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Angiotensin II diminishes the effect of SGK1 on the WNK4-mediated inhibition of ROMK1 channels

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ROMK1 channels are located in the apical membrane of the connecting tubule and cortical collecting duct and mediate the potassium secretion during normal dietary intake. We used a perforated whole-cell patch clamp to explore the effect of angiotensin II on these channels in HEK293 cells transfected with green fluorescent protein (GFP)-ROMK1. Angiotensin II inhibited ROMK1 channels in a dose-dependent manner, an effect abolished by losartan or by inhibition of protein kinase C. Furthermore, angiotensin II stimulated a protein kinase C-sensitive phosphorylation of tyrosine 416 within c-Src. Inhibition of protein tyrosine kinase attenuated the effect of angiotensin II. Western blot studies suggested that angiotensin II inhibited ROMK1 channels by enhancing its tyrosine phosphorylation, a notion supported by angiotensin II's failure to inhibit potassium channels in cells transfected with the ROMK1 tyrosine mutant (R1Y337A). However, angiotensin II restored the with-no-lysine kinase-4 (WNK4)-induced inhibition of R1Y337A in the presence of serum–glucocorticoids-induced kinase 1 (SGK1), which reversed the inhibitory effect of WNK4 on ROMK1. Moreover, protein tyrosine kinase inhibition abolished the angiotensin II-induced restoration of WNK4-mediated inhibition of ROMK1. Angiotensin II inhibited ROMK channels in the cortical collecting duct of rats on a low sodium diet, an effect blocked by protein tyrosine kinase inhibition. Thus, angiotensin II inhibits ROMK channels by two mechanisms: increasing tyrosine phosphorylation of the channel and synergizing the WNK4-induced inhibition. Hence, angiotensin II may have an important role in suppressing potassium secretion during volume depletion.

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ROMK1 channels are located in the apical membrane of the connecting tubule and the cortical collecting duct (CCD) and have an important role in mediating K secretion under normal dietary K intake.[1–3] Our previous study has demonstrated that Src-family protein tyrosine kinase (PTK) is involved in increasing inhibition of ROMK1 channels during K restriction.[3] The effect of PTK on ROMK1 channels is at least partially the result of increasing tyrosine phosphorylation of ROMK1 channels on tyrosine residue 337.[4,5] Consequently, tyrosine-phosphorylated ROMK1 channels are subjected to endocytosis.[6] In addition, ROMK1 channels are also inhibited by with-no-lysine kinase (WNK) family kinases including WNK1, WNK3, and WNK4.[7–10] It has been demonstrated that WNK1 and WNK4 stimulate the internalization of ROMK channels by a mechanism involving intersection, a scaffolding protein, and clathrin.[10] Recently, we have reported that Src-family PTK is also involved in modulating the interaction between serum–glucocorticoids-induced kinase 1 (SGK1) and WNK4 by diminishing the SGK1-mediated phosphorylation of WNK4.[11] Consequently, SGK1 failed to reverse the inhibitory effect of WNK4 on ROMK channels in the presence of Src-family PTK. However, the physiological factor that stimulates Src-family PTK is not known. Our previous study has demonstrated that stimulation of angiotensin II (AngII) type I receptor (AT1R) inhibited ROMK channel activity through a PTK-dependent pathways in the native CCD of rats on a low K diet.[12] Moreover, AngII has been shown to stimulate Src-family PTK in vascular tissue.[13] Therefore, we speculate that AngII may be one of the upstream factors that stimulate Src-family PTK and modulate the effect of SGK1 on WNK4 under physiological conditions. Hence, the goal of this study is to test whether AngII also inhibits ROMK channels by enhancing the inhibitory effect of WNK4 on ROMK1 channels and to test whether AngII decreases ROMK channels in the native CCD.

RESULTS

We used HEK293T cells transfected with green fluorescent protein (GFP)-ROMK1 to study the effect of AngII on ROMK channels. Figure 1a is a typical western blot carried out with lysates from HEK293T cells and rat kidney (cortex) and demonstrates the expression of endogenous AT1R in HEK 293T cells. We measured Ba^{2+}-sensitive K currents with the perforated whole-cell recording technique under control

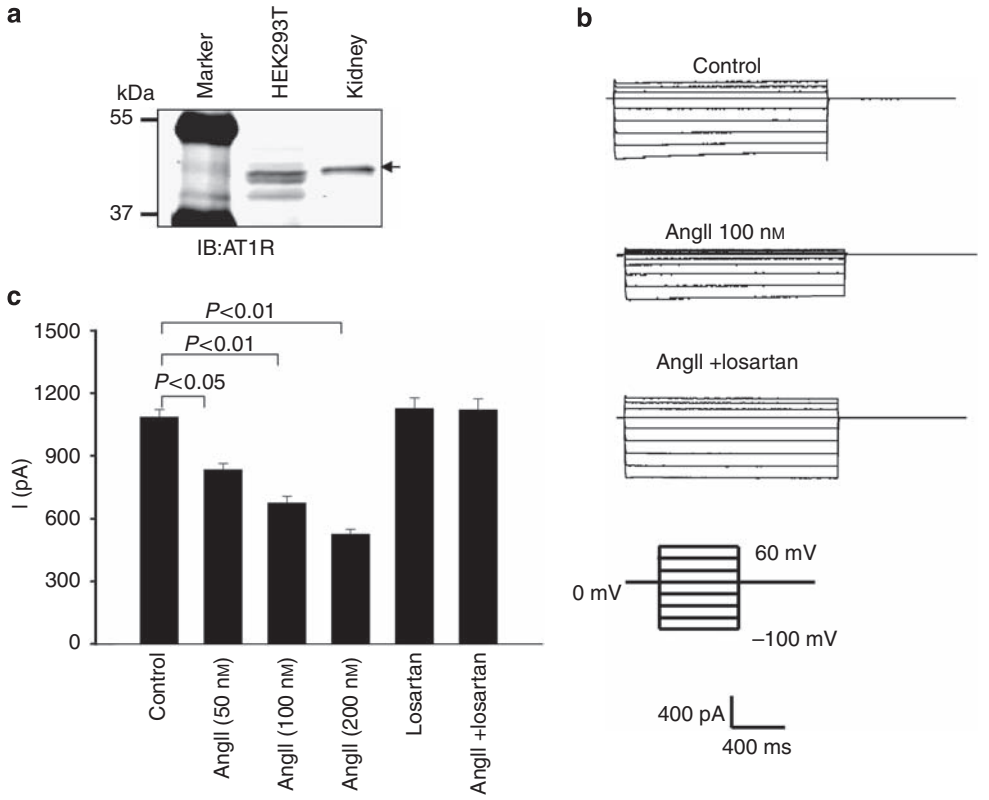

Figure 1 | Angiotensin II (AngII) inhibits ROMK1 channels. (**a**) A western blot showing the expression of type 1 Ang II receptor (AT1R) in HEK293T cells and the rat kidney. (**b**) Whole-cell Ba^{2+}-sensitive K currents in HEK293T cells transfected with green fluorescent protein (GFP)-ROMK1 in the absence of AngII (control) and in the presence of 100 nM AngII and AngII + losartan (20 μM), respectively. K currents were measured with the perforated whole-cell patch clamp from −100 to 60 mV at a step of 20 mV. (**c**) A bar graph summarizes the inhibitory effect of AngII on ROMK1 channels at different concentrations in the absence of or presence of losartan (20 μM). K currents were measured with perforated whole-cell patch clamp at −100 mV in HEK293T cells transfected with GFP-ROMK1. I (pA), currents (pA).

conditions and in the cells treated with AngII (100 nM) for 20 min. Figure 1b is a whole-cell patch recording showing that K currents in cells transfected with GFP-ROMK1 were inward rectified and that application of AngII (100 nM) inhibited ROMK1 channels. We also measured the cell capacitances, which varied between 24.5 and 26 pF. The value was used to normalize the K currents to that of a cell with 25 pF capacitance. The effect of AngII on ROMK channels was dose dependent: application of 50, 100, and 200 nM AngII decreased K currents from 1086 ± 35 pA ($n = 6$) to 833 ± 30 pA ($n = 6$), 678 ± 30 pA ($n = 6$), and 527 ± 22 pA ($n = 6$), respectively (Figure 1c). The effect of AngII on ROMK channels was the result of stimulation of AT1R because losartan completely abolished the inhibitory effect of AngII on the ROMK channels (Figure 1b). Data summarized in Figure 1c show that losartan (20 μM) *per se* has no significant effect on K currents (1127 ± 50 pA, $n = 6$) in comparison with the control value. However, 100 nM AngII failed to decrease K currents (1120 ± 52 pA, $n = 6$) in the presence of losartan.

After demonstrating that stimulation of AT1R inhibited ROMK channels, we examined the effect of AngII (100 nM) on ROMK1 channels in HEK293T cells pretreated with protein kinase C (PKC) inhibitors, calphostin C (100 nM), for 20 min. Although inhibition of PKC had no significant effect on ROMK channel activity, it abolished the effect of AngII. As calphostin C may have an effect other than inhibiting PKC, we repeated the experiments with GF109203x (5 μM). Data summarized in Figure 2 show that treatment of HEK293T cells transfected with ROMK1 with either calphostin C (100 nM) or GF109203x (5 μM) abolished the inhibitory effect of AngII on ROMK channels (control, 1090 ± 40 pA; calphostin C + AngII, 1110 ± 40 pA. GF109203x + AngII, 1100 ± 40 pA). Thus, the present results are in consistence with experiments performed in the rat CCD, in which inhibition of PKC blocked the effect of AngII on ROMK channels.[12] In that study the effect of AngII was also suppressed by blocking PTK.[12] To examine the role of PTK in mediating the effect of AngII on ROMK channels, we studied whether AngII increases tyrosine phosphorylation of c-Src on tyrosine residue 416, an indication of activated c-Src, which was used as a representative member of Src-family PTK. Figure 3a is a typical western blot from five experiments demonstrating that application of AngII at 5, 10, 25, 50, and 100 nM within 30 min enhanced the tyrosine phosphorylation of c-Src on the residue 416, whereas AngII did not change the total c-Src expression. Also, inhibition of PKC with calphostin C (100 nM) attenuated the

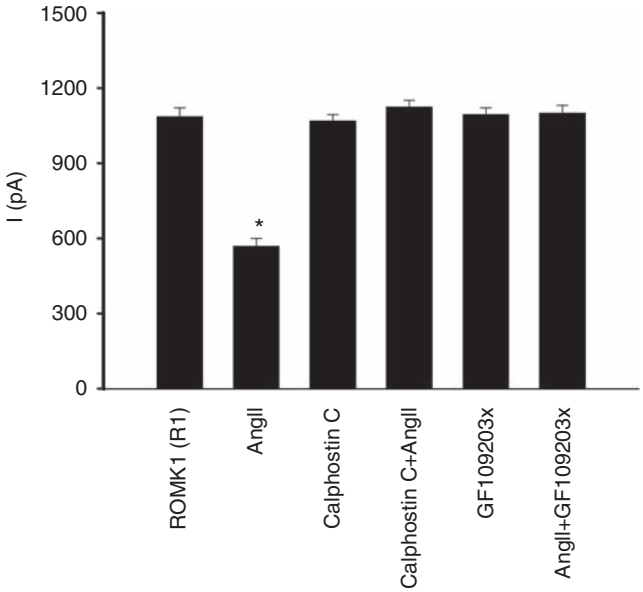

Figure 2 | Inhibition of protein kinase C abolishes the inhibitory effect of angiotensin II (AngII). A bar graph summarizes the effect of AngII on ROMK1 channels in the absence of or in the presence of calphostin C (100 nM) or GF109203x (5 μM). HEK293T cells transfected with green fluorescent protein-ROMK1 were pretreated with calphostin C or GF109203x. The K currents were measured with the perforated whole-cell recording at −100 mV. Asterisk (*) indicates a significant difference from the control value.

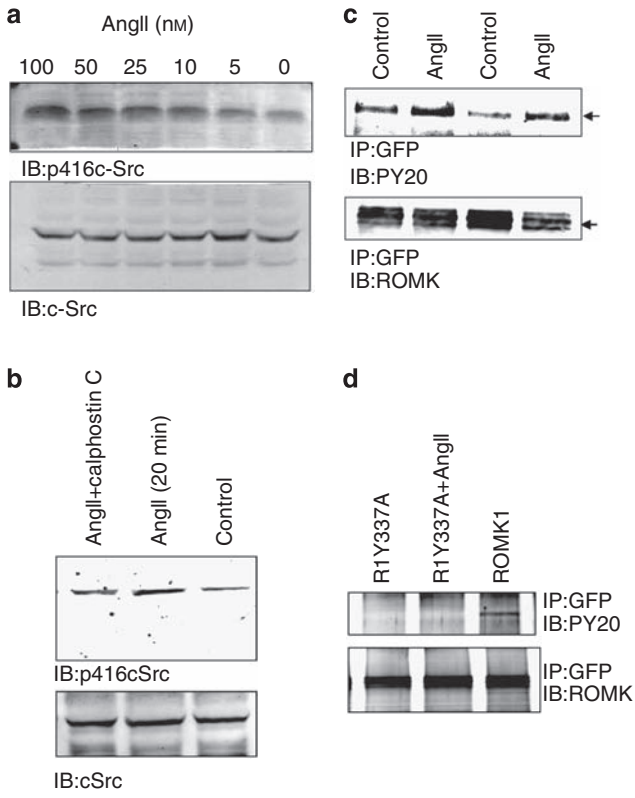

Figure 3 | Angiotensin II (AngII) stimulates c-Src phosphorylation and tyrosine phosphorylation of ROMK1. (**a**) A western blot showing that treatment of HEK293T cells with AngII for 20–30 min increases the tyrosine phosphorylation of c-Src on tyrosine residue 416. (**b**) A western blot demonstrating the effect of AngII on tyrosine phosphorylation of c-Src on tyrosine 416 in the HEK293T cells pretreated with calphostin C. A western blot shows the effect of AngII (100 nM) on the tyrosine phosphorylation of ROMK1 in cells transfected with green fluorescent protein (GFP-ROMK1) (**c**) or with GFP-R1Y337A (**d**). ROMK1 proteins were harvested by immunoprecipitation with anti-GFP antibodies and tyrosine phosphorylation of ROMK1 was detected with PY20.

AngII-induced stimulation of tyrosine phosphorylation of c-Src on tyrosine residue 416 ($n = 3$; Figure 3b), suggesting that AngII stimulates Src-family PTK by a PKC-dependent mechanism. Because ROMK1 channel is the substrate of Src-family PTK, increasing Src-family PTK activity by AngII is expected to stimulate tyrosine phosphorylation of ROMK1 channels. Therefore, we examined the effect of AngII on the tyrosine phosphorylation of ROMK1 channels in the HEK293T cells treated with AngII (100 nM) for 30 min. ROMK1 proteins were harvested by immunoprecipitation with anti-GFP antibodies and tyrosine phosphorylation of ROMK1 was detected by PY20, which reacts with tyrosine-phosphorylated proteins. Figure 3c is a western blot showing that AngII increased ROMK1 tyrosine phosphorylation. In contrast, the effect of AngII on ROMK tyrosine phosphorylation is absent in cells transfected with R1Y337A (Figure 3d). The notion that AngII-induced inhibition of ROMK1 channels was, at least partially, the result of enhancing tyrosine phosphorylation of ROMK1 channels was further tested by examining the effect of AngII on ROMK1 channels in the presence of herbimycin A. Figure 4a is a whole-cell channel recording showing that coexpression of c-Src or application of AngII decreased K currents measured with the perforated whole-cell recording technique and that inhibition of PTK with herbimycin A completely abolished the effect of c-Src and AngII on ROMK1 channels. Coexpression of c-Src enhanced c-Src expression is demonstrated in Figure 4b. Figure 4c summarizes the results from five experiments showing that AngII decreased K currents from 1090 ± 40 to 675 ± 30 pA, whereas c-Src expression reduced K currents from 1100 ± 40 to 530 ± 32 pA. In contrast, neither AngII nor c-Src expression decreased K currents in cells treated with herbimycin A (AngII + herbimycin A, 1300 ± 50 pA; c-Src + herbimycin A, 1350 ± 45 pA). The effect of AngII on ROMK1 channel is possibly mediated through stimulating phosphorylation of ROMK1 in tyrosine 337 because AngII failed to inhibit ROMK1 mutant, R1Y337A in which the tyrosine residue 337 was mutated (Figure 4d). Similar results were obtained with small interfering RNA (siRNA) of c-Src and Figure 5a is a whole-cell recording demonstrating the effect of down-regulation of c-Src on ROMK1 channels. To downregulate endogenous c-Src, we used two c-Src siRNAs, $siRNA_{832}$ and $siRNA_{1430}$, which target nucleotide sequence 832 and 1430 of c-Src, respectively. Figure 5b is a western blot demonstrating the expression of c-Src in HEK293T cells treated with two

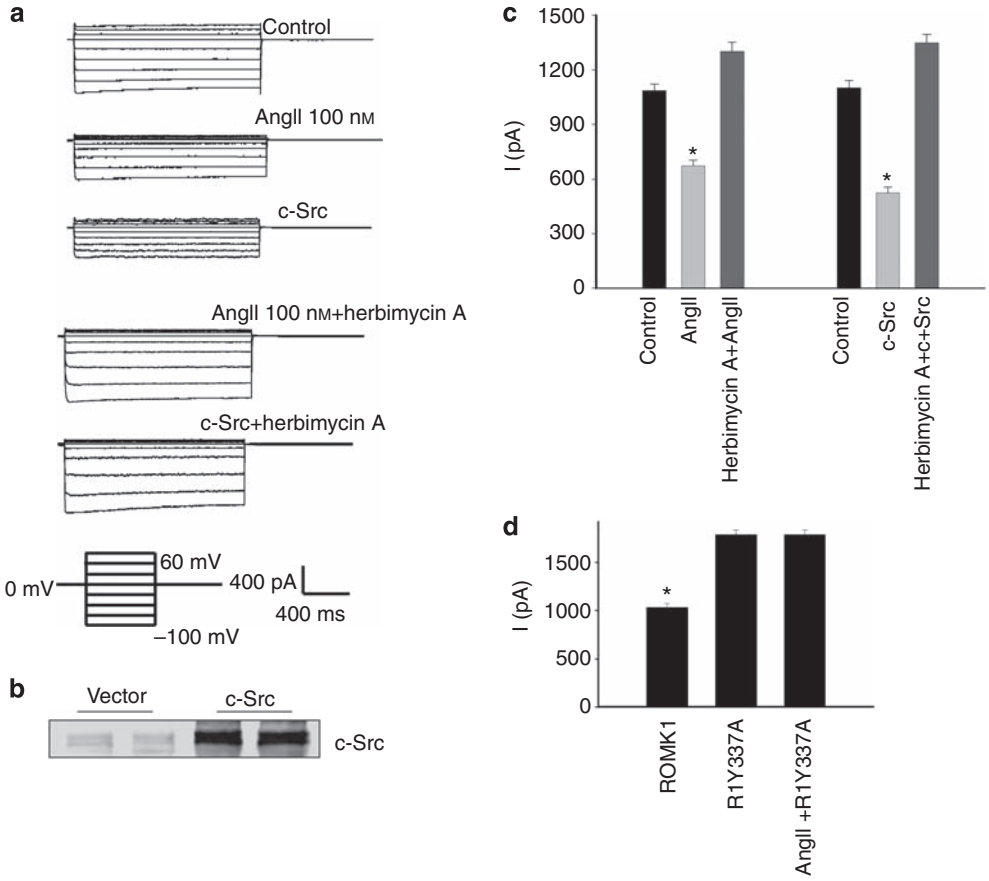

Figure 4 | Inhibition of protein tyrosine kinase abolishes the effect of Angiotensin II (AngII). (**a**) The whole-cell recording demonstrates the effect of c-Src and AngII treatment on K currents in HEK293T cells transfected with green fluorescent protein-ROMK1 with or without herbimycin A. (**b**) A western blot showing the expression of c-Src in HEK293T cells transfected with or without exogenous c-Src. (**c**) A bar graph summarizes the effect of c-Src expression or 100 nM AngII treatment on ROMK1 channels in the presence of herbimycin A (1 μM). (**d**) Effect of AngII (100 nM) on K currents in HEK cells transfected with R1Y337A. The K currents were measured with perforated whole-cell patch recording at −100 mV. Asterisk (*) indicates a significant difference from the control value.

kinds of c-Src-siRNA. It is apparent that both c-Src siRNAs specifically downregulate c-Src by 80 ± 15% ($n = 4$) but failed to change the expression of PKCε, AT1R, and actin. Data summarized in Figure 5c demonstrate that suppression of endogenous c-Src expression significantly increased K currents in cells transfected with ROMK1 from 1080 ± 40 to 1615 ± 40 pA (siRNA$_{832}$, $n = 5$) or 1557 ± 30 pA (siRNA$_{1430}$, $n = 5$), suggesting that ROMK1 channels were suppressed by endogenous c-Src. Moreover, AngII failed to inhibit ROMK channels in HEK293T cells treated with c-Src siRNA (1600 ± 40 pA for siRNA$_{832}$, 1516 ± 30 pA for siRNA$_{1430}$). Hence, these results support the role of c-Src in mediating the effect of AngII on ROMK1 channels.

PTK decreases ROMK channel activity not only by increasing tyrosine phosphorylation of ROMK channels but also by inhibiting the stimulatory effect of SGK1 on WNK4.[11] Therefore, it is possible that AngII may also inhibit ROMK channels by modulating the effect of SGK1 on WNK4 thereby enhancing WNK4-induced inhibition. This hypothesis was tested by examining whether AngII synergizes the inhibitory effect of WNK4 by reversing the effect of SGK1 on WNK4. We used the perforated whole-cell patch recording to measure the K currents in HEK293T cells transfected with GFP-R1Y337A, a ROMK1 mutant in which a tyrosine phosphorylation site was mutated to alanine. The advantage of using R1Y337A for the study is that AngII had no effect on ROMK1 channels, thereby making the interpretation straightforward. Figure 6a is a set of whole-cell patch recordings illustrating the K currents under different experimental conditions. Data summarized in Figure 6b shows that the expression of SGK1 not only significantly increased K currents in cells transfected with GFP-ROMK1Y337A from 1700 ± 50 to 2570 ± 90 pA ($n = 6$) but also abolished the inhibitory effect of WNK4 on ROMK channels: SGK1 increased K currents in cells transfected with WNK4 + GFP-ROMK1Y337A from 830 ± 30 pA ($n = 6$) to 1734 ± 70 pA ($n = 6$), a value not different from the control. Also, 100 nM AngII alone had no effect on ROMK1Y337A in cells transfected with SGK1 + ROMK1Y337A. However, in cells transfected with ROMK1Y337A + WNK4 + SGK1, 100 nM AngII decreased K currents from 1734 ± 70 to 950 ± 30 pA ($n = 6$), a value not significantly different from

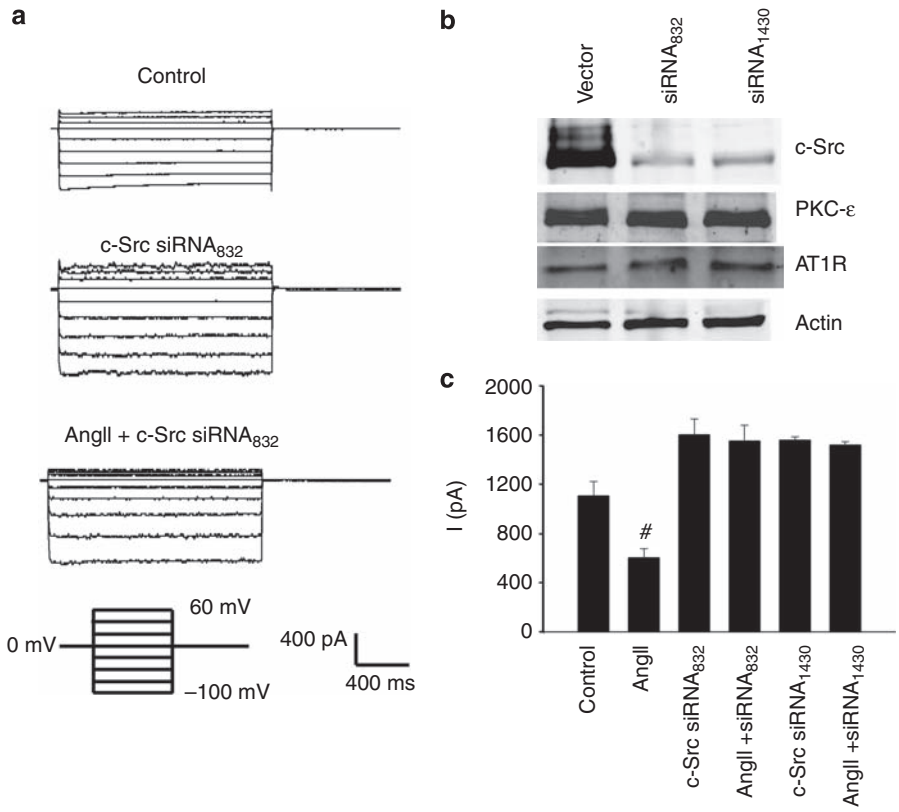

Figure 5 | Downregulation of endogenous c-Src abolishes the effect of angiotensin II (AngII). (**a**) The whole-cell recording demonstrates the effect of downregulating c-Src with small interfering RNA (siRNA), increasing K currents and abolishing the effect of AngII on ROMK1 channels in HEK293T cells transfected with green fluorescent protein-ROMK1. (**b**) A western blot showing the effect of c-Src siRNA on the expression of c-Src, protein kinase Cε (PKCε), AngII type I receptor (AT1R), and actin in HEK293T cells. (**c**) A bar graph summarizes the effect of siRNA of c-Src on AngII-induced inhibition of ROMK1 channels. The K currents were measured with perforated whole-cell patch recording at −100 mV. '#' Indicates significance from rest of groups.

those with WNK4 (830 ± 30 pA), and significantly lower than those with WNK4 + SGK1 (1730 ± 70 pA). The role of c-Src in mediating the effect of AngII was indicated by the observation that herbimycin A (1 μM) abolished the effect of AngII in cells transfected with WNK4 + SGK1 + ROMK1Y337A. The results, therefore, strongly indicate that AngII inhibited ROMK1 channels also by abolishing the effect of SGK1–WNK4 interaction.

The mechanism by which AngII inhibits ROMK channels in the presence of SGK1 may have a role in suppressing K secretion during volume depletion, which is known to stimulate aldosterone and SGK1 activity. Therefore, we used the patch clamp experiment to examine the effect of AngII on ROMK channels in the CCD of rats on a low Na diet to demonstrate the physiological relevance of the present finding. We confirmed the previous report that ROMK channel activity in rats on a low Na diet was not significantly different from those on a control diet.[14] Moreover, application of 50 nM AngII decreased ROMK channel activity from 1.1 ± 0.1 to 0.1 ± 0.05 ($n = 4$; Figure 7a). Figure 7b is a channel recording showing that AngII (50 nM) inhibited ROMK channels in the CCD of rats on a low Na diet. We then examined the effect of AngII on ROMK in the CCD treated with herbimycin A. Although herbimycin A also inhibits heat shock protein, HSP90, and is not a specific c-Src blocker,[15] Herbimycin A stimulated ROMK only in the animals on a K-deficient diet but not on a high K (HK) diet, which has a low c-Src activity.[16] This suggests that the effect of herbimycin A on ROMK channels was relatively specific and the result of inhibiting src-family PTK under our experimental conditions. Figure 7a summarizes the results of four experiments showing that treatment of the CCD with 1 μM herbimycin A for 20 min not only significantly increased channel activity (NP_o, 2.0 ± 0.3) but also abolished the effect of AngII on ROMK channels (NP_o, 2.05 ± 0.3). This suggests that AngII inhibited ROMK in the CCD through a PTK-dependent mechanism.

DISCUSSION

The main finding of this study is that AngII inhibited ROMK channels by a Src-family PTK-dependent pathway. The role of Src-family PTK in mediating the effect of AngII is supported by two lines of evidence: (1) AngII stimulates tyrosine phosphorylation of c-Src at tyrosine residue 416; (2) inhibition of PTK with herbimycin A or downregulation of c-Src with siRNA abolishes the effect of AngII on ROMK

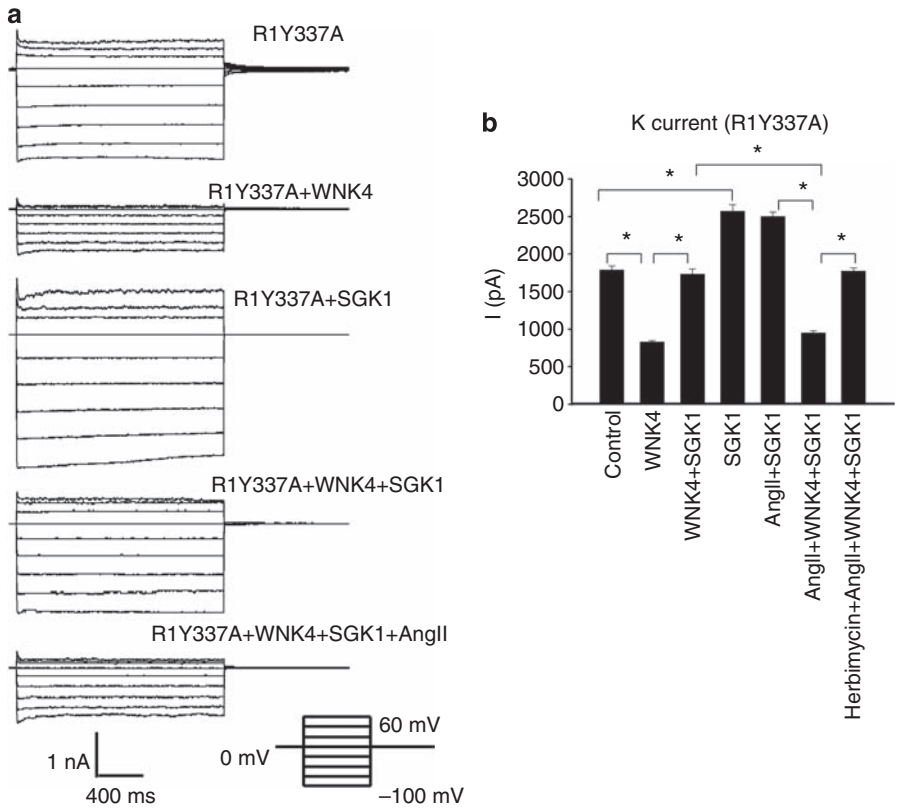

Figure 6 | Angiotensin II (AngII) modulates the effect of serum–glucocorticoids-induced kinase 1 (SGK1) and with-no-lysine kinase-4 (WNK4) on ROMK channels. (**a**) Recording showing the Ba^{2+}-sensitive K currents measured with perforated whole-cell patch clamp at −100 mV to 60 mV under different conditions. (**b**) A bar graph summarizes the experiments in which the K currents were measured at −100 mV in HEK293T cells transfected with green fluorescent protein-ROMK1Y337A (R1Y337A), WNK4 + R1Y337A, SGK1 + WNK4 + R1Y337A, and SGK1 + R1Y337A in the presence or absence of 100 nM AngII. Asterisk (*) indicates the significant difference ($P < 0.05$).

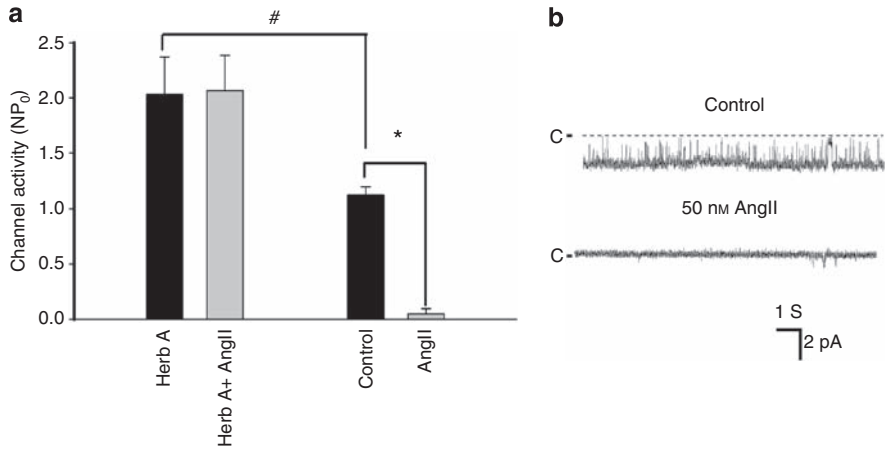

Figure 7 | Inhibition of PTK abolishes the effect of AngII on ROMK. (**a**) A bar graph summarizes the effect of 50 nM angiotensin II (AngII) on ROMK channels in the cortical collecting duct of rats on a low Na diet for 1 week in the presence or in the absence of herbimycin A (Herb A). The statistical significance is indicated by '#' ($P < 0.05$) and '*' ($P < 0.01$). (**b**) A channel recording showing the effect of AngII on ROMK channels in the cortical collecting duct of a rat on a low Na diet. Experiments were performed in cell-attached patches and the holding potential was 0 mV.

channels. Therefore, the present results are consistent with the previous study performed in the rat CCD, in which AngII inhibited ROMK channels by a PTK-dependent mechanism.[12] Moreover, this experiments strongly suggests that Src-family PTK inhibits ROMK channels not only by stimulating tyrosine phosphorylation of ROMK channels but also by abolishing the effect of SGK1–WNK4 interaction on ROMK channels. Our previous studies have shown that ROMK1 channels were phosphorylated by Src-family PTK on tyrosine residue 337 and that increase in tyrosine

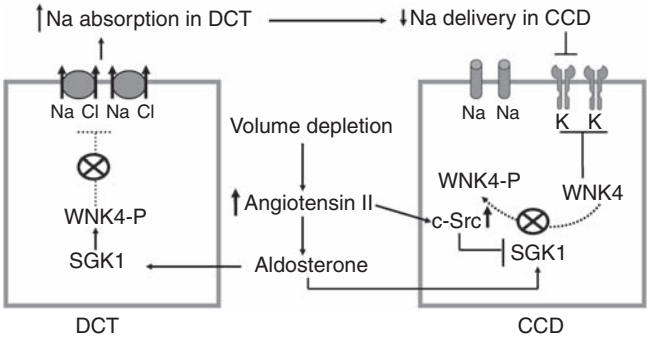

Figure 8 | A cell scheme illustrating the role of angiotensin II in regulating ROMK channels during the volume depletion. A dotted line means a diminished effect. Abbreviations: CCD, cortical collecting duct; DCT, distal convoluted tubule; SGK1, serum–glucocorticoids-induced kinase 1; WNK4, with-no-lysine kinase-4.

phosphorylation of ROMK1 channels facilitated the endocytosis.[5,6] The observation that AngII increased the tyrosine phosphorylation of ROMK strongly supports the notion that the inhibitory effect of AngII on ROMK1 channels is, at least partially, the result of increasing tyrosine phosphorylation. Relevant to the second possibility that AngII affects SGK1–WNK4 interaction is the finding that AngII restored the inhibitory effect of WNK4 on K channels in the presence of SGK1 in cells transfected with R1Y337A, which was not sensitive to either AngII or c-Src in the absence of WNK4.[11] This suggests that AngII enhances WNK4-induced inhibition of ROMK channels by suppressing the effect of SGK1 on WNK4. However, the effect of AngII in regulating ROMK channels *in vivo* has only been demonstrated in severe K-restricted animals and may not have a major role in inhibiting ROMK channels under control conditions.[12] Also, the concentrations used in this experiments were higher than physiological concentrations. Moreover, it has been reported that AngII has a biphasic effect on bicarbonate transport in the proximal tubules.[17] Because the sensitivity of AT1R to AngII may be different between native tissue and cultured cells, the aim of this experiments is to prove the principal that AngII has a role in modulating the effect of WNK4–SGK1 interaction on ROMK channels.

WNK4 is expressed in the aldosterone-sensitive distal nephron (ASDN) including connecting tubule and the CCD.[18] A large body of evidence has demonstrated that WNK4 inhibited ROMK channels by facilitating clathrin-dependent endocytosis.[10,19,20] The missense mutations of WNK4 cause type II pseudohyperaldosteronism, a disease characterized by hypertension and hyperkalemia.[21,22] However, WNK4-induced inhibition of ROMK channels can be reversed by SGK1, which phosphorylates WNK4 on serine residues 1169 and 1196.[20,23] Thus, the domain containing serine residues 1169 and 1196 is thought to be an aldosterone-dependent switch domain, which controls K secretion in ASDN.[20] Recently, we demonstrated that Src-family PTK is involved in suppressing SGK1-mediated serine phosphorylation of WNK4, thereby enhancing WNK4-mediated inhibition of ROMK channels.[11] However, the upstream signaling that stimulates Src-family PTK is not known. This study showed that AngII might be one of signaling pathways, which stimulate Src-family PTK hereby modulating the interaction between SGK1 and WNK4.

AngII has been shown to increase Src-family PTK activity by stimulating nicotinamide adenine dinucleotide phosphate oxidase (NOX) and increasing superoxide anion generation in vascular tissue.[13,24,25] PKC has been reported to mediate the stimulatory effect of AngII on NOXII activity in vascular tissue[25] by phosphorylating $p47^{Phox}$ and stimulating the activity of NOXII.[26,27] Phosphorylation of $p47^{Phox}$ is an important step for the activation of NOXII because phosphorylation of $p47^{Phox}$ results in the translocation of cytosolic complexes to the plasma membrane.[28] Because superoxide anions stimulate Src-family PTK,[29] PKC can increase Src-family PTK through increasing generation of superoxide anion. Our previous study has shown that AT1R has an important role in generating superoxide anions in the kidney[30] and that AngII-induced inhibition of ROMK channels in the CCD depends on PKC and NOX.[12] Moreover, the role of PKC in mediating the effect of AngII on Src-family PTK is also supported by the finding that inhibition of PKC diminished the effect of AngII on c-Src tyrosine phosphorylation.

A large body of evidence indicates that AngII has an important role in the regulation renal membrane transport in the distal nephron segments.[12,31–34] AngII has been shown to stimulate ENaC[32] and increase α-ENaC protein abundance.[31] Also, AngII has been reported to stimulate Cl absorption in the CCD of mice through a pendrin-dependent mechanism.[33] The role of AngII in the regulation of renal K excretion is also supported by micropuncture study, in which luminal AngII inhibits K secretion in the distal nephron from the distal convoluted tubule to the initial CCD.[35] Although AngII inhibits ROMK channels in the CCD, blocking AT1R may not affect the net renal K excretion because AngII has opposite effects on Na and K transport in the CCD. Thus, AngII-induced stimulation of ENaC would increase the driving force for K secretion and offset the AngII-induced inhibition of ROMK channels in the CCD. On the other hand, inhibiting renin–AngII is expected to decrease Na transport but increase apical K channel activity. Moreover, signaling pathways other than AngII may also be involved in activating Src-family PTK. For instance, both insulin-like growth factor and prostaglandin-2-dependent pathway are present in ASDN and stimulation of insulin-like growth factor and prostaglandin-2 pathway could also activate PKC and PTK[36–38] thereby suppressing the interaction of SGK1 and WNK4.

Figure 8 is a scheme illustrating the mechanism by which the volume-depletion stimulates Na absorption but suppresses K secretion. Low Na intake abolishes the inhibitory effect of WNK4 on NaCl cotransporter, thereby increasing Na absorption in the distal nephron and decreasing the Na delivery to the connecting tubule and the CCD. Consequently, a decrease in Na delivery in the connecting tubule and the CCD diminishes the driving force for K secretion. In addition, AngII pathway should also have a role

in preventing K loss during volume depletion, which is expected to increase aldosterone secretion and stimulate Na transport in the ASDN. Both Na restriction and a HK intake have been shown to increase aldosterone and SGK1. Aldosterone augments activity of both Na–K–adenosine triphosphatase and EnaC,[39,40] thereby increasing the driving force for K secretion across the apical membrane. However, aldosterone alone may not be sufficient to stimulate ROMK channel activity in the collecting duct because direct infusion of aldosterone failed to mimic the effect of a HK intake.[41] A HK intake is expected not only to stimulate aldosterone level but also to suppress renin and AngII signaling pathway.[42,43] Consequently, a HK intake stimulates ROMK channel activity and renal K secretion in ASDN. In contrast, Na- restriction stimulates both renin–AngII pathway and aldosterone and SGK1. Thus, an upregulated AngII signaling may be partially responsible for preventing K loss during volume depletion. This speculation is supported by the present finding that AngII inhibits ROMK channels in the CCD of rats on a low Na diet by PTK-dependent mechanism. However, we need further experiments to dissect the effects of PTK on ROMK channels through tyrosine phosphorylation of ROMK or by suppressing SGK1–WNK4 interaction. But, this study clearly suggests the role of AngII in inhibiting K secretion during volume depletion. We conclude that AngII inhibits ROMK channels in ASDN by two mechanisms: increasing tyrosine phosphorylation of ROMK channels and synergizing the WNK4-induced inhibition.

MATERIALS AND METHODS

Cell culture and transient transfection

HEK293T cells were purchased from the American Type Culture Collection and grown in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) in 5% CO_2 and 95% air at 37 °C. All proteins including c-Src and ROMK1 were transiently expressed in HEK293 cells and corresponding complementary DNAs were simultaneously used for the transfection at 50–70% confluence with the complementary DNA using Lipofectamine 2000 transfection reagent (Invitrogen). After transfection the cells were incubated for an additional 24 h before use. The method for treatment of cells with siRNA was similar to those used for transfecting cells with ROMK and siRNA targeting c-Src was incorporated into pSuper expression vectors.

Preparation of CCDs

Male Sprague–Dawley rats (5–6 weeks old, <90 g) were used in the experiments (Taconic Farms, Germantown, NY) and they were fed with control diet (0.4% Na) or low Na diet (Na $<0.0001\%$) (Harlan laboratory, WI) for 7 days. Animals were killed by cervical dislocation and kidneys were removed immediately. The isolated split-open CCD was placed on a 5×5 mm cover glass coated with polylysine and then transfered to a chamber (1000 µl) mounted on an inverted Nikon microscope (Nikon, Melville, NY).

Electrophysiology experiment

Within 24 h after transfection, the cells were treated with trypsin-containing medium (Tryple Ecpresscare, Gibco, Carlsbad, CA) for 10 min to detach the cells. The cell suspension (0.2 ml in volume) was carefully removed to a 5×5-mm cover glass coated with polylysin followed by additional incubation for 30 min to allow the cells to adhere to the cover glass. The cover glass was transferred to a chamber (1 ml) mounted on the stage of a Nikon inverted microscope. We carried out the perforated whole-cell patch clamp experiments at room temperature. The cells were incubated with a bath solution containing 138 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4). Fluorescence signal (an indication of positive transfection) was detected with an intensified video imaging system including SIT 68 camera. Borosilicate glass (1.7-mm optical density) was used to make the patch clamp pipettes that were pulled with a Narishege electrode puller. The pipette had a resistance of 2–4 MΩ when filled with 140 mM KCl. The tip of the pipette was filled with pipette solution containing 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EGTA, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4). The pipette was then back-filled with amphotericin B (2 µg/0.1 ml) containing pipette solution. After forming a high resistance seal (>2 GΩ), the membrane capacitance was monitored until the whole-cell patch configuration was formed. The cell membrane capacitance was measured and compensated. The K currents were measured by an Axon 200A patch clamp amplifier (Axon, Sunnyvale, CA). The currents were low-pass filtered at 1 KHz and digitized by an Axon interface (Digidata 1200, Axon, Sunnyvale, CA) and data were stored in an IBM computer and were analyzed using the pClamp software system 7 (Axon). The method for conducting the patch clamp experiments in the split-open CCD has been described previously.[12]

Preparation of protein samples

The cells were placed in a lysis buffer containing 150 mM NaCl, 50 mM Tris · HCl, 1% Nonidet P-40 (pH 8.0), and protease inhibitor mixture (1%) (Sigma, St Louis, MO), was added to the lysis buffer. The tissues were then homogenized and allowed to sit on ice for an additional 30 min. The sample was subjected to centrifugation at 13,000 rpm for 8 min at 4 °C, and protein concentrations were measured in duplicate using a Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA).

Immunoprecipitation and western blot analysis

The corresponding antibody was added to the protein samples (500 µg) harvested from cell cultures with a ratio of 4 µg/1 mg protein. The mixture was gently rotated at 4 °C overnight, followed by incubation with 25 µl protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 2 h at 4 °C. The tube containing the mixture was centrifuged at 3000 rpm and the agarose bead pellets were mixed with 25 µl $2 \times$ SDS sample buffer containing 4% of SDS, 100 mM of Tris · HCl (pH 6.8), 20% of glycerol, 200 mM of 1,1,1-trichloro-2, 2-bis-(p-chlorophenyl) ethane, and 0.2% of bromophenol blue. After boiling the sample for 5 min, the proteins were resolved by electrophoresis on 8% SDS–polyacrylamide gels followed by transferring them to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, rinsed, and washed with 0.05% Tween20-Tris-buffered saline buffer. The membranes were washed 3 times with PBS and scanned by Odyssey infrared imaging system (LI-COR, Odyssey, Lincoln, NE) at a wave length of 680 or 800 nM.

Experimental materials and statistics

The tyrosine phosphorylation antibody (PY20), PKCε and AT1R antibodies were obtained from Santa Cruz Biotechnology, whereas c-Src antibody was purchased from Millipore/Upstate

(Billerica, MA). Antibodies to GFP and phospho416 c-Src were purchased from Clonetech (Mountain View, CA) and Sigma, respectively. All chemicals were from Sigma. The data are presented as mean ± s.e.m. We used a one-way analysis of variance test to determine the statistical significance. $P<0.05$ was considered to be significant.

DISCLOSURE

All the authors declared no competing interests.

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