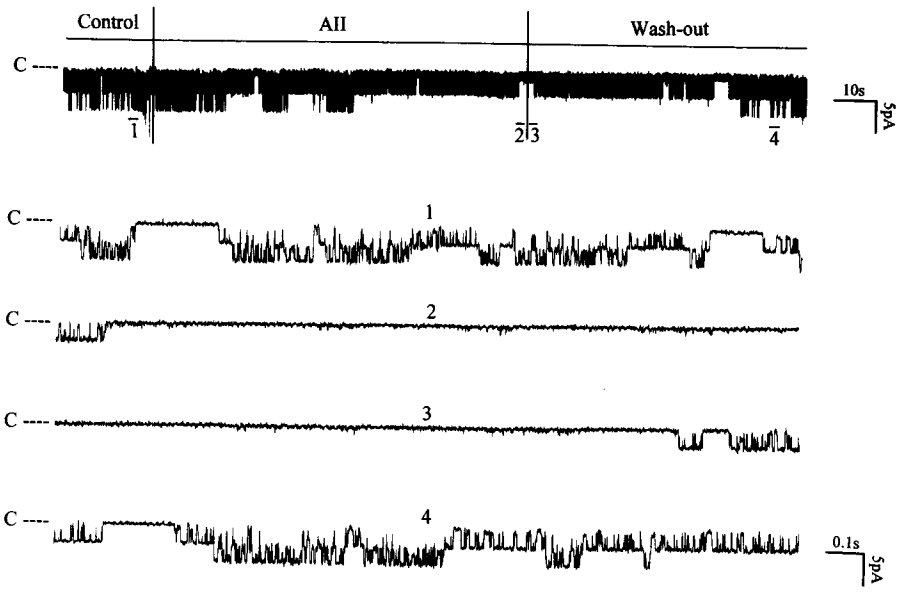

FIGURE 2. A channel recording made in a cell-attached patch shows the effect of 50 pM angiotensin II on the activity of the 70 pS K^+ channel with 140 mM KCl in the pipette with NaCl Ringer in the bath. The channel closed level is indicated by *C* and the pipette holding potential is 0 mV. The top of the figure shows the channel activity at slow time resolution. Four parts of the tracing, which are indicated by short bars and numbers, are extended to show the channel activity at fast time resolution.

Having established that 20-HETE may mediate the inhibitory effect of AII on channel activity, we used GC/MS to measure the intracellular 20-HETE concentration. Fig. 4 is one representative recording out of five such experiments. From inspection of the figure it is apparent that 20-HETE is the major metabolite of AA under both experimental (*a*) and control conditions (*b*). Furthermore, incubation of medullary TAL tubules with 50 pM AII increased the production of 20-HETE to 260 ± 10% of the control value, confirming our hypothesis that 20-

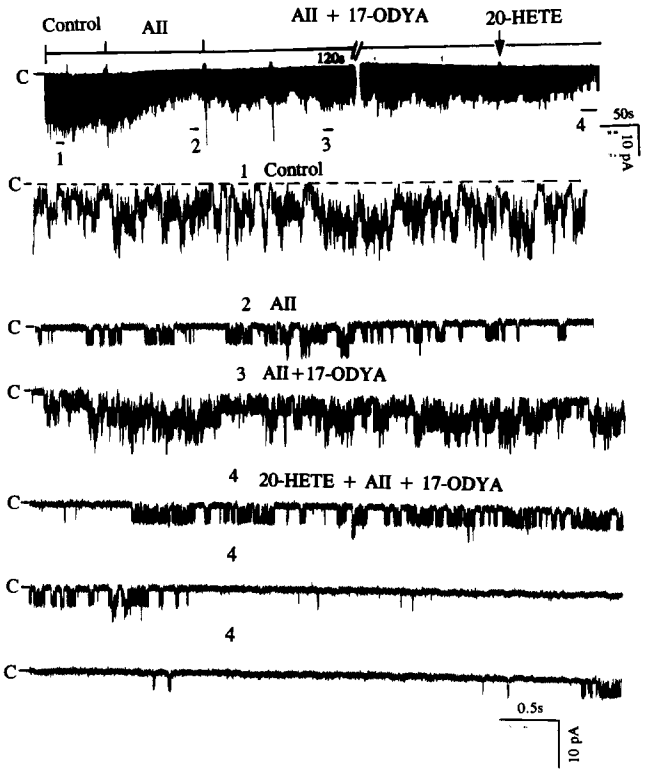

FIGURE 3. A channel recording shows the effect of AII (50 pM) on the activity of the 70 pS K^+ channel in a cell-attached patch in the presence of 5 μM 17-ODYA and 10 nM 20-HETE. The top of the figure shows the channel activity at slow time course. Four parts of the tracing, which are indicated by short bars and numbers, are extended at fast time resolution. The channel closed levels are indicated by *C*, and the holding potential was 0 mV.

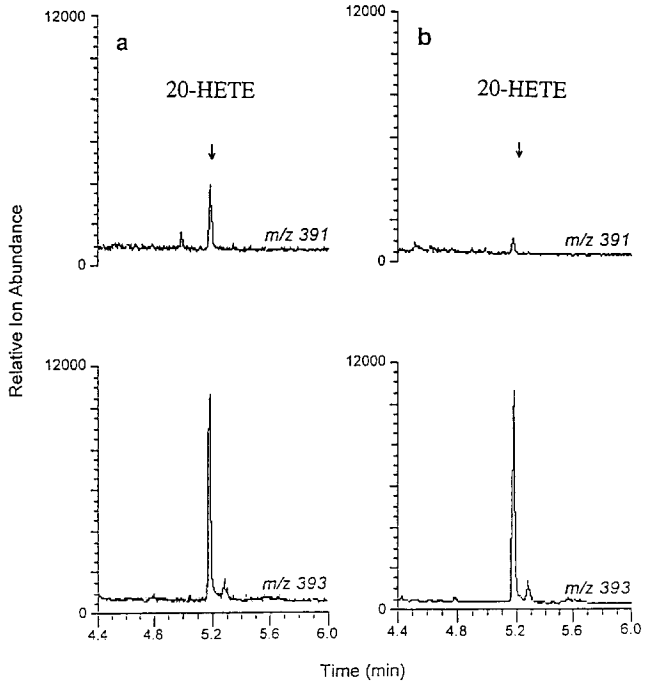

FIGURE 4. GC/MS analyses showing 20-HETE in the mTAL. Top chromatograms correspond to endogenous 20-HETE (ion *m/z* 391); the bottom chromatograms correspond to internal standard (20-HETE-d_2, ion *m/z* 393). The TAL incubated with 50 pM of angiotensin II for 10 min produced 2.6 pg/ μg of protein of 20-HETE (*a*), and the corresponding control value is 0.5 pg/μg of protein of 20-HETE (*b*).

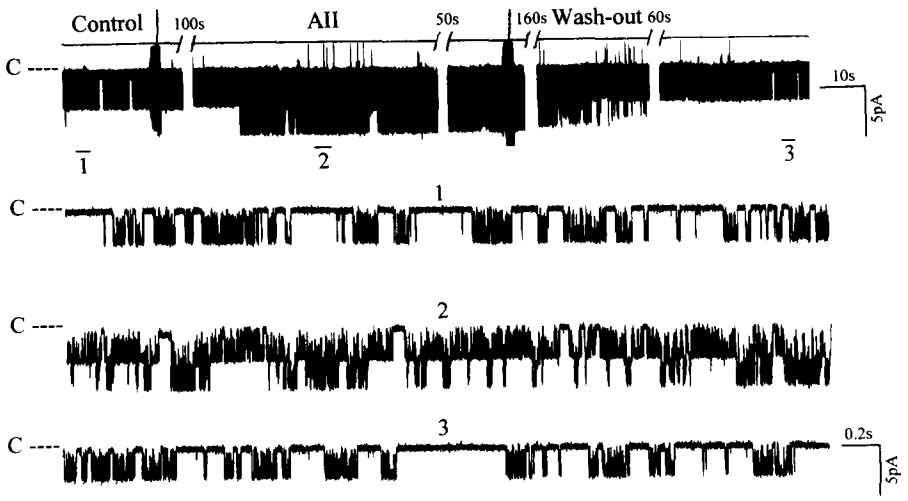

FIGURE 5. A channel recording made in a cell-attached patch shows the effect of 50 nM angiotensin II on the activity of the 70 pS K^+ channel with 140 mM KCl in the pipette and NaCl Ringer in the bath. The channel closed level is indicated by *C*, and the pipette holding potential is 0 mV. The top of the figure shows the channel activity at slow time resolution. Three parts of the tracing, which are indicated by short bars and numbers, are extended to show the channel activity at fast time resolution.

HETE may be responsible for the effect of 50 pM AII. It was shown that AII stimulated epoxygenase activity and accordingly potentiated the formation of EETs from exogenously added AA in rat proximal tubules (Omata et al., 1992). Furthermore, it was recently reported that 11-12 EET stimulated the Ca^{2+}-activated K^+ channel (Zou et al., 1996). Thus, we examined the effect of 11-12 EET on the 70 pS K^+ channel in inside-out patches. Addition of 1 μM 11-12 EET has no significant effect on the activity of the 70 pS K^+ channel in three experiments (data not shown). In addition, GC/MS measurement showed that no EET was present under control conditions and after addition of AII (data not shown). Thus, we exclude the possibility that 11-12 EET could be involved in mediating the effect of AII on channel activity.

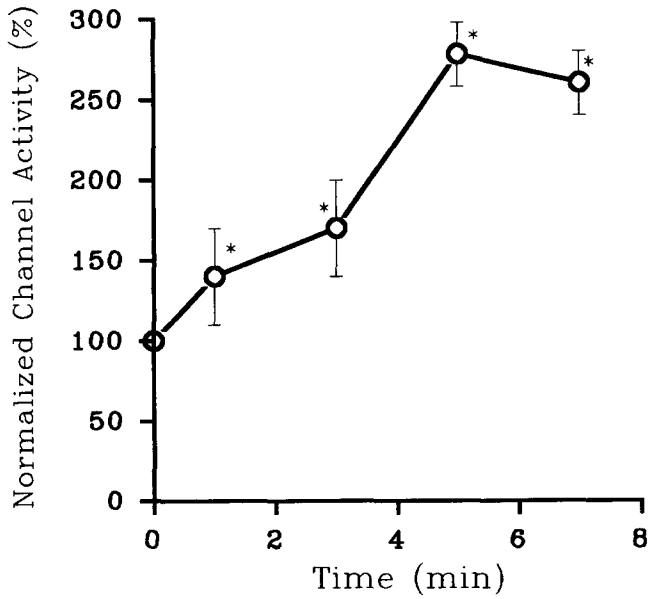

FIGURE 6. The time course of the effect of 50–100 nM AII on the channel activity. The asterisks indicate that the data are significantly different from the control value.

It was demonstrated that the effects of AII on ion transport were biphasic and concentration dependent (Wang et al., 1989). We therefore extended the study to investigate the effect of high concentrations of AII (50–100 nM) on channel activity. Fig. 5 is a typical recording out of 17 experiments showing that addition of

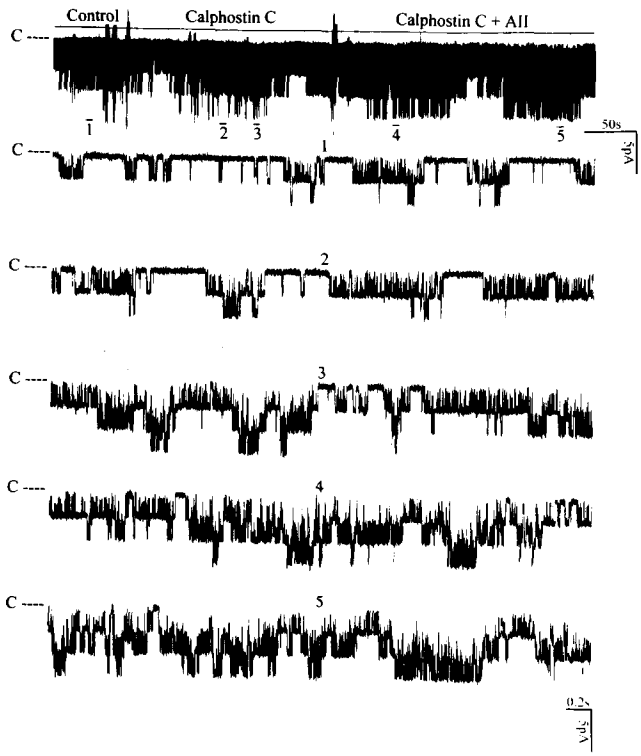

FIGURE 7. A channel recording made in a cell-attached patch shows the effect of 50 nM angiotensin II on the activity of the 70 pS K^+ channel in the presence of 50 nM calphostin C. The channel closed level is indicated by *C*, and the pipette holding potential is 0 mV. The top of the figure shows the channel activity at slow time resolution. Five parts of the tracing, which are indicated by short bars and numbers, are extended to show the channel activity at fast time resolution.

high concentration of AII increased the channel activity in a cell-attached patch. The stimulatory effect of AII was also reversible since wash-out reduced the channel activity to the original level (Fig. 5). From inspection of Fig. 6 it is apparent that the stimulatory effect of AII gradually reached its plateau at 270 ± 15% of the control value in approximately 300 s (Table I).

It has been shown that protein kinase C (PKC) mediates the stimulatory effect of AII on HCO_3^- transport in the proximal tubules (Wang and Chan, 1989). Although we previously demonstrated that stimulation of PKC led to inhibition of K^+ channel activity (Wang et al., 1996), it is still possible that the stimulatory effect of AII on channel activity may be mediated by an additional type of PKC which could either directly or indirectly stimulate the 70 pS K^+ channel. If the stimulatory effect of AII is mediated by PKC, inhibition of PKC should abolish this effect. Fig. 7 is a representative recording to show the effect of 50 nM AII on channel activity in a cell-attached patch in the presence of 50–75 nM calphostin C, an agent that specifically blocks PKC. We confirmed the previous finding (Wang et al., 1996) that addition of calphostin C increased channel activity (194 ± 8% of the control value). However, inhibition of PKC failed to abolish the stimulatory effect of AII, since addition of 50 nM AII further increased the channel activity to 446 ± 30% of the control value (n = 15), suggesting that it is unlikely that PKC plays a major role in mediating the stimulatory effect of AII.

We next examined the possibility that high concentrations of AII inhibit P450 monooxygenase and, accordingly, suppress the production of 20-HETE. Thus, we investigated the effect of 50 nM AII in the presence of a P450 inhibitor, such as 17-ODYA or 12,12-dibromododec-11-enoic acid (DBDD) (Fig. 8). Addition of either DBDD or 17-ODYA increased channel activity to 219 ± 20% of the control value (data not shown) as reported in previous studies (Wang and Lu, 1995). However, addition of 50 nM AII led to further stimulation of the channel activity to 258 ± 20% of the value obtained in the presence of 17-ODYA (n = 9), indicating that the stimulatory effect of AII was not a result of inhibiting the P450 metabolic pathway. This view is further supported by the finding that addition of 50 nM AII also increased the formation of 20-HETE (210 ± 40% of the control value).

Stimulation of AII receptors has been shown to increase the production of nitric oxide (NO) (Kanazawa et al., 1995), and NO has been reported to activate several types of K^+ channels including the basolateral K^+ channel in the collecting duct (Lu and Wang, 1996). Thus, we have examined the possibility that the stimulatory effect of AII on the 70 pS K^+ channel may be mediated by NO. Fig. 9 is a representative recording to show the effect of 50 nM AII on channel activity in a cell-attached patch in the presence of 100 μM L-NAME, an inhibitor of nitric oxide synthase (NOS). The cells were first incubated in a 100 μM L-NAME containing solution for 10 min, then 50 nM AII was added to the bath in the continued presence of the L-NAME while the channel activity was continuously monitored. In contrast to the effect of AII in the absence of L-NAME,

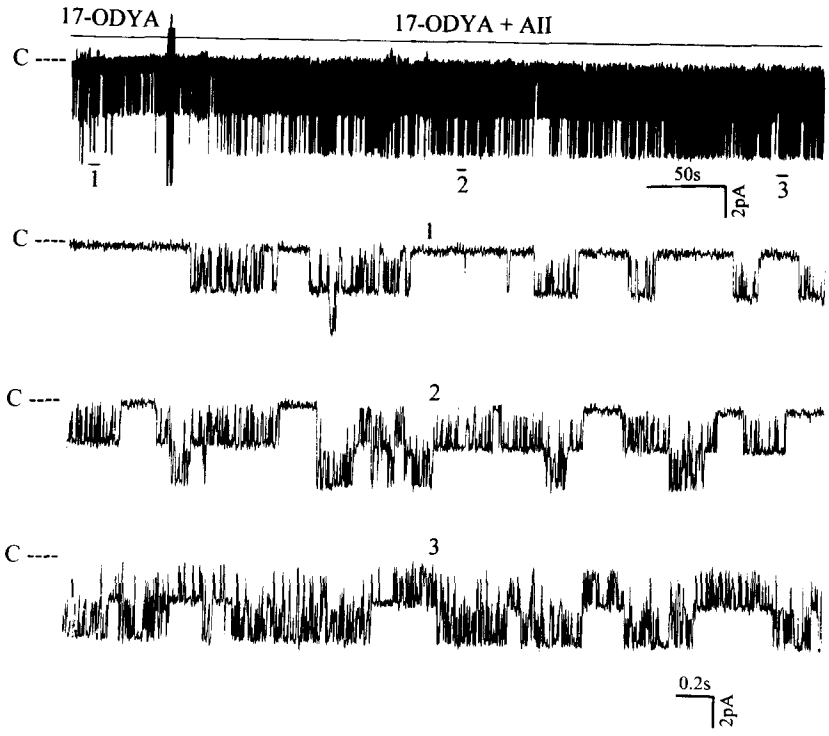

FIGURE 8. A channel recording made in a cell-attached patch shows the effect of 50 nM angiotensin II on the activity of the 70 pS K^+ channel in the presence of 5 μM 17-ODYA. The channel closed level is indicated by *C*, and the pipette holding potential is 0 mV. The top of the figure shows the channel activity at slow time resolution. Three parts of the tracing, which are indicated by short bars and numbers, are extended to show the channel activity at fast time resolution.

addition of 50 nM AII reduced channel activity to 49 ± 16% of the value observed in the presence of L-NAME alone (n = 10, Table I), suggesting that the stimulatory effect of AII is mediated by NO (Fig. 9).

The notion that NO may be involved in mediating the stimulatory effect of AII on channel activity is further supported by results of experiments in which addition of L-NAME reversed the AII-induced stimulation. From inspection of Fig. 10 it is apparent that addition of 100 μM L-NAME abolished the stimulatory effect of AII. In contrast, addition of 10 μM SNAP, an exogenous NO donor, increased the channel activity to 250 ± 20% of the control value (n = 6) in the presence of L-NAME, suggesting that NO was involved in mediating the stimulatory effect of AII.

Since the 70 pS K^+ channel plays an important role in the K^+ recycling across the apical membrane in the TAL, inhibition of channel activity is expected to decrease whereas stimulation of the 70 pS K^+ channel to increase the turn-over rate of the $Na^+/K^+/2Cl^-$ cotransporter. Accordingly, inhibition of the cotransporter should decrease whereas stimulation of the cotransporter should increase the Na^+_i, respectively. We tested this hypothesis by measurement of intracellular Na^+ concentration with SBFI. Fig.11 *a* is a representative recording from four experiments. Application of 50 pM AII decreased Na^+_i from 14 ± 2 mM to 10 ± 1 mM. However, further increase of AII concentration to 1 nM not only abolished the effect of AII but also significantly increased Na^+_i to 16 ± 2 mM. The effect of high concentration of AII (≥1 nM) on the Na^+_i is reversible since wash-out reduced gradually the Na^+_i (data not shown). Fig. 11 *b* is a dose-response curve of the AII effect on Na^+_i. It is apparent that a low concentration of AII (<100 pM) reduced whereas a high concentration (≥1 nM) increased the Na^+_i.

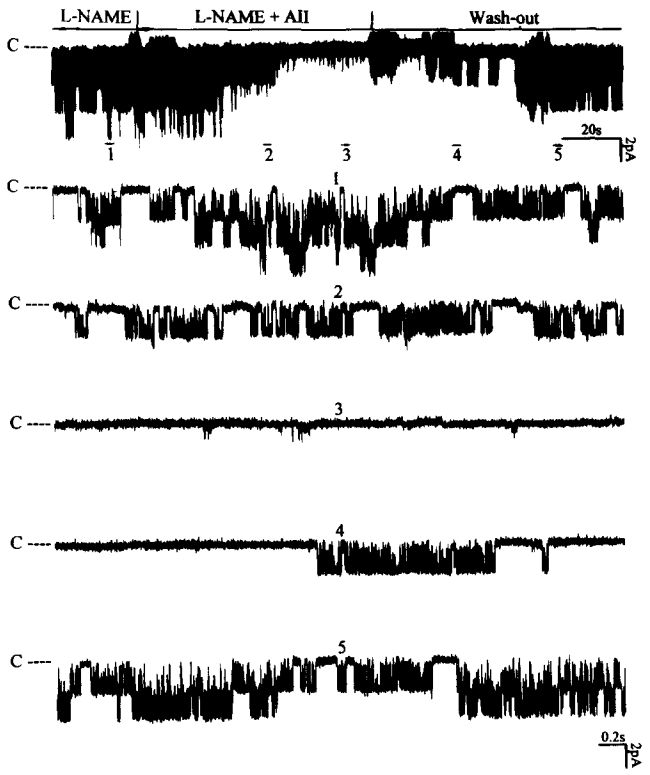

FIGURE 9. A channel recording made in a cell-attached patch shows the effect of 50 nM angiotensin II on the activity of the 70 pS K^+ channel in the presence of 100 μM L-NAME. The channel closed level is indicated by *C*, and the pipette holding potential is 0 mV. The top of the figure shows the channel activity at slow time resolution. Five parts of the tracing, which are indicated by short bars and numbers, are extended to show the channel activity at fast time resolution.

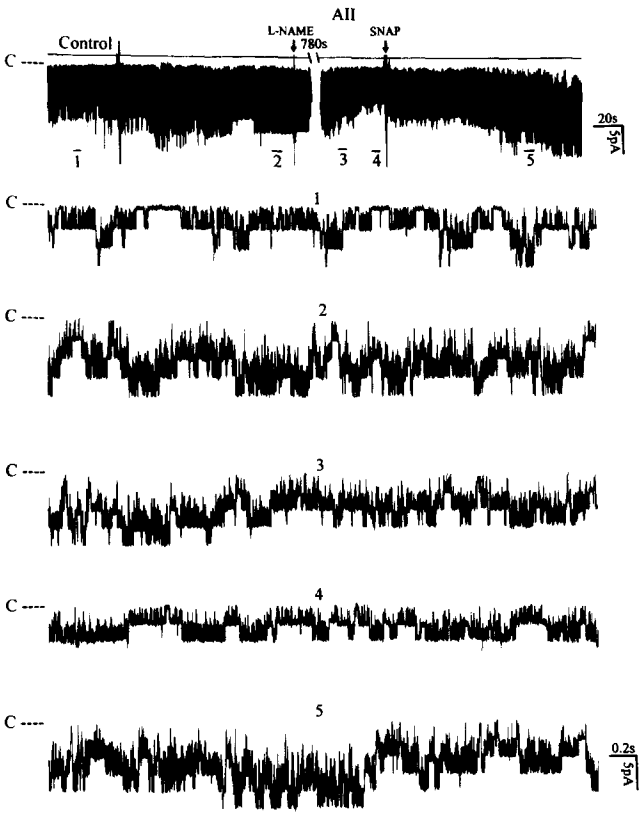

FIGURE 10. A representative channel recording shows the effect of 50 nM AII, 50 nM AII + 100 μM L-NAME, and AII + L-NAME + 10 μM SNAP. The channel closed level is indicated by *C*, and the pipette holding potential is 0 mV. The top of the figure shows the channel activity at slow time resolution. Five parts of the tracing, which are indicated by short bars and numbers, are extended to show the channel activity at fast time resolution.

DISCUSSION

The main finding of the present study is that a low concentration of AII inhibits whereas a high concentration of AII stimulates the 70 pS K^+ channel. Furthermore, we have demonstrated that the stimulatory effect of AII is mediated by NO whereas the inhibitory effect is at

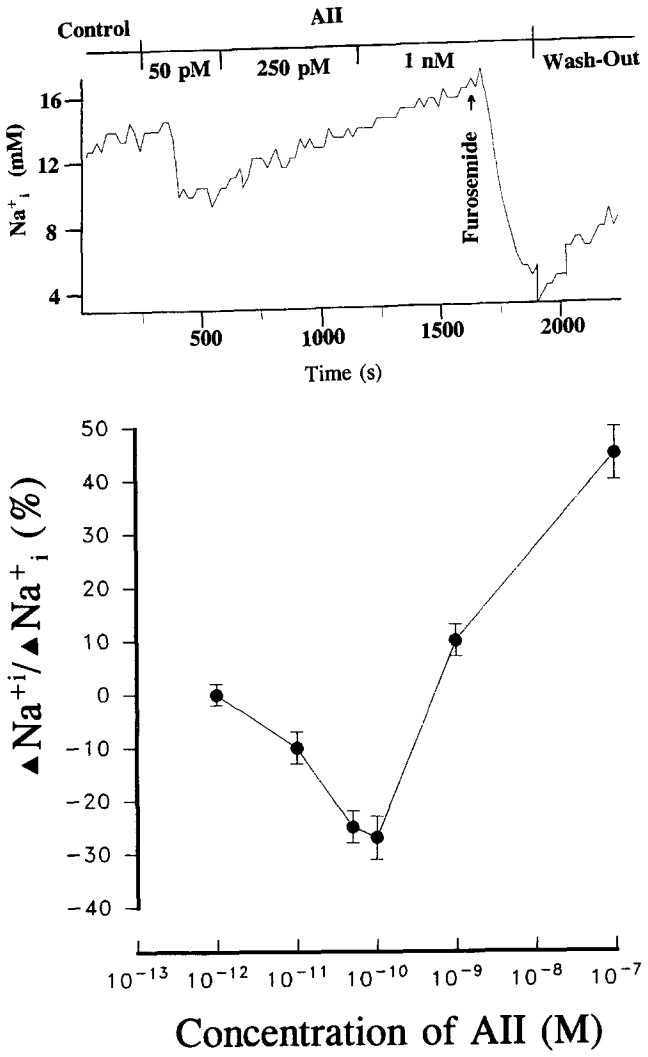

FIGURE 11. (*a*) The effect of AII and furosemide (100 μM) on intracellular Na^+ concentration (Na^+_i) in the TAL. The tubules were split open and loaded with fluorescent dye (SBFI) for 60 min. (*b*) A dose response curve of AII shows the biphasic effect of AII on Na^+_i. Changes in the Na^+_i ($\blacktriangle Na^+_i$) are normalized by using the equation $\blacktriangle Na^+_i/Na^+_i$. Each point, with the exception of 50 pM ($n = 4$), represents three observations that were made separately at each given concentration of AII.

least in part mediated by P450 metabolites of AA. We have also provided evidence showing that AII has a significant effect on Na^+ transport in the TAL and that AII-induced alteration of the apical K^+ conductance is at least partially responsible for the AII effect on Na^+ transport. Although the effect of AII on the 30 pS K^+ channel is not explored in the present study, it is likely that the 30 pS K^+ channel is also regulated by AII and plays a role in mediating the effect of AII on Na^+ transport because the 30 pS K^+ channel contributes at least 20% of the K^+ conductance to the apical membrane of the TAL.

Apical K^+ channels play a key role in maintaining NaCl reabsorption in the TAL (Hebert and Andreoli, 1984; Greger, 1985). First, K^+ recycling across the apical membrane is partially responsible for the positive transepithelial potential, which is the driving force for paracellular reabsorption of Na^+, Ca^{2+}, and Mg^{2+} (Greger, 1985). Hebert et al. (1984) have demonstrated that approximately 50% of the Na^+ reabsorption occurs by the paracellular pathway (Hebert and Andreoli, 1984). Second, K^+ recycling is important for the current flow related to the active reabsorption of Na^+ and Cl^-. Cl^- enters the cell across the apical membrane via the cotransporter and then diffuses across the basolateral membrane through Cl^- channels. Diffusion of Cl^- tends to depolarize the basolateral membrane compromising the electrochemical gradient for Cl^- and tending to reduce the Cl^- reabsorption. However, K^+ recycling across the apical membrane hyperpolarizes the cell membrane and maintains the driving force for Cl^- across the basolateral membrane. Finally, K^+ recycling may be important for providing an adequate supply of K^+ for the cotransporter in the cortical TAL, where K^+ concentration is at least one order of magnitude lower than Na^+ and Cl^-. The importance of K^+ recycling for active Cl^- reabsorption has been well demonstrated by experiments in which removal of luminal K^+ in the presence of Ba^{2+} decreased the Cl^- reabsorption by approximately 90% (Hebert et al., 1984; Greger 1985).

Previous studies showed that the 70 pS K^+ channel was predominant under physiological conditions contributing approximately 80% of apical K^+ conductance (Wang and Lu, 1995). Thus, alteration in the activity of the 70 pS K^+ channel can have a significant effect on the apical K^+ conductance and K^+ recycling. The 70 pS K^+ channel is regulated by several mechanisms including intracellular ATP, cell pH, and PKC (Bleich et al., 1990; Wang et al., 1996). Recently, we have shown that AA is also involved in its regulation and that the effect of AA is mediated by 20-HETE (Wang and Lu, 1995).

Several Lines of evidence have suggested that AII plays an important role in the regulation of tubule transport (Mujais et al., 1986; Wang and Chan, 1989; Geibel et al., 1990; Ichikawa and Harris, 1991). AII has been shown to have an effect on HCO_3^- transport (Wang and Chan, 1989), fluid transport (Wang and Chan, 1989; Li et al., 1994) and Na^+/H^+ exchange (Geibel et al., 1990). AII binding sites, which are presumably AII receptors, have been identified in both the cortical and medullary TALs (Mujais et al., 1986), and AII stimulates HCO_3^- reabsorption in Henle's loop (Capasso et al., 1994). The present studies provide further evidence of involvement of AII in the regulation of ion transport in the TAL.

Three lines of evidence suggest that the inhibitory effect of AII on channel activity is at least partially medi-

ated by P450 metabolites of AA. First, the inhibitory effect of AII was partially reversed by inhibition of the P450 metabolic pathway. Second, addition of 20-HETE in the presence of the inhibitor of P450 monooxygenase reduces channel activity. Finally, 50 pM AII increases the generation of 20-HETE in the mTAL. Although we did not measure 20-HETE production in the cTAL because the yielding of the cTALs is too low to carry out such a measurement, the observation that inhibition of cytochrome P450 can abolish the inhibitory effect of AII on the 70 pS K^+ channel in both cTAL and mTAL supports the notion that 20-HETE is involved in mediating the AII effect. A large body of evidence has supported the view that P450 metabolites of AA, such as 20-HETE, are involved in mediating several cellular events (McGiff, 1991). 20-HETE inhibits the Na^+-K^+-ATPase in the renal proximal tubule (Schwartzman et al., 1985) and $Na^+/2Cl^-/K^+$ cotransporter in the TAL (Escalante et al., 1991). 20-HETE has also been shown to be involved in modulating tubulo-glomerular feedback (Zou et al., 1994). Recent studies have shown that P450 metabolites such as 20-HETE are important second messengers which are involved in mediating the stimulation of the Ca^{2+}-sensing receptor in the TAL (Wang et al., 1996). The present study provides further evidence to support the notion that P450 metabolites of AA such as 20-HETE play a key role in the regulation of tubule transport of the TAL.

Since the release of AA is a rate-limited step for 20-HETE formation (Bonventre and Nemenoff, 1991), it is conceivable that release of AA is enhanced by AII. Two types of AII receptors, AT_1 and AT_2, have been identified in the kidney (Casparo and Levens, 1994). The AT_1 receptor family which is predominant in both proximal and distal nephron has been shown to couple to phospholipase A2, C, or D via G-protein. Stimulation of phospholipase A2, C, or D can enhance the release of AA (Bonventre and Nemenoff, 1991; Douglas and Hopfer, 1994). The mechanism by which AII increases the release of AA is not known and needs to be further explored. The observation that inhibition of the P450 metabolic pathway failed to block the AII effect completely indicates that a mechanism other than increase in production of 20-HETE was also involved. Recently, it has been shown that AA inhibited vasopressin-induced cAMP formation (Firsov et al., 1995). Since the 70 pS K^+ channel is activated by cAMP-dependent protein kinase (unpublished observations), inhibition of cAMP formation by AA could result in decrease in channel activity.

The effect of AII on ion transport in the proximal tubule is biphasic: high-concentrations ($\geq 10^{-7}$ M) of AII inhibit and low concentrations ($\leq 10^{-9}$ M) stimulate the fluid reabsorption (Wang and Chan, 1989). We have also observed the biphasic effect of AII on the activity of the 70 pS K^+ channel. However, it was observed that a low concentration (50 pM) inhibits whereas a high concentration (50–100 nM) stimulates the channel activity, suggesting that the dose response of AII differs between proximal tubule and the TAL.

Several possibilities may explain the stimulatory effect of AII on channel activity. First, a high concentration of AII may suppress the generation of 20-HETE. Second, PKC may be responsible for the stimulatory effect of AII, since PKC has been shown to mediate stimulatory effects on fluid and HCO_3^- transport in the proximal tubule (Wang and Chan, 1989). Finally, NO may be involved in mediating the AII-induced increase in channel activity. The observations that 50 nM AII still increased 20 HETE production and that inhibition of the P450 metabolic pathway failed to abolish the stimulatory effect of AII exclude the first possibility. The second possibility is also largely excluded by the observation that inhibition of PKC failed to abolish the stimulatory effect of AII. In support of the notion that NO may be involved in mediating the stimulatory effect of AII, the AII-induced increase in channel activity was completely abolished by inhibition of NOS and addition of an exogenous NO donor increased channel activity in the presence of the inhibitor of NOS. Interestingly, addition of 50 nM AII led to a decrease of channel activity in the presence of L-NAME instead of the increase observed in its absence, suggesting that the inhibitory effect of AII was suppressed by NO, or alternatively, that the effect of NO overrides the inhibitory effect of AII on channel activity.

Several studies have shown that NO is involved in modifying the action of AII in the kidney (Nicola et al., 1992; Braam and Koomans, 1995). The mechanism by which AII increases the production of NO is not known. AII may stimulate the release of cell Ca^{2+}. Indeed, it has been shown that AII increases intracellular Ca^{2+} concentration in proximal tubular cells (Norman et al., 1987). Since Ca^{2+} is a powerful stimulator for NOS (Knowles and Moncada, 1994), an increase in the NO production is expected.

The importance of NO in the regulation of renal blood flow has been well documented (Raij, 1993). Recently, several studies indicated that NO also plays a key role in the regulation of tubule function (Tojo et al., 1994; Weiner et al., 1995; Lu and Wang, 1996; Stoos et al., 1992). Previous studies have shown that NO activates the basolateral K^+ channel in the cortical collecting duct (Lu and Wang, 1996). The effect of NO on channel activity is mediated by cGMP, since addition of cGMP mimics the effect of NO. Several studies have demonstrated the presence of NOS in the TAL (Terada et al., 1992; Ahn et al., 1994; Morrissey et al., 1994; Bachmann et al., 1995). Although we have shown that NO stimulates the activity of the 70 pS K^+ channel in

the TAL, it is not known whether NO directly or indirectly affects the 70 pS K^+ channel. However, our recent experimental results suggest that the effect of NO is mediated by a cGMP-dependent pathway since cGMP can mimic the effect of NO on the 70 pS K^+ channel (Wang, unpublished observation). We need further experiments to confirm the finding and to explore the mechanism by which NO stimulates the 70 pS K^+ channel in the TAL.

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