Angiotensin II Stimulates Basolateral 10-pS Cl Channels in the Thick Ascending Limb

Peng Wu, Mingxiao Wang, Haiyan Luan, Lili Li, Lijun Wang, Wen-Hui Wang, Ruimin Gu

Abstract—Chloride channels in the basolateral membrane play a key role in Cl absorption in the thick ascending limb (TAL). The patch-clamp experiments were performed to test whether angiotensin II (AngII) increases Cl absorption in the TAL by stimulating the basolateral 10-pS Cl channels. AngII (1–100 nM/L) stimulated the 10-pS Cl channel in the TAL, an effect that was blocked by losartan (angiotension AT1 receptor [AT1R] antagonist) but not by PD123319 (angiotensin AT2 receptor [AT2R] antagonist). Inhibition of phospholipase C or protein kinase C also abolished the stimulatory effect of AngII on Cl channels. Moreover, stimulation of protein kinase C with phorbol-12-myristate-13-acetate mimicked the effect of AngII and increased Cl channel activity. However, the stimulatory effect of AngII on Cl channels was absent in the TAL pretreated with diphenyleneiodonium sulfate, an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Moreover, treatment of the TAL with diphenyleneiodonium sulfate also blocked the effect of phorbol-12-myristate-13-acetate on the 10-pS Cl channel. Western blotting demonstrated that incubation of isolated TAL with AngII increased phosphorylation of p47phox at Ser304, suggesting that AngII stimulates the basolateral Cl channels by increasing NADPH oxidase–dependent superoxide generation. This notion was also supported by the observation that H2O2 significantly increased 10-pS Cl channel activity in the TAL. We conclude that stimulation of AT1R increased the basolateral Cl channels by activating the protein kinase C–dependent NADPH oxidase pathway. The stimulatory effect of AngII on the basolateral Cl channel may contribute to AngII-induced increases in NaCl reabsorption in the TAL and AngII-infuse–induced hypertension. (Hypertension. 2013;61:1211-1217.)

Key Words: angiotensin II receptor ■ ClC-Kb channel ■ hypertension ■ NADPH oxidase ■ protein kinase C

The thick ascending limb (TAL) is responsible for reabsorption of 25% filtered Na and Cl load and plays a key role in urinary concentrating mechanisms.1-3 The transepithelial Cl transport in the TAL is a 2-step process: Cl enters the cell through type II Na-K-Cl cotransporters (NKCC2) in the apical membrane and exits the basolateral membrane through either Cl channels or KCl cotransporters.5 Although type I KCl cotransporter has been shown to be expressed in the basolateral membrane of the TAL,6 the role of KCl cotransporter in mediating Cl exit is not understood. Therefore, it is generally accepted that Cl channels in the basolateral membrane of the TAL play an important role in mediating Cl exit and regulating transepithelial Cl absorption. Patch-clamp experiments have identified a 10-pS Cl channel as the main type of Cl channels in the basolateral membrane of the TAL. Moreover, ClC-K2 is mostlikely the pore-containing component of the 10-pS Cl channel.7,8 A large body of evidence supports the role of angiotensin II (AngII) in modulating renal Na transport in different nephron segments. In the proximal tubule, AngII at low dose has been shown to stimulate fluid and bicarbonate absorption by activating Na/H exchangers.10 Microperfusion studies have shown that AngII stimulated Na, bicarbonate, and fluid absorption in the early distal nephron of rat kidney.11 AngII infusion has been shown to increase furosemide-sensitive oxygen consumption in the TAL of rat kidneys, an indication of augmented NaCl absorption in the TAL.12 However, the NaCl absorption in the TAL requires the involvement of several ion transporters, such as Na-K-ATPase, NKCC2, ROMK, and ClC-K2. The aim of the present study was to examine the hypothesis that AngII-induced stimulation of NaCl transport is partially achieved by activating the basolateral Cl channels in the outer medullary TAL (mTAL).

Methods

Preparation of the TAL

Sprague–Dawley rats of either sex (<90 g) were purchased from the animal facility of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). The animals were kept on a normal rat chow with free access to water. We removed both kidneys after the animal was killed by cervical dislocation. We followed the methods described by Guinamard et al7 for the preparation of the TAL. The kidney was cut into 1-mm-thick slices with a razor blade, and the kidney slices were incubated in a HEPES buffer solution containing collagenase type 1A (1 mg/mL; Sigma, St. Louis, MO) at 37°C for 45 to 60 minutes. After the collagenase treatment, the kidney slices were gently rinsed with a HEPES-buffered solution containing the following (in mmol/L): NaCl 140, KCl 5, MgCl2 1.8, CaCl2 1.8, and HEPES 10 (pH 7.4) at 4°C for 30 minutes. After rinsing, the TAL was dissected from the outer stripe of the outer medulla for the experiments, as described previously.13 The animal protocol was approved by the animal care and use committee of Harbin Medical University.
Patch-Clamp Technique
The method for the patch-clamp experiments has been described previously,13 and the pipette solution contains (in mmol/L) NaCl 140, MgCl₂, 1.8, and HEPES 10 (pH 7.4). Channel activities were defined as \( N_P \), a product of channel open probability (\( P_o \)) and channel number (\( N \)). The \( N_P \) was calculated from data samples of 60-s duration in the steady state as follows:

\[
N_P = \sum (1t_1 + 2t_2 + \cdots + it_i),
\]

where \( t_i \) is the fractional open time spent at each of the observed current levels. The slope conductance of the channel was determined by measuring Cl currents at several holding potentials.

Western Blot
Equal amounts of protein (80 μg) extracted from isolated medullary TAL were separated by electrophoresis using 12% SDS-PAGE and transferred to pure nitrocellulose blotting membranes (Pall Life Sciences). After blocking in 0.1% Tween-Tris-buffered saline (TBS-T) containing 5% nonfat dry milk, the membranes were incubated overnight at 4°C with the corresponding primary antibody. The membranes were washed 4x (10 minutes for each wash) with Tween-Tris-buffered saline, followed by incubation with horseradish peroxidase–conjugated secondary antibodies at room temperature for 1 hour. Protein bands were detected using the enhanced chemiluminescence detection system (Thermo Fisher Scientific Inc) and quantified by densitometry using Quantity One software (Bio-Rad).

Chemicals and Antibodies
Antiphospho-p47phox (P-p47phox)at Ser 304 and anti-p47 phox antibodies were obtained from Sigma. AngII, phorbol-12-myristate-13-acetate (PMA), diphenyleneiodonium sulfate (DPI), apocynin, calphostin C, U73122, losartan, and PD123319 were obtained from Sigma. AngII, phorbol-12-myristate-13-acetate (PMA), diphenyleneiodonium sulfate (DPI), apocynin, calphostin C, U73122, PMA, calphostin C, apocynin, and DPI were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the bath was <0.1% which had no significant effect on channel activity.

Statistical Analysis
Data are shown as mean±SEM. We used paired Student \( t \) tests or 1-way ANOVA test to determine the significance of the difference between the control and the experimental groups. Statistical significance was taken as \( P<0.05 \).

Results
Previous patch-clamp experiments demonstrated the presence of 2 types of Cl channels, a 10 pS and a 20 to 40 pS, in the basolateral membrane of the TAL.7 Moreover, the 10-pS Cl channel was a main type of Cl channel expressed in the basolateral membrane. We confirmed the previous finding and further examined the effect of AngII on basolateral 10-pS Cl channels in the TAL. Figure 1 illustrates a channel recording performed in a cell-attached patch showing that application of AngII (100 nmol/L) stimulated basolateral 10-pS Cl channels. The experiments were performed in a cell-attached patch, and holding potential was −60 mV (hyperpolarization). The top trace shows the time course of the experiments, and 2 parts of the traces indicated by numbers are extended to show the fast time resolution. The channel closed level is indicated by a dotted line and C, and the arrow indicates the addition of AngII. The pipette solution contained 140 mmol/L NaCl, 1.8 mmol/L MgCl₂, and 10 mmol/L HEPES (pH 7.4), and the bath solution was composed of 140 mmol/L NaCl, 5 mmol/L CaCl₂, 1.8 mmol/L KCl, 1.8 mmol/L CaCl₂, 1.8 mmol/L MgCl₂, and 10 mmol/L HEPES (pH 7.4).

Figure 1. Angiotensin II (AngII) stimulates the basolateral 10-pS Cl channels in the thick ascending limb. A channel recording shows the effect of 100 nmol/L AngII on the basolateral 10-pS Cl channels. The experiment was performed in a cell-attached patch, and holding potential was −60 mV (hyperpolarization). The top trace shows the time course of the experiments, and 2 parts of the traces indicated by numbers are extended to show the fast time resolution. The channel closed level is indicated by a dotted line and C, and the arrow indicates the addition of AngII. The pipette solution contained 140 mmol/L NaCl, 1.8 mmol/L MgCl₂, and 10 mmol/L HEPES (pH 7.4), and the bath solution was composed of 140 mmol/L NaCl, 5 mmol/L CaCl₂, 1.8 mmol/L KCl, 1.8 mmol/L CaCl₂, 1.8 mmol/L MgCl₂, and 10 mmol/L HEPES (pH 7.4).

2B). However, it completely abolished the effect of AngII on the 10-pS Cl channels. Figure 3A illustrates a channel recording performed in a cell-attached patch showing that application of AngII failed to stimulate the Cl channels. Results from 7 experiments are summarized in Figure 2B, demonstrating that \( N_P \) was 1.04±0.07 in the presence of 100 nmol/L AngII in the TAL treated with losartan. In contrast, inhibition of AT₂R failed to abolish the effect of AngII. Figure 3B illustrates a channel recording showing that AngII was still able to stimulate the Cl channels in the presence of PD123319. Results summarized in Figure 2B show that AngII increased channel activity from 1.02±0.1 (PD123319 alone) to 2.04±0.1 (AngII+PD123319) in the TAL treated with 10 μmol/L PD123319 (n=7). Therefore, the results strongly suggest that AT₂R was responsible for the stimulatory effect of AngII on the 10-pS Cl channels.

Stimulation of AT₂R has been shown to activate phospholipase C through the G₉ protein.14 Thus, we examined the role of phospholipase C in mediating the effect of AngII on the basolateral 10-pS Cl channels. The experiments were performed in cell-attached patches, and the results are summarized in Figure 4. Inhibition of phospholipase C with 5-μmol/L U73122 had no significant effect on the Cl channels (\( N_P \) = 1.03±0.07; n=5). However, it blocked the stimulatory effect of AngII because channel activity in the presence of AngII (0.99±0.07; n=5) was not different from those in the absence of AngII. Next, we examined the role of protein kinase C (PKC) in mediating the effect of AngII on the Cl channels by investigating the effect of AngII in the TAL treated with
calphostin C. Inhibition of PKC did not significantly affect the Cl channel activity (NPo = 1.01±0.05; n=4); however, it abolished the effect of AngII on the Cl channels. Results summarized in Figure 4 demonstrate that AngII failed to increase the channel activity in the presence of calphostin C (NPo = 1.02±0.05; n=4). The role of PKC in stimulating 10-pS Cl channels was also demonstrated by examining the effect of PMA on the Cl channels. Figure 5 illustrates a channel recording showing that the addition of 10-μmol/L PMA stimulated the 10-pS Cl channels in the TAL. Figure 4 summarizes the results from 8 experiments showing that treatment of the TAL with PMA significantly increased channel activity to 1.82±0.1. Therefore, the results strongly suggest that AngII stimulates 10-pS Cl channels by phospholipase C and PKC pathways.

AngII has been shown to activate NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NOX) by a PKC-dependent pathway. Moreover, increases in superoxide anions play a role in stimulating Na transport in the TAL. To test whether AngII stimulates the 10-pS Cl channels by activating NOX, we examined the effect of AngII on the phosphorylation of p47phox at serine residue 304 (Ser304), which is a PKC phosphorylation site and serves as an indication of activation NOX. The isolated medullary TALs were incubated with 100 nmol/L AngII for 5 minutes and the proteins from TAL lysates were resolved by SDS gel. Figure 6A illustrates Western blot analysis, showing that AngII incubation increased the phosphorylation of p47phox (P-p47phox) by 145±25% (n=5), an effect that was abolished by calphostin C. Results summarized in Figure 6B show that calphostin C had no significant effect on the phosphorylation of p47phox (115±10% of the control). However, in the presence of calphostin C, AngII failed to increase the phosphorylation of p47phox (122±10% of the control). Moreover, incubation of the TAL with PMA also increased the phosphorylation of p47phox by 90±20% (n=5). Thus, AngII is able to stimulate NOX by increasing PKC-dependent phosphorylation of p47phox.

Next, we investigated whether the stimulatory effect of AngII on the Cl channels was induced by activating NOX by...
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examining the effect of AngII on the Cl channels in the TAL treated with DPI, an inhibitor of NOX.19 Figure 7 illustrates a recording, showing that inhibition of NOX with 10-μmol/L DPI did not significantly change the Cl channel activity. However, addition of AngII failed to stimulate the 10-pS Cl channels in the TAL treated with DPI. Results summarized in Figure 8 show that unlike the effect of AngII on the Cl channels in the absence of DPI, AngII did not increase Cl channel activity \( (NP_o=0.98\pm0.05; n=5) \) in the presence of DPI. Because DPI has been shown to have an effect other than inhibiting NOX,20 we also used apocynin, which has a different chemical structure and inhibits NOX, to examine the role of NOX in mediating the effect of AngII on the 10-pS Cl channels. Figure 8 shows that inhibition of NOX with apocynin did not significantly affect the Cl channel activity \( (NP_o=1.10\pm0.05; n=4) \), but it abolished the effect of AngII on the Cl channels in the TAL \( (NP_o=0.99\pm0.1; n=4) \). Also, inhibition of NOX with DPI blocked the effect of PMA on the Cl channels \( (NP_o=1.03\pm0.05; n=7; \text{Figure 8}) \). Thus, results suggest that the stimulation of NOX by PKC plays an important role in mediating the stimulatory effect of AngII on the 10-pS Cl channels in the TAL. This notion was also supported by experiments in which the effect of \( H_2O_2 \) on the Cl channels was examined. Figure 9 illustrates a channel recording performed in a cell-attached patch showing that application of 10-μmol/L \( H_2O_2 \) activated the 10-pS Cl channels and increased channel activity from \( 1.06\pm0.05 \) to \( 1.83\pm0.1 \) \( (n=9) \). Data suggest that superoxide anion–related products stimulate the basolateral Cl channel activity in the TAL.

Discussion

Basolateral 10-pS Cl channels play an important role in the regulation of transepithelial Cl absorption in the TAL because they provide the major pathway for Cl exit. It has been reported that 10-pS Cl channel activity was observed in >80% patches in the basolateral membrane of forskolin-treated TALs.7 CIC-K2 is most likely to be the pore-forming component of the 10-pS Cl channel because it shares the biophysical properties of CIC-K221 and its regulatory mechanisms such as pH-sensitivity and stimulation by cAMP.8 Immunostaining and in situ hybridization confirmed that CIC-K2 was overwhelmingly expressed in the basolateral membrane of the TAL in the rat kidney,22 whereas CIC-K1 was mainly expressed in the thin ascending limb.23 The role of basolateral Cl channels in maintaining NaCl absorption in the TAL is best demonstrated by the observation that defective gene products encoding human basolateral Cl channel (CIC-Kb) and Barttin, a subunit of CIC-Kb, caused type III and IV Bartter syndrome, respectively.5 On the contrary, gain-of-function mutations of CIC-Kb have been reported to have predisposition to hypertension.24 Hence, the regulation of basolateral Cl channels is an important component for modulating epithelial transport in the TAL.

The main finding of the present study is that AngII stimulates the 10-pS Cl channels in the basolateral membrane of the TAL. We demonstrated that AngII, at concentration as low as 1 nmol/L, significantly increased the 10-pS Cl channel activity, suggesting that AngII plays a role in stimulating basolateral Cl channels under physiological conditions. The effect of AngII on the Cl channels was mediated by AT1R rather than AT2R because losartan abolished the effect of AngII. Our previous study showed that AngII also stimulated apical ROMK channels in the TAL.25 Because stimulation of ROMK channels is expected to enhance the K recycling across the apical membrane, thereby increasing NKCC2 activity, the observation that AngII stimulated the basolateral Cl channels in the...
TAL is consistent with the notion that AngII may stimulate transepithelial NaCl absorption. This is consistent with the report that AngII infusion enhanced furosemide-sensitive oxygen consumption in the TAL. However, a flux study performed in inner stripe of the outer medullary TAL demonstrated that AngII inhibited Cl absorption. The cause of the discrepancies between these 2 studies was not clear. One possibility is that different segments of the medullary TAL were used (the inner stripe versus the outer stripe used in the present study).

Two lines of evidence suggest that AngII stimulates the 10-pS Cl channels by activating PKC-dependent pathways: (1) the effect of AngII on the Cl channels was abolished by calphostin C and (2) PMA mimicked the effect of AngII and stimulated the Cl channels. Although we could not exclude the possibility that PKC-mediated phosphorylation of the Cl channels was involved in stimulating Cl channel activity, the stimulatory effect of PKC on the 10-pS Cl channels was, at least partially, the result of stimulation of NOX. This notion was supported by the finding that AngII stimulated phosphorylation of p47phox. The phosphorylation of p47phox is expected to facilitate the translocation of phosphorylated p47phox from cytosolic complexes to the plasma membrane, thereby interacting with gp91phox and p22phox complex and activating NOX. The role of PKC in mediating the stimulatory effect of AngII on NOX activity has been suggested in a variety of tissues.

Figure 7. Inhibition of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase diminishes the effect of angiotensin II (AngII) on the 10-pS Cl channels. A channel recording shows the effect of 100-nmol/L AngII on the basolateral 10-pS Cl channels in the thick ascending limb treated with 10-μmol/L diphenyleneiodonium sulfate (DPI). The experiment was performed in a cell-attached patch, and holding potential was −60 mV (hyperpolarization). The top and bottom traces were recorded from the same patch. The channel closed level is indicated by C and a dotted line.

Figure 8. Inhibition of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase abolishes the effect of angiotensin II (AngII) and phorbol-12-myristate-13-acetate (PMA) on the 10-pS Cl channels. A bar graph summarizes the experiments in which the effect of AngII or PMA on the 10-pS Cl channels was examined in the thick ascending limb treated with diphenyleneiodonium sulfate (DPI) or apocynin. All experiments were performed in cell-attached patches at −60 mV, and the channel activity was determined at the steady state of each treatment.

Figure 9. H2O2 stimulates the 10-pS Cl channel. A channel recording shows the effect of H2O2 on the basolateral 10-pS Cl channels. The experiment was performed in a cell-attached patch, and holding potential was −60 mV (hyperpolarization). The top trace shows the time course of the experiments, and 3 parts of the traces indicated by numbers are extended to show the fast time resolution. The channel closed level is indicated by a dotted line and C.
Moreover, 2 additional pieces of evidence supported the role of NOX in mediating the effect of AngII on the 10-pS Cl channels: (1) inhibition of NOX abolished the effect of AngII on the Cl channels and (2) addition of H2O2 activated the Cl channels. In addition to being an activator, however, PKC could be a mediator for superoxide-induced effect. It has been suggested that superoxide anions or their related products could activate PKC by oxidizing N-terminal regulatory domain containing zinc-binding, cysteine-rich motifs, thereby stimulating PKC. It is possible that H2O2-induced stimulation of Cl channel was the result of activating a different type of PKC, which further stimulates the 10-pS Cl channel by phosphorylation.

AngII has been shown to stimulate the generation of superoxide anions in a variety of tissues, such as heart, vascular tissue, and kidney. AngII-induced increases in superoxide anions play an important role in AngII-mediated hypertension and organ damage. We speculate that AngII-induced stimulation of basolateral Cl channels in the TAL contributes to AngII-induced hypertension. Moreover, we and others have demonstrated that the effect of AngII on superoxide generation is mediated by AT1R. It has been shown that NOXII and NOX4 are mainly expressed in the kidney. We have previously demonstrated that the generation of superoxide and related products was significantly attenuated in gp91phox(−/−) mice, suggesting that NOXII plays a role in generating superoxide in the renal tubule. Accumulated evidences have indicated that superoxide anion–related products play a role in stimulating renal Na absorption. It has been reported that superoxide anion–related products stimulated luminal Na/H exchange and Na/K/2Cl cotransporter in the TAL. We now demonstrated that superoxide anion–related products stimulate the basolateral Cl channels. Although the mechanism by which H2O2 activates the Cl channel is not known, it could activate the Cl channels directly by oxidative modulation of the Cl channel protein or by activating PKC as discussed above. However, we could not speculate whether superoxide or H2O2 is the likely activator of Cl channel in vivo. Stimulation of Cl channels should facilitate the Cl exit across the basolateral membrane, thereby decreasing the intracellular Cl concentration. A low intracellular Cl concentration has been shown to enhance the phosphorylation of NKCC2 by Ste20-related proline/alanine-rich kinase and to activate NKCC2. Therefore, it is conceivable that superoxide anion–induced stimulation of NaCl absorption in the TAL may result from stimulating both NKCC2 and the basolateral 10-pS Cl channels.

**Perspectives**

The physiological importance of the present study is to illustrate that AngII stimulated the 10-pS Cl channel in the basolateral membrane of the TAL. The AngII-induced increases in Cl channel activity may contribute to AngII-dependent salt-sensitive hypertension and may also play a role in augmenting NaCl absorption during Na-restriction. Figure 10 is a cell scheme illustrating a possible mechanism by which AngII activates the basolateral Cl channels in the TAL. Stimulation of AT1R by AngII activates PKC, which, in turn, activates NOX and increases superoxide anions. An increase in superoxide anions and related products activates Cl channels in the TAL. Thus, it is conceivable that enhanced salt absorption induced by high level of superoxide anions is partially achieved by stimulating basolateral Cl channels in the TAL.

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**Disclosures**

None.

**References**

Angiotensin II (AngII) stimulates the basolateral 10-pS Cl channels in the thick ascending limb. The finding provides a mechanism by which AngII stimulates NaCl absorption in the thick ascending limb.

**Novelty and Significance**

**What Is New?**

- Angiotensin II (AngII) stimulates the basolateral 10-pS Cl channels in the thick ascending limb.

- The effect of AngII on the Cl channels is the result of increasing superoxide anions.

**What Is Relevant?**

- This mechanism should be relevant for AngII-induced hypertension.

**Summary**

We illustrate the mechanism by which stimulation of AT1R activates the basolateral Cl channels. AngII-induced activation of NOX plays a role in stimulating basolateral Cl channels in the thick ascending limb. Upregulated Cl channel activity in the thick ascending limb induced by AngII may play a role in AngII-dependent hypertension.
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